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Pesticides in the Modern World

Pests Control and Pesticides Exposure and Toxicity Assessment

Edited by Margarita Stoytcheva

PESTICIDES IN THE MODERN WORLD – PESTS CONTROL AND PESTICIDES EXPOSURE AND TOXICITY ASSESSMENT

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Meet the editor

Professor Margarita Stoytcheva graduated from the University of Chemical Technology and Metallurgy of Sofia, Bulgaria, with titles of Chemical Engineer and Master of Electrochemical Technologies. She has a Ph.D. and DSc. degrees in chemistry and technical sciences. She has acted in research and teaching in several Universities in Bulgaria, Algeria and France. From 2006. to the present she has participated in activities of scientific research, technological development and teaching in Mexico at the University of Baja California, Institute of Engineering, Mexicali, as a full time researcher. Since 2008. she has been a member of the National System of Researchers of Mexico. Her interests and areas of research are analytical chemistry and biotechnology.

Contents

Preface

Volume 5 of the book series "Pesticides in the Modern World" is a collection of selected original research articles and reviews dedicated to the following main topics: biocontrol of pests, pesticides biomarkers, and pesticides toxicity.

The first section (Chapters 1-8) covers a large spectrum of issues associated with the ecological, molecular, and biotechnological approaches to the understanding of the biological control, the mechanism of the biocontrol agents action, and the related effects.

Three examples, given in Chapter 1, illustrate the development of effective and safe biocontrol strategies, namely: control of soil-borne pathogens on medical plants under organic conditions in Egypt, control of fungal pathogens in banana in Uganda, and control of a multi-species disease in the Styrian oilseed pumpkin. In Chapter 2 is summarized the current knowledge on RNA interference research on insects, and the potential application of RNAi in integrated pest management. Using of plant extracts for termites repelling in Pakistan is the subject of Chapter 3. The effects of botanical insecticides on digestive and on detoxifying enzymes, as well as on the immunological system of insects are discussed in Chapter 4. Useful data for the further development of *Pseudomonas spp*. cultivation process in the large-scale production and the commercial use of the biological pesticide pyoluteorin are provided in Chapter 5. The action of *Bacillus thuringiensis israelensis* toxins after ingestion by mosquito larvae and the diversity of mechanisms involved in mosquito resistance are described in Chapter 6. The results of the screening of biocontrol agents against *Rhizoctonia solani* causing web blight diseases of groundnut are reported in Chapter 7. In Chapter 8 are presented experimental data helpful for the optimization of the process of development of the insect-specific baculoviruses, used as biological insecticides.

The second book section (Chapters 9-14) provides recent information on biomarkers research for pesticides exposure assessment. The biomarkers currently used to evaluate pesticide exposure, effects, and the genetic susceptibility of aquatic organisms, terrestrial invertebrates and human populations are revised in Chapters 9-11. Some antioxidant enzymes and vitamins as biochemical markers for pesticide toxicity are examined in Chapter 12. The inhibition of the cholinesterases as a specific *biomarker* for organophosphate and carbamate pesticides is commented in Chapters 13 and 14.

The third book section addresses a variety of pesticides toxic effects and related issues. Chapter 15 is intended to summarize the increasing data regarding the molecular mechanisms involved in pesticides-induced toxicity, with relevance to the progression of the most frequent diseases. Several three-dimensional structural models of cuticle-degrading serine proteases secreted by nematophagous fungi, helpful for exploiting these enzymes as effective bio-control agents are described in Chapter 16. Investigations on fish histopathological, physiological, and DNA changes induced by pesticides exposure are reported in Chapters 17 and 18, thus contributing to the understanding of the toxicological risks caused by pesticides to ecosystems. Data presented in Chapter 19 demonstrate the hazardous effects of the pesticides glyphosate and methidathion on D. magna by studying the changes in the gene expressions of five stress responsive genes, including Dhb, Arnt, Vtg, CYP4, and CYP314. Chapter 20 provides details on anticoagulant rodenticides mode of action and on the strategies for evaluating and managing pesticides resistance in rodents. The potential of the cholinesterase inhibiting organophosphorus and carbamate pesticides is discussed in Chapters 21 and 22. A comprehensive overview of the side-effects of pesticides including discussion on the testing strategies employed to evaluate pesticide compatibility on bumblebees is provided in Chapter 23. The effects of pesticides on spiders and scorpions, the techniques applied for chemical control of arachnids, and the biology of these arthropods are reviewed in Chapter 24. The metabolic fate of xenobiotics such as pesticide-derived aromatic amines and the strategies for bioremediation of contaminated soils are discussed in Chapter 25.

The adequate and up-to-date information related to pesticides control, assessment, and toxicity provided in this book should be of interest for specialists, involved in pest control decisions.

Thanks are extended to each of the authors for their efforts in contributing the series “Pesticides in the Modern World”.

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Part 1

Biocontrol of Pests

1

Using Ecological Knowledge and Molecular Tools to Develop Effective and Safe Biocontrol Strategies

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1. Introduction

Today's farming systems undermine the well-being of communities in many ways: farming has destroyed huge regions of natural habitats, which also implies a loss of species and their ecosystem services (Sachs et al., 2010). Plant protection measures also causes problems for human health (Horrigan et al., 2002), and agriculture is responsible for about 30% of greenhouse-gas-emission (IPCC, 2007). Furthermore, emerging, re-emerging and endemic plant pathogens continue to challenge our ability to safeguard plant growth and health worldwide (Miller et al., 2009). Therefore, one of the major challenges for the 21st century will be an environmentally sound and sustainable crop production.

Microbial inoculants containing microorganisms with beneficial plant-microbe interactions have a great potential to contribute to this objective (Berg, 2009; Bhattacharjee et al., 2008). Over the past 150 years, research has demonstrated repeatedly that bacteria and fungi have an intimate interaction with their host plants and are able to promote plant growth as well as to suppress plant pathogens (Compant et al., 2005; Lugtenberg & Kamilova, 2009; Weller et al., 2002; Weller, 2007; Whipps, 2001). All plant-associated microenvironments, especially the rhizosphere, are colonized in high abundances by antagonistic microbes (Berg et al., 2005a). Between 1 and 35% of the microbial inhabitants showed antagonistic capacity to inhibit the growth of pathogens *in vitro* (Berg et al., 2002, 2006). The proportion of isolates, which express plant growth promoting traits is much higher in general, and was found up to 2/3 of the cultivable population (Cattelan et al., 1999; Fürnkranz et al., 2009; Lottmann et al., 1999). Diverse microbial inoculants, which were selected from this promising indigenous potential, are already on the market. In recent years, the popularity of microbial inoculants has increased substantially, as extensive and systematic research has enhanced their effectiveness and consistency (Berg, 2009).

New molecular and microscopic techniques are one reason for progress in biocontrol research. These techniques have enhanced our understanding about the plant and especially

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the rhizosphere as a microbial ecosystem and resulted into more effective screening strategies for bioactive microbes. In this chapter we will discuss these points first in general and in a second part with three representative examples.

2. Molecular and microscopic tools in biocontrol research

Molecular and microscopic tools can be used to study the ecology of single plant growth promoting rhizobacteria (PGPR) or biological control agent (BCA) strains or to analyse the structure and function of the target microbial community. In a first step we will analyse the use of methods for single strains (Table 1). Here, molecular fingerprints using repetitive elements in the genome (Rademaker & de Bruijn, 1997) can be used at several levels of biocontrol research. While the functions of many of these repetitive sequence elements are still unknown, they have proven to be useful as the basis of several powerful tools for use in microbial ecology. The repetitive, sequence-based PCR or rep-PCR DNA fingerprint technique uses primers targeting several of these repetitive elements and PCR to generate unique DNA profiles or 'fingerprints' of individual microbial strains (Ishii & Sadowsky, 2009). In screening strategies, these fingerprints can be applied to differentiate strains at population level and to select only unique isolates (Berg et al., 2006; Faltin et al., 2004). In a later stage, these highly reproducible fingerprints can be used for identity check and quality control. Genome sequencing also offers a tool to study PGPRs in great detail. Strains of *Pseudomonas fluorescens*, one of the dominant and cosmopolitan plant-associated species (Weller, 2007), were the first sequenced strains (Paulsen et al., 2005). Genomic information allowed the analysis of the mode of action, detailed investigations of interactions as well as optimisation of fermentation and formulation processes (rev. in Gross & Loper, 2009). De Bruijn et al. (2007) used genome mining to discover unknown gene clusters and traits that are highly relevant in the life style of *P. fluorescens* SBW25. Proteomic and transcriptomic studies are interesting to study the function of BCAs. For example, Garbeva et al. (2011) studied transcriptional and antagonistic responses of *Pseudomonas fluorescens* Pf0-1 to phylogenetically different bacterial competitors (*Bacillus, Brevundimonas* and *Pedobacter*), which demonstrated that Pf0-1 shows a species-specific response to bacterial competitors. In another transcriptomic study published by Hassan et al. (2010), a whole genome oligonucleotide microarray was developed for *P. fluorescens* Pf-5 and used to assess the consequences of a gacA mutation: GacA significantly influenced transcript levels of 10% of the 6147 annotated genes in the Pf-5 genome including genes involved in the production of hydrogen cyanide, pyoluteorin and the extracellular protease. Transcriptomic studies can also lead to new insights into plant responses on BCAs: *Pseudomonas*-primed barley genes indicated that, as is the case in dicots, jasmonic acid plays a role in host responses (Petti et al., 2010). A new tool is metabolomics, which allow the analysis of metabolites *in situ*. This is not only a technique to answer questions about the activity *ad planta*, it is also important for registration procedures, which are still a high hurdle on the way to the market. Frimmersdorf et al. (2010) used a metabolomic approach to show how *Pseudomonas aeruginosa* adapts to various environments. In addition, analysis of the mobilome of strains can result in interesting findings for biocontrol research as shown for *P. fluorescens* Pf-5 by Mavrodi et al. (2009), in which mobile genetic elements contain determinants that contribute to Pf-5's ability to adapt to changing environmental conditions and/or colonize new ecological niches. Studying the colonisation of plants has been greatly facilitated by the application of fluorescent proteins which are used as vital markers and reporter genes (rev. in Bloemberg, 2007). These new insights have changed our understanding about

colonisation; many of the strains analysed showed an endophytic life style (Chin-A-Woeng et al., 1997; Zachow et al., 2010), and the "root shield", which was hypothesized in former times, was rarely found in contrast to single cells and micro-colonies. Raman-FISH combines stable-isotope Raman spectroscopy and fluorescence *in situ* hybridization for the single cell analysis of identity and function (Huang et al., 2007a). This potential has been demonstrated through the discriminant functional analysis of Raman spectral profiles (RSP) obtained from the soil and plant-associated bacterium *P. fluorescens* SBW25; results suggests that SBW25 growth in the phytosphere is generally neither carbon-catabolite-repressed nor carbon-limited (Huang et al., 2007b).

Molecular tools were also used to analyse target habitats of biocontrol studies (Table 1). Cultivation-based methods to analyse plant-associated bacteria only address the culturable fraction, which are thought to represent only a small proportion (0.1 to 10%) of the total bacteria present in soil and in the rhizosphere (Amann et al., 1995). The analysis of nucleic acids directly extracted from plant microenvironments opened the chance to study a much broader spectrum of microbes (Table 1). Most frequently ribosomal RNA gene fragments are amplified from total community DNA and subsequently analysed by fingerprinting techniques: Terminal restriction fragment length polymorphism (T-RFLP), single-strand conformation polymorphism (SSCP), denaturing/temperature gradient gel electrophoresis (D/TGGE) using universal/specific primers (Schwieger & Tebbe, 1998; Smalla et al., 2007). Application of these fingerprinting techniques resulted in important findings such as plant-specific microbial communities (Smalla et al., 2001), the impact of cultivars on microbial communities (Milling et al., 2004) or the structure of endophytic communities (Rasche et al., 2006). Fingerprinting techniques are often used to analyse the structure of plant-associated communities and can also be used to study functional aspects. For example, Briones et al. (2002) found cultivar-specific differences for ammonia-oxidizing bacteria (AOB) in rice rhizospheres by a multiphasic approach including DGGE of the amoA gene, analysis of libraries of cloned amoA, fluorescently tagged oligonucleotide probes targeting 16S rRNA of

Objective/Level	Isolates: BCAs and pathogens	Microbial communities
Molecular fingerprints	Rep-PCR (BOX)	T-RFLP, SSCP, D/TGGE using universal/specific primers
Genomic information	Genome sequencing	Metagenome
Functions Functional diversity	Transcriptomics (RNA-based) Proteomics (Protein-based)	Metatranscriptome Metaproteome
Bioactive compounds	Metabolome	Metabolome
Adaptation/evolution	Mobilome	Metamobilome
Visualisation/activity	GFP/DsRed labelled strains, CLSM Raman spectroscopy and fluorescence *in situ* hybridization (FISH)	FISH-CLSM

Table 1. Molecular and microscopic tools in biocontrol research.

AOBs as well as metabolism rates obtained by the 15N dilution technique. Other techniques have a great impact on our functional understanding; this was shown for example for transcriptome profiling (Mark et al., 2005; Yuan et al., 2008), microarrays (Sanguin et al., 2006; Weinert et al., 2011) *in vivo* expression technology and differential fluorescence induction promoter traps as tools for exploring niche-specific gene expression (Rediers et al., 2005), new methods for the *in situ* analysis of antifungal gene expression using flow cytometry combined with green fluorescent protein (GFP)-based reporter fusions (de Werra et al., 2008), barcode pyrosequencing (Gomes et al., 2010), and ultra deep sequencing (Velicer et al., 2006). Stable isotope probing (SIP) used to determine bacterial communities assimilating each carbon source in the rhizosphere of four plant species resulted in plant species specific patterns (Haichar et al., 2008). Metagenomic approaches have been established to analyse the plant-soil interface (Erkel et al., 2006; rev. in Leveau, 2007).

3. Using ecological knowledge to screen and evaluate biocontrol agents

The advanced techniques discussed above should be integrated into strategies to screen and evaluate biocontrol agents (Fig. 1). Of primary importance is the life cycle of the pathogen. This can result in new targets for biocontrol; one example is the impact of zoospores on pathogenic oomycetes, which are primary targets for suppression (de Bruijn et al., 2007; Raaijmakers et al., 2010). Furthermore, it is also important to understand the target microenvironment of plants. Plant specificity is one critical point but also knowledge about the structure and function of the microbial communities. There are strategies to select BCAs from the indigenous antagonistic potential as well as to use ubiquitous, cosmopolitan BCAs (Zachow et al., 2010). If a BCA is selected, an evaluation strategy is needed to assess their potential for commercialization.

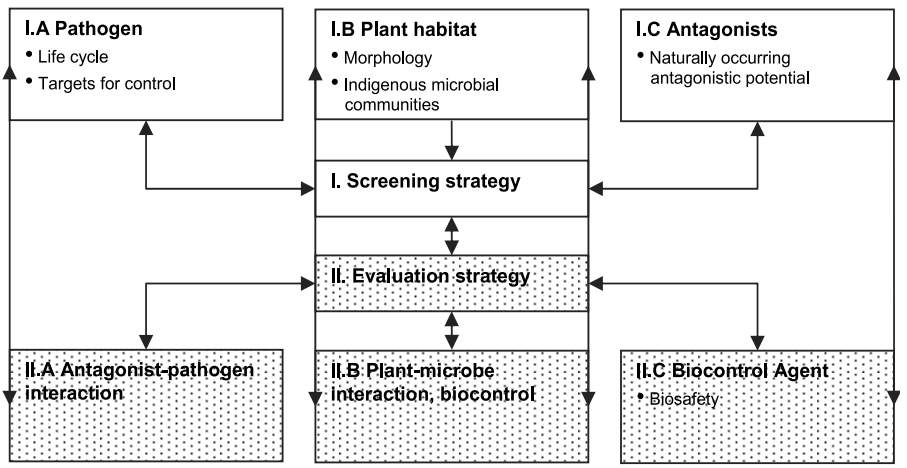

Fig. 1. Integration of ecological knowledge into screening and evaluation strategies.

Knowledge about the effect of BCAs under greenhouse and field conditions presents the basis for this evaluation. However, often inconsistent effects make the decision difficult. Detailed analyses of plant-microbe and pathogen-microbe interactions under different environmental conditions can help to optimize the biocontrol effect under practical conditions. Another aspect, which should be integrated in an early phase of evaluation, is

biosafety. Many BCAs fail here due to problems with human or environmental health. Due to the fact that the whole program to investigate toxicology is time-consuming and expensive, alternative test systems should be used, e.g. the *Caenorhabditis elegans* assay (Zachow et al., 2009) or Duckweed (*Lemna minor*) as a model plant system for the study of human microbial pathogenesis (Zhang et al., 2010).

4. Examples for screening and evaluation strategies

4.1 Strategy to control soil-borne pathogens on medical plants under organic conditions in Egypt

On the SEKEM farms in Egypt desert land was converted into arable land, and biodynamic agriculture is operated for over 30 years now (www.sekem.com). Today SEKEM is carrying out organic agriculture on more than 4100 hectares and has the largest market for organic products outside Europe and North America. They produce organic foods, spices, tea, cotton textiles and natural remedies. However, the cultivation especially of medical plants is more and more affected by soil-borne phytopathogens, which lead to significant yield losses. The objective of our study was to develop a specific biocontrol strategy for desert farming.

An important factor was to find out, whether and how the highly specialized natural microbial communities of the desert soil are affected by agriculture and watering. To examine the impact of organic agriculture on bacterial diversity and community compositions in desert soil, soil from a SEKEM farm in comparison to the surrounding desert soil were assessed by a pyrosequencing-based analysis of partial 16S rRNA gene sequences. When appropriate primers are chosen, in a pyrosequencing analysis with short reads the microbial diversity is represented almost as reliably as with near-full-length sequences (Will et al., 2010). Fragments encompassing the V4-V5 region of the 16S rRNA gene provide estimates comparable to those obtained with the nearly complete fragment (Youssef et al., 2009). In desert soil 19244 and in agricultural soil 33384 quality sequences with a read length of ≥ 150 bp were recovered. Using different data bases, 83.0% of all quality sequences could be classified below the domain level, in the range of the percentage of classified 16S rRNA gene sequences of other pyrosequencing-based studies (Lauber et al., 2009; Lazarevic et al., 2009; Will et al., 2010). The computed Shannon indices of diversity (H') (Shannon, 1997) were much higher for agricultural soil than for desert soil (H' at a dissimilarity level of 20%: SEKEM soil 4.29; desert soil 3.54); this indicates a higher bacterial diversity in soil due the agricultural use of the desert. A comparison of rarefaction analyses with the number of operational taxonomic units (OTUs) estimated by the Chao1 richness estimator (Chao & Bunge, 2002; Will et al., 2010) revealed that at this genetic distance the surveying effort in both soils covered almost the full extent (over 97% in both soils) of taxonomic diversity. This was also shown by a clear saturation of both curves in the rarefaction analysis (data not shown). The 43673 classifiable sequences obtained from both soil types together were affiliated with 18 different phyla. Dominant groups were especially Proteobacteria (30.2%), Firmicutes (27.3%) and Actinobacteria (10.5%). These dominant phyla were present in both soils. In detail, Firmicutes were highly enriched in agricultural soil (from 11.3% in desert soil to 36.6% in SEKEM soil), Proteobacteria (46.0% in desert soil and 21.0% in SEKEM soil) and Actinobacteria (20.7% in desert soil and 4.6% in SEKEM soil) occurred in SEKEM in lower abundances than in the surrounding desert. In addition, in both soils Bacteroidetes (4.6% and 5.3%) and Gemmatimonadetes (1.4% and 1.9%) were

present. Whereas Acidobacteria (7.9%) and Planctomycetes (1.1%) were only present in the agricultural soil, *Deinococcus-Thermus* (1.1%) was only detectable in the desert sand. These abundances of the phyla are coextensive with results from previously reported meta-analysis of bacterial community composition in soils and, despite the specific soil type of the desert, the composition covers rather well with studies of completely different soils (Hansel et al., 2008; Janssen, 2006; Lauber et al, 2009; Will et al., 2010). However, greatly different from all reported studies was the high abundance of Firmicutes. Janssen (2006) reported them to contribute only a mean of 2% (range 0 – 8%) in the total bacterial soil community. Most of the Firmicutes sequences were classified as belonging to the genus *Bacillus*; in the agricultural soil also the phylogenetically related genus *Paenibacillus* was found (5% of classified Firmicutes). In desert soil, *Ochrobactrum* was the most abundant genus within the (Alpha-)Proteobacteria (79% of classified Proteobacteria) and *Rhodococcus* among the Actinobacteria (90% of classified Actinobacteria). The Acidobacteria in the agricultural soil are affiliated only with subdivision 6.

Additionally to the pyrosequencing analysis, the composition of the bacterial as well as fungal community in the two different soil types was investigated by SSCP analysis of rRNA gene fragments (Bassam et al., 1991; Schwieger & Tebbe, 1998). Furthermore, the composition of the microbial community in rhizosphere and endorhiza of three different species of medical plants (*Matricaria chamomilla* L., *Calendula officinalis* L. and *Solanum distichum* Schumach. & Thonn.) grown under organic conditions on SEKEM farms were examined. According to the cluster analysis prepared on the basis of SSCP community fingerprints, the agricultural soil in bacterial as well as in fungal community composition strongly differed from the desert soil. As shown in the pyrosequencing analysis, in comparison to the desert in soil of the SEKEM farm an impressive diversity of bacteria, expressed as various bands in the gel, was found (data not shown). In the bacterial community of the desert soil, two dominant bacterial bands could be detected, which were also visible in all samples from the endorhiza of all three investigated medical plants. This shows that bacteria are taken up by the plants from the soil, and that soil is the main reservoir for biological control agents. The two dominant bands were identified by partial 16S rRNA gene sequence analysis as *Ochrobactrum* sp. (closest database match *O. grignonense*) and *Rhodococcus* sp. (closest database match *R. erythropolis*). Further, nearly in all samples *Bacillus* sp. was found (closest database match *B. subtilis*). By SSCP analysis and also by the pyrosequencing approach, *Ochrobactrum* and *Rhodococcus* could be detected as dominant bacteria. However, both genera include opportunistic human pathogens (*O. anthropi*, *R. equi*). Several studies provided evidence that similar or even identical functions are responsible for beneficial interactions with plants and virulence in humans (Berg et al., 2011). For *Ochrobactrum* was already detected the production of plant growth hormones and siderophores and also an antifungal activity towards several phytopathogens was described (Chakraborty et al., 2009). *Ochrobactrum* was found in diverse environmental niches, like rhizosphere, soil, sediments and activated sludge (Berg et al., 2005b). *Rhodococcus* could also be found in a broad range of environments, including soil, water and eukaryotic cells. This genus includes also a phytopathogenic species causing leafy gall formation on a wide range of host plants, *R. fascians* (Goethals et al., 2001). The fungal community fingerprints included a quite high diversity in all microenvironments. As an example, SSCP profiles of fungal communities in rhizosphere and endorhiza are shown in Figure 2. A dominant band, which was found nearly in all samples, was identified as

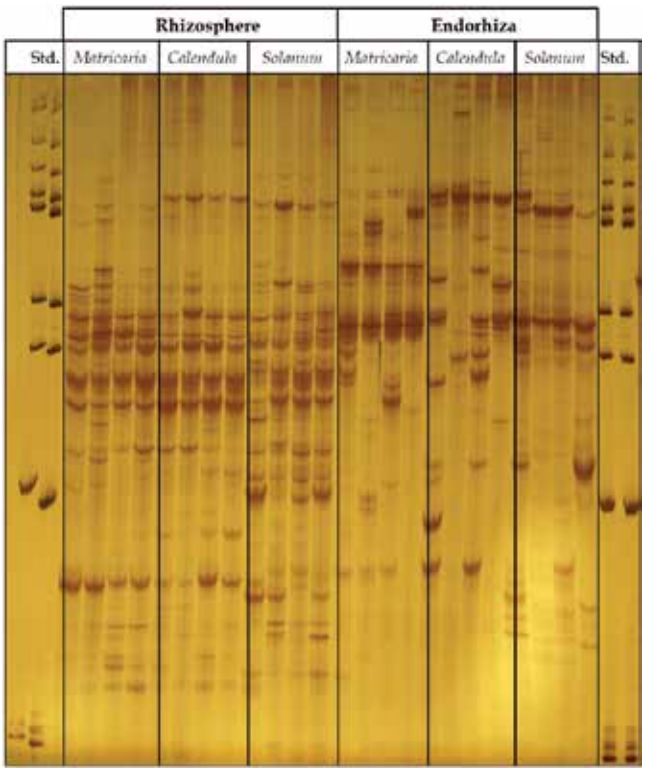

Fig. 2. SSCP profiles of the fungal communities in rhizosphere and endorhiza of the medical plants. Four independent replicates per plant and microenvironment were loaded onto the gel. Std.: 1 kb DNA ladder.

Verticillium dahliae, which is one of the mainly occurring soil-borne phytopathogens on the SEKEM farms. In general, mainly potential plant pathogens were found within the fungal communities. The obligate root-infecting pathogen *Olpidium,* belonging to the fungal phylum Chytridiomycota, was found especially in the rhizosphere and endorhiza of *Matricaria chamomilla*. *Alternaria* and *Acremonium* were found primarily in the rhizosphere samples. According to the generated dendrograms, a clear plant specificity of the bacterial and fungal communities in the rhizosphere as well as in the endorhiza was found (Fig. 3). Furthermore, microenvironment-specific SSCP patterns of the bacterial and the fungal communities were detected (data not shown). There were significant differences between the rhizosphere and the endorhiza of the medical plants. In general, samples from the rhizosphere generated more bands than samples from the endorhiza of the medical plants, which indicate that a sub-set of rhizobacteria was able to invade the root.

The major problems in the cultivation of plants on SEKEM farms are caused by the soil-borne pathogenic fungi *Verticillium dahliae* Kleb., *Rhizoctonia solani* J.G. Kühn and *Fusarium culmorum* (Wm.G. Sm.) Sacc. as well as by the soil-borne pathogenic bacterium *Ralstonia solanacearum*. Although grown in organic agriculture, which aims to minimize the impact on the environment by practices such as crop rotation, using pathogen resistant cultivars, and the use of organic manure (compost) instead of synthetic fertilizers (Schmid et al., 2011), they have an increasing importance. One reason is an intensive growing of a limited number

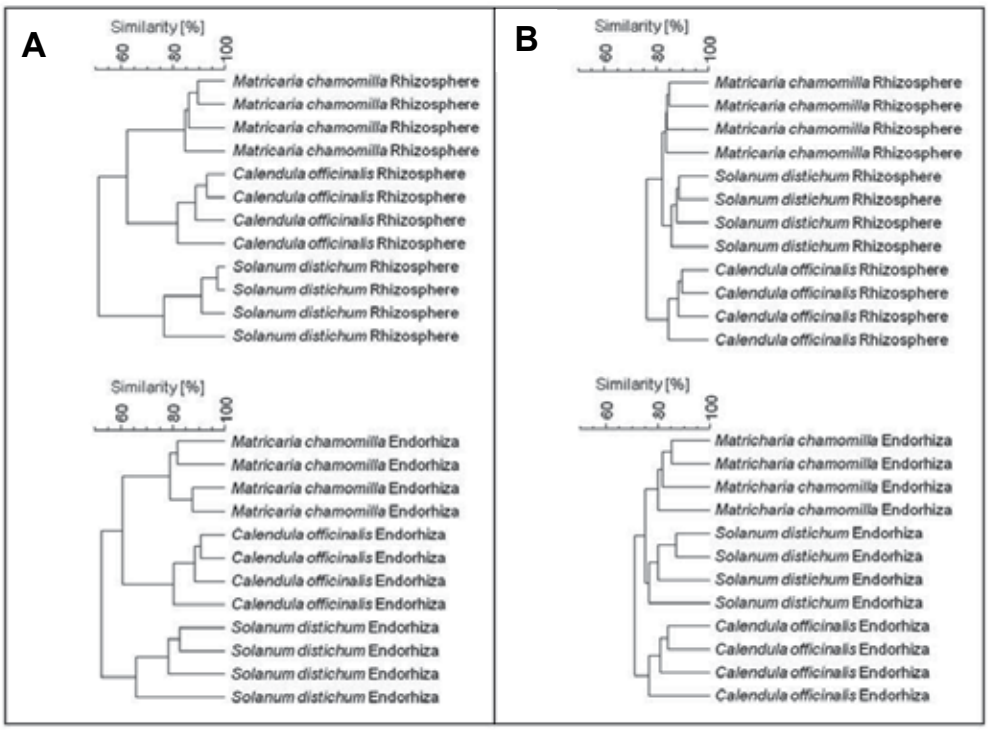

Fig. 3. UPGMA dendrograms of bacterial (A) and fungal (B) communities in rhizosphere and endorhiza of the medical plants. The dendrograms were generated from the SSCP community profiles with GelCompar II. The following settings were used: dendrogram type: unweighted pair group method with arithmetic mean (UPGMA); similarity coefficient: band based: dice; position tolerances: optimization: 4%, position tolerance: 1%.

of crops in short rotations. Here, biocontrol agents should solve these problems and help to suppress soil-borne pathogens on a natural way. Although BCAs are already on the market, our biocontrol product will be optimized for desert farming – regarding soil, weather, pathogen species, etc. For this reason, autochthonous bacteria were isolated from rhizosphere and endorhiza of medical plants as well as from bulk soil collected in SEKEM farms, and were evaluated for their potential for biocontrol. In a first step, the dual-culture assay was used to find out the antagonistic potential towards the pathogenic fungi (Berg et al., 2002, 2005a). A total of 1589 bacterial isolates were screened for their ability to inhibit *in vitro* the growth of *Verticillium dahliae*, *Rhizoctonia solani* and *Fusarium culmorum*. Bacterial isolates obtained from the soil of the SEKEM farm exhibited a higher *in vitro* antagonistic potential towards soil-borne phytopathogenic fungi in comparison to the bacteria isolated from the desert soil (SEKEM 21.6 ± 0.8%; desert 12.4 ± 0.7%). From the agricultural soil 17.4% (27 isolates) demonstrated antagonism towards all three pathogens, from the desert soil 10.6% (21 isolates) were able to suppress the growth of all fungi tested. Already the desert soil harbours a high proportion of antagonists, which were augmented by organic agriculture in SEKEM soil. The soil from the farm seems to be supplied with antagonists in such an optimal way, that there was no detectable enrichment of antagonists in the rhizosphere and endorhiza of the investigated medical plants. In general, *Matricaria*

chamomilla and *Solanum distichum* showed a better antagonistic potential than *Calendula officinalis*. Especially the endorhiza from *Matricaria chamomilla* harbours a high proportion of antagonists. Whereas in the soil and in the rhizosphere could be found most antagonistic bacteria towards *Fusarium culmorum*, in the endorhiza of the medical plants most antagonists were found towards *Verticillium dahliae*.

In a next step, the antagonistic mechanisms of all isolates, which showed an activity towards at least two of the investigated pathogenic fungi (162 isolates), were investigated *in vitro* with a special focus on fungal cell wall degrading enzymes (β-1,3-glucanase, chitinase and protease) (Chernin et al., 1995; Grube et al., 2009) and siderophore-production (Schwyn & Neilands, 1987). Production of chitinase could be detected for 8.0% of the antagonists; *Lysobacter enzymogenes* followed by all isolates of *Streptomyces* showed a high chitinolytic activity. Glucanase activity was shown for nearly all isolated antagonists (93.8%); only the isolates of the *Bacillus cereus* group were not able to degrade β-1,3-glucan. Casein degradation by protease could be shown at 80.9% (*Bacillus* sp. and *Lysobacter* sp.). The production of siderophores was shown for all antagonists except the isolates of *Paenibacillus* sp. (93.2%).

To avoid investigations with genetically similar strains, amplified rRNA gene restriction analysis (ARDRA) of the 16S rRNA gene with the restriction endonuclease *Hha*I (Zachow et al., 2008) and BOX polymerase chain reaction fingerprints (Berg et al., 2002; Rademaker & de Bruijn, 1997) of the antagonistic isolates were performed. A representative selection of promising biological control agents was identified by partial 16S rRNA gene sequencing. The use of ARDRA of the 16S rRNA gene with the restriction enzyme *Hha*I led to the separation of isolates clustered into five groups (data not shown); within groups the similarity of the band patterns was 100% identical: *Bacillus subtilis* group, *Bacillus cereus* group, *Paenibacillus*, *Streptomyces* and *Lysobacter*. Except *Lysobacter* (only one isolate from the rhizosphere of *Matricaria chamomilla*) only gram-positive antagonists were found. All microenvironments were dominated by antagonists from the Firmicutes branch. *Bacillus* and *Paenibacillus* could be isolated from all habitats. Antagonistic isolates of the genus *Streptomyces* were found exclusively in desert soil. Especially within the large ARDRA cluster of the *Bacillus subtilis* group containing 123 isolates, analysis of the BOX PCR fingerprints showed a high genotypic diversity. At a cutoff level of 80%, they could be divided into 39 genotypic groups. The genus *Paenibacillus* could be divided into 11 BOX clusters, *Streptomyces* was subdivided in three genotypes. According to the ARDRA and BOX dendrograms, 46 preferably genotypically different strains were selected to test them on their antibacterial activity towards *Ralstonia solanacearum* (Adesina et al., 2007) and *Escherichia coli*. The cluster of the *Bacillus cereus* group was completely excluded for further investigations, because of some human pathogenic strains belonging to this taxonomic group. Most isolates of the genus *Paenibacillus* (identified as *P. brasilensis* and *P. polymyxa*) were able to inhibit *in vitro* the growth of *E. coli* (7 of 11 isolates), but these strains showed no antagonistic activity towards *R. solanacearum*. The growth of *R. solanacearum* was inhibited by 32.6% of the selected antagonists: most isolates of *Streptomyces* (3 of 4 isolates) and some strains of the *Bacillus subtilis* group (12 of 30 isolates).

Organic amendments like manure, compost and cover crops positively affected the disease suppressiveness of SEKEM soil. During decomposition of organic matter in soil, the ecosystem is subjected to oligotrophication. The ratio of oligotrophic to copiotrophic organisms changes during microbial succession, and this has been associated with general disease suppression (van Bruggen & Semenov, 2000; Garbeva et al., 2004). Our cultivation-

independent approaches showed an extraordinary high Firmicutes level in SEKEM soils. By cultivation and characterization, the antagonistic role of *Bacillus* and *Paenibacillus* (both Firmicutes) was identified. Both are well-known and potent in biocontrol (Berg, 2009; Schisler et al., 2004; Tupinambá et al., 2008). These gram-positive bacteria have a natural formulation advantage due to their ability to form durable, heat-resistant endospores (Emmert & Handelsman, 1999). *Lysobacter* was the only gram-negative genus identified (Park et al., 2008). This is in contrast to the majority of other studies, where members of the *Pseudomonas* genus play a major role (Haas & Défago, 2005; Weller et al. 2007). Due to the fact that the proportion of antagonistic strains in soil and root is already high, biocontrol strategies could aim to enhance the diversity of the antagonistic community by application of *Lysobacter, Pseudomonas* or *Serratia* strains. However, in our study we selected promising candidates, which will be tested *ad planta* in comparison to these often used antagonists.

4.2 Strategy to control Fusarium wilt in bananas in Uganda

The banana family Musaceae includes monocotyledonous plants of the genera *Ensete*, *Musa* and *Musella*. Most important is the genus *Musa* comprising 50 to 100 species and cultivars including those with edible fruits like dessert or cooking banana, species with inedible fruits like ornamental bananas or those used for fibres production (Li et al., 2010). In many countries in Africa, Latin America, Asia or the Caribbean, banana production is an important source of income. Banana is the fourth important staple food after rice, wheat and milk in Uganda, the country with the highest per capita consumption per year of cooking banana and the second largest producer after India in the world. Farmers have to deal with several problems as plant pests and diseases, climate change or soil depletion. Diseases caused by fungi, bacteria and viruses are the most limiting factors of high quality production. Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *cubense* (*Foc*), is the most severe disease in banana plants, which leads to high yield losses (Ploetz, 2006). An infestation with the phytopathogen compromises the water and nutrient transport that can cause, in the worst case, the death of the plant. *Foc* belongs to the *F. oxysporum* species complex, which is distributed in a broad range of soils and causes serious symptoms on numerous host plants. Despite its ubiquitous occurrence, a morphological identification is difficult and is based primarily on the structure and abundance of asexual reproductive structures and on cultural characterizations (Fourie et al., 2011). The species is divided into more than 150 *formae specialis* and further subdivided in races, depending on the affected plant cultivars. *F. oxysporum* persists in soil as immobile chlamydospore until germinating by utilizing nutrients released from plant roots. The life cycle of the fungus commences with a penetration of the spore germ tube or the mycelium of the plants root tip. Further, wounds facilitate the endophyte an entrance of the potential host. When the mycelium entered the xylem vessel, it travels upwards through the plant. In later stages, microconidia are produced, which are distributed in the vessel system and germinate when their movement is stopped. This decreases water and nutrient transport, resulting in severe wilt and eventually death of the plant. Early symptoms of an infestation are reddish brown colouration of the xylem, a yellowing of old leaves and a beginning of wilt. In advanced stages, pseudostem coating leaves collapse and die. The pseudostem sometimes splits. Internally, xylem vessels of the roots and the rhizome turn reddish-brown as the fungus grows through the tissue (Aboul-Soud et al., 2004; Daly & Walduck, 2006). Different studies with bananas and banana plants *in vitro* and *in vivo* have shown that plants harbour fungal and bacterial organisms with antagonistic potential towards plant pathogens (Cao et al.,

2005; de Costa et al., 1997; Lian et al., 2008). However, an efficient strategy to control fungal pathogens especially *Foc* is still missing. In our study, we used molecular techniques to study banana-associated microbial communities in detail and focus on endophytes, which have a great potential for biocontrol of vascular diseases.

For screening of antagonists the rhizosphere, the endosphere and bulk soil of Ugandan banana plants were analysed. The term endosphere refers to the pseudostem of the plant, which is not lignified. Bananas grown in four different fields (variants) in Central Uganda characterized by different manure systems and/or agro-forest systems were sampled. In the first step, bacterial and fungal abundances in the microhabitats were examined. Surprisingly, the highest bacterial abundances with $\log_{10}$ 9.4 ± 0.1 g^{-1} fw were calculated for the endosphere followed by the rhizosphere with $\log_{10}$ 8.4 ± 0.3 g^{-1} fw and soil with $\log_{10}$ 7.7 ± 0.3 g^{-1} fw from R2A medium. Similar values for all microhabitats ranging from $\log_{10}$ 6.2 ± 0.2 g^{-1} fw for rhizosphere followed by soil and endosphere with almost same abundances of $\log_{10}$ 5.5 ± 0.3 g^{-1} fw and $\log_{10}$ 5.4 ± 0.3 g^{-1} fw were estimated for fungal isolates on synthetic nutrient-poor agar (SNA). A total of 1152 bacterial isolates from different media as R2A, MacConkey (for enrichment of Enterobacteriaceae) and King's B medium (for enrichment of *Pseudomonas*) and 586 fungi from SNA medium were randomly selected and screened *in vitro* for their antagonistic potential towards the pathogens. The target pathogen was also isolated from bananas in Uganda. Interestingly, different fungal species were identified: *F. oxysporum* f.sp. *cubense, Fusarium chlamydosporum,* and *Colletotrichum musae.* The latter are known as "low" pathogens; however, strains of all three species were integrated in the screening strategy. The antagonistic activity of bacteria or fungi towards the pathogen evaluated by the method of Berg et al. (2006) ranged from 3 – 6%. Altogether 37 highly active bacterial and 36 fungal strains were further characterized. ARDRA genotyping was able to distinguish bacteria on genus level into *Pseudomonas*, *Bacillus*, *Burkholderia* and *Serratia*. With repetitive BOX PCR a further characterization on population level was performed. Members of the genus *Burkholderia* were more diverse than those of *Serratia* (Fig. 4).

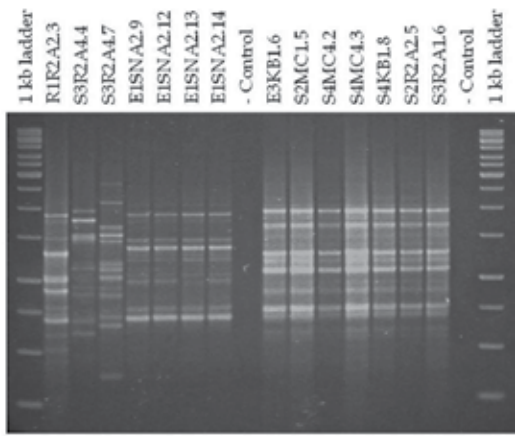

Fig. 4. BOX analysis on species level of bacterial antagonists. First seven isolates were identified as *Burkholderia* species and the other seven as *Serratia marcescens*. For identification of isolates the following abbreviations were used: a) habitat with R for rhizosphere, S for soil and E for endosphere, b) number of variant from 1 to 4, c) medium isolated from MC for MacConkey agar, KB for King's B agar, R2A for R2A agar and SNA for synthetic nutrient-poor agar d) number of replicate from 1 to 4 and e) number of isolate from 1 to 14.

Additionally, the best antagonists were screened for their ability to produce lytic enzymes like glucanase or protease, which are known for their positive influence in combating fungal pathogens by enzymatic degradation of the cell wall (Kamensky et al., 2003). Further, the production of siderophores, short-chained quorum sensing molecules and the auxin indole-3-acetic-acid (IAA) was investigated, which are involved in plant growth promoting processes. The results indicated that 100% of the tested isolates produced an active protease, while only a single isolate, which was identified as *Bacillus indicus*, was able to degrade glucan. Nearly all strains (94.6%) produced siderophores but only 21.6% isolates, belonging to the genera *Pseudomonas* and *Burkholderia*, released quorum sensing molecules. Seven isolates were positively tested for production of IAA, all of them identified as *Serratia marcescens*. To characterize fungal isolates, morphological groups were identified. Sequencing analysis of the ITS region indicated, that the majority of isolates belong to the genera *Penicillium*, *Paecilomyces*, *Fusarium* and *Mortierella*. All of them include known biocontrol strains, some actually tested in *Musa* spp. like non-pathogenic *F. oxysporum* strains (Kidane, 2008).

Cultivation independent analyses include the fingerprint method SSCP, quantitative PCR (qPCR), metagenome analysis and confocal laser scanning microscopy (CLSM) in combination with fluorescence *in situ* hybridization (FISH). Using SSCP fingerprints, a high specificity was shown for each microenvironment of banana, particularly for the endosphere. The patterns obtained from the bacterial community using universal primers were highly diverse, especially for rhizosphere and soil. This is a typical picture for environmental samples, especially for soil. A detection of bacterial species ranges up to 100 most dominant ones. This problem can be solved by using of more specific primers, e.g. for *Pseudomonas* or Enterobacteriaceae. Using both in analyses, specific patterns for each habitat appeared. Surprisingly, comparing all fields with different treatments or environmental influence, bacterial, enteric and fungal community didn't show distinct patterns. This could be explained by a high specificity of banana-associated bacteria independent from the site. The *Pseudomonas* community was more sensitive, but each site showed an individual pattern. In our study, we found that Enterobacteriaceae were extraordinarily present in and around cultivated banana plants. Therefore, further investigations on the microhabitat-specific communities were performed using a metagenomic approach. The sequences (1944 – 23800) obtained after pyrosequencing were aligned with databases and identified on genus level. In Figure 5 taxa including more than 1% of the totally analysed community were presented. Each habitat harboured a specific arrangement of genera. In the two rhizosphere variants, more than 40% of the identified genera are members of the *Enterobacter* community, followed by *Serratia*, *Pantoea* and *Klebsiella* with almost 40% and some other genera making up less than 20%. The bacterial composition in the endosphere differed from the rhizosphere samples with a lower number of *Enterobacter* and higher presence of the genus *Raoultella*. The highest species richness was shown for the soil sample, with the dominant genus *Pantoea* with known plant growth promoting species (Bonaterra et al., 2005; Braun-Kiewnick et al., 2000). *Serratia*, *Klebsiella* and *Enterobacter* represented together more than 40% of the analysed species. The analysis illustrates that depending on the investigated microhabitat, different species dominated. For the majority of the listed genera, species with growth promoting abilities are described. In different parts of the plant, diverse species play a key role, like *Enterobacter* in rhizosphere or *Pantoea* in soil and endosphere. To complement pyrosequencing data, a further assessment of *Pseudomonas* and Enterobacteriaceae was performed with quantitative PCR. Similar results were measured for both communities; the highest copy numbers g^{-1} fresh material of enterics and pseudomonads were detected in endosphere with $\log_{10}$ 8.4 ± 0.5 for *Pseudomonas* and $\log_{10}$ 7.9 ± 0.2 for Enterobacteriaceae

followed by rhizosphere with $\log_{10}$ 7.2 ± 0.6 and $\log_{10}$ 6.8 ± 0.4 and last with $\log_{10}$ 6.3 ± 0.8 for enterics in soil. In the *Pseudomonas*-specific analysis, no data for soil were received due to values under the detection limit. With confocal laser scanning analysis (CLSM) detection of different bacterial classes as Alpha-, Beta- and Gammaproteobacteria was performed to illustration our data. Due to the fact, that the number of enterics was extraordinary high in prior analyses, the focus in microscopy was also set on *Enterobacteriaceae*. The microscopic analysis confirmed the previous results, with the detection of a high number of *Enterobacteriaceae* in the endosphere and also lower detection in rhizosphere.

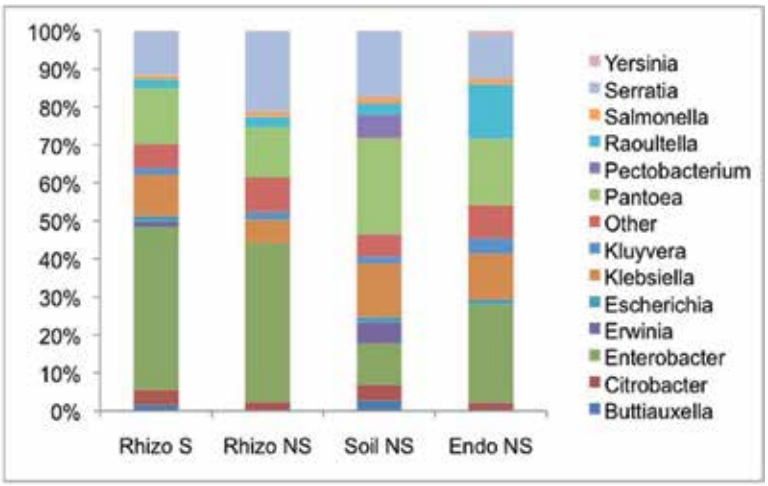

Fig. 5. Genera of the Enterobacteriaceae community associated with banana plants. Two rhizosphere samples under influence of agro-forest (shaded = S) and not (non-shaded = NS) and one sample from soil and endosphere in comparison. DNA was amplified with enterics-specific primers and analysed by pyrosequencing and identification with the web server SnoWMAn 1.7. The pipeline used was BLAT, NCBI database was selected and included taxa covering more than 1%. Phylogenetic groups accounting for ≤ 1 % of all quality sequences are summarized in the artificial group others.

This multiphasic approach showed that the pseudostem of banana – the endosphere – is a unique microenvironment in plants. It is characterized by extremely high microbial abundances, a high diversity and specificity, but a low proportion of antagonistic strains. Enterics play a key role in the bacterial community; they are dominant and represent a cluster of antagonists. However, they also contain human and plant pathogenic species. The endosphere should be the target habitat for biocontrol strategies: the number of strains with a beneficial plant impact should be enhanced here. We have isolated promising strains of *Pseudomonas*, *Bacillus*, *Burkholderia* and *Serratia,* which are interesting candidates for *ad planta* experiments. However, it is necessary to pay attention to the enteric community in bananas, especially to the pathogens.

4.3 Strategy to control a multi-species disease in the Styrian oilseed pumpkin

Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) is a pumpkin variety that bears its name according to its origin of cultivation that is the Austrian district Styria. The specialty of this cultivar is the absence of a wooden seed shell that facilitates the production of pumpkin seed oil. Beside the culinary aspect of this dark green oil it is

famous as a very healthy nutritional supplement containing high levels of polyunsaturated fatty acids, antioxidants, vitamins A, B1, B2, B6, C, D, E and counteracts diseases of bladder and prostate.

In recent years, dramatic yield losses of Styrian oil pumpkin were reported in Styria due to black rot of pumpkins caused by *Didymella bryoniae* (Auersw.) Rehm, anamorph *Phoma cucurbitacearum* (Fr.) (Huss et al., 2007). The ascomycete has a broad host range within the Cucurbitaceae and causes symptoms on vegetative plant parts known as gummy stem blight (Keinath et al., 1995). It spreads from temperate to tropical regions of the world (Sitterly & Keinath, 1996). Fruits, leaves and flower scars are invaded by the pathogen and it can also be seed-borne (Lee et al., 1984; Ling et al., 2010; de Neergaard, 1989; Sitterly & Keinath, 1996). By cultivation-independent SSCP fingerprinting of the fungal ribosomal internal transcribed spacer (ITS) region in combination with DNA sequencing and BLAST analysis (Altschul et al., 1997), it was detected as well in roots of oil pumpkin (data not shown). This underlines the potential establishment of the pathogen even in soils (Bruton, 1998). The analysis of the phenotypic and genotypic variability of the pathogen across different oil pumpkin fields in Styria resulted in a remarkable high morphological versatility in contrast to a low genetic diversity (Zitzenbacher, pers. communication). Styrian oil pumpkins are also affected by bacterial pathogens *Pectobacterium carotovorum* subsp. *carotovorum* and subsp. *atrosepticum*, *Pseudomonas* spp. and *Xanthomonas cucurbitae* causing soft rot of pumpkins and leaf diseases (Huss, 2011). The transport of these bacterial phytopathogens by the fungus was observed in vitro (Zitzenbacher, pers. communication) suggesting synergistic interactions between them in the course of co-infections.

In order to manage microbial diseases of Styrian oil pumpkin based on autochthonous bacterial and fungal antagonists, initial studies to discover the microbial diversity associated with this host plant were conducted. Roots, female flowers and fruit pulp from three different oil pumpkin cultivars ("Gleisdorfer Ölkürbis", "Gleisdorfer Diamant" and "GL Maximal") at a field site in Styria were collected. Root samples were taken at three time points (before flowering, time of flowering, fruits well developed). Bacterial genera *Pseudomonas* and *Bacillus* that are known for their plant beneficial interactions (Haas & Défago, 2005) were analysed by SSCP analysis. Data revealed a greater impact of the microhabitat on community structure for *Pseudomonas*, whereas the plant stage had a stronger impact for *Bacillus* populations. Female flowers as possible gates for bacterial and fungal infections were analysed in more detail. For *Bacillus* and *Pseudomonas* and ascomycete communities, no effect of the plant cultivar on population structure was observed. However, in the flower, the communities are well-structured. FISH-CLSM studies revealed a dense bacterial colonisation of pollen grains that act as propagation vehicles between pistils especially for Alphaproteobacteria (Fig. 6) and shaped in this way the bacterial community structure of the oil pumpkin anthosphere.

To obtain oil pumpkin-associated microorganisms for testing their antagonistic properties against *D. bryoniae* and bacterial pathogens, bacterial and fungal strains were isolated from oil pumpkin cultivars and microhabitats as described above. Endophytes were cultivated from roots and fruit pulp. In addition, seed borne microbial strains were obtained from aforementioned varieties by the isolation from roots, stems and leaves from plants that seeds were surface sterilized and grown under gnotobiotic conditions. Finally 2320 isolates (1748 bacteria and 572 fungi) were subjected to dual culture assays against *D. bryoniae* A-220-2b to test their antagonistic potential against this pathogen. Of tested bacteria, 7.3% inhibited growth, whereas 12.4% of observed fungi showed either growth inhibition or overgrowth of *D. bryoniae* (Fig. 7).

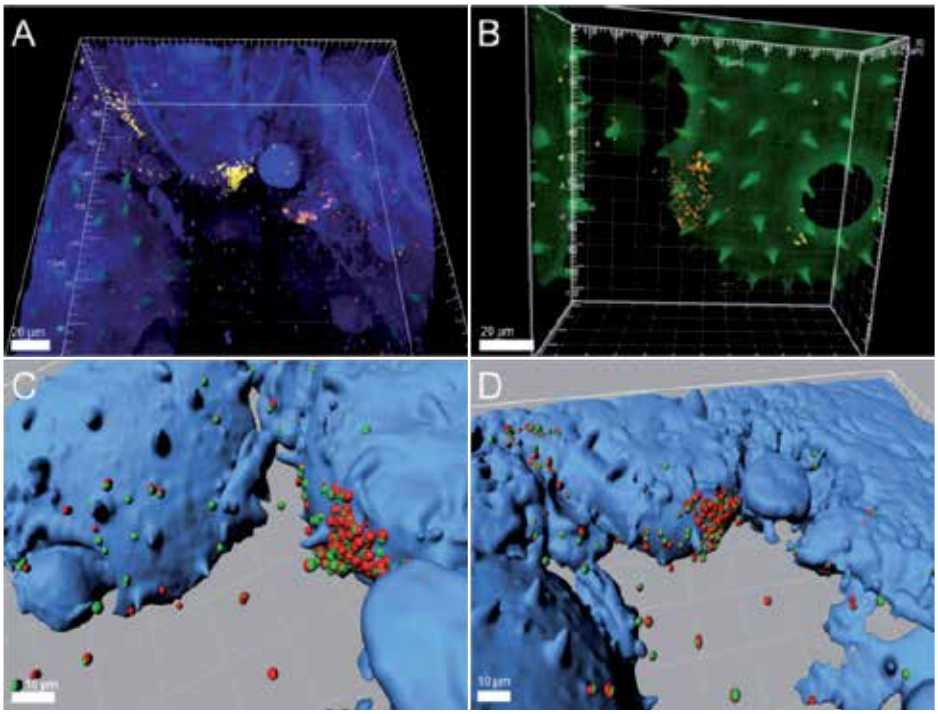

Fig. 6. FISH-stained bacteria colonising pollen grains located on pistils of oil pumpkin (GL Opal) visualized by CLSM. A) Alphaproteobacteria (in yellow) and not taxonomically classified bacteria (in red) labelled with ALF968-Cy5 and EUB338MIX-Cy3. B) Alphaproteobacteria labelled with ALF968-Cy5 (yellow), Firmicutes labelled with LGC354MIX-FITC (pink) and taxonomically undefined bacteria (in red) labelled with EUB338Mix-Cy3. C,D) 3D rendered image (Imaris software) of overall bacterial communities (in red) labelled with EUB338MIX-Cy3 and Alphaproteobacteria (red and green) labelled with ALF968-Cy5.

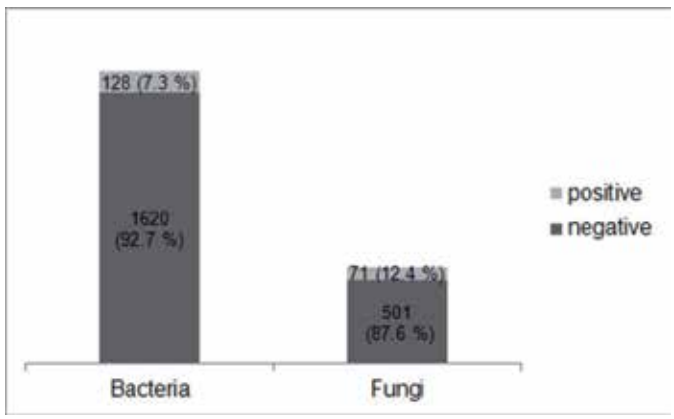

Fig. 7. Amount of oil pumpkin-associated bacterial and fungal isolates positively or negatively tested for *in vitro* antagonism against *D. bryoniae* A-220-2b.

Potential antagonists (128 bacteria and 71 fungi) were subsequently screened *in vitro* for effects on growth inhibition of *Pectobacterium carotovorum* subsp. *atrosepticum* 25-2, *Pseudomonas viridiflava* 2d1 and *Xanthomonas cucurbitae* 6h4 to find broad-spectrum antagonists. Altogether, 32% of fungal as well as 49% of bacterial *D. bryoniae* antagonists were positively tested against at least one, 34% of tested prokaryotes against at least two and 6% of investigated bacterial strains against all three bacterial phytopathogens, whereas no fungal *D. bryoniae* antagonist was effective against more than one bacterial pathogen (Fig. 8).

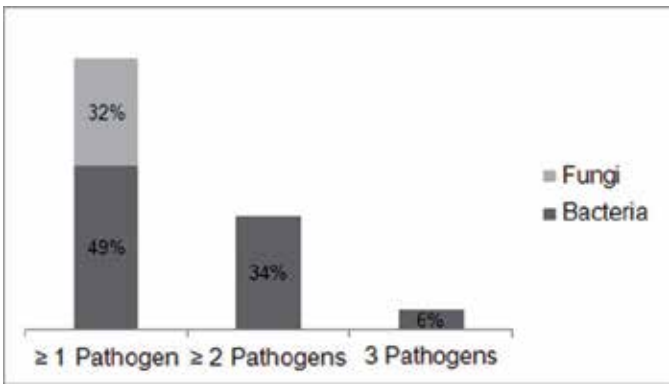

Fig. 8. Percentage of fungal and bacterial *D. bryoniae* antagonists positively tested against at least one, two or all three bacterial pathogens *Pectobacterium carotovorum* subsp. *atrosepticum* 25-2, *Pseudomonas viridiflava* 2d1 *and Xanthomonas cucurbitae* 6h4.

Broad-spectrum antagonists that have the potential to suppress *D. bryoniae* as well as at least two bacterial phytopathogens of oil pumpkin were characterized genotypically by ARDRA. This resulted in a grouping of 43 bacterial isolates into four different genera: *Pseudomonas*, *Paenibacillus*, *Serratia* and *Lysobacter*. As a relative high number of isolates belong to *Paenibacillus* and *Lysobacter* they were further analysed by BOX PCR (Rademaker & de Bruijn, 1997) to get insight into the intra-genera diversities. Within the group of *Paenibacillus* a negligible variability between BOX patterns was observed in contrast to strains of *Lysobacter* that were divided into five groups. Finally five potential broad-spectrum antagonists were chosen for further analysis: one representative for *Pseudomonas*, *Paenibacillus* and *Serratia* and two representatives from the *Lysobacter* cluster. Partial sequencing of 16S rRNA genes with subsequent BLAST analysis (Altschul et al., 1997) was performed for their identification and the following species could be affiliated to respective strains: *Pseudomonas chlororaphis* P34, *Paenibacillus polymyxa* PB71, *Serratia plymuthica* S13, *Lysobacter antibioticus* L175 and *L. gummosus* L101. To learn more about the mode of antagonism of chosen broad-spectrum antagonists against *D. bryoniae*, dual culture assays in which growth inhibition of *D. bryoniae* A-220-2b by either soluble or volatile antimicrobial compounds secreted by the five test strains was assessed were performed. Results suggest a high capability of broad-spectrum antagonists to synthesize bioactive compounds: sterile culture supernatants from *P. chlororaphis* P34, *L. gummosus* L101 and *P. polymyxa* PB71 as well as volatile organic compounds (VOCs) excreted from these bacteria and *S. plymuthica* S13 as well suppressed growth of the fungus significantly compared to control treatments (ANOVA; LSD, $p < 0.05$; data not shown).

Performances of broad-spectrum antagonists in terms of promoting plant growth and health will facilitate the selection of bacterial strains that will be analysed for the production of a biological strengthener for Styrian oil pumpkin. Studies with the model organism *C. elegans* (Zachow et al., 2009) will give insight into the potential pathogenicity of remaining test strains. The manufacture of the final product will further depend on the finding of an appropriate formulation procedure that guarantees a high stability of the ultimate BCAs/PGPRs.

5. Conclusion

Advanced ecological knowledge about plant-associated microorganisms and interactions of the biocontrol agent(s) with abiotic and biotic factors support the development of efficient biocontrol strategies. As shown in three examples, specific strategies have to be developed adapted to the life cycle of the pathogen and the autochthonous microbial communities in the target habitat. The latter varied strongly dependent on the plant species, microenvironment and climate.

6. Acknowledgement

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7. References

Aboul-Soud, M.A., Yun, B.W., Harrier, L.A. & Loake G.J. (2004). Transformation of *Fusarium oxysporum* by particle bombardment and characterisation of the resulting transformants expressing a GFP transgene. *Mycopathologia* 158, 475-482

Adesina, M.F., Lembke, A., Costa, R., Speksnijder, A. & Smalla K. (2007). Screening of bacterial isolates from various European soils for *in vitro* antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum*: site-dependent composition and diversity revealed. *Soil Biol Biochem* 39, 2818-2828

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J.H., Zhang, Z., Miller, W. & Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25, 3389-3402

Amann, R.I., Ludwig, W. & Schleifer, K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* 59, 143-169

Bassam, B.J., Caetano-Anolles, G. & Gresshoff, P.M. (1991). Fast and sensitive silver staining of DNA in polyacrylamid gels, *Anal Biochem* 196, 80-83

Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A. & Smalla, K. (2002). Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. *Appl Environ Microbiol* 68, 3328-3338

Berg, G., Krechel, A., Ditz, M., Sikora, R.A., Ulrich, A. & Hallmann, J. (2005a). Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. *FEMS Microbiol Ecol* 51, 215-229

Berg, G., Eberl, L. & Hartmann, A. (2005b). The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environ Microbiol* 7, 1673-1685

Berg, G., Opelt, K., Zachow, C., Lottmann, J., Götz, M., Costa, R. & Smalla, K. (2006). The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus *Verticillium* differs depending on plant species and site. *FEMS Microbiol Ecol* 56, 250-261

Berg, G. (2009). Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl Microbiol Biotechnol* 84, 11-18

Berg, G., Zachow, C., Cardinale, M. & Müller, H. (2011). Ecology and human pathogenicity of plant-associated bacteria, In: *Regulation of biological control agents*, Ehlers, R.U., 175-189, Springer, Dordrecht

Bhattacharjee, R.B., Sing, A. & Mukhopadyay, S.N. (2008). Use of nitrogen-fixing bacteria as biofertilizer for non-legumes: prospects and challenges. *Appl Microbiol Biotechnol* 80, 199-209

Bloemberg, G.V. (2007). Microscopic analysis of plant–bacterium interactions using auto fluorescent proteins. *Eur J Plant Pathol* 119, 301-309

Bonaterra, A., Camps, J. & Montesinos, E. (2005). Osmotically induced trehalose and glycine betaine accumulation improves tolerance to dessication, survival and efficacy of the postharvest biocontrol agent *Pantoea agglomerans* EPS125. *FEMS Microbiol Lett* 250, 1-8

Braun-Kiewnick, A., Jacobsen, B.J. & Sands, D.C. (2000). Biological control of *Pseudomonas syringae* pv. *syringae,* the causal agent of basal kernel blight of barley, by antagonistic *Pantoea agglomerans*. *Phytopathology* 90, 368-375

Briones, A.M., Okabe, S., Umemiya, Y., Ramsing, N.B., Reichardt, W. & Okuyama, H. (2002). Influence of different cultivars on populations of ammonia-oxidizing bacteria in the root environment of rice. *Appl Environ Microbiol* 68, 3067-3075

van Bruggen, A.H.C. & Semenov, A.M. (2000). In search of biological indicators for soil health and disease suppression. *Appl Soil Ecol* 15, 13-24

de Bruijn, I., de Kock, M.J., Yang, M., de Waard, P., van Beek, T.A. & Raaijmakers, J.M. (2007). Genome-based discovery, structure prediction and functional analysis of cyclic lipopeptide antibiotics in *Pseudomonas* species. *Mol Microbiol* 63, 417-428

Bruton, B.D. (1998). Soilborne diseases in *Cucurbitaceae*: pathogen virulence and host resistance, In: *Cucurbitaceae '98*, McCreight, J.D., 143-166, ASHS Press, Alexandria

Cao, L., Qiu, Z., You, J., Tan, H. & Zhou, S. (2005). Isolation and characterization of endophytic streptomycete antagonists of Fusarium wilt pathogen from surface-sterilized banana roots. *FEMS Microbiol Lett* 247, 147-152

Cattelan, A.J., Hartel, P.G. & Fuhrmann, J.J. (1999). Screening for plant growth-promoting rhizobacteria to promote early soybean growth. *Soil Sci Soc Am J* 63, 1670-1680

Chakraborty, U., Chakraborty, B.N., Basnet, M. & Chakraborty, A.P. (2009). Evaluation of *Ochrobactrum anthropi* TRS-2 and its talc based formulation for enhancement of growth of tea plants and management of brown root rot disease. *J Appl Microbiol* 107, 625-634

Chao, A. & Bunge, J. (2002). Estimating the number of species in a stochastic abundance model. *Biometrics* 58, 531-539

Chernin, L., Ismailov, Z., Haran, S. & Chet, I. (1995). Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Appl Environ Microbiol* 61, 1720-1726

Chin-A-Woeng, T.F.C., de Priester, W., van der Bij, A. & Lugtenberg, B.J.J. (1997). Description of the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* biocontrol strain WCS365, using scanning electron microscopy. *Mol Plant Microbe Interact* 10, 79-86

Compant, S., Duffy, B., Nowak, J., Clement, C. & Barka, E.A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl Environ Microbiol* 71, 4951-4959

de Costa, D.M., Amaradasa, B.S. & Wegiriya, R.N.B.P.M.R.C.L. (1997). Anatgonists *of Colletotrichum musae* associated with banana fruit skin. *J Natn Sci Coun Sri Lanka* 25, 95-104

Daly, A. & Walduck, G. (2006). Fusarium wilt of bananas (Panama disease) (*Fusarium oxysporum* f.sp. *cubense*). *Agnote* 151, Available from: https://transact.nt.gov.au/ebiz/dbird/TechPublications.nsf/1717DB3D7CD580C06925723C004EA6DD/$file/786.pdf?OpenElement

Emmert, E.A. & Handelsman, J. (1999). Biocontrol of plant disease: a (gram-) positive perspective. *FEMS Microbiol Lett* 171, 1-9

Erkel, C., Kube, M., Reinhardt, R. & Liesack, W. (2006). Genome of rice cluster I archaea – the key methane producers in the rice rhizosphere. *Science* 313, 370-372

Faltin, F., Lottmann, J., Grosch, R. & Berg, G. (2004). Strategy to select and assess antagonistic bacteria for biological control of *Rhizoctonia solani* Kühn. *Can J Microbiol* 50, 811-820

Fourie, G., Steenkamp, E.T., Ploetz, R.C., Gordon, T.R. & Viljoen, A. (2011). Current status of the taxonomic position of *Fusarium oxysporum formae specialis cubense* within the *Fusarium oxysporum* complex. *Infect Genet Evol*, In press

Frimmersdorf, E., Horatzek, S., Pelnikevich, A., Wiehlmann, L. & Schomburg, D. (2010). How *Pseudomonas aeruginosa* adapts to various environments: a metabolomic approach. *Environ Microbiol* 12, 1734-1747

Fürnkranz, M., Müller, H. & Berg, G. (2009). Characterization of plant growth promoting bacteria from crops in Bolivia. *J Plant Dis Protect* 116, 149-155

Garbeva, P., van Veen, J.A. & van Elsas, J.D. (2004). Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. *Annu Rev Phytopathol* 42, 243-270

Garbeva, P., Silby, M.W., Raaijmakers, J.M., Levy, S.B. & Boer, W.D. (2011). Transcriptional and antagonistic responses of *Pseudomonas fluorescens* Pf0-1 to phylogenetically different bacterial competitors. *ISME J*, In Press

Goethals, K., Vereecke, D., Jaziri, M., van Montagu, M. & Holsters, M. (2001). Leafy gall formation by *Rhodococcus fascians*. *Annu Rev Phytopathol* 39, 27-52

Gomes, N.C., Cleary, D.F., Pinto, F.N., Egas, C., Almeida, A., Cunha, A., Mendonça-Hagler, L.C. & Smalla, K. (2010). Taking root: enduring effect of rhizosphere bacterial colonization in mangroves. *PLoS ONE* 5, e14065

Gross, H. & Loper, J.E. (2009). Genomics of secondary metabolite production by *Pseudomonas* spp. *Nat Prod Rep* 26, 1408-1446

Grube, M., Cardinale, M., de Castro, J.V. Jr., Müller, H. & Berg, G. (2009). Species-specific structural and functional diversity of bacterial communities in lichen symbioses. *ISME J* 3, 1105-1115

Haas, D. & Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol* 3, 307-319

Haichar, F.Z., Marol, C., Berge, O., Rangel-Castro, J.I., Prosser, J.I., Balesdent, J., Heulin, T. & Achouak, W. (2008). Plant host habitat and root exudates shape soil bacterial community structure. *ISME J* 2, 1221-1230

Hansel, C.M., Fendorf, S., Jardine, P.M. & Francis, C.A. (2008). Changes in bacterial and archaeal community structure and functional diversity along a geochemically variable soil profile. *Appl Environ Microbiol* 74, 1620-1633

Hassan, K.A., Johnson, A., Shaffer, B.T., Ren, Q., Kidarsa, T.A., Elbourne, L.D., Hartney, S., Duboy, R., Goebel, N.C., Zabriskie, T.M., Paulsen, I.T. & Loper, J.E. (2010). Inactivation of the GacA response regulator in *Pseudomonas fluorescens* Pf-5 has far-reaching transcriptomic consequences. *Environ Microbiol* 12, 899-915

Horrigan, L., Lawrence, R.S. & Walker, P. (2002). How sustainable agriculture can address the environmental and human health harms of industrial agriculture. *Environ Health Perspect* 110, 445-456

Huang, W.E., Stoecker, K., Griffiths, R., Newbold, L., Daims, H., Whiteley, A.S. & Wagner, M. (2007a). Raman-FISH: combining stable-isotope Raman spectroscopy and fluorescence *in situ* hybridization for the single cell analysis of identity and function. *Environ Microbiol* 9, 1878-1889

Huang, W.E., Bailey, M.J., Thompson, I.P., Whiteley, A.S. & Spiers, A.J. (2007b). Single-cell Raman spectral profiles of *Pseudomonas fluorescens* SBW25 reflects *in vitro* and *in planta* metabolic history. *Microb Ecol* 53, 414-425

Huss, H., Winkler, J. & Greimel, C. (2007). Der Pilz *Didymella bryoniae* schädigt steirischen Ölkürbisanbau: Fruchtfäule statt Kernöl. *Der Pflanzenarzt* 60, 14-16

Huss, H. (2011). Krankheiten und Schädlinge im Ölkürbisbau. *Der fortschrittliche Landwirt* 3, 30-33

IPCC (2007). Fourth assessment report of the intergouvernmental panel on climate change, In: *Climate Change 2007*, Cambridge University Press, Available from: www.ipcc.ch

Ishii, S. & Sadowsky, M.J. (2009). Applications of the rep-PCR DNA fingerprinting technique to study microbial diversity, ecology and evolution. *Environ Microbiol* 11, 733-740

Janssen, P.H. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl Environ Microbiol* 72, 1719-1728

Kamensky, M., Ovadis, M., Chet, I. & Chernin, L. (2003). Soil-borne strain IC14 of *Serratia plymuthica* with multiple mechanisms of antifungal activity provides biocontrol of *Botrytis cinerea* and *Sclerotinia sclerotiorum* diseases. *Soil Biol Biochem* 35, 323-331

Keinath, A.P., Farnham, W. & Zitter, T.A. (1995). Morphological, pathological, and genetic differentiation of *Didymella bryoniae* and *Phoma* spp. isolated from cucurbits. *Phytopathology* 85, 364-369

Kidane, E.G. (2008). *Management of Fusarium wilt diseases using non-pathogenic Fusarium oxysporum and silicon*, Doctoral Thesis, University of KwaZulu-Natal, Pietermaritzburg

Lauber, C.L., Hamady, M., Knight, R. & Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl Environ Microbiol* 75, 5111-5120

Lazarevic, V., Whiteson, K., Huse, S., Hernandez, D., Farinelli, L., Østerås, M., Schrenzel, J. & François, P. (2009). Metagenomic study of the oral microbiota by Illumina high-throughput sequencing. *J Microbiol Methods* 79, 266-271

Lee, D., Mathur, S.B. & Neergaard, P. (1984). Detection and location of seed-borne inoculum of *Didymella bryoniae* and its transmission in seedling of cucumber and pumpkin. *J Phytopathol* 109, 301-308

Leveau, J.H.J. (2007). The magic and menace of metagenomics: prospects for the study of plant growth-promoting rhizobacteria. *Eur J Plant Pathol* 119, 279-300

Li, L.F., Häkkinen, M., Yuan, Y.M., Hao, G. & Ge, X.J. (2010). Molecular phylogeny and systematics of the banana family (Musaceae) inferred from multiple nuclear and chloroplast DNA fragments, with a special reference to the genus *Musa*. *Mol Phylogenet Evol* 57, 1-10

Lian, J., Wang, Z. & Zhou, S. (2008). Response of endophytic bacterial communities in banana tissue culture plantlets to Fusarium wilt pathogen infection. *J Gen Appl Microbiol* 54, 83-92

Ling, K.S., Wechter, W.P., Somai, B.M., Walcott, R.R. & Keinath, A.P. (2010). An improved real-time PCR system for broad-spectrum detection of *Didymella bryoniae*, the causal agent of gummy stem blight of cucurbits. *Seed Sci Technol* 38, 692-703

Lottmann, J., Heuer, H., Smalla, K. & Berg, G. (1999). Influence of transgenic T4-lysozyme-producing plants on beneficial plant-associated bacteria. *FEMS Microbiol Ecol* 29, 365-377

Lugtenberg, B. & Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annu Rev Microbiol* 63, 541-556

Mark, G.L., Dow, J.M., Kiely, P.D., Higgins, H., Haynes, J., Baysse, C., Abbas, A., Foley, T., Franks, A., Morrissey, J. & O'Gara, F. (2005). Transcriptome profiling of bacterial responses to root exudates identifies genes involved in microbe-plant interactions. *Proc Natl Acad Sci USA* 102, 17454-17459

Mavrodi, D.V., Loper, J.E., Paulsen, I.T. & Thomashow, L.S. (2009). Mobile genetic elements in the genome of the beneficial rhizobacterium *Pseudomonas fluorescens* Pf-5. *BMC Microbiol* 9, 8

Miller, S.A., Beed, F.D. & Harmon, C.L. (2009). Plant disease diagnostic capabilities and networks. *Annu Rev Phytopathol* 47, 15-38

Milling, A., Smalla, K., Xaver, F., Maidl, K., Schloter, M. & Munch, J.C. (2004). Effects of transgenic potatoes with an altered starch composition on the diversity of soil and rhizosphere bacteria and fungi. *Plant Soil* 266, 23-39

de Neergaard, E. (1989). Histological investigations of flower parts of cucumber infected by *Didymella bryoniae*. *Can J Plant Pathol* 11, 28-38

Park, J.H., Kim, R., Aslam, Z., Jeon, C.O. & Chung, Y.R. (2008). *Lysobacter capsici* sp. nov., with antimicrobial activity, isolated from the rhizosphere of pepper, and emended description of the genus *Lysobacter*. *Int J Syst Evol Microbiol* 58, 387-392

Paulsen, I.T., Press, C.M., Ravel, J., Kobayashi, D.Y., Myers, G.S., Mavrodi, D.V., DeBoy, R.T., Seshadri, R., Ren, Q., Madupu, R., Dodson, R.J., Durkin, A.S., Brinkac, L.M.,

Daugherty, S.C., Sullivan, S.A., Rosovitz, M.J., Gwinn, M.L., Zhou, L., Schneider, D.J., Cartinhour, S.W., Nelson, W.C., Weidman, J., Watkins, K., Tran, K., Khouri, H., Pierson, E.A., Pierson, L.S., Thomashow, L.S. & Loper, J.E. (2005). Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. *Nat Biotechnol* 23, 873-878

Petti, C., Khan, M. & Doohan, F. (2010). Lipid transfer proteins and protease inhibitors as key factors in the priming of barley responses to Fusarium head blight disease by a biocontrol strain of *Pseudomonas fluorescens*. *Funct Integr Genomics*, 10, 619-627

Ploetz, R.C. (2006). Fusarium wilt of banana is caused by several pathogens referred to as *Fusarium oxysporum* f.sp. *cubense*. *Phytopathology* 96, 653-656

Raaijmakers, J.M., de Bruijn, I., Nybroe, O. & Ongena, M. (2010). Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiol Rev* 34, 1037-1062

Rademaker, J.L.W. & de Bruijn, F.J. (1997). Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer-assisted pattern analysis, In: *DNA markers: protocols, applications and overviews*, Caetano-Anollés, G. & Gresshoff, P.M., 151-171, John Wiley & Sons, New York

Rasche, F., Velvis, H., Zachow, C., Berg, G., van Elsas, J.D. & Sessitsch, A. (2006). Impact of transgenic potatoes expressing antibacterial agents on bacterial endophytes is comparable to effects of soil, wildtype potatoes, vegetation stage and pathogen exposure. *Can J Microbiol* 43, 555-566

Rediers, H., Rainey, P.B., Vanderleyden, J. & de Mot, R. (2005). Unraveling the secret lives of bacteria: use of *in vivo* expression technology and differential fluorescence induction promoter traps as tools for exploring niche-specific gene expression. *Microbiol Mol Biol Rev* 69, 217-261

Sachs, J., Remans, R., Smukler, S., Winowiecki, L., Andelman, S.J., Cassman, K.G., Castle, D., De Fries, R., Denning, G., Fanzo, J., Jockson, L.E., Leemans, R., Lehmann, J., Milder, J.C., Naeem, S., Nziguheba, G., Palm, C.A., Pingali, P.L., Reganold, J.P., Richter, D.D., Scherr, S.J., Sircely, J., Sullivan, C., Tomich, T.P. & Sanchez, P.A. (2010). Monitoring the world's agriculture. *Nature* 466, 558-560

Sanguin, H., Remenant, B., Dechesne, A., Thioulouse, J., Vogel, T.M., Nesme, X., Moënne-Loccoz, Y. & Grundmann, G.L. (2006). Potential of a 16S rRNA-based taxonomic microarray for analyzing the rhizosphere effects of maize on *Agrobacterium* spp. and bacterial communities. *Appl Environ Microbiol* 72, 4302-4312

Schisler, D.A., Slininger, P.J., Behle, R.W. & Jackson, M.A. (2004). Formulation of *Bacillus* spp. for biological control of plant diseases. *Phytopathology* 94, 1267-1271

Schmid, F., Moser, G., Müller, H. & Berg, G. (2011). Functional and structural microbial diversity in organic and conventional viticulture: organic farming benefits natural biocontrol agents. *Appl Environ Micobiol* 77, In press

Schwieger, F. & Tebbe, C.C. (1998). A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Appl Environ Microbiol* 64, 4870-4876

Schwyn, B. & Neilands, J.B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* 160, 47-56

Shannon, C.E. (1997). The mathematical theory of communication. 1963. *MD Comput* 14, 306-317

Sitterly, R.W. & Keinath, A.P. (1996). Gummy stem blight, In: *Compendium of cucurbit diseases*, Zitter, T.A., Hopkins, D.L. & Thomas, C., 27-28, APS Press, St. Paul

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H. & Berg, G. (2001). Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Appl Environ Microbiol* 67, 4742-4751

Smalla, K., Oros-Sichler, M., Milling, A., Heuer, H., Baumgarte, S., Becker, R., Neuber, G., Kropf, S., Ulrich, A. & Tebbe, C.C. (2007). Bacterial diversity of soils assessed by DGGE, T-RFLP and SSCP fingerprints of PCR-amplified 16S rRNA gene fragments: do the different methods provide similar results? *J Microbiol Methods* 69, 470-479

Tupinambá, G., da Silva, A.J., Alviano, C.S., Souto-Padron, T., Seldin, L. & Alviano, D.S. (2008). Antimicrobial activity of *Paenibacillus polymyxa* SCE2 against some mycotoxin-producing fungi. *J Appl Microbiol* 105, 1044-1053

Velicer, G.J., Raddatz, G., Keller, H., Deiss, S., Lanz, C., Dinkelacker, I. & Schuster, S.C. (2006). Comprehensive mutation identification in an evolved bacterial cooperator and its cheating ancestor. *Proc Natl Acad Sci USA* 103, 8107-8112

Weinert, N., Piceno, Y., Ding, G.C., Meincke, R., Heuer, H., Berg, G., Schloter, M., Andersen, G. & Smalla, K. (2011). PhyloChip hybridization uncovered an enormous bacterial diversity in the rhizosphere of different potato cultivars: many common and few cultivar-dependent taxa. *FEMS Microbiol Ecol* 75, 497-506

Weller, D.M., Raaijmakers, J.M., Gardener, B.B. & Thomashow, L.S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu Rev Phytopathol* 40, 309-348

Weller, D.M. (2007). *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. *Phytopathology* 97, 250-256

de Werra, P., Baehler, E., Huser, A., Keel, C. & Maurhofer, M. (2008). Detection of plant-modulated alterations in antifungal gene expression in *Pseudomonas fluorescens* CHA0 on roots by flow cytometry. *Appl Environ Microbiol* 74, 1339-1349

Whipps, J. (2001). Microbial interactions and biocontrol in the rhizosphere. *J Exper Bot* 52, 487-511

Will, C., Thürmer, A., Wollherr, A., Nacke, H., Herold, N., Schrumpf, M., Gutknecht, J., Wubet, T., Buscot, F. & Daniel, R. (2010). Horizon-specific bacterial community composition of German grassland soils, as revealed by pyrosequencing-based analysis of 16S rRNA genes. *Appl Environ Micobiol* 76, 6751-6759

Youssef, N., Sheik, C.S., Krumholz, L.R., Najar, F.Z., Roe, B.A. & Elshahed, M.S. (2009). Comparison of species richness estimates obtained using nearly complete fragments and simulated pyrosequencing-generated fragments in 16S rRNA gene-based environmental surveys. *Appl Environ Microbiol* 75, 5227-5236

Yuan, Z.C., Liu, P., Saenkham, P., Kerr, K. & Nester E.W. (2008). Transcriptome profiling and functional analysis of *Agrobacterium tumefaciens* reveals a general conserved response to acidic conditions (pH 5.5) and a complex acid-mediated signaling involved in *Agrobacterium*-plant interactions. *J Bacteriol* 190, 494-507

Zachow, C., Tilcher, R. & Berg, G. (2008). Sugar beet-associated bacterial and fungal communities show a high indigenous antagonistic potential against plant pathogens. *Microb Ecol* 55, 119-129

Zachow, C., Pirker, H., Westendorf, C., Tilcher, R. & Berg, G. (2009). The *Caenorhabditis elegans* assay: a tool to evaluate the pathogenic potential of bacterial biocontrol agents. *Eur J Plant Pathol* 125, 367-376

Zachow, C., Fatehi, J., Cardinale, M., Tilcher, R. & Berg, G. (2010). Strain-specific colonization pattern of *Rhizoctonia* antagonists in the root system of sugar beet. *FEMS Microbiol Ecol* 74, 124-135

Zhang, Y., Hu, Y., Yang, B., Ma, F., Lu, P., Li, L., Wan, C., Rayner, S. & Chen, S. (2010). Duckweed (*Lemna minor*) as a model plant system for the study of human microbial pathogenesis. *PLoS ONE* 5, e13527

2

Development of RNAi in Insects and RNAi-Based Pest Control

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1. Introduction

In agricultural systems, insect pests can cause crop damage mainly through loss in yield or quality resulting in a loss in profits for farmers. Worldwide pests cost billions of dollars due to damage and use of pesticides. Chemical pesticides are still the major approach for controlling insect pests, but they are associated with significant hazards to the environment and human health. The alternative commercial biotechnological system relies mostly on the expression of *Bacillus thuringiensis* insecticidal proteins (Cry toxins). Its effectiveness however is threatened by the development of resistance in some species such as *Ostrinia nubilalis* (Lepidoptera, Pyralidae) and *Heliothis virescens* (Lepidoptera: Noctuidae) (Ferre and Van Rie, 2002; Baum *et al*., 2007). As a result, there is an urgent need to develop economically and ecologically sound alternatives for pest control.

Gene silencing has been suggested as one of the new alternatives to reduce damage from insect pests. RNA interference (RNAi) is first described by Fire *et al*. (1998), and its mechanism lies in that a double-stranded RNA (dsRNA) introduced in an organism has the capacity to silence post-transcriptional genes (Hannon, 2002; Geley and Muller, 2004). RNAi is highly conserved in eukaryotic organisms (Fire, 2007). It is considered as a specific type of defence mechanism (Terenius *et al*., 2011). Four different types of RNAi have been described including short interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), endogenous siRNAs (endo-siRNAs or esiRNAs), and microRNAs (miRNAs) (Terenius *et al*., 2011). To date, RNAi has been proven promising for research on gene function determination and gene knockdown in eukaryotes and medical control of cancers and viral disease (Huvenne and Smagghe, 2010).

In insects, studies have mainly targeted the understanding of the RNAi mechanism, and the function, regulation and expression of genes. Introduction of dsRNA into an organism has been tested by using different techniques such as microinjection (Bettencourt *et al*., 2002;

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Tomoyasu and Denell, 2004; Ghanima *et al*, 2007), soaking, or, most preferably, oral feeding of artificial diet (Eaton *et al*., 2002; Turner *et al*., 2006; Baum *et al*., 2007; Mao *et al*., 2007; Chen *et al*., 2008; Tian *et al*., 2009). Transgenic plants producing dsRNAs directed against genes function in Lepidoptera, Coleoptera, and Hemiptera pests are becoming more common (Gordon and Waterhouse, 2007; Baum *et al*., 2007; Mao *et al*., 2007, Chen *et al*., 2010). For example, Chen *et al*. (2010) report the successful feeding of TPS (trehalose-6-phosphate synthase for the synthesis of trehalose, main sugar reserve in haemolymph) dsRNA solutions to silence this gene thus proposing it as a useful pest control agent. These results suggest that over time, new generations of insect-resistant crops will be created to manage agriculturally important insect pests.

In this review chapter, we summarize the current knowledge on the recent RNAi research on insects, including the application of RNAi techniques in research involving functional insect genes and functional genomics, the methods of dsRNA uptake RNAi in insects, the systemic diffusion of RNAi silencing molecules in the insect body and the mechanism underlying this diffusion, and the potential application of RNAi in integrated pest management (IPM). The main purpose of this review is to help entomologists become familiar with RNAi research, a rapidly growing field where new avenues and techniques are being used to investigate insect RNAi mechanisms for the development of pest control.

2. Study on the function of insect genes using RNAi methods

RNAi is a powerful tool for the study on the function of insect genes. It was first used in the study of a model insect, the fruit fly *Drosophila melanogaster* (Lipardi *et al*., 2001). RNAi studies in *D. melanogaster* have laid a solid foundation for the development of insect RNAi technology and the elucidation of the RNAi mechanisms in insects. Recently, Huvenne and Smagghe (2010) have reviewed the definitions of RNAi in insects while Terenius *et al.* (2011) have analyzed the variability and the implications of over 150 published and unpublished studies, mainly focusing the analysis on lepidopteran insects, on the need for further studies on RNAi mechanisms.

In this section, we discuss a selected group of published studies and the main orientations used by researchers in exploring these techniques. Table 1 summarizes the studied functions, methods used for RNAi introduction and the main responses of insects. Besides *D. melanogaster*, 20 other insect species are reported here, including 7 species of Lepidoptera, 3 species of Coleoptera, 3 species of Orthoptera, 2 species of Hymenoptera, 2 species of Homoptera, 1 species of Diptera, 1 species of Isoptera, and 1 species of Hemyptera. These selected papers have all in common the successful use of RNAi mechanisms as potential pest control agent. It is important to note that, except for a few exceptions (e.g. circadian clock gene), most studies have targeted different genes. Responses also greatly vary from minor effects such as disruption in functional rhythm to reduction in fitness and increased mortality. As reported by Terinius *et al*. (2011) for lepidopteran species, most of the studies have helped better understand developmental processes and the immune system.

3. Internal diffusion of RNAi molecules within insect body

The effects of RNAi inside the body of insects are determined by an important factor, the spread of silencing RNA molecules inside the insect body (so-called systemic RNAi). In

Insects	Genes and References	Methods	Effects
Silkworm *Bombyx mori*	Circadian clock gene *per* (Sandrelli *et al.*, 2007)	Transgenics	Disruption of egg-hatching rhythm
	Ecdysis-triggering hormone gene *ETH* (Dai *et al.*, 2008)	Transgenics	Lethal at pharate second-instar larval stage
Egyptian cotton leafworm *Spodoptera littoralis*	*β-actin* gene (Gvakharia *et al.*, 2003)	Injection	Disruption of sperm release
	Circadian clock gene *per* (Kotwica *et al.*, 2009)	Injection	Delayed sperm release
Light brown apple moth *Epiphyas postvittana*	Carboxylesterase gene *EposCXE1* and pheromone binding protein gene *EposPBP1* (Turner *et al.*, 2006)	Feeding	Inhibition of gene expression
Cotton bollworm *Helicoverpa armigera*	Cytochrome P450 gene *CYP6AE14* (Mao *et al.*, 2007)	Feeding	Inhibition of larval growth
	Glutathione-S-transferase gene *GST1* (Mao *et al.*, 2007)	Feeding	Successful inhibition of gene expression
Beet armyworm *Spodoptera exigua*	Chitin synthase gene (Chen *et al.*, 2008)	Injection	Disorder in the insect cuticle, no expansion of the larval trachea epithelial wall, and other larval abnormalities
Japanese pine sawyer *Monochamus alternatus*	Laccase gene *MaLac2* (Niu *et al.*, 2008)	Injection	Pupal and adult cuticle sclerotisation, death at a high dose
Red flour beetle *Tribolium castaneum*	Chitin synthase genes *TcCHS1* and *TcCHS2* (Arakane *et al.*, 2005)	Injection	Disruption in all types of moulting(larva-larva, larva-pupa, and pupa-adult), cessation of ingestion, decrease in larval size, and reduction of chitin content in the midgut
	Chitinase-like proteins *TcCHT5*, *TcCHT10*, *TcCHT7*, and *TcIDGF4* (Zhu *et al.*, 2008)	Injection	Effects on pupal-adult moulting Effects on egg hatching, larval moulting, pupation, and adult metamorphosis. Effects on abdominal contraction and wing/elytra extension. Effects on adult eclosion

Insects	Genes and References	Methods	Effects
Western corn rootworm *Diabrotica virgifera virgifera* LeConte	Vacuolar ATPase (*v-ATP*) (Baum *et al.*, 2007)	Feeding	Delayed larval development and increased mortality
Striped flea beetle *Phyllotreta striolata*	Arginine kinase gene *AK* (Zhao *et al.*, 2008)	Feeding	Delayed development, increased mortality, and reduced fertility
Mediterranean field cricket *Gryllus bimaculatus*	Circadian clock gene *per* (Moriyama *et al.*, 2008)	Injection	Complete loss of circadian control of locomotor activity and electrical activity in the optic lobe
	Nitric oxide synthase gene *NOS* (Takahashi *et al.*, 2009)	Injection	Destruction of long-term memory
German cockroach *Blattella germanica*	*BgRXR* gene (Martin *et al.*, 2006)	Injection	Inhibition of pupal eclosion
	Pigment-dispersing factor gene *pdf* (Lee *et al.*, 2009)	Injection	Effects on insect night activity
American grasshopper *Schistocerca americana*	Eye colour gene *vermilion* (Dong and Friedrich, 2005)	Injection	Suppression of ommochrome formation and systematic expression
Brown planthopper *Nilaparvata lugens*	Trehalose phosphate synthase (*TPS*) (*NlTPS* mRNA) (Chen *et al.*, 2010)	Feeding	Disturbed development through disruption in the TPS enzymatic activity, reduction of insect survival rate
Turnip sawfly *Athalia rosae*	*Ar white* gene (Sumitani *et al.*, 2005)	Injection	White phenocopy in embryonic eye pigmentation
European honey bee *Apis mellifera*	Transcription factor gene *Relish* (Schlüns and Crozier, 2007)	Injection	Inhibition of *Relish* gene expression and reduction in the expression of two other immune genes, *abaecin* and *hymenoptaecin*
Triatomid bug *Rhodnius prolixus*	Salivary nitrophorin 2 gene *NP2* (Araujo *et al.*, 2006)	Injection and feeding	Shortened plasma coagulation time
Savannah tsetse fly *Glossina morsitans morsitans*	*TsetseEP* gene and transferrin gene *2A192* (Walshe *et al.*, 2009)	Feeding	Inhibition of *TsetseEP* gene expression, but no inhibition of *2A192* gene expression

Insects	Genes and References	Methods	Effects
Eastern subterranean termite *Reticulitermes flavipes*	Cellulase enzyme gene *Cell-1* and caste-regulatory hexamerin storage protein gene *Hex-2* (Zhou *et al*., 2008)	Feeding	Reduction in group fitness and increased mortality

Table 1. RNAi research on functional genes in insects

plants, the nematode *Caenorhabditis elegans*, and the planarian *Schmidtea mediterranea*, RNAi is systemic as the RNAi signal spreads throughout the entire biological system by travelling between cells (Fire *et al*., 1998; Newmark *et al*., 2003). In insects, RNAi is not always to be systemic. For example, fly cells take up dsRNA, which cannot spread throughout the entire body (Saleh *et al*., 2006). Whangbo and Hunter (2008) have defined different mechanisms for dsRNA uptake: cell-autonomous and non-cell autonomous. Huvenne and Smagghe (2010) described these two types of RNAi and their level of spread which would be greater in systemic non-cell autonomous RNAi than in the cell autonomous RNAi. While most research in insects has been conducted with cell-autonomous RNAi, it is suggested that studies should focus towards non-cell autonomous RNAi as a better potential for defining agent of insect control.

In summary, studies have shown that the ability to distribute an RNAi signal is different in different insects. The intake of dsRNA by *Drosophila* cells leads to localised gene silencing, without systemic distribution of the RNAi signal (Van Roessel *et al*., 2002; Roignant *et al*., 2003; Dietzl *et al*., 2007). On the other end, *Tribolium* (Tomoyasu *et al*., 2008) and *Schistocerca americana* (Dong and Friedrich, 2005) have strong systemic RNAi reactions. The gene responsible for nematode systemic RNAi is *sid-1* (Winston *et al*., 2002). Correspondingly, the *sid-1* gene is not found in the *Drosophila* genome, whereas the grasshopper has a *sid-1* ortholog (Dong and Friedrich, 2005), and *Tribolium* also has a *sid-1*-like gene (Tomoyasu *et al*., 2008). Further BLAST searches at the NCBI website have identified one species in Coleoptera, one in Lepidoptera, two in Hymenoptera, and three in Hemiptera containing *sid-1* homologs, whereas no homologous gene has been found in Homoptera (Walshe *et al*., 2009).

Further studies have found that the RNAi mechanisms in *Tribolium* and the nematode *C. elegans* are different. *Tribolium* does not have some of the key elements that are required for RNAi in *C. elegans*, such as RNA-dependent RNA polymerase (RdRP) and the RNA channel transporter (SID) (Fire *et al*., 1998; Winston *et al*., 2002). Furthermore, the function of the *sid-1*-like gene of *Tribolium* is not to absorb RNAi but, instead, is similar to the function of the *tag-130* gene of *C. elegans* (Tomoyasu *et al*., 2008). Therefore, further verification is needed to define the function of the *sid-1* gene in insect RNAi. Recent studies have shown that the anti-viral RNAi reaction in *Drosophila* depends on a virus-specific immune signal and systemic spreading (Saleh *et al*., 2009). Further studies need to be conducted to understand the spread of silencing RNA within the insect body and the genes involved in this process. Understanding and revealing the molecular mechanisms of determining how RNA spreads systemically inside the insect body will facilitate the application of RNAi technology for pest control.

4. Methodology of dsRNA uptake in insects

Methods of dsRNA uptake in insects can greatly vary and strongly influence the efficiency of gene silencing, thus their potential as insect pest control agent. It is important to note that

since gene silencing is only limited to cells that are infected, the main challenge is the selection of the delivery system (Terenius *et al*., 2011). In both types, methods of delivery must be first defined, being effectively easier and better understood for cell-autonomous RNAi machinery (Siomi and Siomi, 2009). The main uptake (or delivery) methods include injection, soaking, feeding, transgenic technique, and viral infection. This section examines these various mechanisms and their effectiveness in delivering RNAi and gene silencing in various species.

4.1 Microinjection

Microinjection, i.e. the direct injection of dsRNA into the body of insects, has been one of the most effective delivery methods for systemic RNAi types. Short dsRNA have had the most success with this mechanism (Siomi and Siomi, 2009). In addition, the 5′ end of the dsRNA can affect the effectiveness of RNAi; a phosphorylated 5′ end exhibits better gene silencing rate than does a hydroxylated 5′ end (Boutla *et al*., 2001).

The major advantage of injecting dsRNA into the insect body is the high efficiency of inhibiting gene expression. There are however some limitations with micro-injection. First, the cost for in vitro synthesis and storage of dsRNA is relatively high, and the steps are complicated. In addition, injection pressure and the wound generated inevitably affect the insects. It has been shown that skin damage stimulates the immune response. In practice, this delivery method would have very limited application as pest control agent.

4.2 Soaking

Soaking *D. melanogaster* embryos in a dsRNA solution can inhibit gene expression, and its effectiveness is comparable to the injection method in that it requires a higher concentration of dsRNA (Eaton *et al*., 2002). Soaking *D. melanogaster* S2 cells in *CycE* and *ago* dsRNA solutions has been shown to effectively inhibit the expression of these two genes for cell cycle, thereby elevating levels of protein synthesis (March and Bentley, 2007). The soaking method is suitable only for certain insect cells and tissues as well as for specific insects of developmental stages that readily absorb dsRNA from the solution, and therefore, it is rarely used.

4.3 Feeding of artificial diet

Compared to other methods, dsRNA feeding is the most attractive primarily because it is convenient and easy to manipulate. Since it is a more natural method of introducing dsRNA into insect body, it causes less damage to the insect than microinjection (Chen *et al*., 2010). It is especially popular in very small insects that are more difficult to manipulate using microinjection. Early insect RNAi feeding studies were frustrating; for example, the injection of dsRNA effectively silenced the aminopeptidase gene *slapn*, which is expressed in the midgut of *Spodoptera littoralis*, but feeding with dsRNA did not achieve RNAi (Rajagopal *et al*., 2002).

Fortunately, there are other studies showing that dsRNA feeding can be successful for RNAi studies in insects. Feeding dsRNA to *E. postvittana* larvae has been shown to inhibit the expression of the carboxylesterase gene *EposCXE1* in the larval midgut and also to inhibit the expression of the pheromone-binding protein *EposPBP1* in adult antennae (Turner *et al*., 2006). dsRNA feeding also inhibite the expression of the nitrophorin 2 (*NP2*) gene in the salivary gland of *Rhodnius prolixus*, leading to a shortened coagulation time of plasma

(Araujo *et al*., 2006). dsRNA feeding has also been successful in many other insects, including insects of the orders Hemiptera, Coleoptera, and Lepidoptera (Baum *et al*., 2007; Mao *et al*., 2007).

The main challenge remains that there needs to be a greater amount of material for delivery as silencing has been shown to be incomplete (Chen *et al*., 2010). This phenomenon has been observed after ingestion of *CELL-1* dsRNA by the termite *Reticulitermes flavipes* (Zhou *et al*., 2008), *TPS* dsRNA in *N. lugens* nymphae (Chen *et al*. 2010), *Nitrophorin 2* dsRNA by *Rhodnius prolixus* (Araujoa *et al*., 2006). In addition, different species of insects have different sensitivities to RNAi molecules when delivered orally. For example, *Glossina morsitans* fed with dsRNA may effectively inhibit the expression of *TsetseEP* in the midgut, but cannot inhibit the expression of the transferrin gene *2A192* in fat bodies due to lack of transfer capacity between tissues (Walshe *et al*., 2009). The mechanisms associated with the transfer of gene expression through feeding delivery method still need further study.

In addition, one method that may be better than direct feeding with dsRNA is the use of transgenic plants to produce dsRNA (Baum *et al*., 2007; Mao *et al*., 2007). The advantage of this method is the generation of continuous and stable dsRNA material. Genetically engineered dsRNA-producing yeast strains have also been developed to feed *D. melanogaster*, but gene silencing was not successful (Gura, 2000). However, dsRNA produced in bacteria is effective in *C. elegans* (Timmons and Fire, 1998). Therefore, the use of bacteria, especially insecticidal microorganisms, to produce dsRNA for insect RNAi merits further study.

4.4 Developing transgenic insects

The advantage of using transgenic insects that carry the dsRNA is that as it is inheritable, the expression can be stable and continuous. The technique has been proposed to help either reduce population through introduction of sterile insects or for population replacement. In this case, dsRNA must be first injected in the host insect. Tests are being conducted on several species with promising results but as stated by Scolari *et al*. (2011), there is a need to understand environmental and genetic influences when assessing the potential use of such transgenics. The transgenic method has been first used in *D. melanogaster* with the *GAL4/UAS* transgenic system that leads to the expression of hairpin RNA (Kennerdell and Carthew, 2000; Tavernarakis *et al*., 2000). Subsequently, transgenic technology has generated transgenic *Aedes aegypti* that produces dsRNA (Travanty *et al*., 2004). Through the use of a U6 promoter in *D. Melanogaster*, S2 cells can generate short hairpin RNA (shRNA) to inhibit gene expression (Wakiyama *et al*., 2005). RNAi molecules targeting the circadian clock gene *per* have also introduced into *Bombyx mori* embryos by a piggyback plasmid to obtain gene-silenced transgenic individuals (Sandrelli *et al*., 2007). The transfection technique has been used to silence the *D. melanogaster* mitochondrial frataxin gene *dfh*, generating large-sized, long-lived larvae and short-lived adults (Sandrelli *et al*., 2007). The *GAL4/UAS* transgenic system has also been used in *B. mori* (Sandrelli *et al*., 2007) to allow for induction of the transgenic construct. Therefore, gene function can be studied within a certain time period, and the study of gene functions in development, physiology, and the nervous system is possible.

4.5 Virus-mediated uptake

Virus-mediated RNAi methods involve the infection of the host with viruses carrying dsRNA formed during viral replication and targeting the gene of interest in the host. For

example, recombinant Sindbis virus introduced into *B. mori* cells through electroporation can produce dsRNA to inhibit *BR-C* gene expression, causing the larvae not to pupate or leading to adult defects (Uhlirova *et al*., 2003). Virus-mediated RNAi studies are still rare. However, this method takes advantage of the infection and ability of the virus to spread rapidly in a host population. Virus-mediated RNAi does not require screening for transgenic insects or tissues, and thus, it has unique advantages.

5. RNAi-based pest control

In our struggle to minimizing the damage caused by insect pests, we have to acknowledge that pests cannot be efficiently managed by utilizing a single pest control agent. Several studies have shown that pest resistance to chemical pesticide and more recently to Bt has increased requiring new techniques to be applied to reduce the impacts of pest on crop production. While commonalities regarding the development of resistance to chemical and biological control agents remain to be determined, research suggests that both biochemical and genetic factors can contribute to this resistance. It is therefore crucial to continue examining the potential of integrated pest control or management (IPM) to reduce the threat of pests on agroecosystems. IPM has been suggested as a strategy to control incidence of pests since the 1950's and is based on six components: controlled pest populations, healthy crops, monitoring, mechanical and biological controls, and responsible use of pesticides (Kogan, 1998). The basis of for IPM is to balance ecological gain with economic loss (Southwood and Way, 1970). Over the past decade, the number of studies examining these issues has been increasing with, RNAi, as a novel pesticide-free way, to be integrated into IPM.

Research is still in its infancy to examine the application of RNAi machinery for pest control, as most of the focus has been on functional studies of insect genes. Huvenne and Smagghe (2010) however describe in some detail the potential application of RNAi in insect control through cell-line and feeding-in-plant experiments. Based on the currently available literature, they suggested five important factors largely influencing the silencing effect and the efficiency of RNAi as insect pest control technique: concentration of dsRNA, nucleotide sequence, length of dsRNA fragment, persistence of the silencing effect, and life stage of the target pest.

Through insect gene function studies involving injecting or feeding with dsRNA, we have found that some gene silencing can dramatically affect insect growth and development. Theoretically it would be possible to use RNAi to inhibit insect gene leading to insect control. Already, pest control using transgenic plants expressing dsRNA have been published (Baum *et al*., 2007; Mao *et al*., 2007). Transgenic corn expressing dsRNA against the vacuolar ATPase gene (*v-ATP*) significantly decreases the damage caused by *D. virgifera virgifera* LeConte and, notably, protects corn crops (Baum *et al*., 2007). Introduction of RNAi elements targeting the *CYP6AE14* gene, which is directly related to gossypol detoxification in *Helicoverpa armigera*, into *Arabidopsis* or tobacco inhibits *CYP6AE14* gene expression in *H. armigera* feeding on the transgenic plants and, therefore, increases the toxicity of gossypol (Mao *et al*., 2007). Although there needs to be more testing in the field and at large scale, transgenic insects have also been tested as a mechanism for pest control (Scolari et al., 2011). For both transgenic plants and insects, very few species have been investigated and it is clear that further research is essential to explore the potential use of transgenics as an effective means for pest control. Like any other control mechanisms, risk assessment will be

required to determine whether RNAi technology as a form of pest control will be safe and likely create a new era in pest control.

6. Conclusion

Widespread increase in the application of RNAi technology in insect research has facilitated the identification of insect gene function. Research has shown that while dsRNA is particularly conservative, there are various functions and development factors among insect species. Such variations are yet to be fully understood but certainly can serve as a basis for determining their capacity to control insect genes. The main challenge for moving towards larger scale projects remains the development of effective delivery mechanisms. Feeding is very popular in insect RNAi research and may have the most promising future in pest control, especially with the creation of transgenic plants producing dsRNA. Overtime, the use of transgenic insects will also lead to more efficient pest control.

Our understanding of the types of dsRNA and their spreading mechanisms within an organism can limit our ability to move further. Indeed, existing studies have not provided enough evidence that systemic RNAi, with silencing RNA molecules spreading throughout the entire body, can be achieved in all insects. Which insects have characteristics promoting systemic RNAi? Are the mechanisms underlying systemic RNAi the same in different insect species? Such questions need to be answered before moving further in developing large scale pest control systems. Undoubtedly, there is broad potential for the application of RNAi technology in pest control, mainly if combined into IPM strategies.

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8. References

Anderson PR, Kirby K, Hilliker AJ, Phillips JP, 2005. RNAi-mediated suppression of the mitochondrial iron chaperone, frataxin, in *Drosophila*. *Hum. Mol. Genet.*, 14(22): 3 397–3 405.

Arakane Y, Muthukrishnan S, Kramer KJ, Specht CA, Tomoyasu Y, Lorenzen MD, Kanost M, Beeman RW, 2005. The *Tribolium* chitin synthase genes *TcCHS1* and *TcCHS2* are specialized for synthesis of epidermal cuticle and midgut peritrophic matrix. *Insect Mol. Biol.*, 14(5): 453 – 463.

Araujo RN, Santos A, Pinto FS, Gontijo NF, Lehane MJ, Pereira MH, 2006. RNA interference of the salivary gland nitrophorin 2 in the triatomine bug *Rhodnius prolixus* (Hemiptera: Reduviidae) by dsRNA ingestion or injection. *Insect Biochem. Mol. Biol.*, 36(9): 683–693.

Baum JA, Bogaert T, Clinton W, Heck GR, Feldmann P, Ilagan O, Johnson S, Plaetinck G, Munyikwa T, Pleau M, Vaughn T, Roberts J, 2007. Control of coleopteran insect pests through RNA interference. *Nature Biotechnology*, 25(11): 1 322–1 326.

Boutla A, Delidakis C, Livadaras I, Tsagris M, Tabler M, 2001. Short 5'-phosphorylated double-stranded RNAs induce RNA interference in *Drosophila*. *Curr. Biol.*, 11(22): 1776–1780.

Chen J, Zhang D, Yao Q, Zhang J, Dong X, Tian H, Chen J, Zhang W, 2010. Feeding-based RNA interference of a trehalose phosphate synthase gene in the brown planthopper, *Nilaparvata lugens*. *Insect Molecular Biology*, 19(6): 777–786.

Chen X, Tian H, Zou L, Tang B, Hu J, Zhang W, 2008. Disruption of *Spodoptera exigua* larval development by silencing chitin synthase gene A with RNA interference. *Bulletin of Entomological Research*, 98(06): 613–619.

Dai H, Ma L, Wang J, Jiang R, Wang Z, Fei J, 2008. Knockdown of ecdysis-triggering hormone gene with a binary UAS/GAL4 RNA interference system leads to lethal ecdysis deficiency in silkworm. *Acta Biochimica et Biophysica Sinica*, 40(9): 790–795.

Dietzl G, Chen D, Schnorrer F, Su K-C, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson BJ, 2007. A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature*, 448(7 150): 151–156.

Dong Y, Friedrich M, 2005. Nymphal RNAi: Systemic RNAi mediated gene knockdown in juvenile grasshopper. *BMC Biotechnol.*, 5: 25–32.

Dzitoyeva S, Dimitrijevic N, Manev H, 2001. Intra-abdominal injection of double-stranded RNA into anesthetized adult *Drosophila* triggers RNA interference in the central nervous system. *Mol. Psychiatry*, 6(6): 665–670.

Eaton BA, Fetter RD, Davis GW, 2002. Dynactin is necessary for synapse stabilization. *Neuron*, 34(5): 729–741.

Ferre J, and Van Rie J, 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis. Annu. Rev. Entomol.* 47, 501–533

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC, 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391(6 669): 806–811.

Geley S, Müller C, 2004. RNAi: Ancient mechanism with a promising future. *Exp. Gerontol.*, 39(7): 985–998.

Gura T, 2000. A silence that speaks volumes. *Nature*, 404(6 780): 804–808.

Gvakharia BO, Bebas P, Cymborowski B, Giebultowicz JM, 2003. Disruption of sperm release from insect testes by cytochalasin and beta-actin mRNA mediated interference. *Cell Mol. Life Sci.*, 60(8): 1 744–1 751.

Hannon GJ, 2002. RNA interference. *Nature*, 418(6 894): 244–251.

Huvenne H, Smagghe G, 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. *J Insect Physiol*, 56: 227 - 235.

Kennerdell JR, Carthew RW, 2000. Heritable gene silencing in *Drosophila* using double-stranded RNA. *Nature Biotechnology*, 18(8): 896–898.

Kogan M, 1998. Integrated pest management:Historical perspectives and contemporary developments. *Annual Review of Entomology*, 43: 243-270.

Kotwica J, Bebas P, Gvakharia BO, Giebultowicz JM, 2009. RNA interference of the period gene affects the rhythm of sperm release in moths. *J. Biol. Rhythms*, 24(1): 25–34.

Lee CM, Su MT, Lee HJ, 2009. Pigment dispersing factor: an output regulator of the circadian clock in the German cockroach. *J. Biol. Rhythms.*, 24(1): 35–43.

Lipardi C, Wei Q, Paterson BM, 2001. RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. *Cell*, 107: 297-307.

Mao YB, Cai WJ, Wang JW, Hong GJ, Tao XY, Wang LJ, Huang YP, Chen XY, 2007. Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nature Biotechnology*, 25(11): 1 307–1 313.

March JC, Bentley WE, 2007. RNAi-based tuning of cell cycling in *Drosophila* S2 cells: Effects on recombinant protein yield. *Appl. Microbiol Biotechnol*, 73(5): 1 128–1 135.

Martin D, Maestro O, Cruz J, Mane-Padros D, Belles X, 2006. RNAi studies reveal a conserved role for RXR in molting in the cockroach *Blattella germanica*. *J. Insect Physiol.*, 52(4): 410–416.

Moriyama Y, Sakamoto T, Karpova SG, Matsumoto A, Noji S, Tomioka K, 2008. RNA interference of the clock gene period disrupts circadian rhythms in the cricket *Gryllus bimaculatus*. *J. Biol. Rhythms*, 23(4): 308–318.

Newmark PA, Reddien PW, Cebria F, Alvarado AS, 2003. Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in planarians. *Proceedings of the National Academy of Sciences of the United States of America*, 100(90 001): 11 861 – 11 865.

Niu BL, Shen WF, Liu Y, Weng HB, He LH, Mu JJ, Wu ZL, Jiang P, Tao YZ, Meng ZQ, 2008. Cloning and RNAi-mediated functional characterization of *MaLac2* of the pine sawyer, *Monochamus alternatus*. *Insect Mol. Biol.*, 17(3): 303–312.

Rajagopal R, Sivakumar S, Agrawal N, Malhotra P, Bhatnagar RK, 2002. Silencing of midgut aminopeptidase N of *Spodoptera litura* by double-stranded RNA establishes its role as *Bacillus thuringiensis* toxin receptor. *J. Biol. Chem.*, 277(49): 46 849–46 851.

Roignant JY, Carre C, Mugat B, Szymczak D, Lepesant JA, Antoniewski C, 2003. Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in *Drosophila*. *RNA*, 9(3): 299–308.

Saleh M-C, Tassetto M, van Rij RP, Goic B, Gausson V, Berry B, Jacquier C, Antoniewski C, Andino R, 2009. Antiviral immunity in *Drosophila* requires systemic RNA interference spread. *Nature*, 458(7 236): 346–350.

Saleh MC, van Rij RP, Hekele A, Gillis A, Foley E, O'Farrell PH, Andino R, 2006. The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nature Cell Biology*, 8: 793 – 802.

Sandrelli F, Cappellozza S, Benna C, Saviane A, Mastella A, Mazzotta GM, Moreau S, Pegoraro M, Piccin A, Zordan MA, Cappellozza L, Kyriacou CP, Costa R, 2007. Phenotypic effects induced by knock-down of the period clock gene in *Bombyx mori*. *Genet. Res.*, 89(2): 73–84.

Schlüns H, Crozier RH, 2007. Relish regulates expression of antimicrobial peptide genes in the honeybee, *Apis mellifera*, shown by RNA interference. *Insect Mol. Biol.*, 16(6): 753–759.

Scolari F, Siciliano P, Gabrieli P, Gomulski LM, Bonomi A, Gasperi G, Malacrida AR, 2011. Safe and fit genetically modified insects for pest control: from lab to field applications. *Genetica*, 139: 41–52.

Siomi H, Siomi MC, 2009. On the road to reading the RNA-interference code. *Nature*, 457: 396 – 404.

Sumitani M, Yamamoto DS, Lee JM, Hatakeyama M, 2005. Isolation of white gene orthologue of the sawfly, *Athalia rosae* (Hymenoptera) and its functional analysis using RNA interference. *Insect Biochem. Mol. Biol.*, 35(3): 231–240.

Takahashi T, Hamada A, Miyawaki K, Matsumoto Y, Mito T, Noji S, Mizunami M, 2009. Systemic RNA interference for the study of learning and memory in an insect. *Journal of Neuroscience Methods*, 179(1): 9–15.

Tavernarakis N, Wang SL, Dorovkov M, Ryazanov A, Driscoll M, 2000. Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nature Genetics*, 24(2): 180–183.

Terenius O, Papanicolaou A, Garbutt JS, et al, 2011. RNA interference in Lepidoptera: An overview of successful and unsuccessful studies and implications for experimental design. *J Insect Physiol.* 57: 231-245.

Tian H, Pen H, Yao Q, Chen H, Xie Q, Tang B, et al, 2009. Developmental control of a lepidopteran pest *Spodoptera exigua* by ingestion of bacteria expressing dsRNA of a non-midgut gene. *PLoS One*, 4: 1–13.

Timmons L, Fire A, 1998. Specific interference by ingested dsRNA. *Nature*, 395(6 705): 854.

Tomoyasu Y, Miller SC, Tomita S, Schoppmeier M, Grossmann D, Bucher G, 2008. Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in *Tribolium*. *Genome Biology*, 9(1): R10.1–R10.22.

Travanty EA, Adelman ZN, Franz AWE, Keene KM, Beaty BJ, Blair CD, James AA, Olson KE, 2004. Using RNA interference to develop dengue virus resistance in genetically modified *Aedes aegypti*. *Insect Biochem. Mol. Biol.*, 34(7): 607–613.

Turner CT, Davy MW, MacDiarmid RM, Plummer KM, Birch NP, Newcomb RD, 2006. RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. *Insect Mol. Biol.*, 15(3): 383–391.

Uhlirova M, Foy BD, Beaty BJ, Olson KE, Riddiford LM, Jindra M, 2003. Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of Broad-Complex in insect metamorphosis. *Proceedings of the National Academy of Sciences of the United States of America*, 100(26): 15 607–15 612.

Van Roessel P, Hayward NM, Barros CS, Brand AH, 2002. Two-color GFP imaging demonstrates cell-autonomy of GAL4-driven RNA interference in *Drosophila*. *Genesis*, 34(1–2): 170–173.

Wakiyama M, Matsumoto T, Yokoyama S, 2005. *Drosophila* U6 promoter-driven short hairpin RNAs effectively induce RNA interference in Schneider 2 cells. *Biochem. Biophys. Res. Commun.*, 331(4): 1 163–1 170.

Walshe DP, Lehane SM, Lehane MJ, Haines LR, 2009. Prolonged gene knockdown in the tsetse fly *Glossina* by feeding double stranded RNA. *Insect Mol. Biol.*, 18(1): 11–19.

Whangbo JS, Hunter CP, 2008. Environmental RNA interference. *Trends in Genetics*, 24: 297 – 305.

Winston WM, Molodowitch C, Hunter CP, 2002. Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. *Science*, 295(5 564): 2 456–2 459.

Yang ZX, Wen LZ, Wu QJ, Wang SL, Xu BY, Zhang YJ. 2008. Application of RNA interference in studying gene functions in insects. *Acta Entomologia Sinica*, 51(10): 1 077–1 082.

Zhao Y, Yang G, Wang-Pruski G, You M, 2008. *Phyllotreta striolata* (Coleoptera: Chrysomelidae): Arginine kinase cloning and RNAi-based pest control. *European Journal of Entomology*, 105(5): 815–812.

Zhou X, Wheeler MM, Oi FM, Scharf ME, 2008. RNA interference in the termite *Reticulitermes flavipes* through ingestion of double-stranded RNA. *Insect Biochem. . Mol. Biol.*, 38(8): 805–815.

Zhu Q, Arakane Y, Beeman RW, Kramer KJ, Muthukrishnan S, 2008. Functional specialization among insect chitinase family genes revealed by RNA interference. *Proceedings of the National Academy of Sciences of the United States of America*, 105(18): 6 650–6 655.

3

Evaluation of Plant Extracts on Mortality and Tunneling Activities of Subterranean Termites in Pakistan

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1. Introduction

Plant extracts offer a vast, virtually untapped reservoir of chemical compounds with many potential uses. One of these uses is in agriculture to manage pests with less risk than with synthetic compounds that are toxicologically and environmentally undesirable. Increasing evolution of resistance in pest population further derives the need to search for new bioactive compounds with a wide range of new modes of action. Various experiments using plant extracts in human and animal health protection, agriculture and household pest management have been particularly promising (Pascual-Villalobos & Robledo, 1999; Scott et al., 2004). The apparent societal hope for using plant extracts in place of more traditional pesticides has also increased the attention paid to natural products in the past decade (Duke *et al*., 2003). Plant products have been exploited as insecticides, insect-repellents, antifeedants and insect growth and development regulators (Saxena, 1998). The deleterious effects of phytochemcials or crude plant extracts on insects are manifested in several ways, including suppression of calling behaviour (Khan & Saxena, 1986), growth retardation (Breuer & Schmidt, 1995), toxicity (Hiremath et al., 1997), oviposition deterrence (Zhao et al., 1998), feeding inhibition (Wheeler & Isman, 2001) and reduction of fecundity and fertility (Muthukrishnan & Pushpalatha, 2001).

Many plants have been recognized to have anti-termitic activities (Sakasegawa et al., 2003, Park & Shin, 2005, Jembere et al., 2005, Cheng et al., 2007, Ding & Hu, 2010, Supriadi and Ismanto, 2010) or repellent to the termites i.e., *Eucalyptus globules*, lemmon grass, *Eucalyptus citrodora*, cedar wood, clove bud and vetiver grass (Zhu et al., 2001a, b), *Taiwania cryptomerioides* Hayat (Chang *et al*., 2001), *Dodonaea viscosa* (Purple hop bush) a termite resistant shrub (Anonymous, 2001), *Ocimum basilicum* L., *Cymbopogon winterianus* Jowitt, *Cinammomum camphora*, *Rosmarinus officinalis* (Sbeghen et al., 2002) and *Coleus ambionicus* (Singh et al., 2004) are less extensively studied against termites.

The extracts of plants having anti-termite properties and termite-resistant formulations have been prepared, reported and tested in the laboratory and fields. Substrates in these tests were soil, sand and filter paper. Mortality and inhibition of consumption of wood were indicators of toxic and feeding deterrent activity of these extracts. Those tested in laboratory were extracted in various organic solvents in addition to water. Alcoholic and phenolic compounds in extracts of *Juniperus procera* (Kinyanjui et al., 2000), pine resin and eight of its derivatives (Nunes et al., 2004), 2.0 % chloroform leaf extracts of *Polygonum hydropiper* L. and *Pogostemon parvillorus* against tea termite, *Odontotermes assamensis* Holm. with highest toxic activity (100% mortality) in the extract of *P. hydropiper* (Rahman et al., 2005) are some of the examples. Effects of hexane, ethanol, and petroleum ether extracts of the black pepper fruits, *Piper nigrum*, were studied on the dry-wood termite, *Cryptotermes brevis*. Hexane extract at 0.5% concentration induced 50% mortality, which dropped to 4.76 and 14.28% with ethanol and petroleum ether, respectively, 2 days post treatment (Moein & Farrag, 2000). The termite, *Coptotermes curvignathus*, workers responded differently to soils and pine blocks treated with varying concentrations of *Azadirachta excelsa* leaf extracts in acetone, hexane and methanol. The result showed that extracts from *A. excelsa* leaves had an inhibitory effect on subterranean termites, *C. curvignathus*. The soils treated with the extracts did pose a hindrance to the tunneling activities of the termites (Sajap and Aloysius, 2000). The chloroform extracts of the woods Ipe (*Tabebuia sp.)* and Itauba (*Mezilaurus sp.)* on the dry-wood termite, *Cryptotermes brevis*, applied at a rate of 0.1 g/mL to filter paper to feed the termites and later analyses of substrate consumption rates and mortality by the Kruskal-Wallis method indicated a statistically significant reduction of feeding rates and increased mortality after 30 days (Cabrera et al., 2001). Phytoextracts from *Adhatoda vasica*, *Cynodon dactylon*, *Pongamia pinnata*, *Rauvolfia serpentina*, *Cleistanthus collinus*, *Tamarindus indica* and *Eichhornia crassipes* controlled the termites, *Microcerotermes mycophagus* (Nilanjana & Chattopadhyay, 2003). The biological activity of extracts of Meliaceae in relation with *Heterotermes tenuis* was studied in the laboratory. The effect of aqueous extracts from *Melia azedarach, Trichilia pallida* and *Azadirachta indica* (neem) (1 and 5% w/v), neem oil (1 and 2% v/v), and Nimkol, obtained from neem leaves (0.5 and 1% a.i.), were measured for the survivability of the termites. Nimkol caused a significant mortality of *H. tenuis* after the third day of feeding (1% a.i.) (Castiglioni and Vendramim, 2003). Seed and leaf extract of *Polygonum hydropiper* and *Cannbis sativa* against *Heterotermes indicola* and *Coptotermes heimi* showed more toxicity of seed (52-64% and 70-74% mortality) than leaf extracts (28-54 % and 28-58%) in both species. Crude extracts of various reproductive and vegetative parts of *Calotropis procera* had toxic effects on *H. indicola* (Badshah et al., 2004). *Datura alba*, *D. stramonium* and *Calotropis procera* were the most effective against the termites with 62.5% protection (Bajwa & Rajpar, 2001; Ayodele & Oke, 2003). 5% Chloroform extract of *Lantana camara* var. aculeate at a concentration of 5% was found to be significantly effective against termite workers (Verma & Verma, 2006).

The extracts used in the field were mostly in the form of decoction with water and were used in the soil or poured into the termites' nest directly. Decoction of *Cassia fistula*, *Myrtus communis*, *Sapium sebiferum* and *Thevetia peruviana* at rate of 5% (5g: 100ml) provided significant protection against termites for three months in the field. Fermented extracts of *Tithonia diversifolia* and *Melia azedarach* controlled Isopteran insects when poured into their nest. The ash of *T. diversifolia*, *Cassia siamea* and *C. spectabilis* applied to affected trees provided protection from termites for up to 45 days. *Vernonia amygdalina* and *Agave sisalana*, not only controlled termites and but also contributed to soil fertility (Ghosh, 2009). Soil treated with 2% solution of

Calotropis procera L. and *Azadirachta indica* prevented damage to sugarcane setts by *Odontotermes obesus* (Rambur) controlled the termite (Deka & Singh, 2001; Singh et al., 2002).
Several novel classes of termiticides have been isolated from plants and based on these natural products, more active analogs have been synthesized. Two sesquiterpenes (partheniol and argentone) and a triterpene (incanilin) from guayule resin showed different levels of antifeedant and toxic activity (Gutierrez et al., 1999). The effects of a commercial insecticide formulation (margosan-O) containing 0.3%. Azadirachtin and 14% neem oil on orientation, tunneling, and feeding behaviour of the Formosan subterranean termite have been investigated (Grace & Yates, 1992). Sand treated with vetiver oil or nootkatone at 100 µg/g substrate were effective barriers to the termite, *Coptotermes formosanus* (Maistrello et al., 2001). Thiophenes from four Echinops species and columellarin and sesquiterpene lactone fraction from the heartwood of Australian white cypress (*Callitris glaucophylla*) showed anti-termitic activities against *C. formosanus* Shiraki (Watanabe et al., 2005; Fokialakis et al., 2006)). Vulgarone B (isolated from *Artemisia douglasiana*), apiol (isolated from *Ligusticum hultenii)* and cnicin (isolated from *Centaurea maculosa)* exhibited significantly higher mortalities in Formosan subterranean termite (*C. formosanus*) than in untreated control in the laboratory bioassay (Meepagala et al., 2006).
Oils extracted from plant parts have been applied in a number of situations to protect the substrate from termite infestation. The crude seed oil of *Piper guineense*, each at a 10% concentration at the rate of 18 litres ha^{-1} significantly lowered damage by termites (*Microtermes spp*., *Macrotermes bellicosus* and *M. subhyalinus*) (Umeh & Ivbijaro, 1999). Neem seed oil inhibited growth of termite surface-tunnels (Yashroy & Gupta, 2000). For further references, annual meeting report of IRG can be consulted for efficacy of oils against termites.
Many timbers contain chemicals or complex mixture of chemicals that repel or kill the termites or effect on gut flora in termites (Adams *et al*., 1988); among these are *Pometia pinnata*, *Homalium foetidum*, *Eucalyptus deglupta* and *Alstonia scholaris* (Rokova and Konabe., 1990). Relatively less mentioned other plants with termite control properties are presented below (Anonymous, 2001).

Species	Parts Used	Property
Carya ovata	Bark	Termiticidal
Cedrela odorata	Wood	Termiticidal
Consolida regalis	wood	Termiticidal
Dodonaea viscosa	Leaves, wood / pulp	Termiticidal
Quercus prinus	Bark	Termiticidal
Hardwickia mannii	Stem/ branches	Termiticidal
Pinus strobus	Bark	Termiticidal
Samadera indica	Leaves	Termiticidal
Carica papaya	Fruit, fresh leaves and roots	Insecticidal
Grevillea robusta	Leaves	Insecticidal
Leucaena leucocephala	Used as a leaf mulch	Repellent
Commiphora Africana	Gum/ resin	Repellent
Cassia siamea	Used as a leaf mulch	Repellent
Hyptis spicigera	Aerial parts	Repellent
Ocimum canus	Whole plant	Insecticidal, repellent

Source: HRD Publication UK

Many plant extracts have been found to alter the behaviour of termites. Chemicals showing antifeedant activities had also effect on tunneling of the termites (Ibrahim et al., 2004; Mao & Henderson, 2007).

The foregoing examples are just crust of the copious literature available on this aspect of termite management and control. Previously we have demonstrated some of the above mentioned properties of many extracts of plants, shrubs and trees in our laboratory and have found significant results in controlling termites in the field (Ahmed et al., 2005, 2006, 2007). In order to find out inexpensive alternate to synthetic insecticides, anti termite properties from plants will continue to expand base of the effective molecules to be developed to go well with the ecology of termites.

2. Materials and methods

2.1 Collection of termites

The assorted workers of the termite species, *Microtermes obesi* Holm., in the later instars were collected, within the damaged canes from sugarcane fields and from the corrugated cardboard baits in PVC monitors installed in the fields at different places at the Experimental Area, Department of Agri-Entomology, University of Agriculture, Faisalabad.

2.2 Following plants were selected to obtain their leaf extracts

Botanical name	Family	Common name
Adhatoda vasica (Nees)	Acanthaceae	Malabar nut
Dodonaea viscosa (Linn.) Jacq	Sapindaceae	Hopbush
Thevitia peruviana (Pers) Merr	Apocynaceae	Yellow oleander
Nerium odorum Soland	Apocynaceae	Indian oleander
Salvadora oleiodes Decne	Salvadoraceae	Vann
Alstonia scholaris (R. BR.)	Apocynaceae	Devil tree
Delphinium ajacis Linn.	Ranunculaceae	Larkspur
Papaver somniferum Linn.	Papaveraceae	Garden poppy
Lucaena leucocephala (Lam.) DeWit	Mimosaceae.	Iple iple
Grevilla robusta A.Cunn.Ex.R.Br	Proteaceae	Silky oak
Tephrosia purpurea Linn.	Fabaceae	Wild indigo
Nerium oleander Linn.	Apocynaceae	Rose bay
Jatropha integerrima Jacq.	Euphorbiaceae	Peregrina

2.3 Extraction method

2.3.1 Preparation of leaves for extraction process

Fresh fully developed leaves in the season from middle portion of the plants from Botanical Garden as well as from areas within campus, University of Agriculture, Faisalabad, Pakistan, were collected and these plants were never exposed to pesticides. These leaves were washed with tap water and then air dried in a laboratory for 2 weeks ensuring sufficient air flow to avoid damping. The room-dried leaves were reduced to a powder form by grinding with an electric grinder running at a speed of 6000 rpm for 50-60 sec.

2.3.2 Crude methanolic extract of leaves

One hundred gram (100 g) of powder from each of the plants was extracted in 200 ml of 80% methanol in the ratio of 1:2 (w/v) by following method of extraction (Sadek, 2003). It was kept for 72 hours at room temperature and shaken at intervals to get a better extraction. Thereafter, the extract was filtered through Whatman filter No. 42. After filtering, the methanol was removed at 60°C using rotary evaporator, to obtain solid extract, dried in vacuum desiccator. The final yield of dry material was used to prepare percent solution of crude extract with 2% methanol.

2.3.3 Aqueous extracts of leaves

To get the aqueous extracts, above procedure was followed except powder was extracted in distilled water. The filtrates were stored in a refrigerator at 5°C for subsequent use in bioassays.

2.4 Bioassay

2.4.1 Soil preparation for bioassay

The soil used in bioassays was sandy clay loam (52.6% sand, 24.8% silt and 20.6% clay). There had been no known applications of agro-chemicals in this soil for the control of termites. The soil was sieved through a 30-mesh screen and moisture was determined with the help of a moisture meter. Water was added in this soil to simulate 50% of water holding capacity, to avoid mortality of termites due to dehydration during assays.

2.4.2 Bioassays by mixing leaf extract in the soil

Antitermitic sugarcane strip bioassays (ASSB) using different leaf extracts were done in Petri dishes (95 × 15 mm) containing 20 g sifted sterilized soil and strips of sugarcane (1.5 cm × 6 cm) to keep the termites alive. Every treatment with 10%, 20% and 40% of extracts and control (without extract) were repeated thrice in Completely Randomized Design (CRD). 20 g of sifted soil in Petri dish having sugarcane strip was wetted/ mixed with respective concentration of the extract. 50 active workers and 5 soldiers were released in the Petri dishes having treated and untreated soil.

2.4.3 Filter paper bioassay

Whatman filter paper No. 42, 9 cm in diameter was treated with 10, 20 and 40% concentrations of leaf extracts at the rate of 31 µl/cm^2 and placed in Petri dishes (95 × 15 mm). 50 workers and 5 soldiers of were released in the Petri dishes having treated and untreated filter paper.

The Petri dishes having filter paper and/or soil bioassay were placed in growth chamber under controlled conditions of 28±2°C and 80%±5 humidity. Data for mortality were recorded after every 2 hours up to 12 hours, and then after every 12 hours until all workers and soldiers died. Each treatment was repeated three times.

2.5 Formation of Galleries (FG)

Members of family Termitidae make galleries during foraging. This shows the activity of termites in the soil. The termites started making tunnel along the bottom of each Petri dish around the sugarcane strip. Termite's response towards galleries formation for each plant extract at each concentration after 5, 10 and 15 hours was determined by plotting the tunnels

on the cellophane paper and measured the length in mm^2 with the help of planimeter. The values were correlated with the chemical concentrations. Tunnelling activities were analyzed by Factorial Analysis (CRD).

2.6 Statistical analysis

LT_{50}s in soil and filter paper bioassay was determined using Kaplan Meier Survival Test. In all tests, values of LT_{50}s among replication was non significant and were thus taken as mean LT_{50}. Tunnelling activities analyzed by Two way ANOVA, however, data are represented as mean activity of all time intervals at each concentration. The difference among concentrations was determined by Duncan Multiple Range Test (DMR) at $p<0.05$.

3. Results

The activity of methanolic and aqueous extracts of *D. viscosa*, *A. vasica*, *N. odorum*, *T. peruviana*, *D. ajacis*, *S. oleiodes* and *A. scholaris*, applied on filter paper and / or mixed into soil at various concentrations is expressed as LT_{50} (hours) and is shown in Tables 1 to 4. Extracts have exhibited the activity of causing morality in the termites, *M. obesi*, and were significantly different from control treatments. The activities were concentration dependent and LT_{50} decreased with increase in concentrations. Methanolic extract of *D. viscosa* at 40% (27.5 hours), *D. ajacis* at 10% (47.0 hours) and *N. odorum* at 20% (34.3 hours) concentrations were effective, being shown as lowest LT_{50}s on filter paper (Table 1).

Concentrations	LT_{50} (hours)						
(%)	*D. viscosa*	*A. vasica*	*N. odorum*	*T. peruviana*	*D. ajacis*	*S. oleiodes*	*A. scholaris*
0	291. 5	282. 8	278. 9	281.0	278. 8	263.0	269. 2
10	52.3	63.5	57.6	67.7	47.0	64.8	64.3
20	34.8	42.6	34.3	41.7	36.4	48.2	47.5
40	27.5	29.9	27.2	35.0	32.5	39.4	37.4

Table 1. LT_{50} values with methanolic extracts of *D. viscosa*, *A. vasica*, *N. odorum*, *T. peruviana*, *D. ajacis*, *S. oleiodes*, *A. scholaris* leaves at different concentrations against *M. obesi* in Petri dishes having treated filter paper.

Concentrations	LT_{50} (hours)						
(%)	*D. viscosa*	*A. vasica*	*N. odorum*	*T. peruviana*	*D. ajacis*	*S. oleiodes*	*A. scholaris*
0	308. 4	309. 3	282. 5	300.0	292.0	314. 8	257. 3
10	67.5	66.7	71.1	111.6	77.3	98.0	107.5
20	37.9	45.6	50.4	65.1	52.8	74.5	74.1
40	35.2	40.9	35.8	39.8	39.6	40.5	55.8

Table 2. LT_{50} values with methanolic extracts of *D. viscosa*, *A. vasica*, *N. odorum*, *T. peruviana*, *D. ajacis*, *S. oleiodes*, *A. scholaris* leaves at different concentrations against *M. obesi* in Petri dishes having treated soil.

A. vasica at 10% (66.7 hours) and *D. viscosa* at 20 and 40% concentrations (37.9 and 35.2 hours, respectively) had lowest LT_{50} when these were mixed in the soil (Table 2). Aqueous

extract of *N. odorum* had lowest LT_{50}s (48.9, 33.9 and 27.0 hours) at 10, 20 and 40% concentrations on filter paper (Table 3).). Aqueous extract of *D. ajacis* at 10 and 20% and *A. vasica* at 40% concentrations (36.0 hours) had lowest LT_{50}s (114.2 and 72.1 hours) when mixed in the soil (Table 4).

Concentrations	LT_{50} (hours)						
(%)	*D. viscosa*	*A. vasica*	*N. odorum*	*T. peruviana*	*D. ajacis*	*S. oleiodes*	*A. scholaris*
0	332.2	287.8	293.4	287.5	320.3	293.6	320.5
10	71.5	69.6	48.9	53.9	70.7	89.2	111.2
20	55.7	40.9	33.9	37.9	44.2	69.8	78.7
40	41.1	33.0	27.0	28.6	37.7	59.3	56.6

Table 3. LT_{50} values with aqueous extracts of *D. viscosa*, *A. vasica*, *N. odorum*, *T. peruviana*, *D. ajacis*, *S. oleiodes*, *A. scholaris* leaves at different concentrations against *M. obesi* in Petri dishes having treated filter paper.

Concentrations	LT_{50} (hours)						
(%)	*D. viscosa*	*A. vasica*	*N. odorum*	*T. peruviana*	*D. ajacis*	*S. oleiodes*	*A. scholaris*
0	328.9	293.5	325.0	353.1	290.7	351.3	308.6
10	115.8	101.8	119.1	148.7	114.2	139.1	136.4
20	68.8	78.2	85.1	92.7	72.1	98.3	85.0
40	46.2	36.0	60.9	61.0	50.3	63.3	62.6

Table 4. LT_{50} values with aqueous extract of *D. viscosa*, *A. vasica*, *N. odorum*, *T. peruviana*, *D. ajacis*, *S. oleiodes*, *A. scholaris* leaves at different concentrations against *M. obesi* in Petri dishes having treated soil.

The methanolic extract of *P. somniferum* at 10 and 20% and *G. robusta* at 40% concentrations had lowest LT_{50}s (26.5, 18.6 and 12.5 hours, respectively) (Fig. 1a) on filter paper, whereas *G. robusta* had lowest LT_{50}s at all concentration when mixed in the soil (Fig. 1b). Aqueous extract of *P. somniferum* at 10% (42.1 hours) and of *T. purpurea* at 20 and 40% concentrations (33.3 and 22.7 hours, respectively) had lowest LT_{50}s on filter paper (Fig. 2 a). In soil, *G. robusta* at 10% (75.5 hours) and of *P. somniferum* at 20 and 40% concentrations (44.3 and 21.4 hours, respectively) yielded lowest LT_{50}s (Fig. 2b). Two types of plant extracts have shown 40 to >80% % of LT_{50} on filter paper and in the soil when compared with control treatments.

In other studies, leaf extracts of *Jatropha integerrima*, *N. oleander* and *Lucaena leucocephala* in acetone, methanol, petroleum ether and aqueous solvents showed activity in terms of mortality of termite workers at different concentrations when mixed in the soil in Petri dishes and is represented in Tables 5-7. *J. integerrima* was the most effective among three plants and showed lowest LT_{50} at 10% (9.72 hours) in acetone and then in petroleum ether at 20 and 40% concentrations (6.53 and 4.99 hours, respectively). Lowest LT_{50} of two other plants was shown in acetone extract at 40% concentration (47.8 hours). It is interesting to note that *N. oleander* has shown less activity than *N. odorum*, in addition to acetone and petroleum ether, in methanol and aqueous extracts as well.

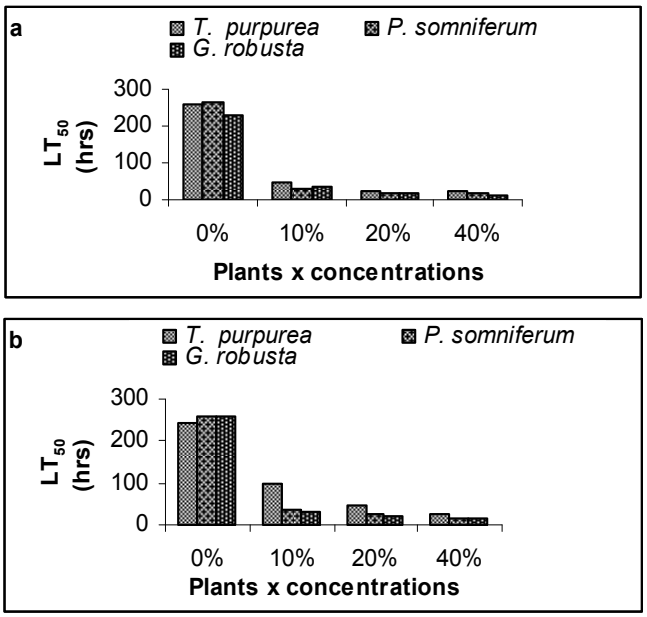

Fig. 1. LT_{50} values with methanolic extract of *Tephrosia purpurea, Papaver somniferum, Grevilla robusta* leaves at different concentrations against *M. obesi* on (a) filter paper (b) mixed in the soil.

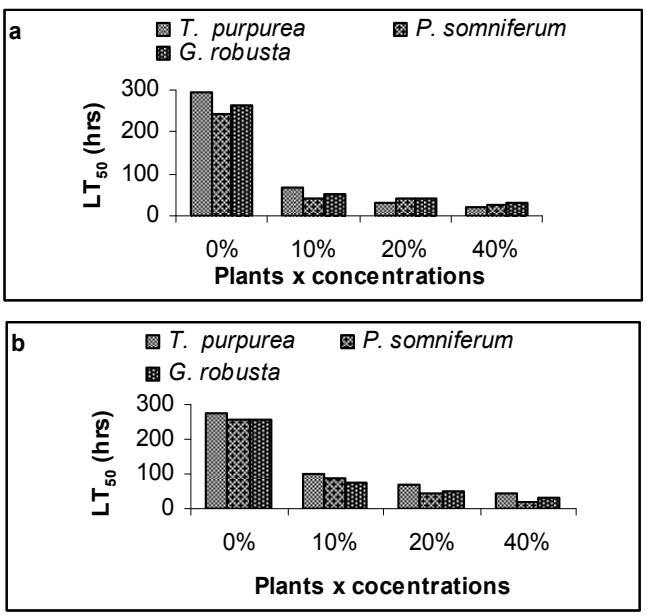

Fig. 2. LT_{50} values with aqueous extract of *Tephrosia purpurea, Papaver somniferum, Grevilla robusta* leaves at different concentrations against *M. obesi* in Petri dishes having treated (a) filter paper (b) soil.

Concentrations	LT_{50} (hours)			
(%)	acetone	methanol	aqueous	petroleum ether
0	396.9	483.5	457.4	483.5
10	13.8	9.7	79.6	108.6
20	8.6	5.4	17.9	6.5
40	6.7	4.5	15.9	4.9

Table 5. LT_{50} values of various extract of *Jatropha integerrima* at different concentrations against *M. obesi* in Petri dishes having treated soil.

Concentrations	LT_{50} (hours)			
(%)	acetone	methanol	aqueous	petroleum ether
0	154.0	174.8	162.2	177.5
10	123.1	165.2	128.2	143.2
20	85.8	130.0	127.0	142.0
40	85.6	116.2	94.9	138.7

Table 6. LT_{50} values of various extract of *Nerium oleander* at different concentrations against *M. obesi* in Petri dishes having treated soil.

Concentrations	LT_{50} (hours)			
(%)	acetone	methanol	aqueous	petroleum ether
0	149.0	167.4	182.0	176.9
10	84.5	152.0	133.7	147.8
20	64.6	146.0	125.7	147.5
40	47.8	135.7	99.0	133.0

Table 7. LT_{50} values of various extract of *Leucaena leucocephala* at different concentrations against *M. obesi* in Petri dishes having treated soil.

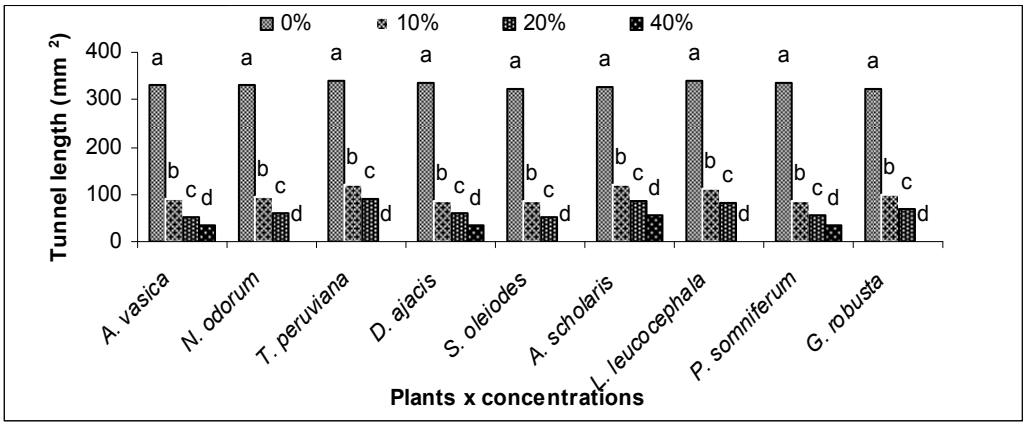

Fig. 3. Comparison of tunnel length at different concentrations of leaf methanol extracts of various plants.

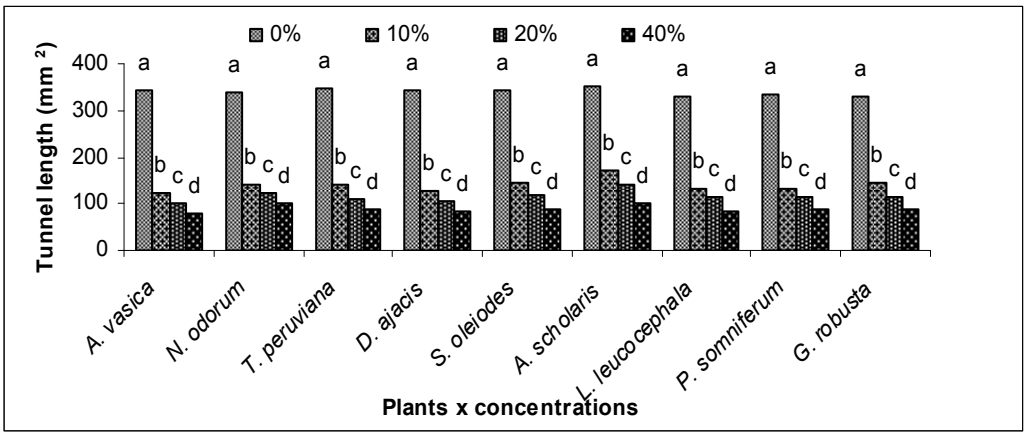

Fig. 4. Comparison of tunnel length at different concentrations of leaf aqueous extracts of various plants.

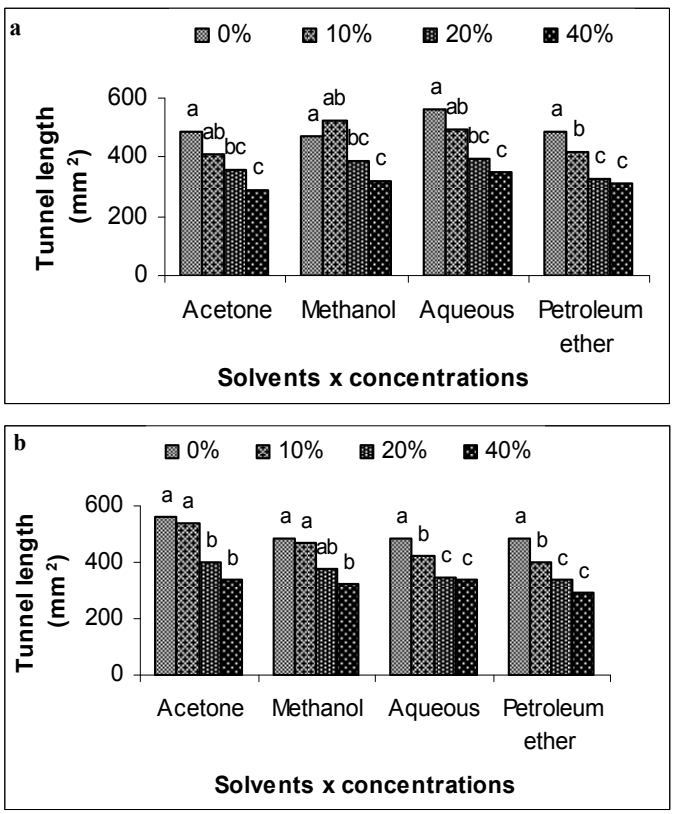

Fig. 5. Comparison of termites' tunnel length in leaf extracts in various solvents of (a) *Nerium oleander* (b) *Lucaena leucocephala*.

Various concentrations had significant difference among them with respect to the tunnelling (tunnel length) by the termites when mixed with the soil at various time intervals (5-15

minutes). After 15 minutes, it was difficult to draw the tunnel length on the paper, however, data are shown as mean tunnel length of the time intervals but not of the concentrations. *A. vasica, N. odorum, S. oleiodes, T. purpurea* and *G. robusta* leaf extracts in methanol at 40% did not show any tunnelling, nevertheless, termites had mined in the aqueous extracts of the same plants at 40% concentration (Figs. 3 & 4). In contrast to *N. odorum*, other species of the same plant *N. oleander* could not prevent tunnel formation as in case of former species, but tunnel length at 40% concentrations had significant difference than that at 10 and 20% concentrations depending upon type of solvent (Fig. 5 a & b).

4. Discussion

Treatment of soil with natural/synthetic compounds to control the termites is common method. Insecticides have been used to form barrier in soil against subterranean termites to prevent their tunnelling and reaching to food sources. Chlorpyrifos, bifenthrin, fipronil and many others have been extensively used for this type of barrier against termites (Su et al., 1997). Criteria used to evaluate potential soil termiticides have been termites' ability to tunnel through treated soil and toxicity of material (plant extracts) in laboratory experiments (Grace et al., 1993; Su et al., 1993).

The results exhibited herein are mostly the confirmation of the results obtained elsewhere for the medicinal plant extracts having anti-termite properties and termite-resistant formulations (Singh et al., 2001; Ding & Hu, 2010). *Dodonaea viscosa* (Purple hop bush) has been reported as a termite resistant shrub (Anonymous, 2001), but bioassay of its extracts with termites has been investigated for the first time in this report. The extracts from *Adhatoda vasica* and *Nerium oleander* are some of the above mentioned plants which have been tested for same purpose (Nilanjana & Chattopadhyay, 2003).

The results revealed two important aspect of toxicological inference (i) LT_{50} irrespective of medium for feeding and movement was almost equal and non-significant depending upon fiducial limits (ii) LT_{50} of methanol extract was shorter than aqueous extract. Many studies have shown activity of plant extracts when applied on filter paper and / or mixed in soil to determine mortality (Blaske & Hertel, 2001; Blaske et al., 2003, Jembere et al., 2005) and concluded that plant extracts have the potential for under- and above-ground application for the termite control.

The present results showed that the tunnelling activities are the function of time and concentration. All concentrations of aqueous and methanolic leaf extracts have less tunnelling activities of *M. obesi* as compared to control. Means of the tunnelling activities of *M. obesi* in methanolic extract treated soil were less than the means of the tunnelling activities in aqueous extract treated soil. There was no tunnelling in leaf methanol extracts of *A. vasica*, *N. odorum*, *S. oleiodes*, *T. purpurea* and *G. robusta* at their 40% concentrations. These results are confirmation of earlier reports mentioined elsewhere depending upon species and kind of plant parts being studied. It is evident from these results that extracts did pose a hindrance to tunnelling activities of the termites (Sajap & Aloysius, 2000; Maistrello et al., 2001; Peterson & Ems-Wilson, 2003; Mao & Henderson, 2007), but the termites, however, may become insensitive towards the extracts upon longer period of exposure and this period depends upon termites species.

The extracts usually oils have been reported for toxicity and tunnelling inhibition studies, however, extracts in water and organic solvents have also yielded results for the above properties for termites' control. It has been summarized from various studies that extracts

having both properties of being toxic and inhibit tunnelling is good for various types of application and can be used in baiting and media application. There is no denying that potential application against termites would require large volume of plant materials, thus a number of plants should be studied to use alternatively. The water extract may be used for delivery into the soil to directly kill termites, or a paint-on material which may prevent termites from infesting wood. Inhibition of tunnelling may be exploited in a number of situations in agricultural ecosystem where seed or plant parts may be prevented from access of termites. One such example is setts protection from plant extract against termites where significant germination of setts of sugarcane in plots treated with plant extracts than in control plots (Ahmed et al. 2005; 2007).

5. Conclusions

1. Laboratory bioassays with a range of plant extracts in particular indicated the potential of some of them as termiticides.
2. Plant extracts used in the present studies had repellency for termites as these checked the tunnelling activities of termites in the soil, and may be used to keep the termites away from plants and ultimately, saving the plants from damage.
3. Leaf extracts of *D. viscosa D. ajacis* and *N. oleander* can be the good candidates for further process of isolation, and characterization of active compounds in the extracts.

6. References

Adams, R.P., McDaniel, C.A. & Carter, F.I. (1988). Termiticidal activities in the heartwood, bark/sapwood and leaves of Juniperus species from the United States. *Biochemical Systematics and Ecology*, Vol. 16, pp. 453-456, ISSN 0305-1978.

Ahmed, S., Naseer, A. & Fiaz, S. (2005). Comparative efficacy of botanicals and insecticides on termites in sugarcane at Faisalabad. *Pakistan Entomologist*, Vol.27, pp. 23-25, ISSN 1017-1827.

Ahmed, S., Khan, R.R. & Riaz, M.A. (2007). Some studies on the field performance of plant extracts against termites (*Odontotermes guptai* and *Microtermes obesi*) in sugarcane at Faisalabad. *International Journal of Agriculture and Biology,* Vol.9, pp. 398–400, ISSN 1560-8530.

Ahmed, S., Riaz, M.A. & Shahid, M. (2006). Response of *Microtermes obesi* (Isoptera: Termitidae) and its gut Bacteria towards some plant extracts. *Journal of Food Agriculture & Environment*, Vol. 4, No.1, pp. 317-320, ISSN 1459-0255.

Anonymous, 2001. Termite conrol without chemicals. HDRA-the organic organisation, pp: 10-12.

Ayodele, M.S. & Oke, O.A. (2003). Studies on the potential of some plant-based community pest management strategies in Southwest Nigeria. An investigation of the anti-termite potency of *Datura stramonium* L. An International Journal of Agricultural Science, Science, Environment and Technology, (ASSET) Series B: Vol.2, No.2, pp.153-159, ISSN 1595-9694.

Badshah, H., Farmanullah, Salihah, Z., Aur Saljoqi & Shakur, M. (2004). Toxic effects of AK (*Calotropis procera*) plant extracts against termites (*Heterotermes indicola* and *Coptoternes heimi*) (Isoptera: Rhinotermitidae). *Pakistan Journal of Biological Science*, Vol.7, No.9, pp.1603-1606, ISSN 10288880

Bajwa, G.A & Rajpar, M.N. (2001). Biological activity of extact of different plants against termites, nettle tree leaf beetle and amaltas leaf stitcher. *Pakistan Journal of Forestry*, Vol.51, No.2, pp.31-41, ISSN 0030-9818.

Blaske, V.U. & Hertel, H. (2001). Repellent and toxic effects of plant extracts on subterranean termites (Isoptera: Rhinotermitidae). *Journal of Economic Entomology*, Vol.94, No.5, pp.1200–1208, ISSN 0022-0493.

Blaske, V.U., Hertel, H. & Forschler, B.T. (2003). Repellent effect of isoborneol on subterranean termites (Isoptera: Rhinotermitidae) in soils of different composition. *Journal of Economic Entomology*, Vol.96, No.4, pp.1267-1274, ISSN 0022-0493.

Breuer, M. & G.H. Schmidt, G.H. (1995). Influence of a short period treatment with *Melia azedarach* extract on food intake and growth of the larvae of *Spodoptera frugiperda* (Lepidoptera; Noctuidae). *Journal of Plant Diseases and Protection*, Vol.102, pp.633-654, ISSN 1866-3829.

Cabrera, R.R., Lelis, A.T. & Berti-Filho, E. (2001). Effects of extracts of woods *Tabebuia* sp. (bignoniaceae) and *Mezilaurus* sp. (Lauraceae) on the dry-wood termite, *Cryptotermes brevis* (Isoptera: Kalotermitidae). Arquivos-do-Instituto-Biologico-Sao-Paulo, Vol.68, No.1, pp.103-106, ISSN 0020-3653

Castiglioni, E. & Vendramim, J.D. (2003). Evaluation of repellence of *Heterotermes tenuis* (Isoptera: Rhinotermitidae) to Meliaceae by-products. Agrociencia. Montevideo, Vol.7, No.1, pp.52-58, ISSN 1510-0839.

Chang, S.T., Cheng, S.S. & Wang, S.Y. (2001). Antitermitic activity of essential oils and components from Taiwania (*Taiwania cryptomerioides). Journal of Chemical Ecology*, Vol.27, No.4, pp.1267-1274, ISSN 0098-0331.

Cheng, S.S., Chang, H.T., Wu, C.L. and Chang, S.T. (2007). Anti-termitic activities of essential oils from coniferous trees against *Coptotermes formosanus. Bioresource Technology*, Vol.98, pp.456-459, ISSN 0960-8524.

Deka, M.K. and Singh, S.N. (2001). Neem formulation in the management of sugarcane insects and pests. *Proceeding of 63rd American Convention Sugar Technologist Association*, pp. 33-38. 27th – 28th August, Jailpur, India.

Ding, W. & Hu, X.P. (2010). Antitermitic effect of the *Lantana camara* plant on subterranean termites (Isoptera: Rhinotermitidae). *Insect Science*, Vol.17, No.5, pp. 427–433, ISSN 1744-7917.

Duke, S.O., Baerson, S.R., Dayan, F.E., Rimando, A.M., Scheffler, B.E., Tellez, M.R., Wedge, D.E., Schrader, K.K., Akey, D.H., Arthur, F.H., Lucca, A.J., Gibson, D.M., Harrison, H.F., Peterson, J.K., Gealy, D.R., Tworkoski, T., Wilson, C.L. & Morris, J.B. (2003). ARS Research on Natural Products for Pest Management. *Pest Management Science*, Vol. 59, No.6-7, pp.708-717, ISSN 1526-498X.

Fokialakis, N., Osbrink, W.L.A., Mamonov, L.K., Gemejieva, N.G., Mims, A.B., Skaltsounis, A.L., Lax, A.R. & Cantrell, C.L. (2006). Antifeedant and toxicity effects of Thiophenes from four *Echinops* species against the Formosan subterreanean termite, *Coptotermes formosanus. Pest Management Science*, Vol.62, No.9, pp.832-838, ISSN 1526-498X.

Ghosh, G.K. (2009). Termite control in Kenya. In: Biopesticide and Integrated Pest Management. pp. 199-204, Aph Publishing Corporation. India. ISBN 8176481351.

Grace, J.K. & Yates, J.R. (1992). Behavioural effects of neem insecticide on *Coptotermes formosanus* (Isoptera: Rhinotermitidae). *Tropical Pest Management*, Vol.38, No.2, pp.176-180, ISSN 0143-6147.

Grace, J.K., Yates, J.R., Tamashiro, M. & Yamamoto, R.T. (1993). Persistence of organochlorine insecticides for Formosan susterranean termite (Isoptera: Rhinotermitidae) control in Hawaii. *Journal of Economic Entomology*, Vol.86, No.3, pp. 761-766, ISSN 0022-0493.

Gutierrez, C., A. Gonzalez-Coloma, A. & Hoffmann, J.J. (1999). Antifeedant properties of natural products from *Parthenium argentatum, P. argentatum* X *P. tomentosum* (Asteraceae) and *Castela emoryi* (Simaroubeaceae) against *Reticulitermes flavipes* industrial. *Industrial Crops and Products*, Vol.10, No.1, pp.35-40, ISSN 0926-6690.

Hiremath, I.G., Youngjoon, A., Soonll, K., Ahn, Y.J. & Kim, S.I. (1997). Insecticidal activity of Indian plant extracts against *Nilaparvata lugens* (Homoptera: Delphacidae). *Applied Entomology and Zoology*, Vol.32, No.1, pp.159-166, ISSN 0021-4914.

Ibrahim, S., Kambham, S., Henderson, G. & Jayasimha, P. (2004). Toxicity and repellency of sesame oil on Formosan subterranean termites (Isoptera: Rhinotermitidae) in treated sand. *Proceedings of the National Conference on Urban Entomology*, 61-63, Phoenix, AZ. 20-22 May, USA.

Jembere, B., Getahun, D., Negash, M. & Sevoum, E. (2005). Toxicity of Birbira (*Milletia ferruginea*) seed crude extracts to some insect pests as compared to other botanical and synthetic insecticides. 11th NAPRECA (Natural Products and Drug Delivery) Symposium Book of Proceeding, Astanarivo, Madagaskar, pp. 88-96.

Khan, Z.R. & Saxena, R.C. (1986). Effect of steam distillate extracts of resistant and susceptible rice cultivars on behaviour of *Sogatella furcifera* (Homoptera: Delphacidae). *Journal of Economic Entomology*, Vol.79, No.4, pp.928-935, ISSN 0022-0493.

Kinyanjui, T., Gitu P.M. & Kamau, G.N. (2000). Potential antitermite compounds from *Juniperus procera* extracts. *Chemosphere*, Vol.41, No.7, pp.1071-1074, ISSN 0045-6535.

Mao, L. & Henderson, G. (2007). Antifeedant activity and acute and residual toxicity of alkaloids from *Sophora flavesces* (Leguminosae) against Formosan subterranean termites (Isoptera: Rhinotermitidae). *Journal of Economic Entomology,* Vol.100, No.3, pp. 866-870, ISSN 0022-0493.

Maistrello, L., G. Henderson, G. & R.A. Laine, R.A. (2003). Comparative effects of vetiver oil, nootkatone and disodium octaborate tetrahydrate on *Coptotermes formosanus* and its symbiotic fauna. *Pest Management Science*, Vol.59, No.1, pp.58-68, ISSN 1526-498X.

Maistreollo, L., G. Henderson, G. & R.A. Laine, R.A. (2001). Efficacy of vetiver oil and nootkatone as soil barriers against Formosan subterranean termite (Isoptera: Rhintermitidae). *Journal of Economic Entomology*, Vol.94, No.6, pp.1532-1537, ISSN 0022-0493.

Meepagala, K.M., Osbrink, W., Sturtz, G. & Lax, A. (2006). Plant- derived natural products exhibiting activity against Formosan subterranean termites. *Pest Management Science*, Vol.62, No.(6), pp.565-570, ISSN 1526-498X.

Moein, S.I. & Farrag, R.M. (2000). Susceptibility of the dry-wood termite *Cryptotermes brevis* Walker to the black pepper extracts. *Egyptian Journal of Agricultural Research*, Vol.78, No.3, pp.1135-1140, ISSN 1110-6336.

Muthukrishnan, J. & Pushpalatha, E. (2001). Effects of plant extracts on fecundity and fertility of mosquitoes. *Journal of Applied Entomology*, Vol.125, pp.31-35, ISSN 0931-2048.

Nilanjana, D. & Chattopadhyay, R.N. (2003). Control of termites through application of some phyto-extracts – a new approach in forestry. *Indian Forester*, Vol.129, No.12, pp.1538-1540, ISSN 0019-4816.

Nunes, L., Nobre, T., Gigante, B. & Silva, A.M. (2004). Toxicity of pine resin derivatives to subterranean termites (Isoptera: Rhinotermitidae). *Environment Quality Management*, Vol.15, No.5, pp.521-528, ISSN 1520-6438.

Park, I.K. & Shin, S.C. (2005). Fumigant activity of plant essential oils and components from garlic (*Allium sativum*) and clove bud (*Eugenia caryophyllata*) oils against the Japanese termite (*Reticulitermes speratus* Kolbe). *Journal of Agriculture, Food and Chemistry*, Vol.53, pp. 4388-7392, ISSN 0021-8561.

Pascual-Villalobos, M. & Robledo, A. (1999). Anti-insect activity of plant extracts from the wild flora in south-eastern Spain. *Biochemical Systematics and Ecology*, Vol.27, pp.1-10, ISSN 0305-1978.

Peterson, C.J. & Ems-Wilson, J. (2003). Catnip essential oil as a barrier to subterranean termites (Isoptera: Rhinotermitidae) in the laboratory. *Journal of Economic Entomology*, Vol.96, No.4, pp.1275-1282, ISSN 0022-0493.

Rahman, I., Gogoi, I., Dolui, A.K. & Handique, R. (2005). Toxicological study of plant extracts on termite and laboratory animals. *Journal of Environmental Biology*, Vol. 26, No.2, pp.239-241, ISSN 0254-8704.

Rokova, M. & Konabe, C. (1990). Assessment of untreated Papua New Guinea timbers for resistance to subterranean termites. Papua New Guinea Forest Research Institute. *Klinkii*. Vo.4, No.2. pp.19-27.

Sadek, M.M., (2003). Antifeedant and toxic activity of *Adhatoda vasica* leaf extract against *Spodoptera littoralis* (Lep. Noctuidae). *Journal of Applied Entomology*, Vol.127, pp.396-404. ISSN 0931-2048.

Sajap, A.S. & Aloysius, F. (2000). Effects of leaf extracts of *Azadirachta excelsa* on *Coptotermes curvignathus* (Isoptera: Rhinotermitidae). *Sociobiology*, Vol.36, No.3, pp.497-503, ISSN 0361-6525.

Sakasegawa, M., Hori, K. & Yatagi, M. (2003). Composition and anti-termite activities of essential oils and Melaleuca species. *Journal of Wood Science*, Vol.49, pp. 181-187, ISSN 1435-0211.

Saxena, R. C. (1998). Botanical pest control, In: Critical issues in Insect Pest Management,, Dhaliwal G.S. & Heinrichs, E.A. (Eds), 115-179, New Delhi, India.

Sbeghen, A.C., Dalfovov, V., Serafini, L.A. & De-Barros, N.M. (2002). Repellence and toxicity of basil, citronella, ho-sho and rosemary oils for the control of the termite, *Cryptotermes brevis* (Isoptera: Kalotermitidae). Sociobiology, Vol.40, No.3, pp.585-594, ISSN 0361-6525.

Scott, I.M., Jensen, H., Nicol, L., Bradbury, R., Sanchez-Vindas, P., Poveda, L., Arnason, J.T. & Philogene, B.J.R. (2004). Efficacy of piper (Piperaceae) extracts for control of common home and garden insect pests. *Journal of Economic Entomology*, Vol.97, No.4, pp. 1390-1403, ISSN 0022-0493.

Singh, G., Singh, O.P., Prasad, Y.R., de-Lampasona, M.P. & Catalan, C. (2004). Chemical and insecticidal investigations in leaf oil of *Coleus amboinicus* Lour. *Journal of Flavour and Fragrance*, Vol.17, No.6, pp.440-442, ISSN 0882-5734.

Singh, Y., Ranawat, B.S., Verma, R.K. & Nayal, S.S. (2001). Termite control with medicinal plant products. *Journal of Medicinal and Aromatic Plant Sciences*, Vol. 22/23, No.4A/1A, pp.151-153, ISSN 6253-7125.

Singh, M., Lal, K. Singh, S.B. & Singh, M. (2002). Effect of Calotropis (*Calotropis procera*) extract on infestation of termite (*Odontotermes obesus*) in sugarcane hybrid. *Indian Journal of Agricultural Sciences*, Vol.72, No.7, pp.439-441, ISSN 0019-5022.

Su, N.Y., Chew, V., Wheeler, G.S. & Scheffrahn, R.H. (1997). Comparison of tunneling responses into insecticide-treated soil by field populations and laboratory groups of subterranean termites (Isoptera: Rhinotermitidae). *Journal of Economic Entomology*, Vol.90, No.2, pp.503-509, ISSN 0022-0493.

Su, N.Y., Scheffrahn, R.H. & Ban, P.M. (1993). Barrier efficacy of pyrethroid and organophophate formulations against subterranean termites (Isoptera: Rhinotermitiae). *Journal of Economic Entomology*, Vol.86, No.3, pp.772-776, ISSN 0022-0493.

Supriadi and Ismanto, A. (2010). Potential Use of Botanical Termiticide. *Perspektif*, Vol.9, No.1, pp. 12-20. ISSN: 1412-8004.

Umeh, V.C. & Ivbijaro, M.F. (1999). Effect of termite damage to maize of seed extracts of *Azadirachta indica* and *Piper guineense* in farmers' field. *Journal of Agricultural Science*, Vol.133, No.4, pp.403-407, ISSN 0021-8596.

Verma, R.K. & Verma, S.K. (2006). Phytochemical and termiticidal study of *Lantana camara* var. aculeata leaves. *Fitoterapia*, Vol.77, No.6, pp.466-468, ISSN 0367-326X.

Watanabe, Y., Mitsunaga, T. & Yoshimura, T. (2005). Investigating antitermitic compounds from Australian white cypress heartwood (*Callitris glaucophylla*) against *Coptotermes formosanus* Shiraki. *Journal of Essential Oil Research*, Vol.17, No.3, pp.346-350, ISSN 1041-2905.

Wheeler, D. A. & Isman, M. (2001). Antifeedant and toxic activity off *Trichilia americana* extract against the larvae of *Spodoptera litura*. *Entomologia Experimentalis et Applicata*, Vol.98, pp.9-16, ISSN 0013-8703.

Yashroy, R.C. & Gupta, P.K. (2000). Neem-seed oil inhibits growth of termite surface-tunnels. *Indian Journal of Toxicology*, Vol.7, No.1, pp.49-50, ISSN 0971-6580.

Zhao, B., Grant, G.G., Langevin, D. & MacDonald, L. (1998). Deterring and inhibiting effects of quinolizidine alkaloids on the spruce budworm (Lepidoptera: Tortricidae) oviposition. *Environmental Entomology*, Vol.27, pp. 984-992, ISSN 0046-225X.

Zhu, B.C.R., Henderson, G., Chen, F., Fei, H. & Laine, R.A. (2001a). Evaluation of vetiver oil and seven insect active essential oils against the Formosan subterranean termite. *Journal of Chemical Ecology*, Vol. 27, No.8, pp.1617-1625, ISSN 0098-0331.

Zhu, B.C.R., Henderson, G., Chen, F., Maistrello, L. & Laine, R.A. (2001b). Nootkatone is a repellent for Formosan subterranean termite (*Coptotermes formosanus*). *Journal of Chemical Ecology*, Vol. 27, No.3, pp.523-531, ISSN 0098-0331.

4

Botanical Insecticides and Their Effects on Insect Biochemistry and Immunity

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1. Introduction

Some concerns, especially environmental ones, lead the researchers to find new avenues of insect control in agriculture. Considering negative effects of synthetic pesticides especially on non-target organisms caused a general perception that natural compounds are better products or Generally Regarded As Safe (GRAS) (Scott et al., 2003). So, researches has been concentrated on the plant kingdom for solutions leading to the production of a myriad of secondary compounds that can have toxic, growth reducing, and antifeedant properties against insects (Berenbaum & Zangerl, 1996). The use of plant extracts (botanical insecticides) to protect crops and stored products is as old as crop protection. Indeed, prior to the development and commercial success of synthetic insecticides beginning in the 1940s, botanical insecticides were major weapons in the farmer's arsenal against crop pests (Isman, 2008). Four major types of botanical insecticides are being used for insect control including pyrethrum, rotenone, neem, and essential oils along with three others in limited use (Isman, 2006).

Pyrethrum is an oleoresin extracted from the dried flowers of the pyrethrum daisy, *Tanacetum cinerariaefolium* (Asteraceae) that its active ingredients are three esters of chrysanthemic acid and three esters of pyrethric acid (Isman, 2006). The insecticidal action of the pyrethrins is characterized by a rapid knockdown effect, particularly in flying insects, and hyperactivity and convulsions in most insects. These symptoms are the result of the neurotoxic action of the pyrethrins, which block voltage-gated sodium channels in nerve axons. Azadirachtin is an extraction from Indian neem tree, *Azadirachta indica*has that has two profound effects on insects (Schmutter, 2002). Azadirachtin, apart from its antifeedant effects on insects, inhibited the synthesis and release of ecdysteroids from the prothoracic gland resulting incomplete ecdysis in immature insects and sterility in adult females (Isman, 2006). Rotenone is a type of isoflavonoids extracted from the roots or rhizomes of the tropical legumes like *Derris*, *Lonchocarpus*, and *Tephrosia* (Isman, 2006). Rotenone is a mitochondrial poison by blocking the electron transport chain leading to inhibition of energy production (Hollingworth et al., 1994). Acetogenin extracts from seeds of *Annona squamosa* known as annonin I, or squamocin, and a similar compound, asimicin were isolated from the bark of the American pawpaw tree, *Asimina triloba* (Johnson et al., 2000). Although, there are many plant extracts widely use against insects but here one of them, *Artemisia*, is discussed. The genus Artemisia is a member of a large plant family Asteracea

(Compositae) encompassing more than 300 different species of this diverse genus. Several isolated compounds from this species have shown anti-malarial, antibacterial, anti-inflammatory, plant growth regulatory and cytotoxicity (antitumor) activities (Akhtar and Isman, 2004).

2. Effect of botanical insecticides on digestive enzymes of insects

Digestion is a process in which ingested macromolecules by insects break down to smaller ones to be absorbable via epithelial cells of midgut. Several enzymes based on food materials have critical roles in this process. Any disruption in their activity disables insects to provide their nutrients for biological requirements. Several studies demonstrated the effect of botanical insecticides on feeding parameters of insects by demonstrating food consumption [CR = I/DT], approximate digestibility of consumed food [%AD = 100(I–F)/I], efficiency of converting the ingested food to body substance [%ECI = 100 G/I], efficiency of converting digested food to body substance [%ECD = 100G/(I–F)] and consumption index [CI = I/W] (Shekari et al., 2008). The fact underlying these changes is inhibitory effects of botanical insects on digestive enzymes (Zibaee and Bandani, 2010a).

Starch in plants and glycogen in animals are the storage carbohydrates that amylases are necessary to digest them in herbivorous and carnivorous insects, respectively. *a*-Amylases (EC 3.2.1.1) catalyze the endohydrolysis of long a-1,4-glucan chains such as starch and glycogen (Terra and Ferriera, 2005). Saleem & Shakoori (1987) showed that sublethal concentrations of pyrethroids decreased the *a*-amylase activity in the larval gut of the beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae). Shekari et al. (2008) demonstrated that a-amylase activity level in elm leaf beetle treated by *A. annua* extract decreased after 24 h and sharply increased after 48 h. Zibaee & Bandani (2010a) showed that *Artemisia annua* extract caused the reduction of a-amylase activity in Eurygaster integriceps Puton (Hemiptera: Scutelleridae), and this reduction increased by higher concentrations of plant extract.

After amylase, glycosidases digest carbohydrate oligomers to monosaccharides (Terra and Ferriera, 2005; Zibaee et al., 2008a; Zibaee et al., 2009a). On the other hands, glycosidases catalyze the hydrolysis of terminal, non-reducing 1, 4-linked alpha-Dglucose residues with releasing of alpha-D-glucose. Treating the adults of *E. integriceps* by different concentrations of *A. annua* extract showed the reduction in the activity of α- and β-glucosidases so that increasing of plant extract concentrations enhanced the enzyme inhibition that emphasais disruption of consumption rates and food conversion efficiencies (Zibaee & Bandani, 2010a). Hemmingi & Lindroth (1999, 2000) determined the effect of phenolic components on gypsy moth (Lepidoptera, Lymantriidae) and forest tent caterpillar (Lepidoptera, Lasiocampidae), founding reduction of the glucosidase activities in both treated larvae.

lipases (EC 3.1.1) are enzymes that preferentially hydrolyze the outer links of fat molecules and have been studied in few insects. Although, there a few studies on insect digestive lipases but the enzyme activity significantly changes due to using botanical insecticides. Senthil Nathan et al. (2006) showed that treating *Cnaphalocrocis medinalis* (Guenee) (Lepidoptera: Pyralidae), the rice leaffolder, with Btk, NSKE and VNLE (azadirachtin and neem components) sharply decreased the activity level of lipase in the midgut. Zibaee et al. (2008b) found inhibition of lipase activity in the midgut of *Chilo suppressalis* Walker (Lepidoptera: Pyralidae) when they add *A. annua* extract to enzyme samples *in vitro*. Zibaee & Bandani (2010a) found similar results when adults of *E. integriceps* fed on food containing *A. annua* extract.

Proteases have a crucial role in food digestion by insects. Different types of proteases are necessary to do this because the amino acid residues vary along the peptide chain (Terra & Ferriera, 2005). There are three subclasses of proteinases involved in digestion classified according to their active site group (and hence by their mechanism): serine, cysteine, and aspartic proteinases. The oligopeptides resulting from proteinase action are attacked from the N-terminal end by aminopeptidases and from the C-terminal end by carboxypeptidases. Studies by Johnson et al. (1990), Senthil-Nathan et al. (2006) and Zibaee and Bandani (2010a) inferred that Botanical insecticides may interfere with the production of certain types of proteases and disable them to digest ingested proteins.

Zibaee et al. (2010) investigated the sole and combined effect of *A. annua* and *Lavandula stoechas* on digestive enzyme activity in *Hyphantria cunea* Drury (Lepidoptera: Arctiidae) (Table 1 and 2). *A. annua* treatment decreased digestive enzyme activities in larvae feed on both mulberry and sycamore in a dos-related manner. Also, treatment of leaves by *L. stoechas* demonstrated a slightly decrease on digestive enzymes except for protease and lipase. However, the effect of L. stoechas extracts on enzyme activities on sycamore was more with regard to mulberry.

Treatment (%)	Esterase		Glutathuion *S*-transferase		Acethylcholinesterase	Alkaline phosphatase	Acid phosphatase
	α-naphtyl	*β*-naphtyl	CDNB	DCNB			
Control	3.76±0.062a	3.34±0.035a	2.82±0.036a	2.84±0.036a	7.56±0.027a	4.92±0.024a	3.93±0.018a
10	4.11±0.021b	3.50±0.012a	2.85±0.022b	2.92±0.022b	7.32±0.020b	4.91±0.020b	3.88±0.046b
15	4.27±0.083b	3.86±0.048ab	3.17±0.031bc	3.27±0.031bc	6.01±0.052c	5.11±0.034c	4.15±0.026c
25	4.75±0.095c	4.22±0.055b	3.49±0.025c	3.45±0.025c	5.31±0.031d	5.46±0.027c	4.35±0.021c

Table 1. Effect of *A. annua* extract on detoxifying enzyme of *E. integriceps* hemolymph after 24 h.

Zibaee & Bandani (2010a) performed analysis of Lineweaver-Burk plots to provide information regarding the mode of action of *A. annua* extract against *E. integriceps* digestive enzymes. In the majority of enzymes, the presence of the plant extract decreased the value of Vmax and increased Km. Since Km has an inverse relationship with the substrate concentration required to saturate the active sites of the enzyme, this indicates decreased enzyme affinity for the substrate (Wilson & Goulding, 1986). In other words, Km is the measurement of the stability of the enzyme-substrate complex and a high Km would indicate weak binding while a low Km would indicate strong binding (Stryer, 1995). The effect of *A. annua* extract on Vmax showed that it interferes with the rate of break down of the enzyme-substrate complex. Thus, the plant extracts inhibit the enzymes by increasing Km and decreasing affinity of the enzyme to substrate. Plant extracts also diminished the Vmax value, which further indicates that they interfered with the rate of breakdown of the enzyme-substrate complex (Morris, 1978). These results showed a mixed inhibition of plant extract on the enzyme activities of the Sunn pest. In this type of inhibition, plant extracts can bind to the enzyme at the same time as the enzyme binds to the substrate, and this binding affects the binding of the substrate and vice versa (Stryer, 1995; Zibaee and Bandani, 2010a). Although it is possible for mixed-type inhibitors to bind in the active site, this type of inhibition generally results from an allosteric effect, where the inhibitor binds to a different site on an enzyme. Inhibitor binding to this allosteric site changes the conformation (i.e. tertiary structure e or three-dimensional shape) of the enzyme so that the affinity of the substrate for the active site is reduced (Morris, 1978; Stryer, 1995; Zibaee and Bandani, 2010a).

Treatment[1]	*a*-amylase		*a*-Glucosidase		*β*-Glucosidase		Protease		Lipase	
	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore
Control	1.87±0.09a	1.75±0.28a	2.05±0.54a	1.90±0.4a	3.88±1.03a	2.67±0.28a	3.80±0.00a	2.36±0.00a	3.43±0.00a	3.34±0.00a
LD_{10}	1.44±0.05b	1.69±0.05a	1.39±0.10b	1.55±0.27b	2.48±0.07c	2.50±0.39b	3.16±0.00ab	1.62±0.00ab	2.79±0.00b	2.61±0.00ab
LD_{30}	1.13±0.00c	1.19±0.04ab	1.24±0.28b	±0.950.09c	1.39±0.14c	1.55±0.21c	1.88±0.00b	0.80±0.00b	2.00±0.00c	2.01±0.00b
LD_{50}	0.84±0.06c	0.99±0.03b	0.52±0.19c	0.14±0.08d	0.28±0.49d	1.25±0.31d	0.76±0.00c	0.50±0.00c	1.47±0.00d	0.56±0.00c

[1]. Concentrations of plant extract are 0.09, 0.22 and 0.42 on mulberry and 0.13, 0.28 and 0.48 on sycamore as . LD_{10}, LD_{30} and LD_{50}.

[2]. Means (SEM±) followed by the same letters above bars indicate no significant difference ($p < 0.05$) according to the Tukey test.

Table 3. Effect of *Artemisia annua* extract on the digestive enzymes profile (*μmol/min/mg* protein) of *Hyphantaria cunea* larvae in the presence of two different host.

Treatment[1]	*a*-amylase		*a*-Glucosidase		*β*-Glucosidase		Protease		Lipase	
	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore	Mulberry	Sycamore
Control	2.08±0.01a	1.97±0.03a	2.20±0.20a	1.49±0.91a	2.89±0.33a	2.61±0.21a	3.64±0.00a	3.51±0.00a	3.25±0.00a	2.77±0.02a
LD_{10}	2.05±0.03a	1.61±0.02b	1.77±0.65b	1.62±0.23	2.42±0.68a	2.46±0.48a	3.65±0.00a	3.43±0.00a	3.18±0.00a	2.49±0.00a
LD_{30}	1.89±0.03b	1.38±0.08b	2.37±0.74a	1.53±0.30a	2.71±0.12a	1.79±0.70a	3.42±0.00a	3.20±0.00a	2.91±0.00a	2.42±0.00a
LD_{50}	1.71±0.02b	1.11±0.05c	2.15±0.75a	1.54±0.34a	2.45±0.23a	1.41±0.23b	3.39±0.00a	3.25±0.00a	2.69±0.00a	2.38±0.00a

[1]. Concentrations of plant extract are 0.02, 0.11 and 0.32 on mulberry and 0.13, 0.38 and 0.79 on sycamore as . LD_{10}, LD_{30} and LD_{50}.

[2]. Means (SEM±) followed by the same letters above bars indicate no significant difference ($p < 0.05$) according to the Tukey test.

Table 4. Effect of *Lavandula stoechas* extract on the digestive enzymes profile (*μmol/min/mg* protein) of *Hyphantaria cunea* larvae in the presence of two different host.

3. Botanical insecticides and detoxifying enzymes

Four types of detoxifying enzymes have been found to react against botanical insecticides including general esterases (EST), glutathione *S*-transferase (GST) and phosphatases.

Esterase (EST) is an important detoxifying enzyme *in vivo* which hydrolyzes the esteric bond in synthetic chemicals. Also, esterase is one of the enzymes showing the strongest reaction to environmental stimulation (Hemingway & Karunatne 1998). The responses of EST to botanical insecticides were significantly due to using different concentrations of extract and long exposure. In the early stage, plant extract stimulated the expression of EST body to increase the detoxification ability (Zibaee and Bandani, 2010b). In the late stage, because of a toxic effect and time EST activity was suppressed.

Glutathione *S*-transferases (GST) are the mainly cytosolic enzymes that catalyze the conjugation of electrophile molecules with reduced glutathione (GSH), potentially toxic substances become more water soluble and generally less toxic (Grant and Matsumura 1989). GSTs play an important role in insecticide resistance and are involved in the metabolism of organophosphorus and organochlorine compounds (Zibaee et al., 2009b). Other xenobiotics such as plant defence allelochemicals against phytophagous insects induce GST activity (Yu, 1982; Vanhaelen *et al.* 2001). By treating *A. annua* extracts on *E. integriceps* adults, Zibaee and Bandani (2010) reported that activity level of GST in 24 h post-treatment increased significantly for both substrates (CDNB, DCNB) of the enzyme. Its (two or one) activity was dose-dependent and increased by exposuring higher concentration of plant extract. Vanhaelen *et al.* (2001) showed that Brassicacea secondary metabolites induced GST activity in *Myzus persicae* and several Lepidopteran species such as *Heliothis virescens* Fabricius, *Trichoplusia ni* Hubner and *Anticarsia gemmatalis* Hubner. The influence of plant

allelochemicals on GST activity is not limited to the herbivores and was observed in several predators, too (Francis *et al.* 2000).

Alkaline phosphatase (ALP, *E.C.3.1.3.1*) and acid phosphatase (ACP, *E.C.3.1.3.2*) are the hydrolytic enzymes, which hydrolyze phosphomonoesters under alkaline or acid conditions, respectively. ALP is primarily found in the intestinal epithelium of animals and its major function is to provide phosphate ions from mononucleotide and ribonucleoproteins for a variety of metabolic processes. ALP is involved in the transphosphorylation reaction and the midgut has the highest ALP and ACP activity as compared to other tissues (Sakharov *et al.* 1989). The overall activity of ALP and ACP decreased due to increasing of plant extract concentrations so that there were significant differences among control and three treatments. These findings coincided with other reports of plant extract treatments of insects. For example, Senthil Nathan (2006) showed that treatment of rice plants with *Melia azedarach* Juss (Meliaceae) extracts decreased the activity level of ALP in *Cnaphalcrocis medinalis* (Guenee). These authors reported that feeding *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) on *Ricinus communis* L. treated with azadirachtin decreases the amount of this enzyme in the midgut (Senthil Nathan & Kalaivani 2005). Changes in ALP and ACP activities after treatment with *A. annua* extract indicating changes of the physiological balance in the midgut.

4. Botanical insecticides and acethylcholine esterase (AChl)

AChE is a key enzyme that terminates nerve impulses by catalyzing the hydrolysis of neurotransmitter, acetylcholine, in the nervous system of various organisms (Oehmichen & Besserer 1982; Grundy & Still 1985; Wang *et al.* 2004). Zibaee and Bandani (2010b) demonstrated that *A. annua* extract inhibited the AChE activity in higher doses which coincided with other reports about effect of botanical insecticides on AChE inhibition. The alteration of AChE was observed in the cockroach, *Periplaneta americana* L., at 4 ppm of AZA, (Shafeek *et al.* 2004) and the snail, *Limnaea acuminate* Lamarck, at 40% and 80% concentrations of neem oil (Singh & Singh 2000). It was also observed that 25 g of distilled water extracts of the botanicals *Punica granatum* L., *Thymus vulgaris* L., and *Artemisia absinthium* L., significantly inhibited the AChE activity of nematodes at 100% concentrations (Korayem *et al.* 1993). Senthil Nathan *et al.* (2008) demonstrated that LC50 concentrations of AZA significantly inhibited the activity of AChE compared with control.

5. Botanical insecticides and insect immunity

5.1 Introduction

Similar to vertebrates, insects have a capable immunity against microbial infections exposing in their environment. This immunity based on involved components known as cellular and humeral defenses (Beckage, 2008). Cellular immunity consists phagocytosis of aggressive microorganisms by hemocytes, nodule formation and encapsulation. Humoral responses comprises factors related to the recognition of invading microorganisms, melanization and coagulation as well as killing factors such as antimicrobial peptides (AMPs), reactive oxygen species and reactive nitrogen intermediates, including nitric oxide, prostaglandins and eicosanoids (Boman, 1998; Stanley, 2006; Beckage, 2008).

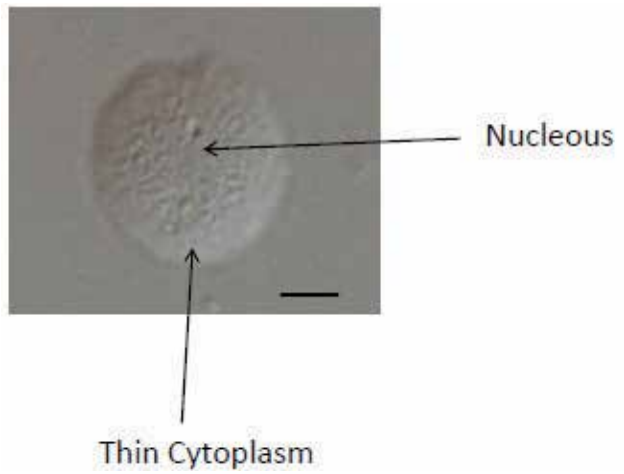
Mentioned immune reactions are initiated by pattern recognition molecules allowing insects to distinguish self-components from nonself-ones. Studies have been identified specific

pattern recognition receptors responding to components in microorganisms such as peptidoglycans and lipopolysaccharides that are main compounds in the cell walls of bacteria and fungi (Theopold et al., 1999; Dziarski, 2004). Peptidoglycan recognition proteins (PGRPs) have been identified in several insect species as activating cascade of melanization on invasive microorganisms (Rolff & Reynolds, 2010). There are specific PGRPs for gram-positive, gram-negative and fungi in the hemolymph of insects. Two signaling pathways namely *Toll* and *Imd* have been activated after recognition of gram-positive microorganisms and fungi as well as gram-negative ones, respectively (Rolff & Reynolds, 2010). These signaling pathways lead to activation of cellular immunity and antimicrobial peptides via final Dif and Relish molecules in nucleus of hemocytes (Tzou et al., 2002; Leihl et al., 2006).
Different environmental factors can definitely affect immune reactions of insects that elucidation of these factors is a significant part to clarify various aspects of these mechanisms. Temperature, different ions and insecticides are some of the most important affecting factors (Zibaee et al., 2009c). In agriculture, combined tactics (as Integrated Pest management) are used to obtain efficient control of insects by considering the lowest disruption in environment. Several studies have been conducted to find combined effect of insecticides, highlighted by botanical materials, and microbial agents on insects. Results revealed that botanical compounds decrease immune ability of insects against microbial agents that describes in forward sections.

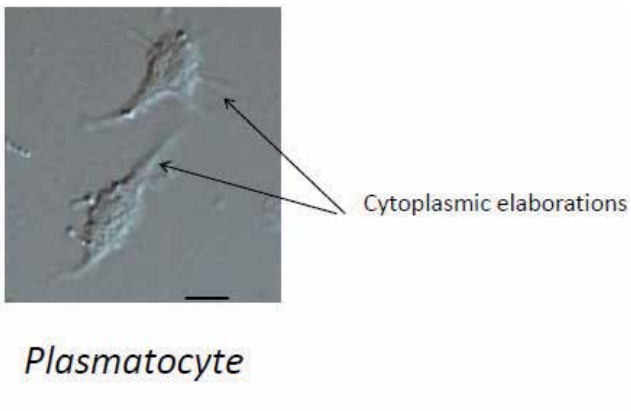
5.2 Effect of botanical insecticides on morphology, number of hemocytes and Phagocytosis

In insect immunity, circulating hemocytes have crucial roles in both cellular mechanisms and producing antimicrobial components. Five basic types of hemocytes have been identified as prohemocytes, plasmatocytes, granulocytes, adipohemocytes and oenocytoids (Lavine & Strand, 2002). Prohemocytes as the smallest one are the basic hemocytes that developed to plasmatocytes and granulocytes when an infectious challenge appeared in the hemolyph. They recognized as large central nucleus and narrow cytoplasm (Lavine & Strand, 2002; Zibaee & Bandani, 2010a) (Figure 2a). Plasmatocytes and granulocytes are the important hemocytes in immune responses to pathogens via phagocytosis (Granulocytes and relatively Plasmatocytes), nodule formation and encapsulation (Strand, 2008). They discriminate each other by spindle shape of plasmatocytes and rounded granulus granulocytes (Figure 2b and c). Adipohemocytes contain lipid droplets so some literature consider them as fat bodies instead of hemocyte (Figure 2d) (Beckage, 2008). Oenocytoids have two specific shape based on intact and immune challenged insects. In normal situation, oenocytoids are spherical cells with peripheral nucleus and crystalline inclusions without any granules (Figure 2e). When an immune challenge occurred, nucleus is going to be smaller and granules appear showing their crucial roles in phenoloxidase[1] (PO) cascade (Strand, 2008; Beckage, 2008).
Different environmental factors could affect insect hemocytes both morphologically and functionally. For example, elevation of environmental temperature increases numbers of plasmatocytes and granulocytes up to 30-40 °C in addition their nodulation ability (Zibaee et al., 2009). Also, different divalent cations have positive effect on hemocytes to provide a cellular network entrapping pathogens in the hemolymph (Willot et al., 2002; Willot and Tran, 2002; Zibaee et al., 2009c) (Figure 3).

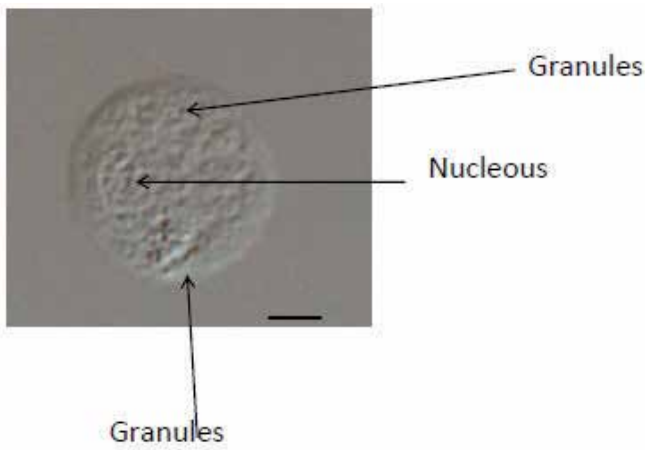
[1] Phenoloxidases have crucial role in immune recognition pathways and melanization of nodules and capsules around an pathogens.


Nucleous
Thin Cytoplasm

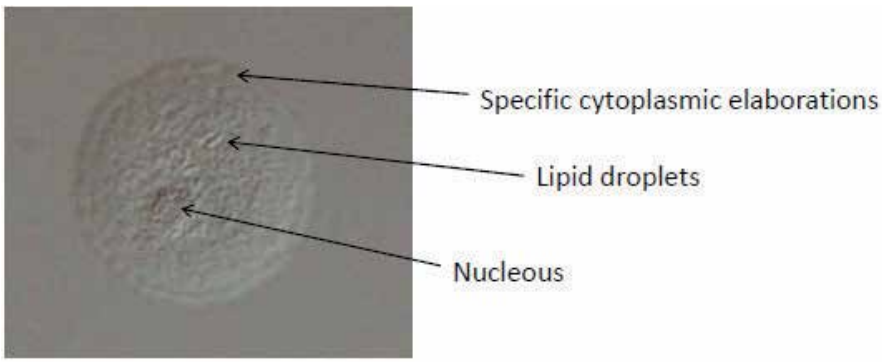
(a)


Cytoplasmic elaborations
Plasmatocyte

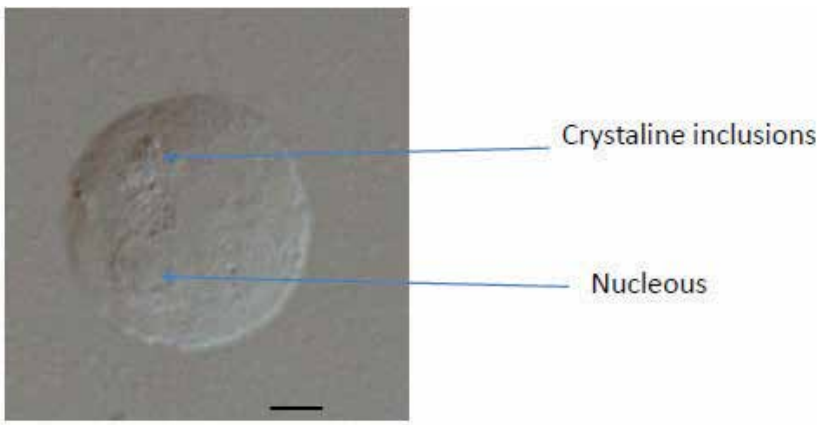
(b)


Granules
Nucleous
Granules

(c)



(d)



(e)

Fig. 2. (a-e) Light microscopy of *E. integriceps* hemocytes. (a) A prohemocyte with a large nucleus (thin arrow) and a thin cytoplasm (b) A plasmatocyte exhibiting a spindle shape and cytoplasmic elaborations. (c) A granulocyte filled with the typical granules in the cytoplasm (arrow) and large nucleus (arrow). (d) An adipohemocyte with lipid droplets spreading in the cytoplasm and specific cytoplasm elaborations (e). An oenocytoid with a round eccentric nucleus and crystalin inclusions. Magnification 40X with the exception of (b) (60X). Bar= 50 µm, with the exception of (b), 33 µm. (Zibaee, A., Bandani, A. R., Talaei-Hassanlouei, R. & Malagoli, D. et al., Unpublished data).

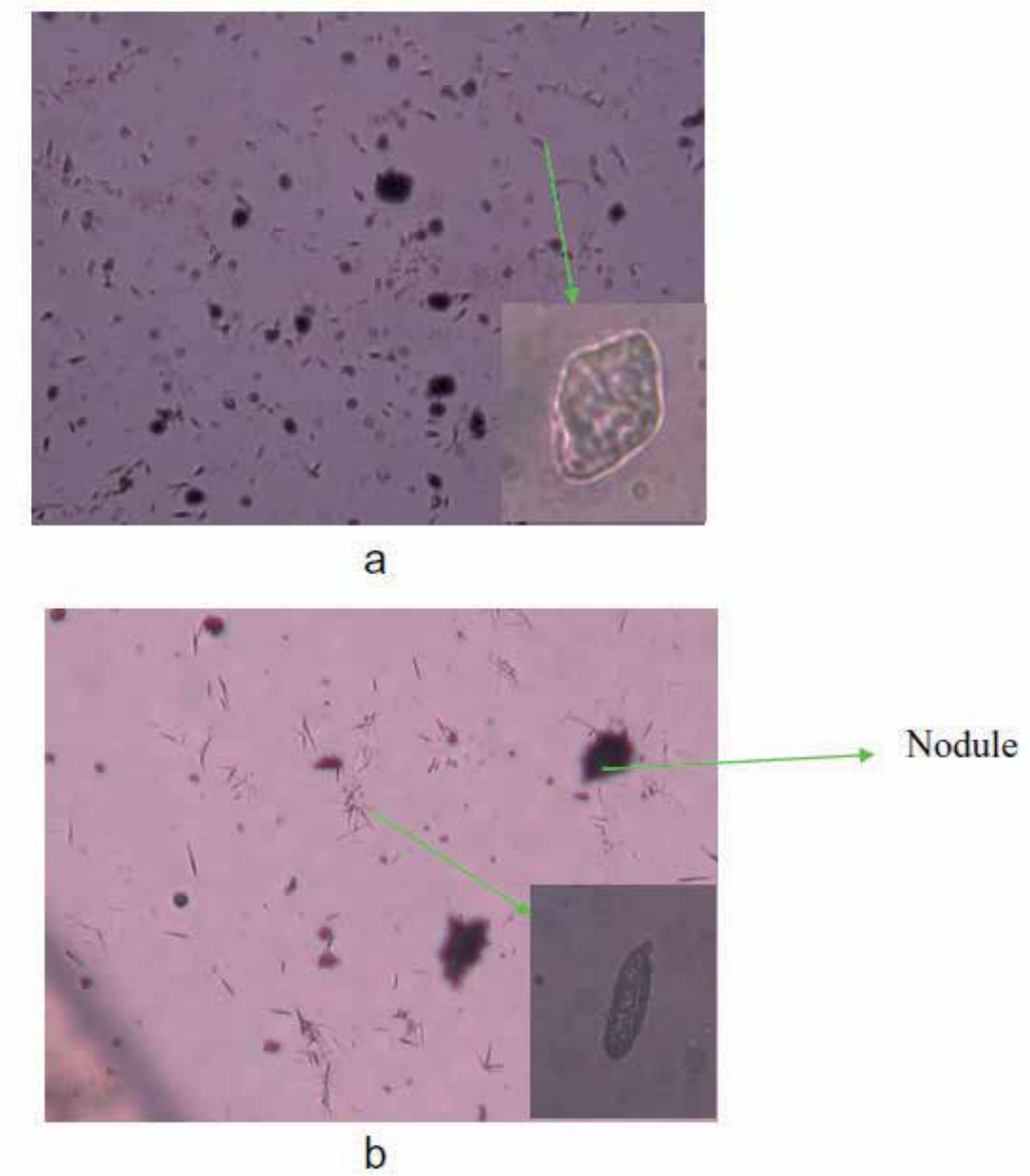


Fig. 3. Phase contrast microscopy of plasmatocytes incubated 12 h by calcium. (a) Control plasmatocytes without incubation by calcium. (b) plasmatocytes incubated by 5 mM concentration of calcium (Zibaee et al., 2009c; Entomological Research; Wiley-Blackwell publishing).

In addition of these positive factors on hemocytes of insects, several other factors, mainly insecticides, have negative effects on number and morphology of them (Figure 4). There are some reports on effects of plant products on the hemocytes such as *Periplaneta americana* L. (Blattodea: Blattidae) (Qadri & Narsaiah, 1978), *Dysdercus koenigii* Fabricius (Hemiptera: Pyrrhoeoridae) (Saxena & Tikku, 1990; Tikku et al., 1992), *Cyrtacanthacris tatarica* L.

(Orthoptera: Acrididae) (Peter & Ananthakrishnan, 1995) and *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) (Sharma et al., 2001, 2003, 2008). Studies by scan electron microscopy demonstrated the complete loss of filopods in plasmatocytes and cytoplasmic projections in granular hemocytes of *S. litura* larvae treated with Neem gold (Sharma et al., 2003). Sharma et al. (2008) also find similar results on effect *Artemisia calamus* oil on larvae of *S. litura* as loss of cytoplasmic projections in granular hemocytes. Interestingly, they observed vacuolization in the cytoplasm and degeneration of the organelles, both in plasmatocytes and granular hemocytes (Sharma et al., 2003). So, it was concluded that rapid degeneration of granular hemocytes, initiated by vacuolization and loss of firmness of organelles leading to degranulation and a degenerative transformation within a period of 48 h, subsequently resulting the total collapse of immunity-building mechanism of *S. litura* (Sharma et al., 2008).

Atemisia annua extract altered number of hemocytes and their phagocytic activity in *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae). Zibaee & Bandani (2010a) reported that treatment of *E. integriceps* with *A. annua* extract affected the total number of hemocytes circulating in the hemolymph indicating that the responses could be due to the toxic effect on the immune cells reducing number of hemocytes attached to fungal spores. Meanwile, an extremely low phagocytic activity was observed in these bioassay experimental groups. Since the attachment of fungal spores to the hemocyte surface is an essential prerequisite to the triggering of phagocytic responses, suggesting that the cellular activity or recognition of spore by hemocyte receptors may be compromised in the hemocytes of insects treated with *A. annua*. Phagocytosis of microbial cells involves interactions between lections on phagocytic cells and sugars on microbial surfaces (Nayar & Knight, 1997). Since *A. annua* extracts suppress phagocytosis (and also nodule formation and PO activity) at different concentrations, this suggests that it may interfere with the ligend-receptor interactions that are likely to occur at the plasma membrane of specific hemocytes because the majority of interactions between cellular and humeral components of the insect immune system are receptor-mediated (Ratcliffe & Rowley, 1987). Therefore, plant extracts at the sub-lethal levels might be enough to interfere with the function of specific receptors, e.g. b-1,3-glucan-specific protein of many insect-species hemocytes, or cause ultrastructural alteration which interfere with normal hemocyte function (Vey et al., 2002).

Garcia et al. (2006) reported significantly higher numbers of *Trypanosoma rangeli* in the hemolymph of *Rodnius prolixus* L. (Hemiptera: Reduviidae) fed on blood containing physalin B at days 2, 4, and 6 post-injection in contrast to that observed in the control. In fact, their data supported the hypothesis that physalin B is an immunomodulator to *T. rangeli* challenge in *R. prolixus*. They concluded four main points for verifying this hypothesis. (i) Mortality of *R. prolixus* in response to common parasite challenge was expressed in a concentration-dependent way in insects treated with concentrations ranging from 0.01 to 1 μg of physalin B/ml of blood meal the idea was supported by Zibaee & Bandani (2010a). (ii) The death rate was significantly enhanced in insects that received concentrations of 0.1 and 1 μg of physalin B and were infected with flagellates. (iii), the hemocyte microaggregation response and nitrite/nitrate concentration, which represent metabolic products of nitric oxide reactions and RNI metabolism against *T. rangeli* infection, was significantly reduced in the hemolymph of insects treated with physalin B (0.1 μg /ml) when compared with infected untreated controls. (iv) The number of parasites in the hemolymph of treated-insects was significantly higher than that observed in insects feeding on blood without physalin B. Based on these results, they proved that physalin B is a

regulator of microaggregation and nitric oxide reactions to parasite challenge in 5th-instar *R. prolixus* larvae (Garcia et al., 2006).

5.3 Effect of botanical insecticides on nodule formation and phenoloxidase activity

PO enzymes in hemolymph that have tyrosinase-like activity can hydroxylate tyrosine (*EC* 1.14.18.1) and also can oxidize *o*-diphenols to quinones (*EC* 1.10.3.1) (Gorman et al., 2007) so called *o*-phenoloxidases. The quinones produced by PO undergo a series of additional enzymatic and non-enzymatic reactions leading to polymerization and melanin synthesis in the final stages of nodulation and encapsulation against invading microorganisms (Zibaee et al., 2011). In fact, insect PO are synthesized as zymogens called pro PO which are activated by proteolytic cleavage at a specific site in response to infection or wounding (Cerenius & Söderhall, 2004). Active PO catalyzes the formation of quinones, which undergoes further reactions to form melanin (Cerenius and Söderhäll, 2004; Gorman et al., 2007). Zibaee & Bandani (2010a) showed the negative effect of *A. annua* extract on nodule formation and phenoloxidase activity of *E. integriceps* (table 2 and 3). Lineweaver-Burk plots analysis of PO activity after treating insects by plant extract revealed an inhibition on enzyme activity by decreasing V_{max} value and increasing K_m one. Since the K_m has an inverse relationship with the substrate concentration required to saturate the active sites of the enzyme, this indicates decreased enzyme affinity for substrate (Zibaee et al., 2011). In other words, K_m is the measurement of the stability of the enzyme-substrate complex and a high K_m would indicate weak binding and a low K_m strong binding. The effect of *A. annua* extract on the V_{max} shows that it interferes with the rate of break-down of the enzyme-substrate complex. Thus, plant extract inhibit the enzymes by increasing the K_m and decreasing affinity of the enzyme to substrate. Plant extract also diminished the V_{max} value which further indicates that they interfere with the rate of breakdown of the enzyme-substrate complex. These results showed a mixed inhibition of plant extract on the enzyme activities of the Sunn pest. In this type of inhibition, plant extract can bind to the enzyme at the same time as the enzyme binds to substrate and this binding affects the binding of the substrate, and vice versa. Although it is possible for mixed-type inhibitors to bind in the active site, this type of inhibition generally results from an allosteric effect where the inhibitor binds to a different site on an enzyme. Inhibitor binding to this allosteric site changes the conformation (i.e., tertiary structure e or three-dimensional shape) of the enzyme so that the affinity of the substrate for the active site is reduced (Zibaee et al., 2011).

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7. References

Akhtar, Y. & Isman, M. B. (2004) Comparative growth inhibitory and antifeedant effects of plant extract and pure allelo- chemicals on some phytophagous insect species, Journal of Applied Entomology 128, 32–38.

Beckage, N. E., 2008. Insect Immunology. Academic press. 348 pp.

Berenbaum, M. R., Zangerl AR. 1996. Phytochemical diversity. Adaptation or random variation? Rec Adv Phytochem 30:1–24.

Boman, H. G. (2003). Antibacterial peptides: Basic facts and emerging concepts. *J. Intern. Med*. 254, 197–215.

Cerenius, L., Soderhall, K., 2004. The prophenoloxidase-activating system in invertebrates. Immunol. Rev. 198, 116–126.

Dziarski, R. (2004) Peptidoglycan recognition proteins (PGRPs). *Molecular Immunology* 40, 877–886.

Garcia, E.S., Castro, D.P., Ribeiro, I.M., Tomassini, T.C., Azambuja, P., 2006. *Trypanosoma rangeli*: effects of physalin B on the immune reactions of the infected larvae of *Rhodnius prolixus*. Experimental Parasitology 112, 37–43.

Gorman, M. J., An, C., Kanost, M. R., 2007. Characterization of tyrosine hydroxylase from Manduca sexta. Insect Biochem. Mol. Biol. 37, 1327–1337.

Hemmingi, J.D.C. & Lindroth, R.L. (1999) Effects of light and nutrient availability on aspen: growth, phytochemistry and insect performance. Journal of Chemical Ecology 26, 1687–1714.

Hemmingi, J.D.C. & Lindroth, R.L. (2000) Effects of phenolic glycosides and protein on Gypsy Moth (Lepidoptera: Lymantriidae) and Forest tent caterpillar (Lepidoptera: Lasiocampidae) performance and detoxication activities. Environmental Entomology 29, 1108–1115.

Hollingworth, R., Ahmmadsahib, K. & Gedelhak, G. McLaughlin J. (1994) Newinhibitors of complex I of the mitochondrial electron transport chain with activity as pesticides. *Biochemical Society and Transgenesis* 22, 230–33.

Isman, M. B. 2006. Botanical insecticides, deterrents and repellents in modern agriculture and an increasingly regulated world. Annual Review of Entomology. 51:45–66.

Isman, M. B. 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world, Ann. Rev. Entomol. 51 (2006) 45–66.

Isman, M. B. 2008. Botanical insecticides: for richer, for poorer. Pest management science. 64: 8-11.

Johnson, D. E., Brookhart, G. L., Kramer, K. J., Barnett, B. D. & McGaughey, W. H. (1990) Resistance to *Bacillus thuringiensis* by the Indian meal moth Plodia interpunctella: Comparison of midgut proteinase from susceptible and resistant larvae. Journal of Invertebrate Pathology 55, 235–244.

Johnson, H. A., Oberlies, N. H., Alali, F.Q. & McLaughlin, J. E. (2000) Thwarting resistance: annonaceous acetogenins as new pesticidal and antitumor agents. In *Biological Active Natural Products: Pharmaceuticals*, ed. SJ Cutler, JG Cutler, pp. 173–83. Boca Raton, FL: CRC Press.

Lavine, M. D. & Strand, M. R. (2002) Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology* 32, 1295–1309.

Liehl, P., Blight, M., Vodovar, N. *et al.* (2006) Prevalence of local immune response against oral infection in a *Drosophila/Pseudomonas* infection model. *PLoS Pathogens* 2, e56.

Nayar, J.K., & Knight, J.W. (1997) Hemagglutinind in *Anopheles quadrimaculatus* strains susceptible and refractory to *Brugia malayi* and their role in the immune response to filarial parasites. Comparative Biochemistry and Physiology 116B, 109–117.

Peter, A.J., Ananthakrishnan, T.N., 1995. Impact of azadirachtin on the haemodynamics of *Cyrtacanthacris tatarica* (Acrididae: Orthoptera). J. Entomol. Res. 19 (4), 285–290.

Qadri, S.S.H., Narsaiah, J., 1978. Effect of azadirachtin on the moulting processes of last instar nymphs of *Periplaneta americana* (L.). Indian J. Exp. Biol. 16, 1141–1143.

Ratcliffe, N.A., & Rowley, A.F. (1987) Insect response to parasites and other pathogens. pp. 271–332 in Soulsby, E.J.L. (Ed.) Immunology, Immunoprophylaxis and Immunotherapy of Parasitic Infections. Boca Raton, FL, USA, CRC Press.

Rolff, J. and Reynolds, S. E. 2010. Insect infection and immunity (evolution, Rcology and Mechanisms). Oxford university press. 254 pp.

Saleem, M. A. & Shakoori, A. R. (1987) Point effects of Dimilin and Ambush on enzyme activies of *Tribolium castaneum* larvae. Pesticide Biochemistry and Physiology 29, 127–137.

Saxena, B.P., Tikku, K., 1990. Effect of plumbagin on hemocytes of *Dysdercus koenigii* F. Proc. Indian Acad. Sci. (Anim. Sci.) 99 (2), 119–124.

Schmutterer, H. (2002) *The Neem Tree*. Mumbai: Neem Found. 892 pp.

Scott, I. M., Jensen, H., Scott, J. G., Isman, M. B., Arnason, J. T., Philogène, B. J. R. 2003. Botanical Insecticides for Controlling Agricultural Pests: Piperamides and the Colorado Potato Beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae). Archives of Insect Biochemistry and Physiology 54: 212–225.

Senthil Nathan, S., Chunga, P.G. & Muruganb, K. (2006) Combined effect of biopesticides on the digestive enzymatic profiles of *Cnaphalocrocis medinalis* (Guenee) (the rice leaffolder) (Insecta: Lepidoptera: Pyralidae). Ecotoxicology and Environmental Safety 64, 382–389.

Sharma, P. R., Sharma, O. P. and Saxena, B. P. 2008. Effect of sweet flag rhizome oil (Acorus calamus) on hemogram and ultrastructure of hemocytes of the tobacco armyworm, *Spodoptera litura* (Lepidoptera: Noctuidae). Micron 39 (2008) 544–551.

Sharma, P.R., Sharma, O.P., Saxena, B.P., 2001. Ultrastructure of the haemocytes of the tobacco armyworm, *Spodoptera litura* Fab. (Lerpidoptera; Noctuidae). Biol. Bratislava 56 (3), 277–285.

Sharma, P.R., Sharma, O.P., Saxena, B.P., 2003. Effect of neem gold on haemocytes of the tobacco armyworm, Spodoptera litura (Fabricius) (Lepidoptera; Noctuidae). Curr. Sci. 84 (5), 690–695.

Shekari, M., Jalali Sendi, J., Etebari, K., Zibaee, A. & Shadparvar, A. (2008) Effects of *Artemisia annua* L. (Asteracea) on nutritional physiology and enzyme activities of elm leaf beetle,*Xanthogaleruca luteola* Mull (Coleoptera: Chrysomellidae). *Pesticide, Biochemistry and Physiology* 91, 66-74.

Stanley, D. W. (2006a). Prostaglandins and other eicosanoids in insects: Biological significance. *Annu. Rev. Entomol.* 51, 25–44.

Strand, M. (2008) The insect cellular immune response. *Insect Science* 15: 1-14.

Terra, W. R., Ferriera, C., 2005. Biochemistry of digestion. In: Comprehensive molecular insect science by Lawrence I. Gilbert, Kostas Iatrou, and Sarjeet S. Gill, volum 3. Elsevier. Pp 171-224.

Theopold, U., Rissler, M., Fabbri, M., Schmidt, O. & Natori, S. (1999) Insect glycobiology: a lectin multigene family in *Drosophila melanogaster*. *Biochemistry Biophysics Research Community* 261, 923-927.

Tikku, K., Saxena, B.P., Satti, N.K., Suri, K.A., 1992. Plumbagin-induced ultrastructural haemocytic response of Dysdercus koenigii (F.). Insect Sci. Appl. 13 (6), 787–791.

Tzou, P., Reichhart, J.M., and Lemaitre, B. (2002) Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immuno-defi cient *Drosophila* mutants. *Proceedings of the National Academy of Sciences USA* 99, 2152–2157.

Vey, A., Matha, V. & Dumas, C. (2002) Effects of the peptide mycotoxin destruxin E on insect haemocytes and on dynamics and efficiency of the multicellular immune reaction. Journal of Invertebrate Pathology 80, 177–187.

Willot, E. and Tran, H. Q. 2002. Zinc and *Manduca sexta* hemocyte functions. Journal of Insect Science. 2.6. Available online: insectscience.org/2.6.

Willott E, Hallberg CA, Tran HQ (2002) Influence of Ca^{2+} on *Manduca sexta* Plasmatocyte Spreading and Network Formation. *Archives of Insect Biochemistry an Physiology* 49: 187– 202.

Zibaee, A. & Bandani, A. R. (2010a) Effects of *Artemisia annua* L. (Asteracea) on digestive enzymes profiles and cellular immune reactions of sunn pest, *Eurygaster integriceps* (Heteroptera: Scutellaridae), against *Beauvaria bassiana*. *Bulletin of Entomological Research* 100, 185-196.

Zibaee, A., Bandani, A. R. & Ramzi, S. (2008b) Lipase and invertase activities in midgut and salivary glands of *Chilo suppressalis* (Walker) (Lepidoptera, Pyralidae), rice striped stem borer. *Invertebrate Survival Journal* 5, 180-189.

Zibaee, A., Bandani, A. R. & Ramzi, S. (2009a) Characterization of *a* and *β*-glucosidases in midgut and salivary glands of *Chilo suppressalis* Walker (Lepidoptera: Pyralidae), rice striped stem borer. *Comptes Rendus Biologies* 332, 633–641.

Zibaee, A., Bandani, A. R., Talaei-Hassanlouei, R. & Malagoli, D. (2009c) Temperature and Ca2 +ion as modulators in cellular immunity of the Sunn pest *Eurygaster integriceps* Puton (Heteroptera: Scutelleridae). *Entomological Research* 39, 364–371.

Zibaee, A., Bandani, A. R., Kafil, M. & Ramzi, S. (2008a) Characterization of *a*-amylase in midgut and salivary glands of *Chilo suppressalis* Walker (Lepidoptera: Pyralidae), rice striped stem borer. *Journal of Asia-Pacific Entomology*. 11, 201-205.

Zibaee, A. & Bandani, A. R. (2010b) A study on the toxicity of the medicinal plant, *Artemisia annua* L. (AStracea) extracts the Sunn pest, *Eurygaster integriceps* Puton (Heteroptera: Scutelleridae). *Journal of Plant Protection Research* 50, 48-54.

Zibaee, A., Sendi, J., Alinia, F., Ghadamyari, M. & Etebari, K. (2009b) Diazinon resistance in different selected strains of *Chilo suppressalis* Walker (Lepidoptera: Pyralidae), rice striped stem borer, in the north of Iran. *Journal of Economic Entomology* 102, 1189-1196.

Zibaee, I., Bandani, A. R., Sendi, J. J., Talaei-Hassanlouei, R. & Kouchaki, B. (2010) Effects of *Bacillus thurengiensis* var. *kurstaki*, and medicinal plants (*Artemisia annua* L.) and (*Lavandula stoechas* L.) extracts on digestive enzymes and Lactate dehydrogenase of *Hyphantria cunea* Drury (Lepidoptera: Arctiidae). *Invertebrate Survival Journal*. 7: 251-261.

Zibaee, A., Bandani, A. R. & Malagoli, D. (2011) Purification and characterization of phenoloxidase from the hemocytes of *Eurygaster integriceps* (Hemiptera: Scutelleridae). *Comparative Biochemistry and Physiology Part B*. 158: 117-123.

5

The Production, Separation and Stability of Pyoluteorin: A Biological Pesticide

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1. Introduction

Pyoluteorin (Plt, 4,5-dichloropyrrol-2-yl 2,6-dihydroxyphenyl), a polyketide antibiotic produced by certain strains of *Pseudomonas* spp., including the rhizobacteria *Pseudomonas fluorescens* CHA0 (Maurhofer et al., 1992), *Pseudomonas fluorescens* Pf-5 (Corbell & Loper, 1995), *Pseudomonas fluorescens* S272 (Yuan et al., 1998) and *Pseudomonas sp*. M18 (Hu et al., 2005) et al., was first identified and separated by Takeda from *Pseudomonas aeruginosa* (Tekeda, 1958). Plt is a yellow crystal composed with a bichlorinated pyrrole linked to a resorcinol moiety (see Fig. 1), it can be completely dissolved in organic solvents such as methanol and chloroform (Wang et al., 2008). Plt can effectively inhibit phytopathogen fungi, including the plant pathogen *Pythium ultimum,* and suppress plant diseases caused by this fungus (Howell & Stipanovic, 1980; Maurhofer et al., 1992; Maurhofer et al., 1994). Moreover, in some instances, it contributes to the ecological competence of the producing strain within Rhizosphere (Howell & Stipanovic, 1980; Carmi et al., 1994; Yuan et al., 1998; Babalola, 2010).

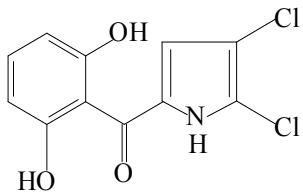

Fig. 1. Chemical structure of Plt

Many studies have focused on the metabolic regulation of Plt biosynthesis to enhance Plt production in cell cultivation. It was reported that the transcriptional activator gene *pltR* linked to Plt biosynthetic genes is required for Plt production (Nowak-Thompson et al., 1999). Whereas the regulator gene *pltZ*, which is located downstream of the Plt gene cluster in the genome of *Pseudomonas sp.* M18, could repress Plt production (Huang et al., 2004). Plt biosynthesis could be enhanced by the amplification of the housekeeping sigma factor *rpoD* in *Pseudomonas fluorescens* CHA0 (Schnider et al., 1995). The two component regulatory system GacS/GacA could positively regulate Plt production (Laville et al., 1992; Whistler et

al., 1998), and inactivation of *rpoS* (Sarniguet et al., 1995) or *lon* (Whistler et al., 2000) also resulted in the overproduction of Plt in *Pseudomonas fluorescens* Pf-5.

The culture conditions are usually important to the yield of any fermentation product. Carbon and nitrogen sources generally play a significant role because these nutrients are directly linked to cell proliferation and metabolite biosynthesis (Park et al., 2001; Casas Lopez et al., 2003; Li et al., 2008). In relation to this, Yuan et al. (Yuan et al., 1998) reported ethanol as a sole carbon source for Plt production by *Pseudomonas fluorescens* S272 cultivation in shake flasks. Duffy and Defago's study showed that Plt production was stimulated by glycerol but was repressed by glucose in cell culture of *Pseudomonas fluorescens* CHA0 (Duffy & Defago, 1999). The influence of other environmental factors on Plt production was also investigated by many researchers. In Brodhagen's study, Plt was found to be induced by itself in cell culture of *Pseudomonas fluorescens* Pf-5 (Brodhagen et al., 2004). In *Pseudomonas fluorescens* S272 cultivation, high NaCl concentration or heat shock could increase the production of Plt (Nakata et al., 1999). Chloride could also increase Plt biosynthesis in cell culture of *Pseudomonas fluorescens* YGJ3 (Matano et al., 2010). The minerals, such as zinc, could enhance Plt production in cell cultivation of both *Pseudomonas fluorescens* CHA0 (Duffy & Defago, 1999) and *Pseudomonas fluorescens* 4-92 (Saikia et al., 2009). However, there have been no reports on the effect of carbon and nitrogen ratio on Plt production through the fermentation of *Pseudomonas* spp. and no work was done on the scale-up fermentation for Plt production.

As a potential biological pesticide, understanding the stability of Plt under different environmental conditions as well as its residue in soil after incorporation into the environment was great important for its commercial use. Some analytical methods of Plt from the fermentation broth based on HPLC have been reported (de Souza & Raaijmakers, 2003; La Fuente et al., 2004). Kim et al. described a method for quantification analysis of Plt in fermentation broth with liquid chromatography–mass spectrometric (LC–MS) (Kim et al., 2003). Wang et al. developed a succinct quantitative method of capillary zone electrophoresis (CZE) for the determination of Plt in fermentation liquor of *Pseudomonas* sp. M18 (Wang et al., 2005). Trace determination of Plt in soil was done by Dong et al. using capillary electrophoresis (CE) (Dong et al., 2011). The degradation of Plt in water under different pH, temperature and light sources were studied by Zhang et al. (Zhang et al., 2010).

Pseudomonas sp. M18 is one of the plant growth promoting rhizobacteria (PGPR) selected in our lab, which can produce the secondary antifungal metabolites of Plt to suppress the root diseases caused by the soil-borne phytopathogens of crop plants (Hu et al., 2005). The chromosomally *rsmA*-inactivated and NTG mutation strain of *Pseudomonas* sp. M18R is a high Plt production strain obtained in our laboratory (Zhang et al., 2005). The identification of an effective medium formulation for Plt production is of great importance because it usually plays a pivotal role in cell growth and the production of metabolites. As a highly efficient anti-fungal metabolite, it is also important to develop the separation and purification method of Plt from its fermentation broth for large-scale preparation.

The medium optimization studies described in the literature have been conducted either by the "one-variable-at-a-time" technique or the "response surface method" (RSM) and "central composite design" (CCD) approach (Chang et al., 2002; Li et al., 2008). In this paper, the carbon or nitrogen sources and their initial concentrations were studied using the "one-variable-at-a-time" method for Plt production from *Pseudomonas* sp.M18R. Central composite design and response surface analysis were then applied to determine the optimal carbon/nitrogen ratio for Plt production. Influence of mineral amendment on Plt production was also investigated,

and the optimized medium was verified in both shake flask and bioreactor cultivation. The separation and purification method for Plt from the fermentation broth of M18R was investigated. The stability of Plt under different conditions were studied (Zhang et al., 2010), and a sensitive analytical method based on capillary electrophoresis (CE) for studying the degradation of trace amounts of Plt in soil samples was developed (Dong et al., 2011). The information obtained is considered fundamental and useful for the development of several *Pseudomonas* strain cultivation processes for efficient large-scale Plt production.

2. Materials and methods

2.1 Bacterial strain and seed culture conditions

The chromosomally *rsmA*-inactivated mutant strain of *Pseudomonas* sp.M18R was obtained as described by Zhang et al. (Zhang et al., 2005). The seed culture medium (with an initial pH of 7.2) consisted of the following components: glycerol, 18 g/L; peptone, 20 g/L; $K_2HPO_4 \cdot 3H_2O$, 0.732 g/L; and $MgSO_4$, 0.514 g/L. The stock culture was maintained on agar slants, which were inoculated with M18R, incubated at 28℃ for 12 hours, and then used for seed culture inoculation. For the seed culture, about 5 mm^2 of the M18R bacterial slants was punched out with a sterilized cutter and was then transferred to a 250 mL Erlenmeyer flask containing 50 mL of the culture medium. The cultivation was maintained at 28°C on a rotary shaker (220 rpm) for 10.5 hours, reaching an OD_{600} at about 1.1.

2.2 Experiments on M18R fermentation

The effects of carbon sources were studied using various carbon sources (18 g/L) such as glycerol, sucrose, glucose, fructose, lactose, maltose, and ethanol. For the investigation of nitrogen sources, 20 g/L of peptone, yeast extract, casein enzymatic hydrolysate, and casein acid hydrolysate were studied. For the investigation of initial carbon concentrations, different glycerol levels were used at 15, 18, 21, and 24 g/L. Furthermore, 12, 16, 20, and 24 g/L peptone was used for the study of initial nitrogen concentrations. In the experiments on the effects of carbon/nitrogen ratios, glycerol and peptone levels in the medium were changed, and a statistical approach was used. For the studies of mineral sources, 0.02 g/L ZnSO4, $CuSO_4$, $CoCl_2$, $FeSO_4$, $MnCl_2$ or NaCl was added individually into the control medium. The cells were harvested on 72 h to analyze Plt production.

Inoculation was done by transferring 7.5 mL of the above seed culture broth to a 150 mL fermentation medium in a 500 mL shake flask. The fermentation medium was the same as the seed culture medium except for the conditions studied. The cultivation was conducted at 28°C on a rotary shaker at 220 rpm. Multiple flasks were run under each condition for all cultures. The cultivation data represent the mean values with the standard deviations from three independent flasks.

The scale-up fermentation was carried out in a 10 L SY-3000E bioreactor (Shiyou Company, Shanghai, China) with 6 L optimized medium. The fermentation was conducted at an aeration rate of 1.6 vvm and an agitation speed of 250 rpm for 96 h and the shake flask culture was done as control.

2.3 Central composite design

RSM was used to optimize the glycerol and peptone ratios for enhanced Plt production based on CCD (Li et al., 2008). The second-order model used to fit the CCD experimental results is shown in Eq. (1):

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where y is the predicted response, β_0 is a constant, β_i is a coefficient for the linear effect, β_{ii} is a coefficient for the quadratic effect, β_{ij} is a coefficient for the interaction effect, and x_i and x_j are the coded levels of variables x_i and x_j, respectively. The fitness of the second-order model was checked using the adjusted coefficient of determination R^2, and its statistical significance was determined by the application of Fischer's F test. The software programs SAS (version 9.1 by SAS Institute Inc., NC, USA) and MatLab (version 7.1 by Mathworks, Inc.) were used for regression and graphical analyses of the data obtained, respectively.

2.4 Measurement of cell growth and Plt production

Cell growth was assayed in terms of optical density at 600 nm (OD600). A 1 mL culture broth was centrifuged at 12000 rpm for 5 min, and the sediments were re-suspended and diluted in distilled water.

The analytical procedures for Plt production were slightly modified as described by Zhang et al. (Zhang et al., 2005). For Plt extraction, 1 mL of each culture broth was mixed with the same volume of ethyl acetate. The upper layer was collected after complete agitation and centrifugation at 8000 g for 5 min. The water layer was then extracted once more with 0.5 mL ethyl acetate. The combined extracts were finally dried in a desiccated vacuum at 40°C and then dissolved in 1 mL of HPLC grade methanol. Using a Shimadzu LC-8A HPLC apparatus equipped with a variable-wavelength UV detector (Shimadzu, SPD-8A), 20 μL sample was analyzed by reverse-phase HPLC. A Zorbax SB-C18 column (250×4.6 mm^2; 5 μm) was used at 25°C. The mobile phase consisted of 70% methanol (vol) and 30% water (vol), with the flow rate kept constant at 1 mL/min. Plt was monitored and quantified at 308 nm, and was then identified by comparison with its authentic sample.

2.5 Plt purification

Firstly, the fermentation broth was extracted with ethyl acetate and the crude Plt extract was obtained. Secondly, the crude Plt extract was separated by silica gel column chromatography with a glass column (700×50 mm^2) packed with 500 g analytical grade silica of 100-200 mesh. The column was eluted with benzene-acetic acid (20:1,vol) and the elution which contained Plt was collected and evaporated at 40°C to dry, and the residue was dissolved in methanol (HPLC grade) for further purification.

Preparative HPLC was conducted for further purification of Plt using a Tigerkin C-18 column (300×20 mm^2; 10 μm) under 25°C with 50% methanol as the mobile phase. The flow rate was kept constant at 15 mL/min and the highest peak containing Plt was collected. Plt crystal was finally obtained after the elution was evaporated at 40°C to dry.

2.6 Trace analysis of Plt in soil samples (Dong et al., 2011)

A series of Gly-NaOH buffers with different pH values and concentrations were prepared. The stock solution of internal standard (IS) was prepared by adding 1.0 mg PCA into 10.0 mL methanol to fix its concentration at 100 μg/mL. Standard solutions are prepared by dissolving Plt with different concentrations in methanol, forming a concentration gradient of 100, 75, 50, 25, 5, 2, 0.5 μg/mL. All solutions and buffers were stored at 4°C.

Plt was extracted from soil samples (air dried, mixed and sieved through a 2-mm sieve) with ethanol. The extraction procedure was performed as follows: the working standard solution

was spiked into 10.0 g soil in an appropriate volume, which was ultrasonic extracted with 50 mL ethanol for 20 min. The extraction liquid was collected and the solid sample was transferred to the Soxhlet extractor for 4 h using another batch of 100 mL ethanol. After mixing and evaporating all organic phases, the dry residue was dissolved in 5 mL methanol and then centrifuged at 3000 rpm for 10 min. A portion of the 500 μL methanol layer was distributed to one Eppendorf tube with an addition of 125 μL PCA (25 μg/mL). All solutions were stored at 4°C before injection into capillary.

The capillary-based experiments were carried out with an ACS 2000 HPCE apparatus (Beijing Cailu Scientific Inc., Beijing, China). The operating system is Windows XP professional SP2 with a HW-2000 chromatography work station (Qianpu Software Co. Ltd., Nanjing, China). Electrophoresis was performed in an untreated fused-silica capillary of 53 cm total length (44 cm effective length) × 75 μm I.D. (375 μm O.D.) (Yongnian Optical Fiber Factory, Hebei, China). Before use, a new fused-silica capillary was pressure-rinsed with 0.1 M NaOH for 20 min, ultra-pure water for 20 min, 0.1 M HCl for 20 min, ultra-pure water for 20 min, and CE running buffer for 30 min orderly. The capillary was rinsed with CE running buffer for 5min between injections and finally stored in water when not in use. The samples were injected in pressure mode at the inlet (13 mbar, for 45 s). The ultimate working voltage was 10 kV.

Plt degradation study in soil was carried out with samples of both near-surface soil (0~10cm in depth) and rhizosphere soil (10 cm below surface), which were initially spiked with Plt at concentration of 500 μg/kg. The extraction and analytical procedure were done as described above.

2.7 Degradation of Plt under different conditions

The degradation of Plt in water was done as describe by zhang et al. (Zhang et al., 2010). Plt solutions were prepared using our purified Plt and pure water, with the Plt concentrations varying between 121.5 mg/L and 626.5 mg/L. Temperature was thermostat-controlled, and irradiation was carried out in a Pyrex vessel, using natural sunlight. Samples were taken from the reactor periodically and were analyzed immediately by HPLC. The sample treatment procedure was as follows: 200 μL ethyl acetate was added to the reactive solutions to stop the reaction and extract Plt. The organic solution was then dehydrated to obtain a powder residue of Plt. All experiments were preformed in duplicate.

3. Results and discussion

3.1 Optimization of M18R cultivation

3.1.1 Experiments on carbon sources and initial carbon concentrations

Carbohydrates are important carbon and energy sources for bacterial growth and metabolite biosynthesis. In the cell culture of *Pseudomonas fluorescens* S272, a high Plt production titer was obtained using ethanol as sole carbon source (Yuan et al., 1998). In the current work, we investigated the effects of ethanol as well as various common carbon sources, such as glycerol, sucrose, glucose, fructose, lactose, and maltose on M18R cultivation. The time profiles of cell growth (OD600) and Plt production are shown in Fig. 2. It can be seen that the cell grew well in glycerol, glucose, and fructose, and the maximum cell density was obtained in glucose after 72 hours of cultivation. A higher Plt production titer was obtained in glycerol and fructose for M18R. The order of maximum Plt production titer was 540.2±20.0, 401.2±24.8, 270.1±20.1, 212.6±12.6, 119.7±11.7, 37.2±3.2, and 45.7±3.7 mg/L in glycerol, fructose, ethanol, glucose, sucrose, lactose, and maltose media, respectively.

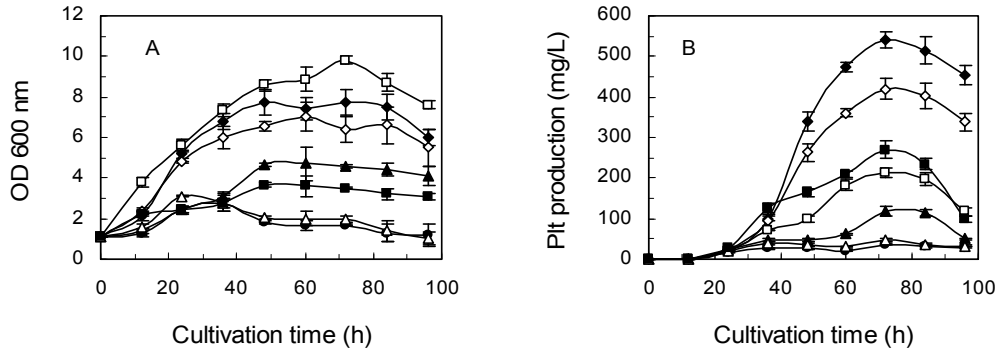

Fig. 2. Time profiles of cell growth (A) and Plt production (B) for the cell culture of *P*. M18R with different carbon sources (18 g/L). Symbols for different carbons: glycerol (dark diamond), fructose (open diamond), sucrose (dark triangle), maltose (open triangle), ethanol (dark square), glucose (open square), and lactose (dark circle). The error bars in the figure indicate the standard deviations from three independent samples.

Initial glycerol concentration (g/L)	Plt production (mg/L)	Plt productivity (mg/L per hour)	Plt yield on carbon (mg/g)
15 (72 h)[a]	460.3± 27.8	6.39± 0.39	30.7± 1.9
18 (72 h)	568.3± 30.1	7.89± 0.42	31.6±1.7
21 (60 h)	464.3± 31.9	7.74± 0.53	22.1± 1.5
24 (60 h)	423.7± 19.8	7.06± 0.33	17.7± 0.8

Table 1. Effects of initial glycerol concentration on Plt production for *P*.M18R cultivation.
[a] Cultivation time when the maximum Plt production was achieved.

Based on the above results, glycerol was selected as the carbon source for the cell culture of M18R. The effects of initial glycerol concentrations on Plt production are shown in Table 1. The highest production and productivity of Plt were obtained at an initial glycerol concentration of 18 g/L. The Plt production was decreased at high initial glycerol concentrations (21 or 24 g/L), the inhibitory effect of high initial carbon concentration on metabolite biosynthesis was also observed in ganoderic acid biosynthesis by *Ganoderma lucidum* (Fang & Zhong, 2002).

3.1.2 Experiments on nitrogen sources and initial nitrogen concentrations

In the cell culture, both carbon and nitrogen sources are very important for cell growth and metabolite production. Various organic nitrogen, including peptone, yeast extract, casein enzymatic hydrolysate, and casein acid hydrolysate, were studied in this paper. The time profiles of cell growth (OD600) and Plt production for the cell culture of M18R in different nitrogen were shown in Fig. 3. It is evident that the cells grew well in peptone, and a maximum Plt production titre of 553.2±27.2 mg/L was achieved after 72 hours of cultivation in the peptone medium. The effects of initial peptone concentrations on Plt biosynthesis were further examined, the results of which are shown in Table 2. The respective maximum Plt production and productivity of 568.3±30.1 mg/L and 7.89±0.42 mg/L per hour were obtained at an initial peptone concentration of 20 g/L.

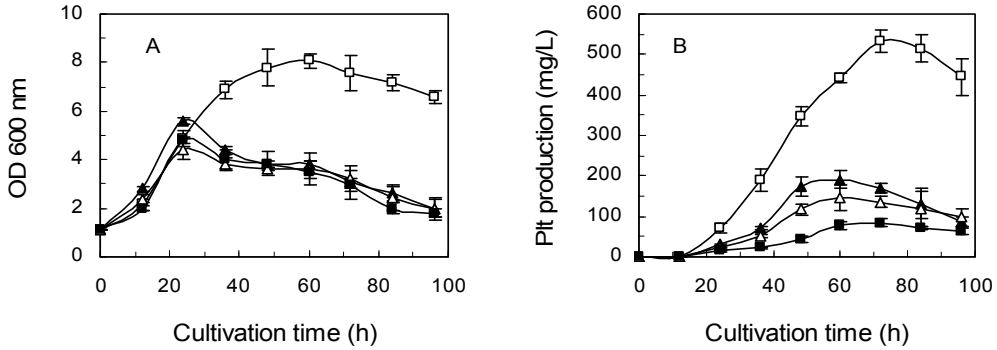

Fig. 3. Time profiles of cell growth (A) and Plt production (B) for the cell culture of *P*. M18R with different nitrogen sources (20 g/L). Symbols for different nitrogens: peptone (open square), yeast extract (dark square), casein acid hydrolysate (open triangle), and casein enzymatic hydrolysate (dark triangle). The error bars in the figure indicate the standard deviations from three independent samples.

Initial peptone concentration (g/L)	Plt production (mg/L)	Plt productivity (mg/L per hour)	Plt yield on carbon (mg/g)
12 (84 h)[a]	393.2± 30.8	4.68± 0.37	21.8± 1.7
16 (72 h)	485.5± 15.0	6.74± 0.21	27.0±0.8
20 (72 h)	568.3± 30.1	7.89± 0.42	31.6±1.7
24 (60 h)	332.3± 12.5	5.54± 0.21	18.5± 0.7

Table 2. Effects of initial peptone concentration on Plt production for the cultivation of *P*.M18R. [a] Cultivation time when the maximum Plt production was achieved.

3.1.3 Experiments on carbon/nitrogen ratio

The combined effect of carbon (glycerol) and nitrogen (peptone) was studied using the CCD because the concentrations of both carbon and nitrogen sources and their ratio are very important for metabolite production (Chang et al., 2002; Li et al., 2008). The levels of variables for CCD experiments were selected according to the results of the one-at-a-time strategy, and the coded (−1.414, −1, 0, 1, and 1.414) and real values of the variables at various levels are listed in Table 3. The experimental responses, along with the predicted response obtained from the regression equation, are also shown in Table 3.

Regression analysis was performed to fit the response function (Plt production) with the experimental data. From the variables obtained (Table 4), the model was expressed by Eq. (2), which represented Plt production (y) as a function of glycerol (x_1) and peptone (x_2) concentrations. Furthermore, the results of the F-test analysis of variance (ANOVA) in Table 5 showed that the regression was statistically significant ($P < 0.05$) at a 95 % confidence level. The model presented a high regression coefficient of 0.9694.

$$y(mg/L) = 577.732 + 71.327x_1 - 54.053x_2 - 46.802x_1^2 - 106.449x_2^2 - 24.010x_1x_2 \quad (2)$$

Runs	x_1 (g/L) Glycerol	x_2 (g/L) Peptone	y (mg/L) Plt production	
			Observed	Predicted
1	21 (+1)	24 (+1)	444.8± 22.1[a]	417.7
2	21 (+1)	16 (-1)	593.9± 35.2	573.9
3	15 (-1)	24 (+1)	311.8± 17.5	323.1
4	15 (-1)	16 (-1)	364.9±20.4	383.2
5	22.242 (1.414)	20 (0)	553.4± 10.9	585.0
6	13.758 (-1.414)	20 (0)	405.9± 25.0	383.3
7	18 (0)	25.696 (1.414)	278.9±14.1	288.4
8	18 (0)	14.304 (-1.414)	441.8± 19.4	441.3
9[b]	18 (0)	20 (0)	548.1	577.7
10	18 (0)	20 (0)	573.6	577.7
11	18 (0)	20 (0)	602.1	577.7
12	18 (0)	20 (0)	589.6	577.7
13	18 (0)	20 (0)	575.1	577.7

Table 3. Experimental design and responses of the central composite design (CCD). [a] Samples were taken at 72h, and the standard deviation was calculated from three independent samples. [b] Runs 9-13 were replicates at the center point.

Parameters	Parameter estimate	Standard error	T value	Pr > |t|
Intercept	577.732	11.746	49.184	<0.0001
β_1	71.327	9.286	7.681	<0.0001
β_2	-54.053	9.286	-5.821	0.001
β_{11}	-46.802	9.958	-4.700	0.002
β_{22}	-106.449	9.958	-10.689	<0.0001
β_{12}	-24.010	13.133	-1.828	0.110

Table 4. Regression results from the data of central composite design (CCD) experiments x_1: glycerol concentration; x_2: peptone concentration

Regression	DF	Sum of squares	F value	Pr > F
Linear	2	64075	46.44	<0.0001
Quadratic	2	86495	62.69	<0.0001
Crossproduct	1	2306	3.34	0.110
Total model	5	152876	44.32	<0.0001

Table 5. ANOVA results for Plt production obtained from central composite design (CCD) experiments

The response surface plot obtained from Eq. (2) is shown in Fig. 4. Based on the canonical analysis produced by the SAS software, it is evident that Plt production reached its maximum at a combination of coded level 0.85 (x_1, glycerol) and -0.35 (x_2, peptone). The model predicted a maximum response of 617.6 mg/L Plt at 20.55 g/L glycerol and 18.6 g/L peptone levels as optimized medium components.

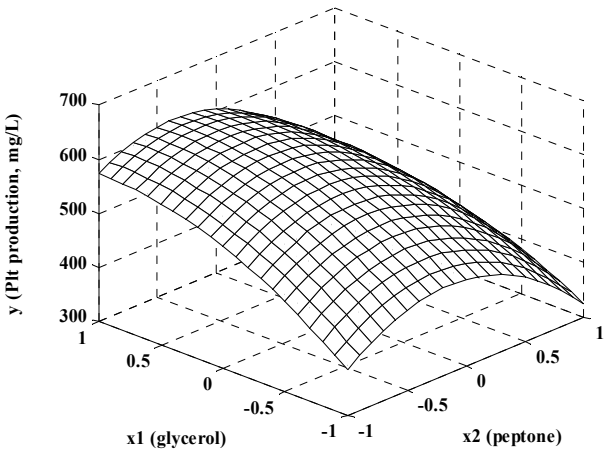

Fig. 4. The response surface curve of the effect of glycerol and peptone on Plt production by *P*.M18R.

To confirm the above prediction, experiments using both optimized (as predicted) and non-optimized media (18 g/L glycerol and 20 g/L peptone) were performed. The results are shown in Fig. 5. Similar time profiles of cell growth (OD600) were observed for both optimized and non-optimized media. A maximum Plt production titre of 648.3±20.1 mg/L for 72 hours of cultivation was obtained for the cells cultured in the optimized medium, which was higher than that in the non-optimized medium.

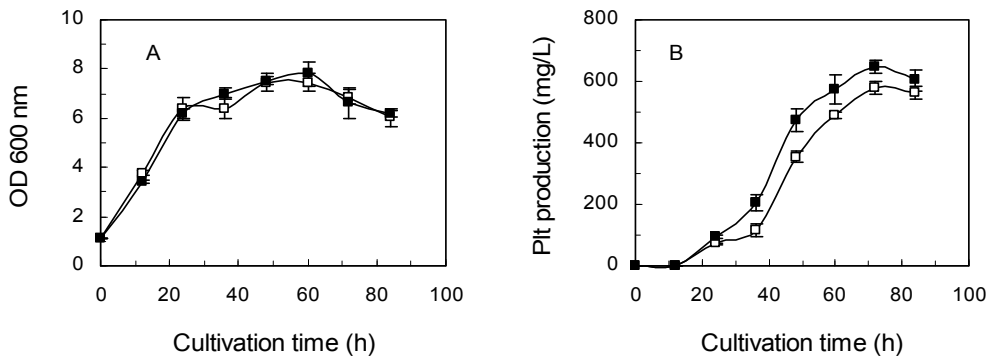

Fig. 5. Time profiles of cell growth (A) and Plt production (B) for the cell culture of *P*. M18R in different media. Symbols: non-optimized medium (open square), optimized medium (dark square). The error bars in the figure indicate the standard deviations from three independent samples.

The effects of major nutrients such as carbon/nitrogen sources as well as the ratios on Plt production were studied for the cell culture of *Pseudomonas sp*. M18R in order to obtain a suitable fermentation medium. The findings show that the most favorable carbon source for Plt biosynthesis in *Pseudomonas sp*. M18R was glycerol. The Plt production titre in a glycerol medium (540.2±20.0 mg/L) was about 1.29, 2.00, 2.54, 4.51, 11.80, and 14.50 times of that in fructose, ethanol, glucose, sucrose, lactose, and maltose media for 72 hours of cultivation, respectively. The same results were obtained in the cell culture of the biocontrol strain *Pseudomonas fluorescens* CHA0. The respective Plt production titre in 48 hours was about 220, 50, and 10 ng/10^8 CFU in glycerol, fructose, and glucose media (Duffy & Defago, 1999). However, ethanol showed the highest Plt production for the cell culture of *Pseudomonas fluorescens* S272. The Plt production titre was about as twice of that in the ethanol medium (56 mg/L) as in the glycerol medium (26 mg/L) for 72 hours. Moreover, no Plt was observed in other carbons such as fructose, glucose, and sucrose, among others (Yuan et al., 1998). These results showed that the effect of carbon source on Plt production seemed to be strain dependent. For the organic nitrogen investigated, the Plt production titer was much higher in peptone than in yeast extract, casein enzymatic hydrolysate, and casein acid hydrolysate for our cell, and only a little Plt was observed in inorganic nitrogen such as ammonium nitrate (data not shown). In the cell culture of *Pseudomonas fluorescens* S272, similar result for the respective Plt production titer of 124, 88, and 31 mg/L in peptone, soybean, and ammonium nitrate medium was obtained (Yuan et al., 1998). According to the central composite design and response surface analysis, maximum Plt production (648.3±20.1 mg/L) and productivity (9.01±0.28 mg/L per hour) were successfully obtained using 20.55 g/L of glycerol and 18.6 g/L of peptone by the cell culture of *Pseudomonas sp*. M18R. The production titer of Plt for the cell culture of M18R obtained in this work increased about 10 times.

3.1.4 Experiments on mineral sources

The effects of mineral sources on Plt production in cell culture of M18R were investigated and the results were shown in Table 6. Plt production was influenced by addition of 0.02g/L $ZnSO_4$, $CuSO_4$, $FeSO_4$ or $CoCl_2$, whereas $MnCl_2$ or NaCl have no effects on it.

Culture medium	Plt Production (mg/L)
Control	609.5± 21.4
$ZnSO_4$	643.0± 26.9
$CuSO_4$	553.0± 25.1
$FeSO_4$	507.8± 17.8
$MnCl_2$	626.3± 33.2
$CoCl_2$	640.2± 30.9
NaCl	614.7± 28.4

Table 6. Effects of different mineral sources on Plt production for *P*.M18R cultivation on 72h.

From Table 6 we could see that $ZnSO_4$ or $CoCl_2$ could enhance Plt production in *P*.M18R, the same results was obtained in *Pseudomonas fluorescens* CHA0 (Duffy & Defago, 1999). Plt production was decreased by the addition of 0.02 g/L $FeSO_4$ in *P*.M18R, but increased by

the addition of 0.15 g/L $FeSO_4$ in *Pseudomonas fluorescens* CHA0 (Duffy & Defago, 1999). High NaCl concentration (15 g/L) could increase the production of Plt in *Pseudomonas fluorescens* S272 (Nakata et al., 1999), however no effects was observed in our research. These results indicated that not only the mineral sources, but also the mineral concentration have obvious effects on Plt production. The optimal concentration for each mineral in cell culture of M18R is still under research.

3.1.5 Scale-up fermentation of M18R

A 10 L bioreactor was adopted to test the optimized medium conditions obtained in shake flask and the Plt production were shown in Table 7. Plt attained a maximal production of 708.1± 35.7 mg/L in 78 h, a little higher than that in flasks. The productivity of Plt in flask and bioreactor cultivation were nearly at the same level.

Culture condition	Plt production (mg/L)	Plt productivity (mg/L per hour)
Flask cultivation (72h)	673.8± 25.6	9.36± 0.57
Bioreactor cultivation (78h)	708.1± 35.7	9.08± 0.78

Table 7. Scale-up fermentation of *P.* M18R for Plt production.

3.2 Purification of Plt from the fermentation broth of M18R

3.2.1 Extraction of Plt from the fermentation broth

The solubility of Plt in different solvents was analyzed (see Table 8), and ethyl acetate was selected as the extract solvent.

Solvent	Plt solubility (g/L)
Methanol	761.7±26.1
Ethanol	750.8±34.5
Acetone	786.7±37.4
Ethyl acetate	520.7±12.8
Chloroform	90.1±3.1
Dichloromethane	13.82±0.06
Carbon tetrachloride	0.88±0.03
Water	0.75±0.01

Table 8. Solubility of Plt in different solvents at 293.2K.

The extract times and the volume ratio of the organic solvent used were studied, and the results were shown in Table 9. Every 5 mL fermentation broth was used for each condition and each experiment was carried out in triplicate. Plt content in the extract increased from 538.7 mg/L to 624.2 mg/L with the increasing of the volume ratio of ethyl acetate and fermentation broth from 0.5:1 to 2:1 for once extraction. When extracted twice, the volume ratio of organic solvent and culture broth have no influence on Plt extraction and all data for twice extraction were higher than once extraction.

Extract methods		Plt (mg/L)
Extract times	Volume ratio of ethyl acetate and fermentation broth	
Once	0.5:1	538.7±4.8
	1:1	597.6±33.9
	2:1	624.2±25.6
Twice	0.5:1	691.9±8.8
	1:1	698.0±60.0
	2:1	687.8±12.3

Table 9. Extract methods of Plt from the fermentation broth

From this result, 2 L fermentation broth was extracted with ethyl acetate twice and 1 L ester were used for each time. The extraction were combined and evaporated at 40°C to dry and 1.204 g of crude Plt was obtained.

3.2.2 Silica gel column chromatography

Samples of Plt extract (1.204 g) was mixed with 2.5 g silica gel and loading onto a glass silica gel column after grinding. The column was eluted with benzene-acetate acid (20:1, vol) and every 50 mL of the elution was collected and evaporated at 40°C to dry. The residues were dissolved in methanol and analyzed by HPLC, the fractions that contained Plt were combined and about 0.953 g of crude Plt was obtained. The HPLC chromatogram shown a purity of Plt was about 89% for this crude sample and further purification need to be done.

3.2.3 High performance liquid chromatography of Plt

Elution conditions of crude Plt were investigated on preparative HPLC column and the chromatogram were shown in Fig. 6. When 70% methanol was used as the mobile phase, the peaks of Plt and the main impurity were very near and their respective retention time were 7.26 min and 5.05 min. Decreasing methanol concentration from 70% to 50% could increase the retention time between Plt and the main impurity from 2.21 min to 17.04 min. Therefore, using 50% methanol as the mobile phase would be beneficial to the separation of massive samples on preparative column.

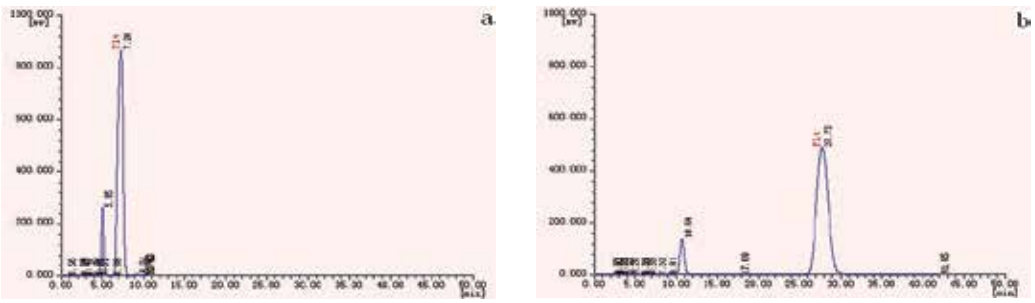

Fig. 6. Optimization of mobile phase on preparative HPLC. Conditions: C-18 column (300×20 mm², 5 μm); flow-rate: 15 mL/min; detection wavelength: 308 nm. a: 70% methanol; b: 50% methanol

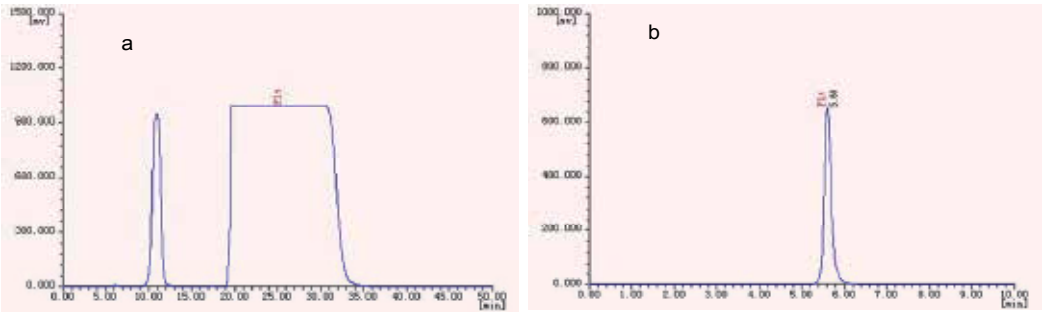

Fig. 7. a: Preparative HPLC chromatogram for Plt purification. Conditions: C-18 column (300 mm × 20 mm i.d., 5 μm); mobile phase: 50% methanol; flow-rate: 15 mL/min; detection wavelength: 308 nm. b: HPLC chromatogram of Plt after purification by preparative HPLC. Conditions: C-18 column (250 mm × 4.6 mm i.d., 5 μm); mobile phase: 70% methanol; flow-rate: 1 mL/min; detection wavelength: 308 nm.

Crude Plt (0.953g) was dissolved in 50% methanol to a total volume of 4 mL and then purified by preparative HPLC. The biggest peak (see Fig. 7a) was collected and evaporated to dry and 0.841g yellow crystal was finally obtained. Structure of the crystal was confirmed to be Plt by means of modern spectroscopic techniques, including UV, MS, 13C NMR and 2H NMR (data not shown). HPLC chromatogram of Plt crystal was shown in Fig. 7b and its purity was about 99%.

The results of Plt purification from the culture broth were shown in Table 10. From the table we could see that 0.841 g Plt was obtained from 2 L culture broth with a total recovery of about 69.8%.

Purification steps	Plt quantity (g)	Plt Purity (%)	Recovery (%)
Extract with ethyl acetate	1.204	38.8	100
Silica gel column chromatography	0.953	89.1	79.2
Preparative HPLC	0.841	98.9	69.8

Table 10. The results of Plt purification

3.3 Trace analysis of Plt by CE

3.3.1 Effect of applied voltage

For the trace analysis of Plt by CE, applied voltage (ranging from 5 to 25kV) has an obvious influence on migration time (Fig.8 (A)), peak height and peak area (Fig.10 (A)). With the decrease of the running voltage, the migration time of Plt increased and their corresponding peak height and peak area were heightened, which represented high detection sensitivity. Effect of applied voltage on separation of Plt and PCA (IS) was also investigated (Fig.9 (A)). The experiment showed no obvious variation on resolution. Considering the requirement for detection sensitivity, 10kV was selected as the optimum applied voltage in this experiment.

3.3.2 Effect of buffer pH values and buffer concentration

The pH of the electrolyte buffer (ranging from 8.3 to 9.0) plays an important role in both electroosmotic flow (EOF) and electrophoretic mobilities of the analytes, so as to impact the resolution (Fig.9 (B)). The experiment demonstrated that the increase of pH value led to a decrease in the migration time of PCA (Fig.8 (B)) and a certain increase in peak height but little change in peak area (Fig.10 (B)). However, the migration time of Plt (Fig.8 (B)) was reduced with pH values between 8.3 and 8.6, and then rose back when the pH value reached 8.6. Besides, the resolution of Plt and PCA decreased as the pH values increased, but Plt and methanol can not be separated with pH values below 8.4. For a comprehensive thought, the pH value of the background electrolyte buffer is controlled at 8.6, to get good resolutions of both Plt and PCA, and Plt and methanol.

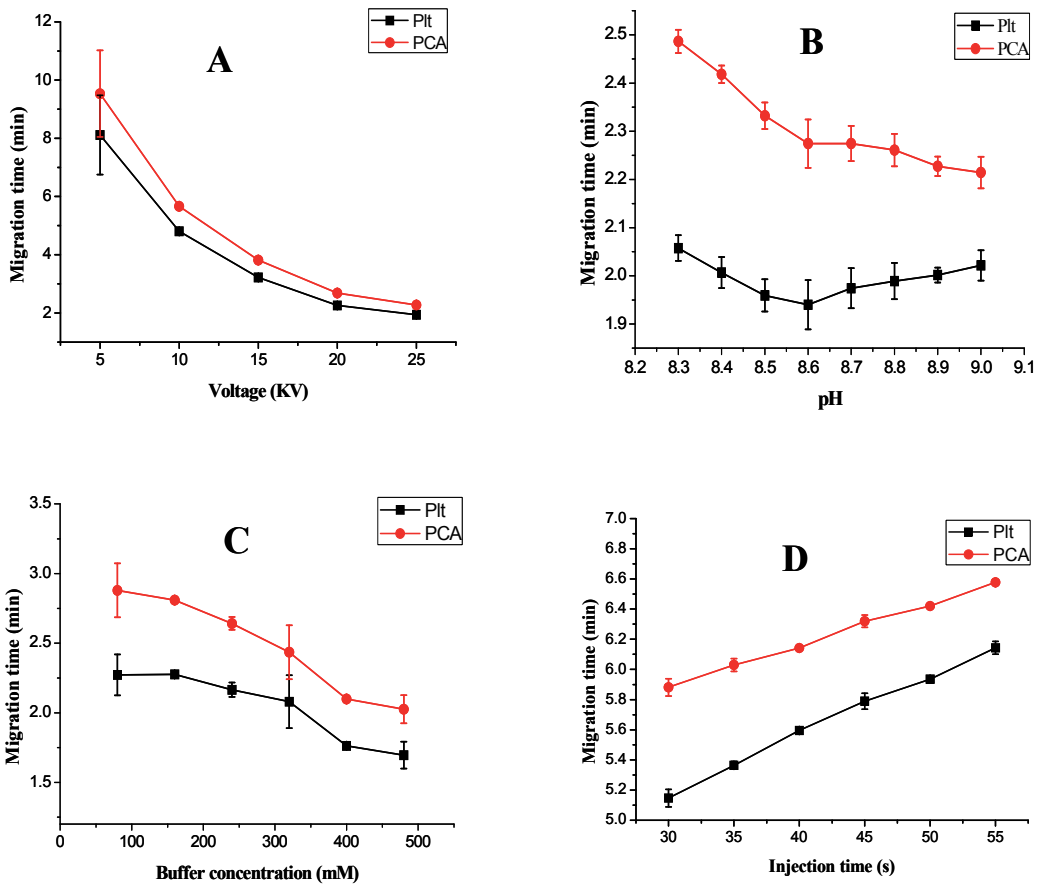

Fig. 8. Effects to the migration time of Plt and PCA in stacking CE by voltage (A), pH value (B), buffer concentration (C) and injection time (D)

3.3.3 Effect of buffer concentration

Different concentrations of Gly-NaOH buffer (ranging from 80 to 480 mmol/L) have evident influence on electric current, resolution and sensitivity. The results showed that the resolution of Plt and PCA (Fig.9 (C)) increased significantly with the increasing buffer concentrations. When the concentration was 240 mmol/L, the highest peak height and a relatively larger peak area of Plt (Fig.10 (C)) were gained. So, 240 mmol/L was selected as the optimum background buffer concentration in view of good detection sensitivity and resolution.

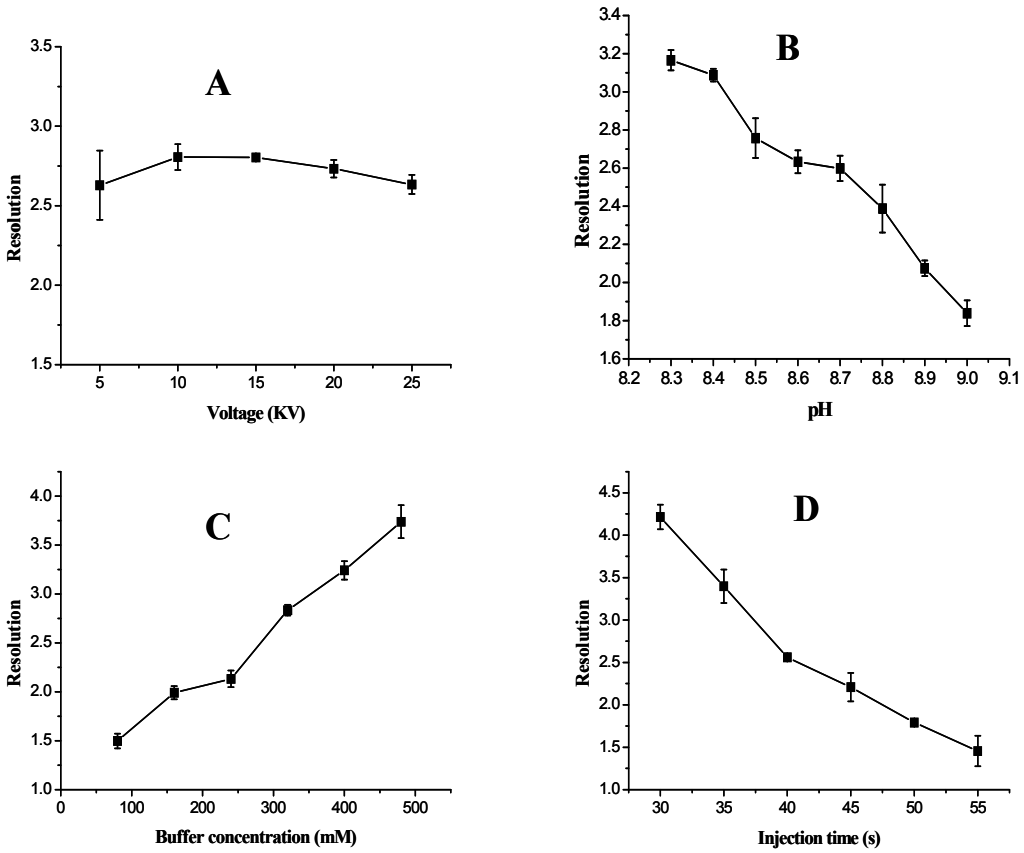

Fig. 9. Effects to the resolution of Plt and PCA in stacking CE by voltage (A), pH value (B), buffer concentration (C) and injection time (D)

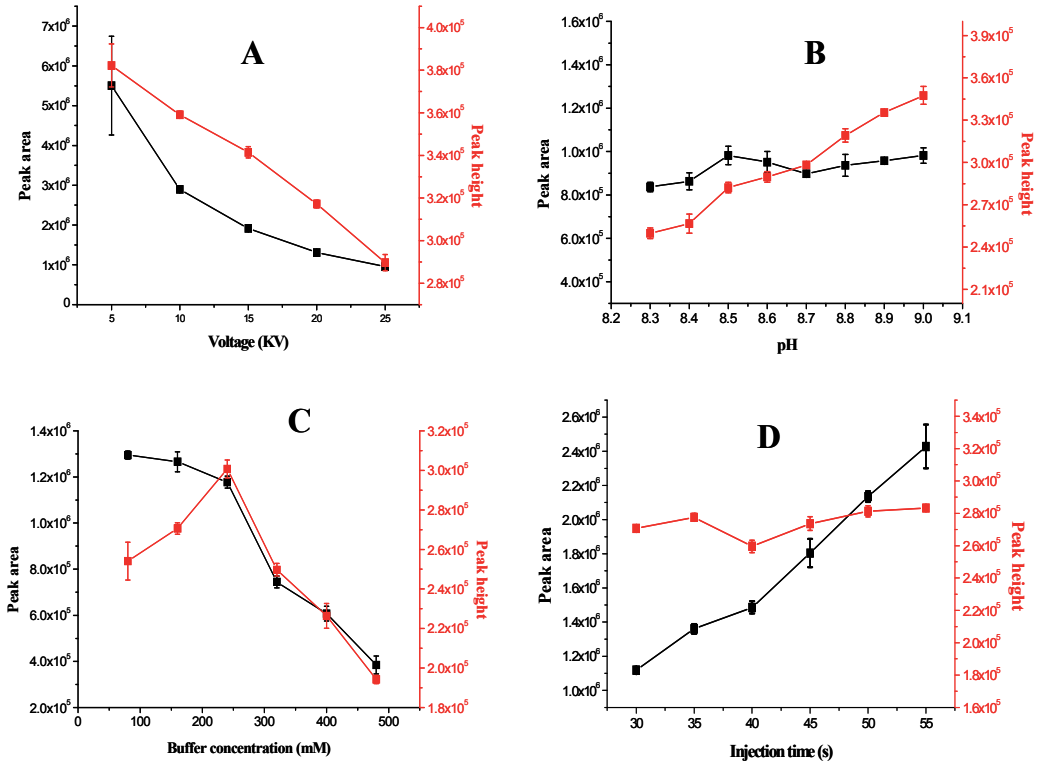

Fig. 10. Effects to the peak area and peak height of Plt in stacking CE by voltage (A), pH value (B), buffer concentration (C) and injection time (D)

3.3.4 Effect of injection time

The injection time (ranging from 30 to 55 s) impacts the sensitivity and resolution significantly. As the injection time increased, the resolution of Plt and PCA (Fig.9 (D)) got poorer (destroyed with injection time at 55 s), a great increase in peak area and little variation in peak height of Plt (Fig.10 (D)) was gained. Considering the requirement for both sensitivity and resolution, a 45 s injection time was selected.

3.3.5 Validity of the developed methods

Finally, we achieved the following optimized CE conditions: 240 mmol/L, pH 8.6 Gly-NaOH buffer, a fused-silica capillary of total length 53 cm×75 μm ID, 375 μm OD, with effective length 44 cm, 10 kV, 13 mbar 45 s pressure sample injection, and 25°C air-cooling for room temperature control. The method was validated for specificity, linearity and precision. The results showed that the method we developed had a good specificity and linearity for trace Plt analysis (Dong et al., 2011).

The precision of the developed method was evaluated by measuring inter-day and intra-day standard deviations (S.D.) and the relative standard deviations (R.S.D.) of both migration time and peak area ratios between the analyte and the IS. The intra-day values of S.D. and

R.S.D. were calculated based on six replicate injections of the four different concentrations within a day, while inter-day values were evaluated with six replicate injections of the same standard concentration in 6 days. The two precise results are listed in Table 11, which imply the stable analytic conditions and good repeatability of this method.

	Sample concentration	Migration time			Peak area ratio		
	(μg/mL)	Mean (min)	S.D.	RSD (%)	Mean (10^6)	S.D. (10^6)	RSD (%)
Intra-day	100	6.295	0.223	3.54	4.585	0.298	3.64
	75	6.468	0.194	2.99	3.696	0.096	2.61
	25	6.323	0.068	1.08	1.171	0.039	3.36
	0.5	6.209	0.121	1.95	0.012	0.000	3.30
Inter-day	100	6.242	0.217	3.42	4.781	0.174	3.63
	75	6.309	0.297	4.58	3.411	0.125	3.65
	25	6.328	0.238	3.65	1.162	0.051	4.39
	0.5	6.002	0.080	1.33	0.014	0.001	4.77

Table 11. Intra-day and inter-day standard deviations of Plt determination (n=6)

CZE is a simple method for the monitor of Plt in fermentation liquor (Wang et al., 2005). However, it suffers from poor sensitivity due to the small sample amounts injected for detection. A field-amplified sample stacking advanced by Chien (Chien & Burgi, 1991) and Burgi (Burgi & Chien, 1991) was applied to overcome the limitation of CE. The analyte is dissolved in a diluted background electrolyte or pure solvent to form a sample solution with lower conductivity compared with CE buffer solution. When the high potential is applied to capillary, the sample zone generates an amplified electric field, which makes the analyte irons move faster until reaching the CE buffer zone. Thus, stacking could be achieved at the boundary between sample and CE zone. The method has been confirmed on sensitivity improvement of CE analysis for environmental samples (Albert et al., 1997; Palmarsdottir et al., 1997; Liu et al., 2008), and received approval in trace determination of pesticides and other environmental pollutants (Nunez et al., 2002; Carabias-Martinez et al., 2003; Lagarrigue et al., 2008).

Herein, we developed a method of CE which combined field-amplified sample stacking to analyze trace amount of Plt. The limit of detection (LOD) of this method was 0.107 μg/mL, by setting the signal-to-noise ratio at 3:1, and the limit of quantification (LOQ) for Plt was 0.36 μg/mL by setting the signal-to-noise ratio at 10:1. The LOD of our method was much lower than that of HPLC (10 μg/mL) (de Souza & Raaijmakers, 2003) and CEZ (0.66 μg/mL) (Wang et al., 2005). This result suggests that this method can meet with the requirement of pesticide residue analysis.

3.4 Degradation of Plt

3.4.1 Effect of light source

When a pesticide is used on farmland, it is exposed to sunlight, which includes both UV light and Vis light. Many compounds are light sensitive, such as aldicarb, parathion, mecoprop, linuron, and chlorpyrifos (Burrows & Canle, 2002; Anfossi et al., 2006). Thus, it is necessary to study the effect of UV/Vis irradiation on the degradation rate of Plt. We used a

fluorescent lamp (390-760 nm), an incandescent lamp (390-760 nm), a high-pressure mercury lamp (250-450 nm), and sunlight (280-800 nm) to investigate the effects of different irradiation on Plt degradation and the results is shown in Fig. 11. The photodegradation rate followed first-order kinetics. The half-life of Plt under fluorescent lamps (23.2 d) and incandescent lamp (23.5 d) showed little difference from that in the dark (24.9 d), whereas the high-pressure mercury lamp (3.1 d) and sunlight (3.5 d) greatly decreased its half-life.

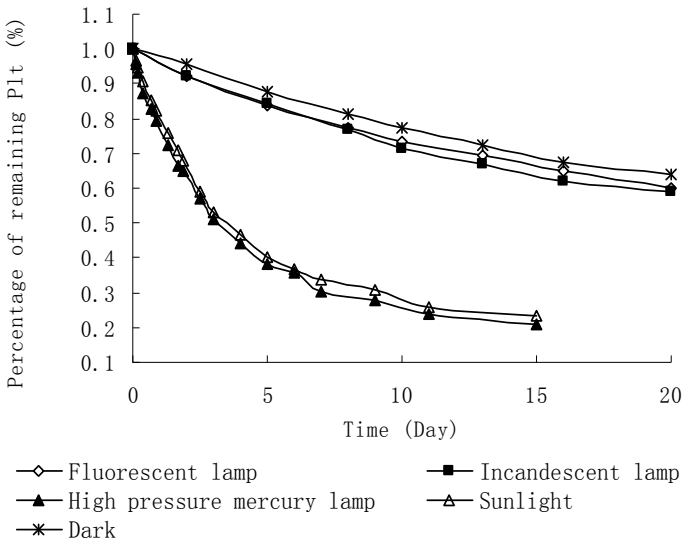

Fig. 11. The degradation of Plt on different light resources (stored at room temperature).

3.4.2 Effect of pH

Fig. 12 shows the photodegradation of Plt in buffered solutions at different pH levels. The degradation rate at different pH also followed first-order kinetics. The half-life of Plt decreased from 1.77 d to 0.42 d with the increasing pH increased from 5.8 to 7.8. In dark conditions, Plt is relatively stable at the pH range from 5.8～7.8 because the half-life were all

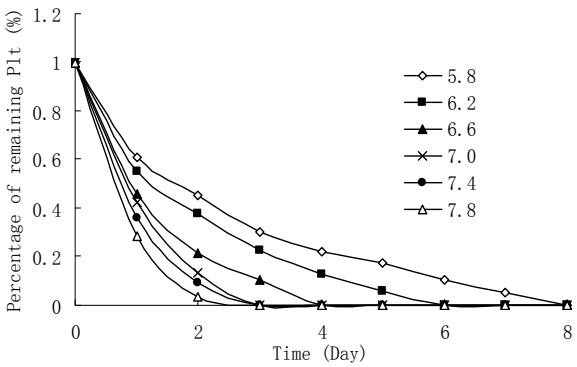

Fig. 12. The photodegradation of Plt at different pH values (room temperature, under the sunlight).

above 20 d (Zhang et al., 2010). This difference might be due to the change of UV–visible spectra of Plt at different solutions (Zhang et al., 2010). The higher efficiency of degradation of Plt under photo irritation at higher pH values can be due to the higher irradiation absorbance of higher-pH solutions.

3.4.3 Effect of water sources

A kinetic model was developed to study the degradation mechanisms and kinetics of Plt, and the results showed that Plt is relatively stable in pure water solutions and at room temperature (Zhang et al., 2010). Here, the stability of Plt in natural water at room temperature was investigated. The degradation of Plt in nature water also followed first-order reaction kinetics as that in pure water and the results were shown in Table 12. The half-life of Plt in nature water was much shorter (4.3 d in lake water and 5.2 d in rain water) than that in pure water (24.7 d), suggestted that Plt is unstable in solutions of natural water.

Water source	Rate constant (d^{-1})	R^2	Half-life (d)
Lake water	0.1605	0.9955	4.3
Rain water	0.1343	0.9783	5.2
Pure water	0.0281	0.9564	24.7

Table 12. Effects of water sources on Plt degradation in the dark at room temperature

The stability of Plt for different treatment of lake water was shown in Table 13, the degradation also followed first-order reaction kinetics. The half-life of Plt in treated lake water (11.4 d in filtered lake water and 16.1 d in heated lake water) was much longer than that in untreated lake water (4.3 d), suggestted that there might be Plt degrading bacteria in natural water.

Treat method	Rate constant (d^{-1})	R^2	Half-life (d)
Untreated	0.1605	0.9675	4.3
Filtered	0.0608	0.9483	11.4
Heated	0.0430	0.9964	16.1

Table 13. Degradation of Plt in treated lake water in the dark at room temperature

3.4.4 Plt degradation in soil (Dong et al., 2011)

Plt degradation study was carried out with samples of both near-surface soil (0~10cm in depth) and rhizosphere soil (10 cm below surface), which were initially spiked with Plt at concentration of 500 μg/kg. From the results shown in Fig. 13, it can be found that the degradation of Plt in rhizosphere soil was faster than that near-surface soil, which was possibly due to the higher microbial activity in rhizosphere soil.

Plt degradation in studied soil tended to follow the first-order kinetics. The respective half-life values for Plt residue in near-surface and rhizosphere soil were 42.26 h and 32.84 h, much shorter than that in pure water.

On the whole the fast degradation of Plt under natural conditions (under sunlight, in soil, in natural water) demonstrated its excellent biological safety as an antimicrobial pesticide, but also revealed the disadvantage of not up to the requirement for a long control efficiency.

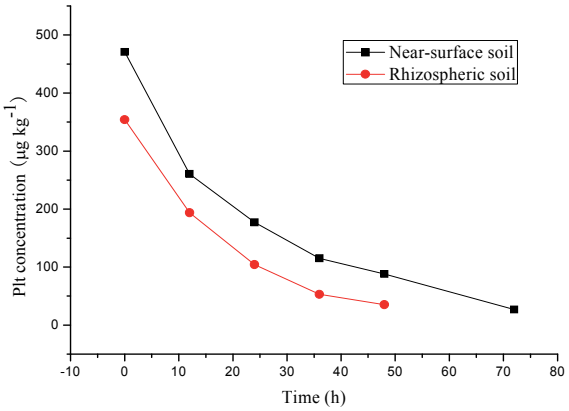

Fig. 13. Degradation curve of PCA in near-surface soil (■) and rhizosphere soil (●)

4. Conclusion

The effects of medium components on the production of Plt through cultivation of the *rsmA* inactivated mutant strain of *Pseudomonas sp.* M18R were investigated in shake flasks. The carbon sources examined were glycerol, sucrose, glucose, fructose, lactose, maltose, and ethanol, among which the 18 g/L glycerol was found to be the most favorable to Plt production. Meanwhile, the nitrogen sources examined were peptone, yeast extract, casein acid hydrolysate, and casein enzymatic hydrolysate, among which the 20 g/L peptone was found to be the most favorable to Plt production. To further enhance Plt production, the effects of carbon/nitrogen ratios were studied using central composite design and response surface analysis. The maximum Plt production titre of 648.3±20.1 mg/L was achieved in a medium with optimized carbon and nitrogen (i.e., 20.55 g/L glycerol and 18.6 g/L peptone). The effects of inorganic components on Plt production were also studied. The optimized medium was conducted in bioreactor cultivation, and a maximal production of 708.1± 35.7 mg/L and productivity of 9.08 ± 0.78 mg/(L · h) was attained after 78 h cultivation.

A simple and rapid method for Plt separation and purification from the fermentation broth of *Pseudomonas* sp. M18R was developed in this paper. Yellow Plt crystal with a purity of about 99% was obtained through ethyl acetate extraction, silica gel column chromatography and preparative HPLC with a total recovery of about 69.8%.

A sensitive analytical method based on CE with field-amplified sample stacking, Soxhlet's extraction and ultrasonic extraction for studying the trace amounts of Plt in soil samples was also developed. The results showed that this method was of high sensitivity, good linearity and good repeatability, and the whole processing procedures were proved with high recovery and convenience of analysis. This method can also be modified and developed to be applied in the determination of Plt in foods and drinking water.

The degradation of Plt under different conditions, such as water sources, pH value of solutions, UV intensity, soil et al., were investigated. Degradation of Plt followed first-order reaction kinetics, and it had a high degradation rate in soil, natural water and UV light. Plt

can decompose quickly in natural conditions suggested that it has good environmental compatibility. However, short residual time brings short reacting time. Thus, the method to improve its stability needs to conduct before its commercial use. The slow-release formulation of Plt in order to prolong its antifungal effects was achieved in our lab (Chen et al., 2010).

Overall, the findings of the study will be beneficial for the further development of the *Pseudomonas* spp. cultivation process in the large-scale production and the commercial use of Plt.

5. Acknowledgment

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6. References

Albert, M.; Debusschere, L.; Demesmay, C. & Rocca, J. (1997). Large-volume stacking for quantitative analysis of anions in capillary electrophoresis II. Large-volume stacking without polarity switching. *Journal of Chromatography A*, 757(1-2), 291-296

Anfossi, L.; Sales, P. & Vanni, A. (2006). Degradation of anilinopyrimidine fungicides photoinduced by iron (III): cpolycarboxylate complexes. *Pest Management Science*, 62(9), 872-879

Babalola, O.O. (2010). Beneficial bacteria of agricultural importance. *Biotechnology letters*, 32(11), 1559-1570

Brodhagen, M.; Henkels, M.D. & Loper, J.E. (2004). Positive autoregulation and signaling properties of pyoluteorin, an antibiotic produced by the biological control organism *Pseudomonas fluorescens* Pf-5. *Applied and Environmental Microbiology*, 70(3), 1758-1766

Burgi, D.S. & Chien, R.L. (1991). Optimization in sample stacking for high-performance capillary electrophoresis. *Analytical Chemistry*, 63(18), 2042-2047

Burrows, H. & Canle, L. (2002). Reaction pathways and mechanisms of photodegradation of pesticides. *Journal of Photochemistry and Photobiology B: Biology*, 67(2), 71-108

Carabias-Martinez, R.; Rodriguez-Gonzalo, E.; Revilla-Ruiz, P. & Dominguez-Alvarez, J. (2003). Solid-phase extraction and sample stacking-micellar electrokinetic capillary chromatography for the determination of multiresidues of herbicides and metabolites. *Journal of Chromatography A*, 990(1-2), 291-302

Carmi, R.; Carmeli, S.; Levy, E. & Gough, F.J. (1994). (+)-(S)-Dihydroaeruginoic acid, an inhibitor of *Septoria tritici* and other phytopathogenic fungi and bacteria, produced by *Pseudomonas fluorescens*. *Journal of natural products*, 57(9), 1200-1205

Casas Lopez, J.; Sánchez Pérez, J.; Fernandez Sevilla, J.; Acién Fernández, F.; Molina Grima, E. & Chisti, Y. (2003). Production of lovastatin by *Aspergillus terreus*: effects of the C: N ratio and the principal nutrients on growth and metabolite production. *Enzyme and Microbial Technology*, 33(2-3), 270-277

Chang, Y.N.; Huang, J.C.; Lee, C.C.; Shih, I.L. & Tzeng, Y.M. (2002). Use of response surface methodology to optimize culture medium for production of lovastatin by *Monascus ruber*. *Enzyme and Microbial Technology*, 30(7), 889-894

Chen, J.; Wang, W.; Xu, Y. & Zhang, X. (2010). Slow-release formulation of a new biological pesticide, pyoluteorin, with mesoporous silica. *Journal of Agricultural and Food Chemistry*, 845-849

Chien, R.L. & Burgi, D.S. (1991). Field amplified sample injection in high-performance capillary electrophoresis. *Journal of Chromatography A*, 559(1-2), 141-152

Corbell, N. & Loper, J. (1995). A global regulator of secondary metabolite production in *Pseudomonas fluorescens* Pf-5. *Journal of Bacteriology*, 177(21), 6230-6236

de Souza, J.T. & Raaijmakers, J.M. (2003). Polymorphisms within the prnD and pltC genes from pyrrolnitrin and pyoluteorin-producing *Pseudomonas* and *Burkholderia spp*. *FEMS microbiology ecology*, 43(1), 21-34

Dong, H.; Gao, B.; Wang, W.; Fan, L.; Xu, Y.; Zhang, X. & Cao, C. (2011). Experimental study on the determination and degradation of pyoluteorin in soil via CE with Soxhlet's extraction and field-amplified sample stacking. *Chromatographia*, 73, 609-612

Duffy, B.K. & Defago, G. (1999). Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *Applied and Environmental Microbiology*, 65(6), 2429-2438

Fang, Q.H. & Zhong, J.J. (2002). Submerged fermentation of higher fungus *Ganoderma lucidum* for production of valuable bioactive metabolites--ganoderic acid and polysaccharide. *Biochemical Engineering Journal*, 10(1), 61-65

Howell, C. & Stipanovic, R. (1980). Suspension of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. *Phytopathology*, 70(8), 712-715

Hu, H.B.; Xu, Y.Q.; Chen, F.; Zhang, X.H. & HUR, B.K.I. (2005). Isolation and characterization of a new fluorescent *Pseudomonas* strain that produces both phenazine 1-carboxylic acid and pyoluteorin. *Journal of microbiology and biotechnology*, 15(1), 86-90

Huang, X.; Zhu, D.; Ge, Y.; Hu, H.; Zhang, X. & Xu, Y. (2004). Identification and characterization of pltZ, a gene involved in the repression of pyoluteorin biosynthesis in *Pseudomonas sp*. M18. *FEMS Microbiology Letters*, 232(2), 197-202

Kim, H.; Roh, H.; Lee, H.J.; Chung, S.Y.; Choi, S.O.; Lee, K.R. & Han, S.B. (2003). Determination of phloroglucinol in human plasma by high-performance liquid chromatography-mass spectrometry. *Journal of Chromatography B*, 792(2), 307-312

La Fuente, L.D.; Thomashow, L.; Weller, D.; Bajsa, N.; Quagliotto, L.; Chernin, L. & Arias, A. (2004). *Pseudomonas fluorescens* UP61 isolated from birdsfoot trefoil rhizosphere produces multiple antibiotics and exerts a broad spectrum of biocontrol activity. *European Journal of Plant Pathology*, 110(7), 671-681

Lagarrigue, M.; Boss¨¦e, A.; B¨¦gos, A.; Delaunay, N.; Varenne, A.; Gareil, P. & Bellier, B. (2008). Field-amplified sample stacking for the detection of chemical warfare agent degradation products in low-conductivity matrices by capillary electrophoresis-mass spectrometry. *Journal of Chromatography A*, 1178(1-2), 239-247

Laville, J.; Voisard, C.; Keel, C.; Maurhofer, M.; Defago, G. & Haas, D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black

root rot of tobacco. *Proceedings of the National Academy of Sciences of the United States of America*, 89(5), 1562-1566

Li, Y.Q.; Jiang, H.X.; Xu, Y.Q. & Zhang, X.H. (2008). Optimization of nutrient components for enhanced phenazine-1-carboxylic acid production by gacA-inactivated *Pseudomonas sp*. M18G using response surface method. *Applied microbiology and biotechnology*, 77(6), 1207-1217

Liu, H.M.; Zhang, X.H.; Huang, X.Q.; Cao, C.X. & Xu, Y.Q. (2008). Rapid quantitative analysis of phenazine-1-carboxylic acid and 2-hydroxyphenazine from fermentation culture of *Pseudomonas chlororaphis* GP72 by capillary zone electrophoresis. *Talanta*, 76(2), 276-281

Matano, I.; Tsunekawa, M.; Shimizu, S.; Tanaka, I.; Mitsukura, K. & Maruyama, K. (2010). The chloride ion is an environmental factor affecting the biosynthesis of pyoluteorin and 2, 4-diacetylphloroglucinol in *Pseudomonas sp*. YGJ3. *Bioscience, biotechnology, and biochemistry*, 74(2), 427-429

Maurhofer, M.; Keel, C.; Haas, D. & Defago, G. (1994). Pyoluteorin production by *Pseudomonas fluorescens* strain CHA0 is involved in the suppression of *Pythium* damping-off of cress but not of cucumber. *European Journal of Plant Pathology*, 100(3), 221-232

Maurhofer, M.; Keel, C.; Schnider, U.; Voisard, C.; Haas, D. & Defago, G. (1992). Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. *Phytopathology*, 82(2), 190-195

Nakata, K.; Yoshimoto, A. & Yamada, Y. (1999). Promotion of antibiotic production by high ethanol, high NaCl concentration, or heat shock in *Pseudomonas fluorescens* S272. *Bioscience, biotechnology, and biochemistry*, 63(2), 293-297

Nowak-Thompson, B.; Chaney, N.; Wing, J.S.; Gould, S.J. & Loper, J.E. (1999). Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. *Journal of Bacteriology*, 181(7), 2166-2174

Nunez, O.; Moyano, E. & Galceran, M. (2002). Solid-phase extraction and sample stacking-capillary electrophoresis for the determination of quaternary ammonium herbicides in drinking water. *Journal of Chromatography A*, 946(1-2), 275-282

Palmarsdottir, S.; Mathiasson, L.; J nsson, J. & Edholm, L.E. (1997). Determination of a basic drug, bambuterol, in human plasma by capillary electrophoresis using double stacking for large volume injection and supported liquid membranes for sample pretreatment. *Journal of Chromatography B: Biomedical Sciences and Applications*, 688(1), 127-134

Park, J.; Kim, S.; Hwang, H. & Yun, J. (2001). Optimization of submerged culture conditions for the mycelial growth and exo-biopolymer production by *Cordyceps militaris*. *Letters in Applied Microbiology*, 33(1), 76-81

Saikia, R.; Varghese, S.; Singh, B.P. & Arora, D.K. (2009). Influence of mineral amendment on disease suppressive activity of *Pseudomonas fluorescens* to *Fusarium* wilt of chickpea. *Microbiological Research*, 164(4), 365-373

Sarniguet, A.; Kraus, J.; Henkels, M.D.; Muehlchen, A.M. & Loper, J.E. (1995). The sigma factor sigma s affects antibiotic production and biological control activity of *Pseudomonas fluorescens* Pf-5. *Proceedings of the National Academy of Sciences of the United States of America*, 92(26), 12255-12259

Schnider, U.; Keel, C.; Blumer, C.; Troxler, J.; Defago, G. & Haas, D. (1995). Amplification of the housekeeping sigma factor in *Pseudomonas fluorescens* CHA0 enhances antibiotic production and improves biocontrol abilities. *Journal of Bacteriology*, 177(18), 5387-5392

Tekeda, R. (1958). *Pseudomonas* pigments: Ⅰ.Pyoluteorin, a new chlorine-containing pigment produced by *Pseudomonas aeruginosa*. *Hakko Kgaku Zasshi*, 36, 281-290

Wang, Q.L.; Zhang, X.H.; Fan, L.Y.; Zhang, W.; Xu, Y.Q.; Hu, H.B. & Cao, C.X. (2005). Quantitative analysis of pyoluteorin in anti-fungal fermentation liquor of *Pseudomonas* species by capillary zone electrophoresis with UV-vis detector. *Journal of Chromatography B*, 826(1-2), 252-256

Wang, W.; Lu, X.; Qin, X.J.; Zhang, X.H. & Xu, Y.Q. (2008). Solubility of pyoluteorin in water, dichloromethane, chloroform, and carbon tetrachloride from (278.2 to 333.2) K. *Journal of Chemical & Engineering Data*, 53(9), 2241-2243

Whistler, C.A.; Corbell, N.A.; Sarniguet, A.; Ream, W. & Loper, J.E. (1998). The two-component regulators *GacS* and *GacA* influence accumulation of the stationary-phase sigma factor sigma S and the stress response in *Pseudomonas fluorescens* Pf-5. *Journal of Bacteriology*, 180(24), 6635-6641

Whistler, C.A.; Stockwell, V.O. & Loper, J.E. (2000). Lon protease influences antibiotic production and UV tolerance of *Pseudomonas fluorescens* Pf-5. *Applied and Environmental Microbiology*, 66(7), 2718-2725

Yuan, Z.; Cang, S.; Matsufuji, M.; Nakata, K.; Nagamatsu, Y. & Yoshimoto, A. (1998). High production of pyoluteorin and 2, 4-diacetylphloroglucinol by *Pseudomonas fluorescens* S272 grown on ethanol as a sole carbon source. *Journal of Fermentation and Bioengineering*, 86(6), 559-563

Zhang, J.; Wang, W.; Lu, X.; Xu, Y. & Zhang, X. (2010). The stability and degradation of a new biological pesticide, pyoluteorin. *Pest Management Science*, 66(3), 248-252

Zhang, X.; Wang, S.; Geng, H.; Ge, Y.; Huang, X.; Hu, H. & Xu, Y. (2005). Differential regulation of *rsmA* gene on biosynthesis of pyoluteorin and phenazine-1-carboxylic acid in *Pseudomonas sp*. M18. *World Journal of Microbiology and Biotechnology*, 21(6), 883-889

6

Using the Bio-Insecticide *Bacillus Thuringiensis Israelensis* in Mosquito Control

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1. Introduction

Mosquito control is a major public health concern, as mosquitoes transmit many severe human diseases such as malaria, filariasis, dengue, yellow fever, West Nile virus and the chikungunya virus. These diseases represent a major health threat and economic burden in disease-endemic countries, and are currently in expansion due to increased worldwide exchanges, urbanization, and global warming. The only effective way of reducing the incidence of these diseases is to control the vector mosquitoes, mainly by application of insecticides to their breeding places. Since the 1950s, the massive use of chemical insecticides has led to undesired toxicity on non-target organisms and the selection of insecticide resistance mechanisms in mosquito populations (Hemingway & Ranson, 2000). A safe alternative to chemical insecticides is to spray toxins produced by the bacteria *Bacillus thuringiensis* subsp. *israelensis* (Bti) over mosquito breeding sites (Lacey, 2007). Bti represents today the best alternative to chemical insecticides in controlling mosquitoes. Bti toxins are safe for non-target species and human health, are believed to show low persistence in the environment, and so far no resistance was detected in mosquito populations. Bti is the only insecticide allowed against mosquito larvae in Europe. To insure a long-term efficiency of this bio-insecticide, it is however necessary to evaluate the risks associated to its intensive worldwide use. The two main risks are (1) the accumulation of spores and toxins in the environment, and possible proliferation of Bti a long time after spraying, which may have an impact on the whole ecosystem functioning, and (2) the evolution of resistance to Bti in mosquitoes, rendering the treatment inefficient. It is therefore necessary to develop monitoring tools to follow the fate of spores and toxins in the environment and the evolution of resistance in target mosquito populations. Here we review recent advances in our understanding of the mechanisms of Bti toxicity and of mosquito resistance. The chapter will be organized in three parts: the first part describes Bti structure and its fate in the environment, the second part describes the action of Bti toxins after ingestion by mosquito larvae and the diversity of mechanisms involved in mosquito resistance, and the third part is dedicated to the challenging objective of managing resistance in the field. We conclude in identifying issues that need further research.

2. Bti in the environment

2.1 What is Bti?

Bacillus thuringiensis subsp. *israelensis* (Bti), serotype H14, is a subspecies of the diversified *Bacillus thuringiensis* species, an entomopathogenic bacterium able to survive in the environment as a spore and producing insecticidal toxins within an inclusion body during the process of sporulation. Bti was first isolated from a water pond in the Negev desert (Goldberg & Margalit, 1977) and was the very first strain described for having insecticidal activity outside Lepidoptera. Bti, like other *B. thuringiensis* subspecies, is a member of the *Bacillus cereus* complex. The characteristic of *B. thuringiensis* is the presence of an inclusion body or crystal (figure 1). The different subspecies are characterized by different flagellar H antigens (serotypes). However, the specificity to a given group of insects is a consequence of the particular set of proteins a strain is producing and there is thus no strict correlation between serotypes, the toxins they produce during sporulation as insecticidal crystal inclusions and the host range. Nevertheless, the specificity of the *B. thuringiensis* insecticidal proteins is central in the wide use of Bt as an alternative to chemical insecticides for the control of insect pests in forestry, agriculture, and public health. The serotype H14, Bti, produces 4 main toxins (Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa) specific to dipterans (mosquitoes, blackflies and chironomous midges). However, the 128-kb conjugative plasmid pBtoxis bearing the toxin genes carries three more genes for insecticidal proteins: Cry10Aa, Cyt2Ba and Cyt1Ca (Berry et al., 2002).

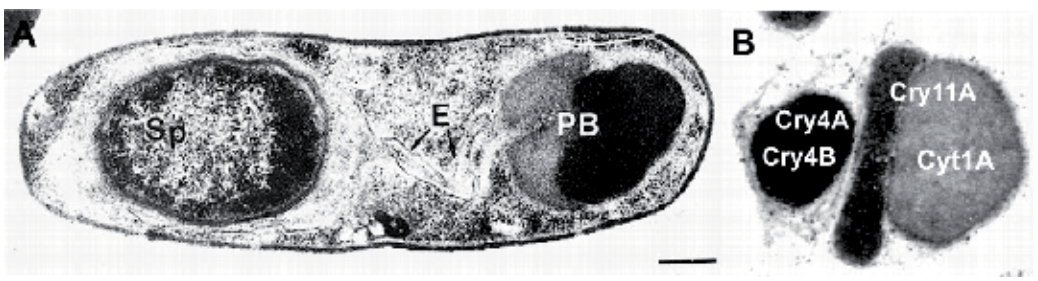

Fig. 1. Ultrastructural section of a sporulated *Bacillus thurigiensis* subsp. *israelensis* cell (A) and of a purified inclusion body (B). Sp: spore; E: exosporium; PB: parasposral body. From Federici et al., 2003.

2.2 Structure and mode of action of Bti toxins.

Like other Bt toxins the mode of action of the Bti toxins is closely related to specific structure-function relationships. One particular feature of Bti is that its insecticidal activity relies on the combination of three distinct groups of toxins with respect to structure-function and thus specific mode of action, i.e. Cry4Aa+Cry4Ba, Cry11A and Cyt1Aa. This also shows at the level of the inclusion body which is a composite entity comprising three different crystal component, one for each the three groups mentioned above. Indeed, each group folds and accumulates separately into a specific sub-inclusion body of different shape assembled into a spherical parasporal body and held together by a lamellar envelope (Ibarra & Federici, 1986, Federici et al., 2003). The organization of the genes on pBtoxis reflects these differing structures with a separate monocistronic organization for each toxin gene. However, coevolution and selection for synergism can also be seen in this organization.

Indeed, the Cyt1Aa1 is cytotoxic also to bacteria and to *B. thur*ingiensis and must be properly folded into an inactivated intermediate state until activation by insect midgut proteases. This proper folding is mediated by a chaperone protein, P20, which is located in the Cry11Aa operon, along with the P19 protein. Interestingly, Cry11Aa does not require the P20 chaperone for folding whereas Cyt1Aa1 which requires it is located in a separate cistron in opposite orientation from the Cry11Aa operon. This strongly suggests a coevolution of all these genes for synergism in mosquitocidal activity which is also underlined by the divergence of the four major Bti toxins in toxicity and host range: Cry4Ba is active primarily against *Anopheles* and *Aedes*, and shows no toxicity to *Culex* species, in contrast to Cry4Aa toxin that is toxic to *Culex* larvae. Cry11 is the most toxic to *Aedes*, and Cyt1Aa shows low (*Aedes*, *Culex*) to non-toxicity at all (*Anopheles*). Cyt1Aa have a strong synergistic effect on the toxicity of Cry toxins in all mosquitoes

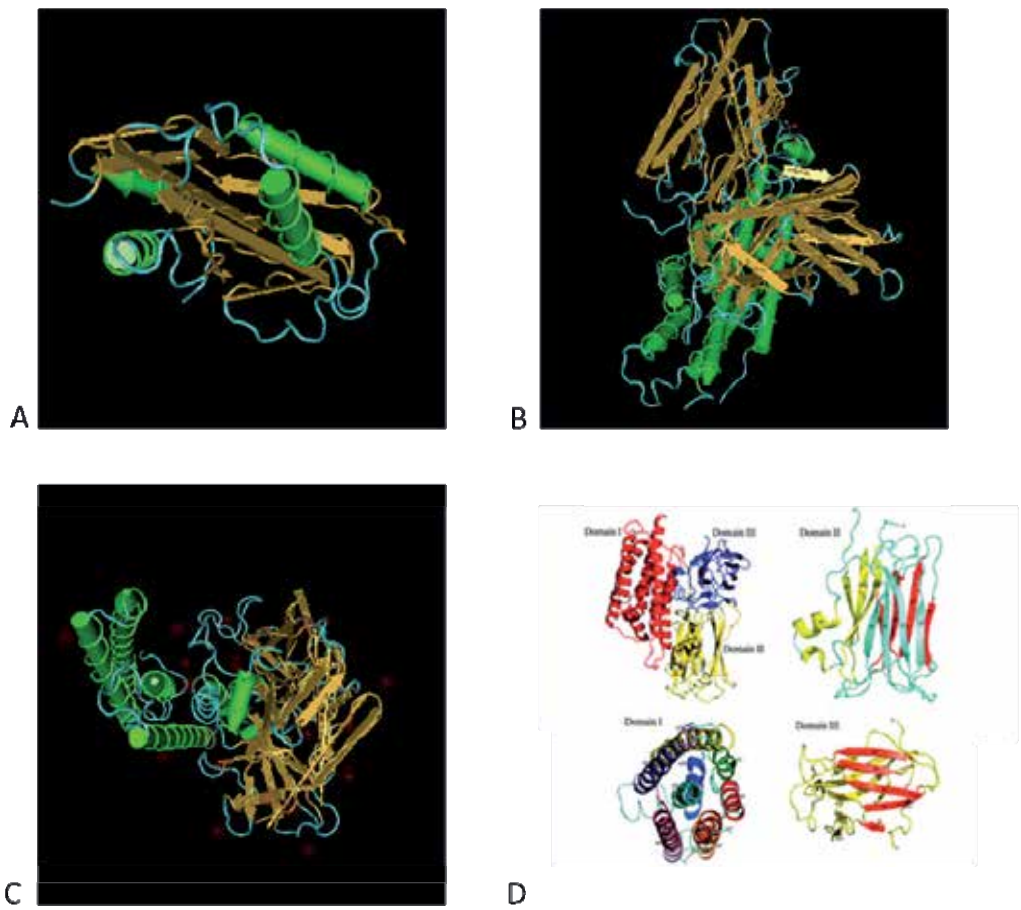

Fig. 2. 3-dimentional structure of *Bacillus thuringiensis* subsp. *israelensis* toxins. A: Cyt2Ba (from Cohen et al 2008). B: Cry4Aa (from Boonserm et al., 2006), C: Cry4Ba (from Boonserm et al., 2005), D: Ribbon view of the complete Cry4Aa toxin and of the separate structural domains (from Boonserm et al., 2006)

The 3-D structure of several Bti crystal proteins has been determined using x-ray chromatography. The crystal structure of Cry4Aa (Boonserm et al., 2006) and Cry4Ba (Boonserm et al., 2005) were analyzed with 2.8 and 1.75 Angström resolution, respectively. The overall structure of Cry4Aa and Cry4Ba resembles that of Cry1Aa (Grochulski et al., 1995) and Cry3Aa (Li et al., 1991; Galitsky et al., 1997) with a three-domain organization (figure 2). Domain I is composed of several α helices (the number varies between Cry4Aa and Cry4Ba) and is involved in the formation of a pore in the midgut epithelial cell membrane following insertion of the α4 and α5 transmembrane hairpin (Boonserm et al., 2005, 2006, Grochulski et al., 1995, Li et al., 1991; Galitsky et al., 1997). Domains II and III are composed of beta sheets and are involved in the specific recognition of the midgut receptors and in stability of the toxin (Boonserm et al., 2005, 2006, Grochulski et al., 1995, Li et al., 1991; Galitsky et al., 1997). Although this structure is very similar to that of Cry1 and Cry3 toxins, several differences are present between Cry4 and other toxins but also between Cry4Aa and Cry4Ab. A first set of differences appear with structural domain I.

Domain I of Cry4Aa comprises seven α-helices, like Cry1Aa or Cry3Aa (Grochulski et al., 1995, Li et al., 1991; Galitsky et al., 1997). However, the structure of the α4-α5 loop is unique with the presence of a specific disulfide bound and a proline-rich motif rigidifying the hairpin and limiting the flexibility of the inter-helix loop (Boonserm et al., 2006). A consequence is that, unlike the very closely related Cry4Ba, Cry4Aa was unable to allow the release of calcein (Boonserm et al., 2006) which suggests a difference of channel behaviour, and thus of mode of action, between Cry4Aa and Cry4Ba. If Cry4Ba displays a α4-α5 loop similar to that of Cry1 and Cry3, its domain I also exhibits a specific structure. Indeed, unlike the structurally related Cry toxins, and unlike Cry4Aa, domain I of Cry4Ba does not display seven α-helices but only five (Boonserm et al., 2005). Alpha helices 1 and 2 are absent, although the DNA sequence encoding this region is present in the toxin gene, which indicates that they are removed by proteolysis, probably in the process of crystallization. Nevertheless, Cry4Ba retains full mosquitocidal activity. A key difference is in structural domain II and more precisely in the loops connecting the β-sheets (Boonserm et al., 2005, 2006). Domain II is the most variable domain among all Cry toxins and these loops are involved in insect receptor specificity. Cry4Aa and Cry4Ba differ significantly in the size, especially loop2, and sequence of the domain II loops and exchanging loop 3 significantly increased toxicity of Cry4Ba to *Culex* while its activity against *Aedes* and *Anopheles* remained (Abdullah et al., 2003). This indicates that specificity for receptor binding in *Culex* is located mainly in loop 3 whereas specificity to *Aedes* and *Anopheles* is located in loops 1 and 3 (Abdullah et al., 2003). These data also further indicate that the closely related Cry4Aa and Cry4Ba toxins display different specificity and structures and that they act synergistically within the Bti inclusion body to increase the insecticidal host range. Cyt2Ba, is the other Bti toxin for which the 3-D structure was determined (Cohen et al., 2008). Unfortunately there is no 3-D structure available for Cyt1Aa, the major cytotoxin from Bti. Another 3-D structure of a Cyt2 toxin is available but not from Bti. It is the Cyt2Aa cytotoxin from *B. thuringiensis* subsp. *kyushuensis* (Li et al., 1996). Nevertheless, these structure analyses provide similar data and conclusion and one can assume that they might also apply to Cyt1Aa owing to the similarity of biochemical traits between Cyt1 and Cyt2 toxins. Unlike Cry4 toxins, the monomeric Cyt2 is organized as a β-sheet made of 6 antiparallel β-strands flanked on each side by two short α-helice (Li et al., 1996; Cohen et al., 2008). Still unlike Cry4 toxins, the transmembrane domain is made by the β-strands and not the α-helices which are involved

in interprotein binding and oligomerisation (Du et al. 1999; Li et al., 1996; Gazit et al., 1997). These data on the structure of Cry and Cyt toxins from Bti clearly indicate major structural and functional differences and must be considered when addressing their respective mode of action and resistance to Bti toxins.

All Bti insecticidal proteins are produced as protoxins and all must be activated *in vivo* by insect midgut proteases prior insecticidal activity. Following this initial activation, the subsequent receptor binding step is still not fully resolved (figure 3).

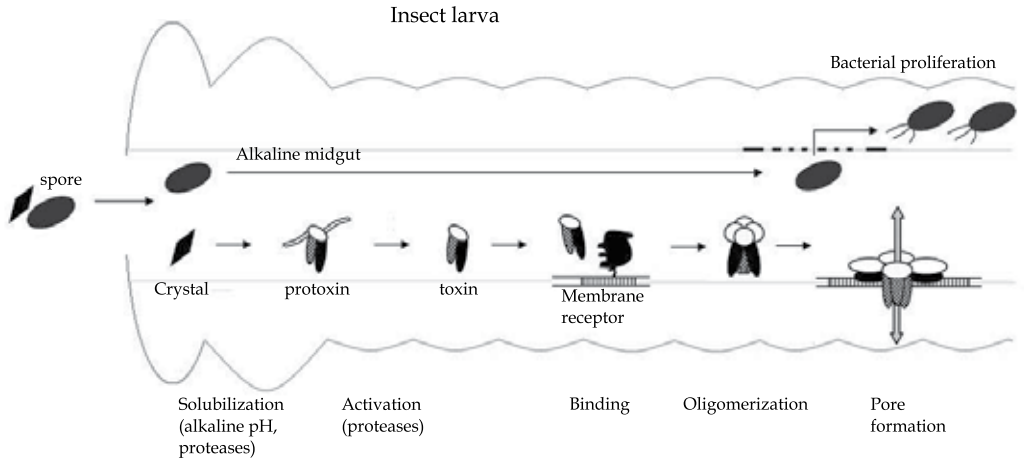

Fig. 3. Model of the mode of action of Bti Cry toxin in insect midgut. The crystal is ingested by larva, protoxins are solubilized in the alkaline midgut and activated to toxins that bind to specific membrane receptors, oligomerize and form a pore allowing the bacteria to proliferate in the host larva. Another model was recently proposed that does not involve oligomerization and pore formation. After the death of the larva, bacteria are liberated in the environment and they sporulate still they encounter another host. The two main resistance mechanisms are modifications in the activity of midgut proteases and in the receptor sites. Adapted from Bravo et al., 2007.

The Cry proteins were shown to bind to midgut receptors but the nature of the receptors is not completely established. There is no report on the receptors recognized by Cry4Aa. However, with respect to Cry4Ba, cadherin AgCad1 of *Anopheles gambiae* (Hua et al., 2008; Park et al., 2009) and alpha-amylase from *Anopheles albimanus* (Fernandez-Luna et al., 2010) were shown to act as receptors. Furthermore, a series of other proteins, i.e. aminopeptidase, several alkaline phosphatase isoforms, flotillin, prohibitin, V-ATPase B subunit and actin were shown to bind Cry4Ba in *Aedes aegypti* (Bayyareddy et al., 2009). Data are also available on the membrane receptors of Cry11Aa. Cadherin (Chen et al., 2009a), aminopeptidase N (Chen et al., 2009b), alkalinephosphatase (Fernandez et al., 2009) and alpha-amylase (Fernandez-Luna et al., 2010) have all been described as Cry11Aa receptors in *Ae. aegypti*. Following binding, these proteins insert in the membrane to form a pore and more precisely an ionic channel triggering osmotic imbalance, cell death and ultimately insect death. However, important and not yet fully resolved steps are involved in this membrane insertion and permeation process. A first intermediate step seems to be oligomerization into a prepore structure with the probable involvement of membrane receptors. However, activated toxins are hydrosoluble intermediate forms which must undergo a conformational

change to expose hydrophobic domains and insert stably into the membrane in order to form a transmembrane ionic channel. The two Cry4 toxins undergo this process in a way similar to that of the Cry1 or Cry3 proteins. The tight structure of the toxin is lost and the structural domain I moves freely from domains II and III allowing the bundle of α helices to reorganize at the contact of the lipid membrane. The highly hydrophobic helix α5 inserts into the membrane dragging along α4 which, with its free charged residues, will conduct ions through the membrane. This mode of action similar to that of Cry1 toxins requires more than one toxin to form a pore (figure 3). Atomic force microscopy analyses showed that four Cry4Ba toxins are required to form a pore (Puntheeranurak et al., 2005) exactly like Cry1Aa (Vie et al., 2001; Laflamme et al., 2008). Although Cry11Aa was shown to form pores in the membrane like Cry4 toxins, the exact mode of membrane insertion and permeation is still not fully described. Data available suggest that Cry11Aa could act in different ways. Proteolysis of the 72-kDa Cry11Aa was reported to result into two inactive subunits of 32 kDa and 36 kDa. However, when these two subunits were mixed in equimolar amounts, full insecticidal activity was restored (Yamagiwa et al., 2002, 2004; Revina et al., 2004). This suggests that Cry11Aa could display at least under some conditions a binary-like action. However, there is no indication of whether this process is the normal mode of action of Cry11Aa, a particular mechanism or an intermediate step. Nevertheless, its mode of action must be rather complex owing to its interaction with Cyt1A. Cry11Aa was indeed reported to bind to Cyt1A which can facilitate pre-pore oligomerization (Perez et al., 2007) and act as an additional membrane-bond receptor, increasing thus the ability of Cry11Aa to insert in the membrane (Perez et al., 2005). Unlike the other Bti toxins, Cyt1A does not recognize a specific membrane receptor. It has the ability to insert by itself in the lipid bilayer (Butko et al., 1996; Gazit et al., 1997; Du et al., 1999) and form cationic channels pores through a process of detergent-like colloid-osmosis (Knowles et al., 1989, Manceva et al., 2005). Recently, domain homology with the *Erwinia* virulence factor (evf) was found which could explain this ability to integrate into lipid bilayers (Rigden, 2009). In addition to its own mosquitocidal and cytotoxic activity, Cyt1A was shown to act synergistically with the other Bti toxins (Wu & Chang, 1985; Ibarra and Federici, 1986; Federici et al., 2003; Crickmore et al., 1995; Perez et al., 2007, Soberon et al., 2010; Canton et al., 2011) but also with *Bacillus sphaericus* toxins (Wirth et al., 2000a). This synergistic effect is at the heart of the mode of action of Cyt1A and is explained by involvement of the N-terminal part of Cyt1Aa in protein-protein interaction with Cry toxins while the C-terminus of Cyt1A is involved in hydrophobic interaction and membrane insertion (Rodriguez-Almazan et al., 2011). Cyt1A binding to Bti Cry toxins provide a means for shunting the natural membrane receptors of these Cry proteins increasing the level of pore formation and membrane disruption, leading thus to synergism, but even more importantly leads immediately to the key aspect in insect control with Bt toxins: preventing and overcoming insect resistance.

2.3 Type and use of Bti formulations

The discovery of the pathogen activity of Bti against Dipteran vectors (mosquitoes and black flies) was rapidly followed by applications. From 1980s', thanks to combined efforts of World Health Organization (WHO), other institutions, and numerous research laboratories, several international programs were developed for the use of Bti and *Bacillus sphaericus* (Bs) (Lecadet, 1996). Primary powder formulation of Bti had virtually no residual effect against mosquito larvae beyond application, although the delta-endotoxin remained chemically stable in neutral and acid waters (Sinègre and al., 1980). Numerous trials and field

Type of Formulation	Product Name	Potency (UTI/mg)	Registered dose/surface unit	Main uses
Bti alone				
Technical powder (TP)	VectoBac® technical Powder Aquabac® primary powder	6000 7000		Manufacturing use product intended for formulation into end-use products
Suspension concentrate (SC)	VectoBac® 12AS AquaBac® XT Teknar®HD-P	1200 1200 1200	0.25-1-2 pts/acre 0.25-1-2 pts/acre 0.25-1-2 pts/acre	All mosquito breeding sites by ground or by air application: irrigation ditches, roadside ditches, flood water, standing ponds, woodland pools, snow melt pools, pastures, catch basins, storm water retention areas, tidal water, salt marshes and rice fields. Standing water containing mosquito larvae, in fields growing crops (alfalfa, almonds, asparagus, corn, cotton, dates, grapes, peaches and walnuts) Polluted water (such as sewage lagoons, animal waste lagoons). Liquid formulations are also recommended for control of black fly in streams and nuisance flies (*Psychoda* spp., *Chironomus* spp.) in sewage treatment facilities utilizing trickling filter systems.
Water dispersible granule (WG)	VectoBac® WDG Aquabac® DF 3000	3000 3000	1.75-7-14 oz/acre 1.75-7-14 oz/acre	
Granule (GR)	VectoBac® G Aquabac® 200G Aquabac® 400G Mosquito Bits®	200 200 400 200	2.5-10-20 lb/acre 2.5-10-20 lb/acre 1.5-5-8 lb/acre 0.5 lb/2178 ft²	
Tablet (TB)	VectoBac® DT	3400	1 tab./50 l	Artificial containers (terracotta, concrete, iron, plastic), flower pots, catch basins, and a variety of small breeding sites.
Briquette (BR)	Mosquito Dunk® Summit Bti Briquets™	7000 7000	1 br/25-100 ft². 1 br/25-100 ft².	Outdoor applications near the household where water collects and remains for periods of time,

				ditches, tree holes, roof gutters for collecting rainwater, flower pots, animal watering troughs, etc. or indoor use such as elevator shafts, basements that flood, sump pumps and any drainage areas within buildings
Bti + *Bacillus sphaericus*				
Granule (GR)	VectoMax® CG VectoMax® G VectoMax® WSP	nc & 50 nc & 50 nc & 50	5-20 lbs/acre (0.5-2 lbs/1000 ft² in used tires) 1 pouch/50 ft²	Sewage effluent, sewage lagoons, oxidation ponds, septic ditches, animal waste lagoons, impounded wastewater associated with fruit and vegetable processing. drainage ditches, roadside ditches, retention, detention, and seepage ponds, tidal water, salt marshes, mangroves, estuaries, natural and man made aquatic sites , waste tires stockpiled in dumps, landfills, recycling plants, and other similar sites. Briquets are recommended in quite small man-made and natural water holding receptacles.
Briquette (BR)	FourStar™ Briquets 45 FourStar™ Briquets 90 FourStar™ Briquets 180	70 & 60 70 & 60 70 & 60	1 br/≤100 ft² 1 br/≤100 ft² 1 br/≤100 ft²	
VectoBac®, Teknar® are the registered trademarks of Valent BioSciences Corp., Illinois, USA ; AquaBac® is the registered trademark of Becker Microbial Products Inc., Florida, USA; Mosquito Dunk®, Mosquito Bits® are the registered trademarks of Summit Chemical Co, Maryland, USA; Fourstar™ is the registered trademark of Fourstar Microbials LLC, New York, USA. nc= not communicated				

Table 1. Formulations currently available on the European and USA market, their respective potency, doses of application, and main uses. The manufacturers recommend the higher dosage rates when late 3rd and early 4th instar larvae predominate, mosquito populations are high, water is heavily polluted, and/or algae are abundant.

observations show a quick decline in efficacy within few days in open conditions and a very low residual activity afterwards. This decline is mainly due to the quick sedimentation outside of the nutrition zone of the larvae, the inactivation by UV light of tryptophan residues essential for insecticidal activity (Pusztai et al., 1991; Padilla et al., 2006) or degradation in polluted or highly organic matter concentrated water. If several authors have reported recycling of Bti in larval cadavers in controlled or simulated conditions (Aly et al., 1985; Khawaled et al., 1990;

Boisvert & Boisvert, 1999), evidence of such recycling under natural field conditions is scarce (but see Tilquin et al, 2008; de Melo-Santos et al, 2009). Numerous papers reviewed by Lacey (2007) have studied the biotic and abiotic factors influencing the larvicidal activity of Bti i.e. the specific susceptibility of the target species and their feeding strategies, the rate of ingestion, the density of larvae and their age, the dosage, temperature, solar radiation, depth of water, turbidity, tannin and organic content, vegetation coverage, etc. If the first Bti-based formulations were a technical powder more or less difficult to use due to the bad miscibility with water, various types of formulations were developed since 1981, adapted to the different mosquito species and habitats to be treated (Table 1).

The different formulations are aimed to favour a better contact with larvae by taking into account their bioecology and the specificity of their habitats. The type of formulation influences highly the efficacy and the persistence of Bti depending on the toxin content, how effectively the material reaches the target, and settling rate, storage conditions, means of application and frequency of treatments, and production factors, especially the medium in which the bacterium is grown. When Bti is applied as a liquid (after mixing a suspension concentrate or a water dispersible granule in water) or a ready-for-use granule formulations directly in open field, its biological efficacy declines generally quickly after 24 to 48 hours. Such formulation are used where short action is required, by instance for the control of univoltine species like *Ochlerotatus caspius* and *Oc. detritus* in Mediterranean or Atlantic temporary flooded saltmarshes or *Oc. rusticus, Aedes vexans*, or *Oc. sticticus* in fresh water breeding sites as in Rhone-alpine or upper Rhine valleys. According of the size of the breeding site, the products are applied by ground using hand, or shoulder-carried manually-operated compression sprayers or knapsack sprayer with gooseneck lance, portable or vehicle-mounted power-operated mistblowers. Airborne applications (helicopters, fixed-wing aircrafts) may be justifiable in emergency situations but also in routine mosquito control where large or inaccessible areas must be treated quickly (World Health Organisation, 1996), by instance in many US counties, along the French Western Mediterranean coast and in Thessalonica plain, in the upper Rhine valley, the Po and Elbra deltas, etc.). On the contrary, ready-for-use tablets, pellets, briquets or specific slow release granules are designed to persist for several weeks. They are above all used in urban or periurban areas for the control of plurivoltine species like *Culex* or *Aedes* spp. (Stegomyia) in small natural or anthropic peridomestic breeding sites like containers, flower pots, catch basins, etc. Bti-based products are now used all over de world. They represent now the main larvicides used in natural areas in Europe. Urban and periurban mosquito vector control strategies under subtropical and tropical conditions depend of the target species and are based on the use of a panel of insecticides for controlling adults and/or larvae. Where they are recommended, larvicides used are generally long lasting chemicals like organophosphates whereas the use of bioinsecticides and insect growth regulators is still limited (WHO, 2009). However, Bti is used in alternance where resistance to chemical insecticides appears, or to replace them when they are prohibited and/or removed from the market as in the French overseas territories. The long-lasting or slow release Bti-based products are now able to compete with them. Different extemporaneous formulations were also tested and used. Before Bti granule formulation was available on the market, some mosquito control operators like EID Rhône-Alpes (France) applied by helicopter sand granule mixed beforehand with Bti-based wettable powder and vegetal oil. Such method was used to improve the penetration of the product into the canopy for the control of

wooded breeding sites. The frozen granule formulation "IcyPearls" produced by Becker (2003) and used against *Ae. vexans* larvae in the upper Rhine River Valley of Germany has certain advantages over Bti sand granules. The ice pellets melt on the water surface where the microbial toxins are slowly released. Applied by helicopter, the ice formulation results in increased swath widths and the cost of application is consequently reduced.

2.4 Effect of environmental variables on Bti persistence and proliferation

Numerous studies have assessed the persistence of Bti toxicity after treatment. Several environmental factors such as solar radiation, temperature, type of substrate, presence of vegetation, salinity, pollution, water height, were shown to influence the persistence of Bti toxicity in the environment (reviewed in Lacey, 2007). Depending on all these factors, Bti toxicity was shown to decrease with highly variable patterns in the field, from a few days to several weeks. In contrast, very few studies looked at the fate of Bti spores in the environment after spraying (Hajaij et al., 2005; Tilquin et al., 2008), and no studies exist so far on the fate of Bti toxins in the environment. Reduced toxicity over time does not mean that Bti spores and toxins are quickly eliminated in the environment: they may accumulate, or even proliferate in soil, or in decaying vegetation at the bottom of mosquito breeding sites (Tilquin et al., 2008; de Melo-Santos, 2009). If many studies have investigated the fate of Cry toxins produced by genetically modified plants (GMPs) and released into agricultural soils (e.g., Clark et al., 2005), such studies are missing for Bti toxins. It is however important to be able to follow the fate of each toxin in the environment after spraying, because the acute toxicity of Bti is due to the synergistic action of toxins. If some toxins are more rapidly eliminated than others in the environment, mosquito populations might be in contact long after spraying with only one or two of the most persistent toxins, thereby favouring the evolution of resistant mechanisms to these toxins, a first step toward the evolution of resistance to Bti. There is therefore an urgent need for developing an easy-to-use immunological test for detecting each of the main Bti toxins in environmental samples. Indeed, if commercial ELISA kits are available for detecting each of the Bt toxins introduced in GMPs, such immunological tests are not available so far for Bti toxins. Until now, few studies have evaluated the persistence of Bti spores in the environment following spraying (Hajaij et al., 2005). In this study, to follow the fate of Bti spores after spraying, environmental samples were collected several times after treatment, plated on a nutrient-rich medium and Bt-like colonies (i.e., sporulate cells containing crystal inclusions visualized using a phase-contrast microscope) were counted. Besides being a fastidious method, it does not allow discriminating between Bti and other Bt strains potentially naturally present in the environment. Recently, a method involving whole DNA extraction from environmental samples and amplification of the Cry4 (A and B) genes present only in Bti by real-time quantitative PCR (RT-qPCR) was proposed for an accurate and precise quantification of the Bti present in treated sites (Guidi et al., 2010); this method does not require bacterial cultivation and allows the quantitative detection of Bti spores directly from environmental samples. This molecular tool allows monitoring the fate of Bti spores and possible recycling in the environment after a treatment.

2.5 Ecological risk

2.5.1 Effect of Bti on non-target species

Bti products present low risk for the human health through direct or indirect exposure. Laboratory studies have demonstrated that Bt and Bt products are non infectious and are

toxic to mammals only at a dose higher or equal to 10^8 colony forming unit per mouse (Siegel, 2001). The pH and presence of receptors in the midgut determine the specificity of the larvicide action of Bti. Since the discovery of its insecticidal potential in 1976, the innocuity of Bti for micro- and macro-invertebrates, fishes, batracians, and other vertebrates sharing the same habitats as mosquito larvae is well established at dosage rates used at the operational scale (Boisvert & Lacoursière, 2004; Lacey & Merritt, 2004). All the studies carried out in laboratory and in field conditions show that Bti has a main target effect on the Diptera Nematocera i.e. Culicidae, Simulidae, and Chironomidae (Ali, 1981, Garcia et al., 1981, Merritt et al., 1989, Boisvert & Boisvert, 2000, He & Ong, 2000). Amongst the 77 scientific papers reviewed by Boisvert and Lacoursière (2004), negative effects (mortality, population reduction) were observed on 15% of the 616 identified taxa of non target aquatic organisms after Bti treatment against mosquitoes or black flies. Amongst these 98 taxa, 62% were exposed to concentrations of Bti 5 to 1000 fold the recommended dosage rate and 45% were chironomids. The other impacted taxa belong to Diptera, Trichoptera, Plecoptera, Ephemeroptera, Lepidoptera, and Hemiptera as well as some worms, Crustacea, Gasteropoda, Fish or Algae, all after exposure to extreme overdosage.

2.5.2 Effect of Bti on ecosystems

The activity persistence of Bti and the induced side-effects on non target organisms depend on the type and the characteristics of the formulation, the frequency of application as well as the environmental factors such as the temperature, the water depth or the vegetation. A 5-years study including three years of intensive Bti treatments (six applications on three months between 1991 and 1993) showed a reduction of the taxonomic richness and the total number of invertebrates (Hershey et al., 1998; Niemi et al., 1999). These changes should have interfered with the trophic network involving the invertebrates but did not affect the populations of zooplancton and nesting birds. In a 4-years study conducted between 1998 and 2002 by Lagadic et al. (2002) in Morbihan, France, the health, the number, and the abundance of non target aquatic invertebrates present in mosquito breeding sites treated annually with Bti (VectoBac®12AS) were monitored. Bti was sprayed by ground using knapsack sprayers. No significant effect was observed on both sentinel species *Nereis diversicolor* and *Chironomus salinarius*. Even analysis at the community level showed that the environmental fluctuations had a more important impact on the community structure and that no Bti effect was detectable. A comparative study with VectoBac® 12AS and VectoBac®WG carried out in the same habitats between 2006 and 2007 did not demonstrate any effect on aquatic invertebrates namely on groups representing a trophic interest for the birds. These results allow considering that Bti treatments at the recommended registered dose did not present unacceptable risks for non target aquatic invertebrates and the trophic chains. In the frame of a in situ study (European project n°LIFE99 ENV/F/000489) carried out from 2000 till 2003 in Grande Camargue, southern France, on the impact of five treatment campaigns with VectoBac®12AS, comparatively with temephos, an organophosphate larvicide, on temporary flooded saltmarshes favourable to *Ochlerotatus caspius* and *Oc. detritus*, no significant effect of Bti on Chironomidae and other invertebrate taxa were observed during the three successive stocking stages characterising these habitats: a first colonization stage by *Ochlerotatus* spp., then Baetidae and Chironomidae is followed by a maturity stage with a dominance of Chironomidae and Baetidae and a higher taxonomic richness, and finally by a senescence stage where sedentary (Crustacean), allochtonous, and predatory taxa are predominant. In another study carried out also in

Camargue, Franquet and Fayolle (2002) did not highlight effect of Bti on flora and non target fauna in similar temporary flooded breeding sites of *Oc. caspius*. No proliferation of phytoplanctonic algae was observed after the disappearance of the mosquito larvae and some other taxa playing the role of filter-feeders. In cases of overdosage at 8 L/ha VectoBac®12AS (1200 UTI/mg), adverse effects on population dynamic of chironomids were observed, immediately after the treatment, or 2 and 8 days after, although the persistence of Bti is low. The dose of 3 L/ha seems to be the maximum acceptable dosage for the fauna in such temporary breeding sites.

A recent study focused on the development and use of methods for assessing the environmental risk of Bti and another bio-insecticide, spinosad, in the context of mosquito control in coastal wetlands of two French ecoregions: Morbihan (south Brittany) and Grande Camargue (southern France) (Duchet et al., 2008, 2010). *Daphnia pulex* and *Daphnia magna* were used as model species in laboratory bioassays and as sentinel species in field studies performed in *in situ* microcosms (enclosures). The scientific approach aimed at determining whether the toxic effects (lethal and non lethal) that affect individuals can be detected at the population level. Modelling with RAMAS GIS was used to simulate effects of the 2 larvicides on population dynamics, and extinction risk of the exposed populations was estimated. Bti showed no significant impact on both *D. pulex* and *D. magna* population dynamics, a result confirmed by experiments conducted in microcosms that showed no impact of Bti on *D. pulex* population even when exposed to the highest concentration of 0.50 $\mu L.L^{-1}$ (Duchet et al., 2008). Combination of laboratory bioassays and field microcosm studies provides a sound and reproducible methodological framework that could be used to define a strategy for the risk assessment of bio-larvicides used for mosquito control in coastal wetlands.

Ecological and structural impact of insecticides on the trophic networks and aquatic and terrestrial communities could result from the massive reduction of one or more organism groups. Some studies have been published on these effects associated to the temporary disappearance of mosquito larvae after Bti treatments. Mosquito larvae are only a part of the diet of aquatic and terrestrial predators. Mammals (e.g. mole, shrew), birds (ducks, seaguls, starling, minah bird, nightjars, swallows), batrachians (frogs, salamanders), fishes (salmons, trouts, stickle back, miller's stumb, perches, brill, bass, zebrafish, chub), spiders, insects (odonates, plecoptera, megalopetra, trichoptera, coleoptera, hymenoptera, diptera) and microinvertebrates (platyhelminthes, leech, crustaceans) can eat larvae and adults of mosquitoes and black flies (Peckarsky, 1984; Crosskey, 1990; Davies, 1991). But even in the case of high abundances, mosquito represents less than 5% of the diet of birds (Bourassa, 2000) probably because their respective activity periods do not overlap. However, using the common house martin *Delichon urbica* species as a model, Poulain et al. (2010) assessed the effect of Bti spraying on foraging rates and chick diet prior to and during three years of Bti spraying in the Camargue, France. Intake of Nematocera (including midges and mosquitoes) and their predators (spiders and dragonflies) decreased significantly at treated sites concurrently with increase of flying ant intake. Small preys were significantly more taken at treated relative to control sites where the foraging rates were also higher. As a result, clutch size and fledgling survival were significantly lower at treated sites relative to control. Bats feed at sunset during the activity period of mosquitoes. However, the bolus of *Myotis daubentoni* contains only 0 to 8.25% Culicidae and the bolus of *Pipistrellus nathusii* 4.86-9.91% Culicidae and Chaoboridae (Arnold et al., 2002). Several studies have shown that invertebrate predators and detritivores eat mosquito larvae killed by Bti without negative impact on their growth and emergence (Aly & Mulla, 1987; Wipfli & Merritt, 1994a, 1994b)

3. Evolution of Bti resistance in mosquitoes

Resistance to Bti toxins is nothing else than the interruption at one place or at several places of the cascade of events known as "mode of action". This interruption of the mode of action, i.e. mechanisms of resistance, can occur at different levels including toxin lack of activation, toxin proteolysis, precipitation of the toxin, modification of the receptor but also through a lack of pore formation after specific binding (Frutos et al., 1999). However, the most frequently encountered mechanisms of resistance were receptor mutation and altered binding on one hand and lack of proteolytic toxin activation on the other hand. It is also important to consider that almost all these accounts are laboratory works under forced artificial conditions where resistance can be easily selected. With respect to Bti, there is, despite a significant amount of research over the last twenty years, no resistance to the whole Bti crystal in laboratory or in the field. The reason for that is a direct consequence of the mode of action of Cyt1Aa and its interaction with the other Bti toxins. Resistance to individual Bti toxins was easily developed under laboratory conditions. High levels of resistance were established for Cry11Aa, Cry4Aa+Cry4Ba and Cry4Aa+Cry4Ba+Cry11Aa and in all cases the addition of Cyt1Aa overcame the resistance established (Georghiou & Wirth, 1997; Wirth et al., 1997, 2005a, 2010; Khasdan et al., 2001; Federici et al., 2003). Conversely, evolution of resistance could not be obtained when Cyt1Aa was present. This positive impact of Cyt1Aa on the prevention of resistance or overcoming of established resistance was also observed with *B. sphaericus* toxins (Wirth et al., 2000a, 2004, 2010). Furthermore, addition of Cyt1Aa to *B. sphaericus* resulted in a 3600-fold increase of toxicity to *Ae. aegypti* (Wirth et al., 2000b). This increase of toxicity of *B. sphaericus* toxins was linked to the Cyt-mediated insertion of *B. sphaericus* Bin toxins in the membrane (Federici et al., 2003). It is this same cooperative effect of Cyt-mediated membrane insertion that prevents resistance to Bti toxins by shunting mutating receptors or making the common resistance mechanism of receptor mutation inefficient. Synergism, combinational mode of action and counter-resistance effect are unique evolutionary traits of Bti which makes it the ideal biocontrol agent and explain why it is still the most efficient tool for mosquito control. A major point to address today is thus how to further improve the product and even more importantly how to make it sustainable.

In natural mosquito populations, no consistent resistance has been detected even after long periods of repeated treatment with Bti toxins, but recent studies suggested that moderate Bti resistance may occur locally (Paul et al., 2005; Boyer et al., 2007; Paris et al., 2010). Furthermore, we obtained in only 22 generations of selection using environmental Bti (i.e., field collected leaf litters containing Bti toxins), a strain of *Ae. aegypti* resistant only moderately to commercial Bti (2-fold), but 4-fold to environmental Bti and up to 30-fold to individual Cry toxins (Paris et al., 2011a). Furthermore, in this leaf litter collected several months after Bti spraying, no Cyt1Aa could be detected using specific antibodies, although high levels of Cry toxins were detected, suggesting that Cyt1Aa toxins might be more prone to rapid degradation than Cry toxins in the environment. This raises concerns about a possible accumulation of Cry toxins in the environment, and the evolution of resistance to Cry toxins in mosquito populations, which is a first step towards the evolution of resistance to the full Bti mixture.

Although resistance mechanisms to Bti toxins have not been well characterized yet in dipteran insects, resistance to other Cry toxins have been intensively studied in lepidopteran insects resistant to transgenic crops producing *Bacillus thuringiensis* Cry toxins (Ferre & Van

Rie, 2002; Bravo et al. 2007). To date, changes in the activity of the midgut proteases involved in toxin activation, and modifications in specific membrane receptors are the two main mechanisms described for resistance to Cry toxins (Bravo et al., 2007; Oppert et al., 1997), but recent studies suggest that genes involved in insect immunity and in membrane cell regeneration might also be involved in resistance to Bti (Paris et al., in prep).

3.1 The role of proteases in resistance

The mode of action of Bti toxins in the gut of susceptible insects is complex, involving many steps in the conversion of protoxins to toxins. In the crystal, protoxins interact through hydrogen bounding, disulfide linkages, and hydrophobic interactions. In the alkaline midgut of insect larvae, the protoxins are hydrolyzed to toxins by proteases, mostly serine-proteases. Both the bacterium and the insect produce proteases able to solubilize and activate Bti protoxins, and in the lab, mammalian trypsin and chymotrypsin can also activate the protoxins (Oppert, 1999). However, insect proteases appear to be key in determining toxin specificity; for example, Bt *aizawai* protoxins are toxic to both lepidopteran and dipteran insects, but their incubation with lepidopteran proteases yields a 55kDa protein toxic only to lepidopterans, while their incubation with dipteran proteases results in a 52kDa protein toxic only to dipterans (Haider et al., 1986). The role of proteases in resistance to Bt toxin Cry1Ca1 was recently demonstrated in *Spodoptera frugiperda*: a serine-protease gene found to be down regulated in the larval midgut of intoxicated larvae was further shown by RNAi-mediated knockdown to be involved both in reduced protoxin activation in the midgut and reduced susceptibility of insects to toxins in bioassays (Rodriguez-Cabrera et al., 2010). In contrast with their role in toxin activation, proteases were shown in several studies to be involved in Bt toxin degradation and/or sequestration in resistant insects, but this mechanism has not been reported so far in mosquitoes. Altogether, the role of proteases in conferring resistance to Bti toxins is ambiguous as both the type and activity level (decreased or increased activity) of gut proteases might be involved in resistance. A comparison of the genes differently expressed in Bti resistant and susceptible mosquitoes was performed using high-throughput sequencing technology (Digital Gene Expression Tag Profiling). This method is based on sequencing of 20 pb fragments in the 5'-terminal region of mRNA anchored on specific restriction sites, long enough to indentify the genes and quantify expression levels, but not long enough to investigate alternative splicing events and sequence variations. Out of a total of 138 genes differently expressed, nearly a quarter of those with known function were proteases, and among them, about half were under-expressed in resistant insects and half were over-expressed (Paris et al, in prep). Further understanding of the role of proteases in resistance will involve investigating the precise role of a set of candidate proteases, either by using specific inhibitors, or gene silencing approach (siRNA specific), or heterologous expression in *E. coli*, and then test the activity of the target protease on Bti protoxins (activation/ degradation). The ultimate validation would be testing toxicity on mosquito larvae after protoxin processing by the candidate protease (bioassays).

3.2 The role of membrane receptors in resistance

Cry toxins bind to the brush border membrane cells in the midgut on specific membrane receptors, leading to pore formation, ionic imbalance, cell lysis, bacterial proliferation in host tissues, and septicemia (figure 3). An alternative mode of action for Cry toxins was recently proposed that involves a cellular signaling pathway following toxin binding

rather than pore formation, and cytotoxicity can eventually be due to the combined effects of osmotic lysis and cell signaling, but initial toxin binding to specific membrane receptors remains a key process (Zhang et al 2006; Pigott & Ellar, 2007; Soberon et al., 2009, 2010). Genetic resistance to Cry toxins involve change in the sequence of the specific receptor gene so that binding is no more effective (Tabashnik et al., 1997), and/or down regulation of the receptor gene so that binding is considerably reduced or suppressed. Alternative splicing of the receptor gene due to transposable elements was shown to confer resistance to *Bacillus sphaericus* binary toxins in *Culex* pipiens populations (Darboux et al., 2007) but such mechanism has not been demonstrated so far for Bti toxins. Cross-resistance mechanisms between different Cry toxins have been reported, presumably involving shared membrane receptors (Siqueira et al., 2004; Zhao et al., 2001; Xu et al., 2010; Likitvivatanavong et al., 2011). In lepidopterans, four types of receptors for Cry1A have been identified: cadherin, glycosylphosphatidylinosytol (GPI)-anchored APN (aminopeptidase N), GPI-anchored ALP (alkaline phosphatase) and glycolipids (reviewed in Pigott & Ellar, 2007). Cry1Ab toxin binds to the abundant but low affinity GPI-anchored proteins ALP or APN and concentrates in the microvilli membrane where it then binds to cadherin receptor with high affinity. This binding mechanism was named 'ping-pong' because it involves going from GPI-anchored proteins to cadherin and back to GPI-anchored proteins before membrane insertion (Gomez et al., 2010). The specific receptors of Bti toxins in mosquito midguts have been much less studied. To date, one cadherin was identified as Cry4B receptor and one APN as Cry11B receptor in *Anopheles* (Hua et al., 2008; Abdullah et al., 2006; Zhang et al., 2008), and one cadherin as Cry11B receptor, one APN as Cry11A receptor and one other ALP as Cry4B receptor in *Aedes* (Chen et al., 2009a, Fernandez-Luna et al., 2006, Bayyareddy et al., 2009). Another cadherin was found to be down-regulated and to exhibit genomic signature of selection in a Bti resistant *Aedes aegypti* strain, suggesting its implication in resistance, but there is not so far validation of its role as a receptor for Cry toxin (Bonin et al., 2009).

3.3 Other mechanisms involved in mosquito resistance

Zhang et al. (2005) suggested that cell death following Cry toxins binding to membrane receptors is a more complex cellular response than the simple osmotic lysis previously assumed. They propose a pathway involving G protein, adenylyl cyclase and adenosine monophosphate, and resulting in the activation of protein kinases A that initiate membrane blebbing, cell swelling and cell lysis (Zhang et al., 2006). The crucial role of protein kinases A in this cell death pathway was demonstrated by using specific inhibitors (Zhang et al., 2006). Using two-dimensional gel electrophoresis and mass spectrometry method, actin was identified as a binding protein for the Cry1Ac toxin in the lepidopteran species *M. sextra* (McNall & Adang, 2003), *Heliothis virescens* (Krishnamoorthy et al., 2007) and *Helicoverpa armigera* (Chen et al., 2010), and for the Cry4B toxin in *Ae. aegypti* (Bayyareddy et al., 2009). Because actin is located within the cell, it is unlikely to be a membrane receptor for Cry toxin, and binding presumably occurs after the penetration of the toxin into the epithelial barrier of the midgut (McNall & Adang, 2003). Toxin binding to actin could lead to disruption of its normal function in maintenance of the cytoskeleton architecture, causing loss of cell shape and integrity (Krishnamoorthy et al., 2007). Changes in the expression of genes involved in membrane cell remodelling and epithelium reparation, and genes involved in immunity, were observed in Bti resistant mosquitoes (Paris et al., in prep). A

more efficient repair or replacement of damaged midgut cells could be involved in resistance. Similarly, a more efficient immune response to bacterial invasion may be selected for in insects fed with Bti, but this mechanism alone is unlikely to confer resistance, because Bti toxins alone are able to kill susceptible mosquito larvae, although radiated spores are 20-30% less efficient than unradiated spores (Becker, 2002).

3.4 The genetic basis of resistance

Because Bti contains many toxins with different modes of action that act in synergy to confer acute toxicity, various mechanisms are likely to be simultaneously involved in resistance: behavioural avoidance of toxins, physiological changes in the larval midgut (pH, protease activity), and genetic changes in specific receptors. Mosquito larvae are particle feeders and Bti tend to sink more or less rapidly in mosquito breeding sites where it is sprayed. In the field, Bti toxicity is lost within a week against *Anopheles* which feeds in surface (Kroeger et al., 1995), whereas *Aedes* species are still killed up to 4 weeks after treatment (Marcombe et al., 2010), suggesting that differences in feeding behaviour might explain this difference in susceptibility (Lacey, 2007). Changes in the type and/or level of expression of the midgut proteases can also be involved, as well as modifications in the expression and/or sequence of the specific membrane receptors. All these mechanisms are likely to act together in resistant insects. For example, a resistant strain of *Ae. aegypti* exhibited slower larval development as compared to the susceptible strain (Paris et al., 2011b). This difference in larval development time might be attributed to differences in feeding behaviour, and/or to changes in proteases modifying metabolic efficiency, and/or to pleiotropic effects of modified receptors. So far the study of resistance to Bt toxins in insects was based on the study of a few candidate proteins, either toxin receptors or proteases, using proteomic approaches (2D-DIGE, ligand binding…). Resistance to Bti can involve both mutations (in specific proteases, specific membrane receptors), transcriptional regulation (up or down regulation of proteases, of membrane receptors and other co-factors involved in toxin binding, of immune genes…) and post-transcriptional changes (alternative splicing of mRNA…). Given the variety of mechanisms involved in resistance to the multiple toxin contained in Bti, a global screening of genomic /transcriptomic/ proteomic changes in resistant insects should be favoured to a candidate gene approach, in order to tackle all the genes simultaneously involved and their interactions (Bonin et al., 2008, 2009, Paris et al., 2010). Bti containing a mixture of toxins with various modes of action, the resistance is likely to involve many loci with various levels of dominance and various degrees of epistatic interactions. The selection of fully resistant genotypes is likely to take longer than for monolocus resistance, because recombination disrupts advantageous resistant allele combinations at each generation. However, the multigenic basis of Bti resistance is not a guarantee that resistance will not evolve in natural populations. Indeed, high resistance levels evolved in field *Culex pipiens* populations treated with *Bacillus sphaericus*, despite the fact that resistance involves at least two recessive loci. Complex interactions between these two loci appear to protect each recessive mutant from disappearing when both are rare, leading to a rapid increase in frequency of the resistant alleles in natural populations (Chevillon et al., 2001). In the case of resistance to Bti in mosquitoes, the genes involved and the dominance level of the resistant alleles are not yet known. Further characterization of mechanisms underlying resistance to Bti is essential in order to develop an effective resistance management of field populations.

3.5 Consequence of resistance on insect fitness: the cost of resistance

When affecting genes with an important function, resistance alleles may have pleiotropic effects and reduce the fitness of resistant individuals in the absence of insecticide. The spread and the evolution of resistance in populations depend not only on the selective advantages linked to level of resistance, but also the negative fitness costs associated with the resistance. Resistance costs can be estimated in two ways. The first one consists of directly comparing the life-history traits associated with fitness, such as survival, reproduction or behavior, of susceptible and resistant individuals (Gassmann et al., 2009). The second way consists of monitoring the changes in resistance allele frequencies in space (through transects between resistant and susceptible populations) or time (over several generations without insecticide). The main advantage of this method is that it takes into account the fitness costs expressed at all the life-stages of the resistant phenotype. The cost of resistance to Cry toxins has been intensively studied in lepidopteran or coleopteran pests resistant to transgenic Bt crops expressing Cry toxin genes (reviewed in Gassmann et al., 2009). About 70% of studies detected costs associated with Cry toxin resistance, affecting various phenotypic and life history traits such as development time, mass, survival or fecundity. In contrast, only two studies of the fitness cost of resistance to Bti toxins in mosquitoes have been undertaken so far, on *Culex pipiens* (Saleh et al., 2003) and *Aedes aegypti* (Paris et al., 2011b) laboratory strains. Larval selection with Bti in *C. pipiens* over 20 generations caused a 44.8% reduction in female fecundity, but no significant reduction in fertility (egg hatchability) or in adult longevity was found; resistance quickly decreased by 58% after 3 generations without selection (Saleh et al., 2003). In *Ae. aegypti* selected for 22 generations using leaf litters containing Bti, 40% reduction in female fecundity, 68% decrease in egg survival after 4 months desiccation, and 17% increase in larval development time were observed; resistance was totally lost after only 5 generations without selection (Paris et al., 2011b). The magnitude of variation in fitness traits measured in the laboratory may be more or less transposed to natural mosquito populations, depending on the ecological conditions experienced in the field. For example, in temperate regions, eggs will usually persist for a long time in the environment (usually during winter), and the fitness cost in terms of egg survival to long-term desiccation will be expressed. In contrast, it is presumably of less importance in tropical regions where the successive mosquito generations usually do not require egg diapause. However, in tropical regions with many successive generations, the costs on larval development time and female fecundity will be expressed. Finally, for a similar period of time (e. g., 4 months), the resistance level is reduced to the level of the susceptible strain in both environments, even though different fitness costs are expressed and involved in the counter-selection of resistant individuals. Therefore, the rapid decline in resistance observed in laboratory conditions is promising as it opens up perspectives for effective management strategies. However, compensatory mutations decreasing fitness costs are likely to be selected for, especially in natural populations with a large standing variation. The selection of allelic combinations conferring both resistance and reduced fitness costs from the genetic diversity already present within the population is the first mechanism to act in recently treated areas. Then, new mutations which decrease fitness costs may appear and accumulate. Finally, the replacement of the costly resistant allele by a less costly resistant allele may occur at the same or at another locus (Roush & McKenzie, 1987; Labbe et al., 2009).

4. Future directions and challenges: managing Bti resistance in the field

4.1 Monitoring resistance in natural populations

4.1.1 Toxicological tests (bio-assays)

So far, no consistent resistance to Bti has been reported in natural populations, even in regions heavily treated since decades (Becker & Ludwig, 1993).The lack of evidence for Bti resistance in treated mosquito populations does not mean that resistance to individual toxins is not arising in the field (Paris et al., 2010). Indeed, resistance to individual toxins might evolve in field populations if toxins have different persistence in the environment. If some toxins are more persistent than others, the selective pressure at play in natural populations may be quite different from the full toxins mixture tested in the laboratory. An *Ae. aegypti* strain selected in the laboratory with leaf litter collected in the field long after Bti treatment (Tilquin et al., 2008) showed high levels of resistance to individual Cry toxins (up to 30-fold resistance) but only limited resistance to Bti mixture (2-fold resistance, Paris et al., 2011a). This resistance to particular Bti toxins might represent a 'first step' toward Bti resistance in regions treated exclusively with this bio-insecticide. In light of these results, we propose to monitor resistance in field populations using separate toxins rather than the full mixture, in order to detect resistance at the earliest step. A preliminary survey of 16 treated populations belonging to 5 mosquito species (*Oc. rusticus*, *Ae. vexans*, *Oc. sticticus*, *Ae. cinereus* and *Culex pipiens*) have shown that each mosquito species presents only limited difference in its level of tolerance to individual Bti toxins (i. e. less than 2-fold difference in LC_{50}). We found evidence for increased resistance to Cry4A and Cry11 in one population of *Ochlerotatus sticticus* out of three (more than 10-fold difference in LC_{50}), although no such evidence was found with Bti. It is therefore important that mosquito control agencies periodically check the level of resistance in treated populations to individual toxins, because it is the first step to resistance to the full Bti mixture. Undertaking bioassays on field populations is not an easy task, because it requires testing many doses with many replicates in order to determine the LC_{50}. We therefore propose to develop a simple toxicological test involving only two diagnostic doses per toxin and per mosquito species. Only when a field collected population of larvae will present more than 10-fold resistance to an individual toxin as compared to populations of the same species, a more rigorous F2 screen test involving to create many F2 progeny from isofemale lines, and test for mortality on F2 larvae using the highest diagnostic dose, will be performed to further evaluate the frequency of resistant alleles in that particular population.

4.1.2 Monitoring change in resistance allele frequencies

Identifying genes involved in resistance to Bti is a challenging ongoing research area, and so far, no diagnostic mutation is known for Bti resistance. When available, such molecular diagnostic tool will allow the mosquito control agencies to efficiently monitor the frequency of resistant alleles in treated populations, and to adapt their treatment strategy in order to delay the evolution of resistance in the field.

4.2 Strategies for resistance management

4.2.1 Taking advantage of the cost of resistance

Although the commercial Bti mix is a combination of several toxins, the evolution of resistant alleles in treated populations will probably occur sooner or later, if no management strategy is in place to slow down their frequency. The high cost of resistance to Bti expressed

at various stages of the mosquito's life opens up perspectives for managing Bti resistance before the resistant alleles spread into populations. The cornerstone of resistance management is the 'high dose-refuge' (HDR) strategy in which some sites are heavily treated while adjacent sites remain untreated, allowing the persistence of a population of susceptible insects. The principle of the HDR strategy is to conserve non treated refuges in proximity of treated areas to promote survival and dispersion of susceptible insects (spatial refuges). Its success depends principally on the counter-selection of resistance alleles in refuges due to fitness cost. Alternatively, the treatment can be interrupted during a period of time, allowing the competition between susceptible and resistant phenotypes to slow down the evolution of the resistant alleles (temporal refuges). This strategy is particularly effective when resistance is at least partly recessive, and relies on the competitive superiority of susceptible individuals in untreated sites (due to the cost of resistance) that will invade the adjacent treated areas and mate with the few surviving resistant individuals. The success of this strategy therefore depends on the cost of resistance, the level of dominance of the resistant alleles, and the migration rate of insects between untreated and treated sites. The latter determines the proportion of refuges required, and their spatial arrangement. The size and disposition of these refuges have been widely modeled but only in situations involving a single, bi-allelic resistance locus (e.g., Tabashnik & Croft, 1982; Caprio, 2001). In the case of resistance to Bti, a mixture of toxins with various modes of action, the resistance is likely to involve many unlinked loci with various levels of dominance (Bonin et al., 2009; Paris et al., 2010). Further characterization of mechanisms underlying resistance to Bti is essential in order to develop an effective resistance management of field populations. Paris et al. (2011b) have shown that the diapause of mosquito eggs for four months is long enough to counter-select resistance alleles suggesting that Bti resistance can be slowed down in temperate climates, at least for mosquito species overwintering as eggs. This could partly explain the lack of resistance detection in European countries that have been using Bti for decades. The fitness costs observed in the laboratory when the selective pressure was relaxed appear to be sufficient to counter-select resistant individuals over just five generations (Paris et al., 2011b). This suggests that in tropical regions with a rapid turnover of generations, just a few generations with no treatment would limit resistance evolution. The ability to easily counter-select resistant individuals by exploiting the resistance costs expressed in tropical or in temperate environments opens up interesting perspectives for ensuring the long-term effectiveness of this environmentally safe bio-insecticide against mosquitoes. However, it would not be possible to stop anti-mosquito treatment altogether for several consecutive generations in tropical regions where mosquitoes represent a major threat to human health. An alternative to HDR strategy is the regular release of susceptible adult males bred in the lab in treated populations. Indeed, mosquito males are not disease vectors and they do not bite mammals, so that their release will not generate any nuisance. Furthermore, Bti targets larvae so that these released susceptible adult males are not affected by treatment, and efficiently compete with local males to mate with females, thereby diluting resistant alleles in the next generation. Finally, combining chemical insecticides against adults together with Bti against the larvae every few generations could contribute to effective Bti resistance management in tropical regions.

4.2.2 Combining the use of Bti with other bio-insecticides

Following the discovery of Bti, other *B. thuringiensis* strains and toxins active against mosquitoes have been searched for intensively. There are today at last 19 type toxins with

mosquitocidal activity in addition to the Cry4, Cry11A and Cyt toxins, i.e. Cry16Aa, Cry17Aa, Cry19Aa, Cry19Ba, Cry20Aa, Cry20Ba, Cry24Aa, Cry24Ba, Cry24Ca, Cry25Aa, Cry27Aa, Cry29Aa, Cry30Aa, Cry30Ba, Cry30Ca, Cry30Da, Cry30Fa, Cry30Ga, Cry39Aa, Cry40Aa, Cry40Ba, Cry44Aa, Cry48Aa, Cry49Aa, Cry52Ba, Cry54Aa, Cry56Aa, Cry60Aa, Cry60Ba, which belong to at least 10 identified *B. thuringiensis* serotypes, i.e. *morrisoni, fukuokaensis, jegathesan, kyushuensis, higo, medellin, entomocidus, sotto, aizawai* and *malayensis*, but also from *Clostridium bifermentans* subsp. *malaysia* and from *Bacillus sphaericus* (Lee & Gill, 1997; Juarez-Perez, et al., 2003; Ito et al., 2006; Beron & Salerno, 2007; Barloy et al., 1996; Hwang et al., 1998; Ohgushi et al., 2005; Tan et al., 2009; Jones et al., 2007; Zhu et al. 2010; Padua & Federici, 1990; Lacey et al., 1988; Lacey, 2007; Federici et al., 2003, Poopathi & Abidha, 2010). However, despite a high number of toxins and strains identified the efficiency of Bti for controlling mosquitoes was not reached. The only strain which could truly compete with Bti was *B. thuringiensis* subsp *morrisoni* strain PG-14 (Padua & Federici, 1990; Lacey et al., 1988; Lacey, 2007). PG-14 produces the same toxins as in Bti in addition to a 144-kDa non-mosquitocidal toxin. However the 144-kDa toxin gene is borne on a different plasmid than those coding for the Bti toxins. PG-14 is therefore an initially non-mosquitocidal strain which acquired mosquitocidal activity by transfer of the conjugative pBtoxis plasmid from Bti. The efficiency of Bti is closely linked to its specific combination of toxins displaying synergisms for both toxicity (acute effect) and sustainability (resistance avoidance). As Bti, all these Bt subspecies produce a cocktail of Cry and Cyt toxins, and the mode of action is likely to involve the same mechanisms if not the same binding receptors: cross-resistance might evolve rapidly if these insecticides are used in combination with Bti. Prospects for improvement might therefore be on complementary products with a mode of action radically different from Bt toxins. Many other bio-insecticides have proven to be efficient against mosquitoes, including the binary toxins produced by *Bacillus sphaericus* (Bs). Although Bs toxins also bind to larval midgut receptors, there is no cross resistance to Bti within Bs resistant *Culex* populations, and there is even evidence for an increased sensitivity to Bti (Rao et al., 1995). Valent BioSciences Corporation (ill., USA) recently developed a new granule product, under the commercial name VectoMax® G (table 1), combining Bti and Bs in a specific toxin ratio into every micro particle. This mixture exhibits extended residual control and good efficacy in polluted water contrary to Bti. The neuro-toxic spinosad produced by the actinomycete *Saccharopolyspora spinosa* also exhibits efficient mosquitocidal activity (Bond et al., 2004). Plant compounds are a main source of natural insecticides (Després et al., 2007) and their efficiency has been evaluated against mosquitoes. Saponins and essential oils with larvicidal, repellent, or oviposition deterrent effects on mosquitoes have been described (Kiran et al., 2007, Senthilkumar et al., 2009). Seed extract from *Moringa oleifera* was shown to efficiently kill mosquito larvae without toxicity to non-target organisms (Ferreira et al., 2009), as well as sodium anacardate extracted from cashew nut shells (Farias et al., 2009). These bio-insecticides present the advantage of showing different specificity towards the various mosquito species co-existing in treated areas, and of action modes and target sites very different from those of Bti. The combined or sequential use of these bio-insecticides with Bti can allow efficient mosquito control together with limited selective pressure for resistance evolution in treated populations.

4.2.3 What future for mosquitocidal Bt products?

An important element to consider when addressing the potential future of mosquitocidal products is that only two biopesticides are currently registered for mosquito control: Bti

and *B. sphaericus*. The immediate consequence is that, considering the time and cost of toxicology analyses and registration, there might be at best little incentive to develop novel products when Bti is a perfect biocontrol agent. Ways for improving Bti are not on the toxins per se for two main reasons. First, there is little room, if any, to improve the activity of a set of toxins capable to synergize and to delay resistance. Secondly, any modification of the toxins would result into a new product to register following a long process of deregulation and delay, eliminating thus the benefits of having a product already registered and relying on 30 years of toxicological and environmental analyses. Ways of improvement are therefore in the mode of delivery and in the increase of the life time of the sprayable product. These aspects have been investigated soon after the development of Bti as a successful biocontrol agent. Expression of Bti toxins in different species of cyanobacteria and fresh-water bacteria such as *Caulobacter*, *Asticcacaulis* or *Ancylobacter* (Angsuthanasombat & Panyim, 1989; Chungjatupornchai, 1990; Liu et al., 1996; Murphy and Stevens, 1992; Sangthongpitag et al., 1996; Soltes-Raket al., 1995; Thanabalu et al., 1992; Xiaoqiang et al., 1997; Xudong et al., 1993; Yap et al., 1994). The objective in this case was to integrate the Bti toxins in a permanent way in the chain food of mosquito larvae without resorting to sprays. Another opportunity offered by genetic engineering is the expression along with the Cry and Cyt toxins of chitinases or chitinase-like genes. These proteins also often referred to as enhancing factors or enhancin, were shown to effectively increase the toxicity of Bt toxins (Thamthiankul et al., 2004; Liu et al., 2002; Fang et al., 2009). However, this approach, although technically efficient is from a regulatory standpoint moving away from a biopesticide approach to a transgenic approach and is thus liable to the GMO regulation. Besides the delivery mode, the other way of engineering explored in the past few years was to improve the level of production and to stack Bti and *B. sphaericus* toxins or extended synergism (Park et al., 2003, 2005; Wu & Federici, 1993, 1995; Federici et al., 2003, 2007). Nevertheless, these approaches also generate recombinant strains which must be considered under the time consuming and expensive GMO regulation. Besides, GMO is still the subject of public debates which may further delay the use of genetically improved products. The solution in the near future might therefore be on the improved management, monitoring and formulation of the existing Bti product which is after all the best mosquito control product currently available.

5. Conclusion

Bti appears to be a safe and efficient bio-insecticide against mosquitoes. Although Bti still comes at a higher cost than chemical insecticides, presumably due to its so far limited use, there is no technical reason for this high cost. The successful use of food processing organic industrial wastes such as chicken feathers as a nutrient medium to grow Bti (Poopathi & Abidha, 2007) opens avenues to a future low-cost production of this bio-insecticide, with the additional benefit of effective recycling of bio-organic wastes from the environment. The most important threat to the long-term use of this efficient mosquito-control tool is the evolution of resistance in treated populations. There is an urgent need to develop efficient, easy-to-use and low-cost diagnostic tools to evaluate the fate of Bti in the environment, and to monitor Bti resistance evolution in mosquito populations.

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7. References

Abdullah, M.A., Alzate, O., Mohammad, M., McNall, RJ., Adang, M.J. & Dean, D.H. (2003). Introduction of Culex toxicity into *Bacillus thuringiensis* Cry4Ba by protein engineering. *Appl. Environ. Microbiol.*, 69, 5343–5353.

Abdullah, M. A. F., Valaitis, A. P., & Dean, D. H. (2006). Identification of a *Bacillus thuringiensis* Cry11Ba toxin-binding aminopeptidase from the mosquito, *Anopheles quadrimaculatus*. *BMC Biochem, 7*, 16.

Ali, A. (1981). *Bacillus thuringiensis* serovar *israelensis* (ABG-6108) against chironomids and some nontarget aquatic invertebrates. *Journal of Invertebrate Pathology*, Vol.38, pp.264-272

Aly, C. & Mulla, M. S. (1987). Effect of two microbial insecticides on aquatic predators of mosquito. *Journal of Applied Entomology*, 103, 113-118

Aly C, Mulla, M.S., & Federici, B.A. (1985). Sporulation and toxin production by *Bacillus thuringiensis* var. *israelensis* in cadavers of mosquito larvae (Diptera: Culicidae). *J Invertebr Pathol*, 46, 251–258

Angsuthanasombat, C., & Panyim, S. (1989). Biosynthesis of 130-kilodalton mosquito larvicide in the cyanobacterium *Agmenellum quadruplicatum* PR-6. *Appl. Environ. Microbiol*., 55, 2428-2430.

Arenas, I., Bravo, A., Soberon, M., & Gomez, I. (2010) Role of Alkaline Phosphatase from *Manduca sexta* in the Mechanism of Action of *Bacillus thuringiensis* Cry1Ab Toxin. *Journal of Biological Chemistry,* 285(17), 12497-12503.

Arnold, A., Häussler, U., & Braun, M. (2002). Comparative study of the diet of two pipistrelle species *Pipistrellus pygmaeus* and *P. pipistrellus* in southwest Germany. *Bat Research News*, Vol.43, p.72

Barloy F., Delecluse A., Nicolas L., & Lecadet M.M. (1996). Cloning and expression of the first anaerobic toxin gene from *Clostridium bifermentans* subsp. *malaysia*, encoding a new mosquitocidal protein with homologies to *Bacillus thuringiensis* delta-endotoxins. *J. Bacteriol*., 17, 3099-3105.

Bassi, D.G., Weathersbee, A.A., Meisch, M.V., & Inman, A. (1989). Efficacy of Duplex and VectoBac against *Psorophora Columbiae* and *Anopheles quadrimaculatus* larvae in Arkansas ricefields. *Journal of the American Mosquito Control Association*, 7, 313-315

Bayyareddy, K., Andacht, T.M., Abdullah, M.A., & Adang, M.J. (2009). Proteomic identification of *Bacillus thuringiensis* subsp. *israelensis* toxin Cry4Ba binding proteins in midgut membranes from *Aedes* (*Stegomyia*) *aegypti* Linnaeus (Diptera, Culicidae) larvae. *Insect Biochem. Mol. Biol*., 39, 279–286

Becker, N. (2002). Sterilization of *Bacillus thuringiensis israelensis* products by gamma radiation. *J. Am. Mosq. Control Assoc.*, 18(1), 57-62.

Becker, N. (2003). Ice granules containing endotoxins of microbial agents for the control of mosquito larvae – a new application technique. *Journal of the American Mosquito Control Association*, Vol. 19, pp.63-66

Becker, N., & Ludwig, M. (1993). Investigation on possible resistance in *Aedes vexans* field populations after a 10-year application of *Bacillus thuringiensis israelensis*. *Journal of the American Mosquito Control Association, 9*(2), 221-224.

Beron, C.M., & Salerno, G.L. (2007). Cloning and characterization of a novel crystal protein from a native *Bacillus thuringiensis* isolate highly active against *Aedes aegypti*. *Curr. Microbiol*., 54, 271-276.

Berry, C., O'Neil, S., Ben-Dov, E., Jones, A.F., Murphy, L., Quail, M.A., Holden, T.G., Harris, D., Zaritsky, A., & Parkhill, J. (2002). Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. *Appl. Environ. Microbiol.,* 68, 5082-5095.

Boisvert, M. & Boisvert, J. (1999). Persistence of toxic activity and recycling of *Bacillus thuringiensis* var. *israelensis* in cold water: field experiments using diffusion chambers in a pond. *Biocontrol Science and Technology*, 9, 507-522.

Boisvert, M. & Boisvert, J. (2000). Effects of *Bacillus thuringiensis* var. *israelensis* on target and nontarget organisms: a review of laboratory and field experiments. *Biocontrol Science and Technology*, 10, 517-561

Boisvert, J. & Lacoursière, J.O. (2004). Le Bacillus thuringiensis et le contrôle des insectes piqueurs au Québec. ENV/2004/0278. Ministère de l'Environnement Québéquois, Québec. 101p.

Bond, J. G., Marina, C. F., & Williams, T. (2004). The naturally derived insecticide spinosad is highly toxic to *Aedes* and *Anopheles* mosquito larvae. *Medical and Veterinary Entomology,* 18(1), 50-56.

Bonin, A., Paris, M., Despres, L., Tetreau, G., David, J. P., & Kilian, A. (2008). A MITE-based genotyping method to reveal hundreds of DNA polymorphisms in an animal genome after a few generations of artificial selection. *Bmc Genomics*, 9.

Bonin, A., Paris, M., Tetreau, G., David, J. P., & Despres, L. (2009). Candidate genes revealed by a genome scan for mosquito resistance to a bacterial insecticide: sequence and gene expression variations. *Bmc Genomics,* 10.

Boonserm, P., Davis, P., Ellar, D.J., & Li, J. (2005). Crystal structure of the mosquito-larvicidal toxin Cry4Ba and its biological implications. *J Mol Biol*., 348, 363-382.

Boonserm, P., Mo, M., Angsuthanasombat, C., & Lescar, J. (2006). Structure of the functional form of the mosquito larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8-angstrom resolution. *J Bacteriol*., 188, 3391-401.

Bourassa, J. P. (2000). Le Moustique : par solidarité écologique. Les Éditions du Boréal, Québec. 240 p.

Boyer, S., Tilquin, M., & Ravanel, P. (2007). Differential sensitivity to *Bacillus thuringiensis* var. *israelensis* and temephos in field mosquito populations of Ochlerotatus cataphylla (Diptera : Culicidae): Toward resistance? *Environmental Toxicology and Chemistry,* 26(1), 157-162.

Bravo, A., Gill, S. S., & Soberon, M. (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon, 49*(4), 423-435.

Butko, P., Huang, F., Pusztai-Carey, M. & Surewicz, W.K. (1996). Membrane permeabilization induced by cytolytic delta-endotoxin CytA from *Bacillus thuringiensis* var. *israelensis*. *Biochemistry,* 35, 11355-11360.

Butko, P., Huang, F., Pusztai-Carey, M., & Surewicz, W.K., (1997). Interaction of the delta-endotoxin CytA from *Bacillus thuringiensis* var *israelensis* with lipid membranes. *Biochemistry.,* 36, 12862–12868.

Canton, P.E., Reyes, E.Z., de Escudero, I.R., Bravo, A., & Soberon, M. (2011). Binding of *Bacillus thuringiensis* subsp. *israelensis* Cry4Ba to Cyt1Aa has an important role in synergism. *Peptides.*, 32, 595-600.

Caprio, M. A. (2001). Source-sink dynamics between transgenic and non-transgenic habitats and their role in the evolution of resistance. *Journal of Economic Entomology,* 94(3), 698-705.

Chen, J., Aimanova, K.G., Fernandez, L.E., Bravo, A., Soberon, M., & Gill, S.S. (2009a). *Aedes aegypti* cadherin serves as a putative receptor of the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*. *Biochem. J.,* 424, 191-200.

Chen, J, Aimanova, K.G., Pan, S., & Gill, S.S. (2009b). Identification and characterization of *Aedes aegypti* aminopeptidase N as a putative receptor of *Bacillus thuringiensis* Cry11A toxin. *Insect Biochem. Mol. Biol.,* 39, 688-696.

Chen, L.Z., Liang, G.M., Zhang, J., Wu, K.M., Guo, Y.Y., & Rector, B.G. Proteomic analysis of novel Cry1Ac binding proteins in *Helicoverpa armigera* (HUBNER). *Archives of Insect Biochemistry and Physiology*, 73, 61-73.

Chevillon, C., Bernard, C., Marquine, M., & Pasteur, N. (2001). Resistance to Bacillus sphaericus in Culex pipiens (Diptera : Culicidae) interaction between recessive mutants and evolution in southern France. *Journal of Medical Entomology,* 38(5), 657-664.

Chungjatupornchai, W. (1990). Expression of the mosquitocidalprotein genes of *Bacillus thuringiensis* subsp. *israelensis* and the herbicide-resistance gene bar in *Synechocystis* PCC6803. *Curr. Microbiol*., 21, 283-288.

Clark, B. W., Phillips, T. A., & Coats, J. R. (2005). Environmental fate and effects of Bacillus thuringiensis (Bt) proteins from transgenic crops: a review. *Journal of Agricultural And Food Chemistry, 53*(12), 4643-4653.

Cohen, S., Dym, O., Albeck, S., Ben-Dov, E., Cahan, R., Firer, M., & Zaritsky, A. (2008). High-resolution crystal structure of activated Cyt2Ba monomer from *Bacillus thuringiensis* subsp. *israelensis*. *J. Mol. Biol., 380*, 820-827.

Crickmore, N., Bone, E.J., Williams, J.A., & Ellar, D.J. (1995).Contribution of the individual components of the d-endotoxin crystal to the mosquitocidal activity *of Bacillus thuringiensis* subsp. *israelensis*. *FEMS Microbiol. Lett*., 131, 249-254.

Crosskey, R.W. (1990). The natural history of black flies. John Wiley & Sons, England, 711 p.

Darboux, I., Charles, J. F., Pauchet, Y., Warot, S., & Pauron, D. (2007). Transposon-mediated resistance to *Bacillus sphaericus* in a field-evolved population of *Culex pipiens* (Diptera : Culicidae). *Cellular Microbiology,* 9(8), 2022-2029.

Davies, D.M. (1991). Additional records of predators upon black flies. Bulletin of the Society of Vector Ecology 16:256-268.

Despres, L., David, J-P., & Gallet, C. (2007). The evolutionary ecology of insect resistance to plant chemicals. *Trends in Ecology & Evolution* 22, 298-307.

de Melo-Santos, M. A. V., de Araujo, A. P., Rios, E. M. M., & Regis, L. (2009). Long lasting persistence of *Bacillus thuringiensis serovar. israelensis* larvicidal activity in *Aedes aegypti* (Diptera: Culicidae) breeding places is associated to bacteria recycling. *Biological Control, 49*(2), 186-191.

Du, J., Knowles, B.H., Li, J., & Ellar, D.J. (1999). Biochemical characterization of *Bacillus thuringiensis* cytolytic toxins in association with a phospholipid bilayer. *Biochem. J.,* 338, 185–193.

Duchet, C., Larroque, M., Caquet, T., Franquet, E., Lagneau, C., & Lagadic, L. (2008). Effects of spinosad and *Bacillus thuringiensis israelensis* on a natural population of *Daphnia pulex* in field microcosms. *Chemosphere* 74:70-77.

Duchet C., Caquet, T., Franquet, E., Lagneau, C., & Lagadic, L. (2010). Influence of environmental factors on the response of a natural population of *Daphnia magna* (Crustacea: Cladocera) to spinosad and *Bacillus thuringiensis israelensis* in Mediterranean coastal wetlands. *Environmental Pollution* 158, 1825–1833

Fang, S., Wang, L., Guo, W., Zhang, X., Peng, D., Luo, C., Yu, Z., Sun, M., (2009). *Bacillus thuringiensis* bel protein enhances the toxicity of Cry1Ac protein to *Helicoverpa armigera* larvae by degrading insect intestinal mucin. *Appl. Environ. Microbiol*., 75, 5237-5243.

Farias, D. F., Cavalheiro, M. G., Viana, S. M., De Lima, G. P. G., Da Rocha-Bezerra, L. C. B., Ricardo, N., et al. (2009). Insecticidal action of sodium anacardate from Brazilian cashew nut shell liquid against *Aedes Aegypti*. *J. Am. Mosq. Control Assoc.,* 25(3), 386-389.

Federici, B.A., Park, H.W., Bideshi, D.K., Wirth, M.C., & Johnson, J.J. (2003). Recombinant bacteria for mosquito control. *J. Exp. Biol.,* 206, 3877-3885.

Federici, B.A., Park, H.W., Bideshi, D.K., Wirth, M.C, Johnson, J.J., Sakano, Y., & Tang, M. (2007). Developing recombinant bacteria for control of mosquito larvae. *J. Am. Mosq. Control Assoc*. 23, 164-175.

Fernandez, L.E., Martinez-Anaya, C., Lira, E., Chen, J., Evans, A., Hernandez-Martinez, S., Lanz-Mendoza, H., Bravo, A., Gill, S.S., & Soberon, M. (2009) Cloning and epitope mapping of Cry11Aa-binding sites in the Cry11Aa-receptor alkaline phosphatase from *Aedes aegypti*. *Biochemistry* 48: 8899–8907.

Fernandez-Luna, M.T., Lanz-Mendoza, H., Gill, S.S., Bravo, A., Soberon, M., & Miranda-Rios, J. (2010). An alpha-amylase is a novel receptor for *Bacillus thuringiensis* ssp. *israelensis* Cry4Ba and Cry11Aa toxins in the malaria vector mosquito *Anopheles albimanus* (Diptera: Culicidae). *Environ Microbiol.*, 12, 746-757.

Franquet, E., & Fayolle, S. (2002). Etude d'impact d'un éventuel traitement au Bti sur le territoire du Parc naturel régional de Camargue. Rapport scientifique. Parc Régional de Camargue. 107p.

Ferre, J., & Van Rie, J. (2002). Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annual Review of Entomology*, 47, 501-533.

Ferreira, P. M. P., Carvalho, A. F. U., Farias, D. F., Cariolano, N. G., Melo, V. M. M., Queiroz, M. G. R., Martins, A. M. C., & Machado-Neto, J. G. (2009). Larvicidal activity of the

water extract of Moringa oleifera seeds against Aedes aegypti and its toxicity upon laboratory animals. *Anais Da Academia Brasileira De Ciencias* 81, 207-216.

Frutos, R., Rang, C., & Royer, M. (1999). Managing insect resistance to plants producing *Bacillus thuringiensis* toxins. *Critical Rev. Biotech.*, 19, 227-276.

Galitsky, N., Cody, V., Wojtczak, A., Ghosh, D., Luft, J.R., Pangborn, W., & English, L. (2001). Structure of the insecticidal bacterial delta-endotoxin Cry3Bb1 of *Bacillus thuringiensis*. *Acta Crystallogr. Sect.,* D 57, 1101-1109.

Garcia, R., B. Des Rochers & W. Tozer, (1981). Studies on the toxicity of *Bacillus thuringiensis* var. *israelensis* against organisms found in association with mosquito larvae. *Proceedings and Papers of the Californian Mosquito and Vector Control Association* 48, 33-36.

Gassmann, A. J., Carriere, Y., & Tabashnik, B. E. (2009). Fitness costs of insect resistance to *Bacillus thuringiensis*. *Annual Review of Entomology*, 54, 147-163.

Gazit, E., Burshtein, N., Ellar, D.J., Sawyer, T., & Shai, Y. (1997). *Bacillus thuringiensis* cytolytic toxin associates specifically with its synthetic helices A and C in the membrane bound state. Implications for the assembly of oligomeric transmembrane pores. *Biochemistry*, 36, 15546-15554.

Georghiou, G.P., & Wirth, M.C. (1997). Influence of exposure to single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). *Appl. Environ. Microbiol.,* 63, 1095-1101.

Goldberg, L.J., & Margalit, J. (1977). A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univitattus*, *Aedes aegypti*, and *Culex pipiens*. *Mosq. News,* 37, 355-358.

Gomez, I., Arenas, I., Pacheco, S., Bravo, A., & Soberon, M. (2010) New insights into the mode of action of Cry1Ab toxin used in transgenic insect-resistant crops. *Southwestern Entomologist,* 35(3), 387-390.

Grochulski, P., Masson, L., Borisova, S., Pusztai-Carey, M., Schwartz, J.-L., Brousseau, R., & Cygler, M. (1995). *Bacillus thuringiensis* CryIA(a) insecticidal toxin crystal structure and channel formation. *J. Mol. Biol.,* 254, 447-464.

Guidi, V., De Respinis, S., Benagli, C., Luthy, P., & Tonolla, M. (2010). A real-time PCR method to quantify spores carrying the *Bacillus thuringiensis* var. *israelensis* cry4Aa and cry4Ba genes in soil. *Journal of Applied Microbiology,* 109(4), 1209-1217.

Haider, M. Z., Knowles, B. H., & Ellar, D. J. (1986). Specificity of *Bacillus thuringiensis* var *colmeri* insecticidal delta-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. *European Journal of Biochemistry,* 156(3), 531-540.

Hajaij, M., Carron, A., Deleuze, J., Gaven, B., Setier-Rio, M.-L., Vigo, G., Thiery, I., Nielsen-Leroux, C., & Lagneau, C. (2005). Low persistence of *Bacillus thuringiensis* serovar *israelensis* spores in four mosquito biotopes of a salt marsh in Southern France. *Microb. Ecol.* 50, 475-487.

He, L.S. & Ong, K.H. (2000). Effects of *Bacillus thuringiensis* H-14 on bloodworms (Diptera : Chironomidae). *Singapore Journal of Primary Industries* 28, 7-12.

Hemingway, J., Hawkes, N.J., McCarroll, L., & Ranson, H. (2004) The molecular basis of insecticide resistance in mosquitoes. *Insect Biochemistry and Molecular Biology* 34, 653-665.

Hershey, A.E., Lima, A.R., Niemi, G.J., & Regal, R.R. (1998). Effects of *Bacillus thuringiensis israelensis* (Bti) and methoprene on nontarget macroinvertebrates in Minnesota wetlands. Ecological Applications 8:41-60.

von Hirsch H. & Becker, N. (2009). Cost-benefit analysis of mosquito control operations based on microbial control agents in the upper Rhine valley (Germany). *Journal of the European Mosquito Control Association, European Mosquito Bulletin*, Vol.27, pp.47-55

Hua, G., Zhang, R., Abdullah, M.A., & Adang, M.J. (2008). *Anopheles gambiae* cadherin AgCad1 binds the Cry4Ba toxin of *Bacillus thuringiensis israelensis* and a fragment of AgCad1 synergizes toxicity. *Biochemistry,* 47, 5101–5110.

Hwang, S.H., Saitoh, H., Mizuki, E., Higuchi, K., & Ohba, M. (1998). A novel class of mosquitocidal delta-endotoxin, Cry19B, encoded by a *Bacillus thuringiensis* serovar *higo* gene. *Syst. Appl. Microbiol.,* 21, 179-184.

Ibarra, J.E., & Federici, B.A. (1986). Isolation of a relatively nontoxic 65-kilodalton protein inclusion from the parasporal body of *Bacillus thuringiensis* subsp. *israelensis*. *J. Bacteriol.,* 165, 527-533.

Ito,T., Ikeya,T., Sahara,K., Bando,H., & Asano, S. (2006). Cloning and expression of two crystal protein genes, cry30Ba1 and cry44Aa1, obtained from a highly mosquitocidal strain, *Bacillus thuringiensis* subsp. *entomocidus* INA288. *Appl. Environ. Microbiol*., 72, 5673-5676.

Jones, G.W., Nielsen-Leroux, C., Yang, Y., Yuan, Z., Dumas, V.F., Monnerat, R.G., & Berry, C. (2007). A new Cry toxin with a unique two-component dependency from *Bacillus sphaericus*. *FASEB J.,* 21, 4112-4120.

Juarez-Perez, V., Porcar, M., Orduz, S., & Delecluse, A. (2003). Cry29A and Cry30A: two novel delta-endotoxins isolated from *Bacillus thuringiensis* serovar *Medellin*. *Syst. Appl. Microbiol.,* 26, 502-504.

Khasdan, V., Ben-Dov, E., Manasherob, R., Boussiba, S., & Zaritsky, A. (2001). Toxicity and synergism in transgenic *Escherichia coli* expressing four genes from *Bacillus thuringiensis* subsp. *israelensis*. *Environ Microbiol.,* 3, 798-806.

Khawaled K., Ben-Dov, E., Zaritsky, A., & Barak, Z. (1990). The fate of *Bacillus thuringiensis* var. *israelensis* in B. thuringiensis var. israelensis-killed pupae of *Aedes aegypti*. *J. Invertebr. Pathol.* 56, 312–316.

Kiran, S.R., & Devi, P.S. (2007) Evaluation of mosquitocidal activity of essential oil and sesquiterpenes from leaves of *Chloroxylon swietenia* DC. *Parasitology Research*, 101, 413-418.

Knowles, B.H., Blatt, M.R., Tester, M., Horsnell, J.M., Carroll, J., Menestrina, G., & Ellar, D.J. (1989) A cytolytic δ-endotoxin from *Bacillus thuringiensis* forms cation-selective channels in planar lipid bilayers. *FEBS Lett.,* 244, 259–262.

Krishnamoorthy, M., Jurat-Fuentes, J. L., McNall, R. J., Andacht, T., & Adang, M. J. (2007). Identification of novel CrylAc binding proteins in midgut membranes from

Heliothis virescens using proteomic analyses. *Insect Biochemistry and Molecular Biology*, 37(3), 189-201.

Kroeger, A. , Horstick, O., Riedl, C., Kaiser, A., & Becker, N. (1995) The potential for malaria control with the biological larvicide *Bacillus thuringiensis israelensis* (Bti) in Peru and Ecuador. *Acta Tropica* 60, 47-57.

Labbe, P., Sidos, N., Raymond, M., & Lenormand, T. (2009). Resistance gene replacement in the mosquito *Culex pipiens*: fitness estimation from long-term cline series. *Genetics, 182*(1), 303-312.

Lacey, L. A. (2007). *Bacillus thuringiensis* serovariety *israelensis* and *Bacillus sphaericus* for mosquito control. *J. Am. Mosq. Control Assoc.*, 23(2), 133-163.

Lacey, L.A., Lacey, C.M., & Padua, L.E. (1988). Host range and selected factors influencing the mosquito larvicidal activity of the PG-14 isolate of *Bacillus thuringiensis* var. *morrisoni. J. Am. Mosq. Control Assoc.*, 4, 39-43.

Lacey, L.A. & Merritt, D.L. (2004). The safety of bacterial microbial agents used for black fly and mosquito control in aquatic environments. *Environmental Impacts of Microbial Insecticides: Need and Methods for Risk Assessment* (eds H.M.T. Hokkanen & A.E. Hajek), pp. 151-168. Kluwer Academic Publishers, Dordrecht.

Lacey L.A. & Smittle, B. (1985). The effects of gamma radiation on spore viability and mosquito larvicidal activity of *Bacillus sphaericus* and *Bacillus thuringiensis* var. *israelensis*. *Bull Soc Vect Ecol* 10:98–101.

Laflamme, E., Badia, A., Lafleur, M., Schwartz, J.L., & Laprade, R. (2008). Atomic force microscopy imaging of *Bacillus thuringiensis* Cry1 toxins interacting with insect midgut apical membranes. *J. Membr. Biol.*, 222, 127-139.

Lagadic, L., Caquet, T., Fourcy, D., & Heydorff, M. (2002). Évaluation à long terme des effets de la démoustication dans le Morbihan. Suivi de l'impact écotoxicologique des traitements sur les invertébrés aquatiques entre 1998 et 2001. Rapport scientifique de fin de programme, Convention de recherche Conseil général du Morbihan, 215p.

Lecadet, M.M. (1996). La lutte bactériologique contre les insectes : une vieille histoire très actuelle. *Annales de l'Institut Pasteur, Actualités* 7(4):207-216.

Lee, H.K., & Gill, S.S. (1997). Molecular cloning and characterization of a novel mosquitocidal protein gene from *Bacillus thuringiensis* subsp. *Fukuokaensis*. *Appl. Environ. Microbiol.,* 63, 4664-4670.

Li, J.D., Carroll, J., & Ellar, D.J. (1991). Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. Nature, 353, 815-821.

Li, J., Koni, P.A., Ellar, D.J. (1996). Structure of the mosquitocidal delta-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. *J Mol Biol.,* 257, 129-152.

Likitvivatanavong, S., Chen, J., Bravo, A., Soberon, M., & Gill, S.S. (2011). Cadherin, alkaline phosphatase, and aminopeptidase N as receptors of Cry11Ba toxin from *Bacillus thuringiensis* subsp. *jegathesan* in *Aedes aegypti. Appl Environ Microbiol.,* 77, 24-31.

Liu, J.W, Yap, W.H., Thanabalu, T., & Porter, A.G. (1996). Efficient synthesis of mosquitocidal toxins in *Asticcacaulis excentricus* demonstrates potential of gram-negative bacteria in mosquito control. *Nature Biotechnol.,* 14, 343-347.

Liu, M., Cai, Q.X., Liu, H.Z., Zhang, B.H., Yan, J.P., & Yuan, Z.M., (2002). Chitinolytic activities in *Bacillus thuringiensis* and their synergistic effects on larvicidal activity. *J. Appl. Microbiol*., 93, 374-379.

Manceva, S.D., Pusztai, M., Russo, P.S., & Butko, P. (2005). A detergent-like mechanism of action of the cytolytic Cyt1A from *Bacillus thuringiensis* var. *israelensis*. *Biochem.* 44, 589-597.

Marcombe, S., Darriet, F., Agnew, P., Etienne, M., Tcha, M. M. Y., Yebakima, A., et al. (2010) Field Efficacy of new larvicide products for control of multi-resistant *Aedes aegypti* populations in Martinique (French West Indies). *American Journal of Tropical Medicine and Hygiene,* 84(1), 118-126.

Merritt, R.W., Walker, E.D., Wilzbach, M.A., Cummins, K.W., & Morgan, W.T. (1989). A broad evaluation of Bti for black fly (Diptera : Simuliidae) control in a Michigan river : efficacy, carry and non target effects on invertebrates and fish. *Journal of the American Mosquito Control Association* 5: 397-415.

McNall, R. J., & Adang, M. J. (2003). Identification of novel *Bacillus thuringiensis* Cry1Ac binding proteins in Manduca sexta midgut through proteomic analysis. *Insect Biochemistry and Molecular Biology, 33*(10), 999-1010.

Murphy, R.C., & Stevens, S.E. (1992). Cloning and expression of the *cryIVD* gene of *Bacillus thuringiensis* subsp *israelensis* in the cyanobacterium *Agmenellum quadruplicatum* PR-6 and its resulting larvicidal activity. *Appl. Environ. Microbiol.,* 58, 1650-1655.

Niemi, G. J., Hershey, A.E., Shannon, L., Hanowski, J.M., Lima, A., Axler, R.P., & Regal, R.R. (1999). Ecological effects of mosquito control on zooplankton, insects and birds. *Environmental Toxicology and Chemistry* vol.18:549-559.

Ohgushi, A., Saitoh, H., Wasano, N., Uemori, A., & Ohba, M. (2005). Cloning and characterization of two novel genes, cry24B and s1orf2, from a mosquitocidal strain of *Bacillus thuringiensis* serovar *sotto. Curr. Microbiol*., 51, 131-136.

Oppert, B., Kramer, K. J., Beeman, R. W., Johnson, D., & McGaughey, W. H. (1997). Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. *Journal of Biological Chemistry, 272*(38), 23473-23476.

Oppert, B. (1999). Protease interactions with Bacillus thuringiensis insecticidal toxins. *Archives of Insect Biochemistry and Physiology, 42*(1), 1-12.

Padilla, C., Pardo-Lopez, L., de la Riva, G., et al. (2006) Role of tryptophan residues in toxicity of Cry1Ab toxin from *Bacillus thuringiensis*. *Applied and Environmental Microbiology*, 72, 901-907.

Padua, L.E., & Federici, B.A. (1990). Development of mutants of the mosquitocidal bacterium *Bacillus thuringiensis* subspecies *morrisoni* (PG-14) toxic to lepidopterous or dipterous insects. *FEMS Microbiol. Lett.,* 54, 257-262.

Paris, M., Boyer, S., Bonin, A., Collado, A., David, J-P., & Despres, L. (2010). Genome scan in the mosquito *Aedes rusticus*: population structure and detection of positive selection after insecticide treatment. *Molecular Ecology, 19*(2), 325-337.

Paris, M., Tetreau, G., Laurent, F., Lelu, M., Despres, L., & J-P. David (2011a). Persistence of *Bacillus thuringiensis israelensis* (*Bti*) in the environment induces resistance to multiple Bti toxins in mosquitoes *Pest Manag Sci,* 67, 122–128.

Paris, M., David, J-P., & Despres L. (2011b) Fitness costs of resistance to Bti toxins in the dengue vector *Aedes aegypti*. *Ecotoxicology,* DOI : 10.1007/s10646-011-0663-8.

Park, H.W., Bideshi, D.K., & Federici, B.A. (2003). Recombinant strain of *Bacillus thuringiensis* producing Cyt1A, Cry11B, and the *Bacillus sphaericus* binary toxin. *Appl. Environ. Microbiol.,* 69, 1331-1334.

Park, H.W., Bideshi, D.K., & Federici, B.A. (2005). Synthesis of additional endotoxins in *Bacillus thuringiensis* subsp. *morrisoni* PG-14 and *Bacillus thuringiensis* subsp. *jegathesan* significantly improves their mosquitocidal efficacy. *J. Med. Entomol.,* 42, 337-341.

Park, Y., Hua, G., Abdullah, M.A., Rahman, K., & Adang, M.J. (2009). Cadherin fragments from *Anopheles gambiae* synergize *Bacillus thuringiensis* Cry4Ba's toxicity against *Aedes aegypti* larvae. *Appl. Environ. Microbiol.,* 75, 7280-7282.

Paul, A., Harrington, L. C., Zhang, L., & Scott, J. G. (2005). Insecticide resistance in Culex pipiens from New York. *Journal of the American Mosquito Control Association, 21*(3), 305-309.

Peckarsky, B.L. (1984). Predator-prey interactions among aquatic insects. pp. 196-254 In: *The ecology of aquatic insects*. V. H. Resh et D. M. Rosenberg (Eds.) Praeger Scientific, 625 p.

Perez, C., Fernandez, L.E., Sun, J., Folch, J.L., Gill, S.S., Soberon, M., & Bravo, A. (2005). *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. *Proc. Natl. Acad. Sci. USA.,* 102,18303-18308.

Perez, C., Munoz-Garay, C., Portugal, L. C., Sanchez, J., Gill, S. S., Soberon, M., & Bravo, A. (2007) *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa enhances activity of Cry11Aa toxin by facilitating the formation of a pre-pore oligomeric structure. *Cell. Microbiol.,* 9, 2931-2937.

Pigott, C. R., & Ellar, D. J. (2007). Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiology and Molecular Biology Reviews,* 71(2), 255.

Poopathi, S., & Abidha, S. (2007). Use of feather-based culture media for the production of mosquitocidal bacteria. *Biological Control,* 43(1), 49-55.

Poopathi, S., & Abidha, S. (2010). Mosquitocidal bacterial toxins (Bacillus sphaericus and Bacillus thuringiensis serovar israelensis): mode of action, cytopathological effects and mechanism of resistance. *J. Physiol. Pathophysiol.* 1(2), 22-38.

Poulin, B., Lefebvre, G., & Paz, L. (2010). Red flag for green spray: adverse trophic effects of Bti on breeding birds. *Journal of Applied Ecology* 47(4), 884-889.

Puntheeranurak, T., Stroh, C., Zhu, R., Angsuthanasombat, C., & Hinterdorfer, P. (2005). Structure and distribution of the *Bacillus thuringiensis* Cry4Ba toxin in lipid membranes. *Ultramicroscopy,* 105, 115–124.

Pusztai, M., Fast, P., Gringorten, L., Kaplan, H., Lessard, T., & Carey, P.R. (1991) The mechanism of sunlight-mediated inactivation of *Bacillus thuringiensis* crystals. *Biochemical Journal* 273, 43-47.

Rao, D. R., Mani, T. R., Rajendran, R., Joseph, A. S., Gajanana, A., & Reuben, R. (1995). Development of a high level of resistance to *Bacillus sphaericus* in a field population

of Culex quinquefasciatus from Kochi, India. *Journal of the American Mosquito Control Association,* 11(1), 1-5.

Revina, L.P., Kostina, L.I., Ganushkina, L.A., Mikhailova, A.L., Zalunin, I.A., Chestukhina, G.G. (2004). Reconstruction of *Bacillus thuringiensis* ssp. *israelensis* Cry11A endotoxin from fragments corresponding to its N- and C-moieties restores its original biological activity. *Biochemistry (Mosc).,* 69, 181-187.

Rigden, D.J. (2009). Does distant homology with Evf reveal a lipid binding site in *Bacillus thuringiensis* cytolytic toxins? *FEBS Letters,* 583, 1555-1560

Rodriguez-Almazan, C., Ruiz de Escudero, I., Canton, P.E., Munoz-Garay, C., Perez, C., Gill, S.S., Soberon, M., & Bravo, A. (2011). The Amino- and Carboxyl-terminal fragments of the *Bacillus thuringiensis* Cyt1Aa toxin have differential roles in toxin oligomerization and pore formation. *Biochemistry,* 50, 388–396.

Rodriguez-Cabrera, L., Trujillo-Bacallao, D., Borras-Hidalgo, O., Wright, D. J., & Ayra-Pardo, C. RNAi-mediated knockdown of a *Spodoptera frugiperda* trypsin-like serine-protease gene reduces susceptibility to a *Bacillus thuringiensis* Cry1Ca1 protoxin. *Environmental Microbiology,* 12(11), 2894-2903.

Roush, R. T., & McKenzie, J. A. (1987). Ecological genetics of insecticide and acaricide resistance. *Annual Review of Entomology*, 32, 361-380.

Saleh, M. S., El-Meniawi, F. A., Kelada, N. L., & Zahran, H. M. (2003). Resistance development in mosquito larvae Culex pipiens to the bacterial agent Bacillus thuringiensis var. israelensis. *Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie*, 127(1), 29-32.

Sangthongpitag, K., Delaney, S.F., & Rogers, P.L. (1996). Evaluation of four fresh-water unicellular cyanobacteria as potential hosts for mosquitocidal toxins. *Biotechnol. Lett.,* 18, 175-180.

Senthilkumar, N., Varma, P., and Gurusubramanian, G. (2009). Larvicidal and adulticidal activities of some medicinal plants against the Malarial Vector, *Anopheles stephensi* (Liston). *Parasitology Research* 104, 237-244.

Scott, A.R., Rapley, L.P., & Benjamin, S. (2010). *Bacillus thuringiensis* var. *israelensis* (Bti) provides residual control of *Aedes aegypti* in small containers. *Am. J. Trop. Med. Hyg.* 82(6):1053-1059.

Siegel, J.P. (2001). The mammalian safety of *Bacillus thuringiensis*-based insecticides. *Journal of Invertebrate Pathology* 77, 13-21.

Siqueira, H. A. A., Moellenbeck, D., Spencer, T., & Siegfried, B. D. (2004). Cross-resistance of CrylAb-selected Ostrinia nubilalis (Lepidoptera : Crambidae) to Bacillus thuringiensis delta-endotoxins. *Journal of Economic Entomology*, 97(3), 1049-1057.

Sinègre, G., Gaven, B., Julien, J.L., & Crespo, O. (1980). Effectiveness of the serotype H-14 of *Bacillus thuringiensis* against the principal species of man-biting mosquitoes on the Mediterranean coast of France. *Parasitologia* 22:223-231

Soberon, M., Gill, S. S., & Bravo, A. (2009). Signaling versus punching hole: How do Bacillus thuringiensis toxins kill insect midgut cells? *Cell Mol Life Sci, 66*(8), 1337-1349.

Soberon, M., Pardo, L., Munoz-Garay, C., Sanchez, J., Gomez, I., Porta, H., & Bravo, A. (2010). Pore formation by Cry toxins. In *Proteins: Membrane Binding And Pore Formation* (Vol. 677, pp. 127-142). Berlin: Springer-Verlag Berlin.

Soltes-Rak, E., Kushner, D.J., Williams, D.D., & Coleman; J.R. (1995). Factors regulating *cryIVB* expression in the cyanobacterium *Synechococcus* PCC-7942. *Mol. Gen. Genet.,* 246, 301-308.

Tabashnik, B. E., & Croft, B. A. (1982). Managing pesticide resistance in crop-arthopod complexes- interactions between biological and operational factors. *Environmental Entomology,* 11(6), 1137-1144.

Tabashnik, B. E., Liu, Y. B., Malvar, T., Heckel, D. G., Masson, L., Ballester, V., et al. (1997). Global variation in the genetic and biochemical basis of diamondback moth resistance to *Bacillus thuringiensis*. *Proceedings of the National Academy of Sciences of the United States of America*, 94(24), 12780-12785.

Tan, F., Zhu, J., Tang, J., Tang, X., Wang, S., Zheng, A., & Li, P. (2009). Cloning and characterization of two novel crystal protein genes, *cry54Aa1* and *cry30Fa1*, from *Bacillus thuringiensis* strain BtMC28. *Curr. Microbiol.*, 58, 654-659.

Thamthiankul, S., Moar, W.J., Miller, M.E., & Panbangred, W., (2004). Improving the insecticidal activity of *Bacillus thuringiensis* subsp. *aizawai* against *Spodoptera exigua* by chromosomal expression of a chitinase gene. *Appl. Microbiol.Biotech.*, 65, 183-192.

Thanabalu, T., Hindley, J., Brenner, S., Oei, C., & Berry, C. (1992). Expression of the mosquitocidal toxins of *Bacillus sphaericus* and *Bacillus thuringiensis* subsp *israelensis* by recombinant *Caulobacter crescentus*, a vehicle for biological control of aquatic insect larvae. *Appl. Environ. Microbiol.*, 58, 905-910.

Tilquin, M., Paris, M., Reynaud, S., Despres, L., Ravanel, P., Geremia, R. A., et al. (2008). Long lasting persistence of *Bacillus thuringiensis* subsp *israelensis* (Bti) in mosquito natural habitats. *Plos One,* 3(10).

Vie, V., Van Mau, N., Pomarede, P., Dance, C., Schwartz, J.L., Laprade, R., Frutos, R., Rang, C., Masson, L., Heitz, F., & Le Grimellec, C. (2001). Lipid-induced pore formation of the *Bacillus thuringiensis* Cry1Aa insecticidal toxin. J. Membr. Biol., 180, 195-203.

Wipfli, M.S. & Merritt, R.W. (1994a). Effects of Bacillus thuringiensis var. israelensis on nontarget benthic macroinvertebrates through direct and indirect exposure. *Journal of the North America Benthological Society* 13:190-205.

Wipfli, M.S. & Merritt, R.W. (1994b). Disturbance to a stream food web by a bacterial larvicide specific to black flies: feeding responses of predatory macroinvertebrates. *Freshwater Biology* 32:91-103.

Wirth, M.C., Delecluse, A., & Walton, W.E. (2003). Cyt1Ab1 and Cyt2Ba1 from *Bacillus thuringiensis* subsp. *medellin* and *B. thuringiensis* subsp. *israelensis* Synergize *Bacillus sphaericus* against *Aedes aegypti* and Resistant *Culex quinquefasciatus* (Diptera: Culicidae). *J. Am. Mosq. Control Assoc.*, 9, 39-46

Wirth, M.C., Georghiou, G.P., & Federici, B.A. (1997). CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito *Culex quinquefasciatus*. *Proc. Natl. Acad. Sci. USA.,* 94, 10536-10540.

Wirth, M.C., Jiannino, J.A., Federici, B.A., & Walton, W.E. (2004). Synergy between toxins of *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus*. *J. Med. Entomol.,* 41, 935-941.

Wirth, M.C., Jiannino, J.A., Federici, B.A. & Walton, W.E. (2005a). Evolution of resistance toward *Bacillus sphaericus* or a mixture of *B.sphaericus*+Cyt1A from *Bacillus*

thuringiensis, in the mosquito, *Culex quinquefasciatus* (Diptera: Culicidae). *J. Invertebr. Pathol.*, 88, 154-162.

Wirth, M.C., Park, H.W., Walton, W.E., & Federici, B.A. (2005b). Cyt1A of *Bacillus thuringiensis* delays evolution of resistance to Cry11A in the mosquito *Culex quinquefasciatus*. *Appl. Environ. Microbiol.,* 71, 185-189.

Wirth, M.C., Walton, W.E., & Federici, B.A. (2000a). Cyt1A from *Bacillus thuringiensis* restores toxicity of *Bacillus sphaericus* against *Culex quinquefasciatus* (Diptera: Culicidae). *J. Med. Entomol.,* 37, 401-407.

Wirth, M.C., Walton, W.E., & Federici, B.A. (2000b). Cyt1A from *Bacillus thuringiensis* synergizes activity of *Bacillus sphaericus* against *Aedes aegypti. Appl. Environ. Microbiol*., 66, 1093-1097.

Wirth, M.C., Walton, W.E., & Federici, B.A. (2010). Inheritance patterns, dominance, stability, and allelism of insecticide resistance and cross-resistance in two colonies of *Culex quinquefasciatus* (Diptera: Culicidae) selected with cry toxins from *Bacillus thuringiensis* subsp. *israelensis. J. Med. Entomol.,* 47, 814-822.

World Health Organisation. (1996). Operational manual on the application of insecticides for control of the mosquito vectors of malaria and other diseases. Division of control of tropical disease. WHO/CTD/VBC/96.1000. 198 pp.

World Health Organisation. (2009). Global insecticide use for vector-borne disease control. 4th ed. WHO/HTM/NTD/WHOPES/GCDPP/2009.6, 83 p.

Wu, D., & Chang, F.N. (1985). Synergism in mosquitocidal activity of 26 and 65 kDa proteins from *Bacillus thuringiensis* subsp. *israelensis* crystal. *FEBS Lett.,* 190, 232-236.

Wu, D., & Federici, B.A. (1993). A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. *J. Bacteriol.,* 175, 5276-5280.

Wu, D., & Federici, B.A. (1995). Improved production of the insecticidal CryIVD protein in *Bacillus thuringiensis* using *cryIA(c)* promoters to express the gene for an associated 20-kDa protein. *Appl. Micro. Biotech.,* 42, 697-702.

Xiaoqiang,W.U., Vennison, J., Huirong, L., Ben-Dov, E., Zaritsky, A., & Boussiba, S. (1997). Mosquito larvicidal activity of transgenic *Anabaena* strain PCC7120 expressing combinations of gene from *Bacillus thuringiensis* subsp *israelensis*. *Appl. Environ. Microbiol.,* 63, 4971-4975.

Xu, L., Wang, Z., Zhang, J., He, K., Ferry, N., & Gatehouse, A. M. R. Cross-resistance of Cry1Ab-selected Asian corn borer to other Cry toxins. *Journal of Applied Entomology, 134*(5), 429-438.

Xudong X, Renqiu, K., & Yuxiang, H. (1993). High larvicidal activity of intact cyanobacterium *Anabaena* sp PCC7120 expressing gene 51 and gene 42 of *Bacillus sphaericus* sp 2297. *FEMS. Microbiol. Lett.,* 107, 247-250.

Yamagiwa, M., Ogawa, R., Yasuda, K., Natsuyama, H., Sen, K., Sakai, H. (2002). Active form of dipteran-specific insecticidal protein cryllA produced by *Bacillus thuringiensis* subsp. *israelensis*. *Biosci. Biotechnol. Biochem*., 66, 516-522.

Yamagiwa, M., Sakagawa, K., & Sakai, H. (2004). Functional analysis of two processed fragments of *Bacillus thuringiensis* Cry11A toxin. Biosci. *Biotechnol. Biochem.,* 68, 523-528.

Yap, W.H., Thanabalu, T., & Porter, A.G. (1994). Expression of mosquitocidal toxin genes in a gas-vacuolated strain of *Ancylobacter aquaticus*. *Appl. Environ. Microbiol.*, 60, 4199-4202.

Zhao, J. Z., Li, Y. X., Collins, H. L., Cao, J., Earle, E. D., & Shelton, A. M. (2001). Different cross-resistance patterns in the diamondback moth (Lepidoptera : Plutellidae) resistant to *Bacillus thuringiensis* toxin CrylC. *Journal of Economic Entomology, 94*(6), 1547-1552.

Zhang, X., Candas, M., Griko, N. B., Rose-Young, L., & Bulla, L. A. (2005). Cytotoxicity of Bacillus thuringiensis Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R-1 expressed in insect cells. *Cell Death and Differentiation,* 12(11), 1407-1416.

Zhang, X. B., Candas, M., Griko, N. B., Taussig, R., & Bulla, L. A. (2006). A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of Bacillus thuringiensis. *Proceedings of the National Academy of Sciences of the United States of America,* 103(26), 9897-9902.

Zhang, R., Hua, G., Andacht, T. M., & Adang, M. J. (2008). A 106-kDa aminopeptidase is a putative receptor for *Bacillus thuringiensis* Cry11Ba toxin in the mosquito *Anopheles gambiae*. *Biochemistry,* 47(43), 11263-11272.

Zhu, J., Zheng, A.P., Tan, F.R., Wang, S.Q., Deng, Q.M., Li, S.C., Wang, L.X., & Li, P. (2010). Characterisation and expression of a novel holotype crystal protein gene, *cry56Aa1,* from *Bacillus thuringiensis* strain Ywc2-8. *Biotechnol. Lett.,* 32, 283-288.

7

Screening of Biocontrol Agents Against *Rhizoctonia solani* Causing Web Blight Disease of Groundnut (*Arachis hypogaea* L.)

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1. Introduction

Soil borne plant pathogenic fungi cause heavy crop losses all over the world. With variable climate from region to region, most of the crops grown in India are susceptible to diseases caused by soil borne fungal pathogens. Among tropical, subtropical land crops groundnut (*Arachis hypogaea* L.) is an important annual oil seed crop, which provides vegetable oil as human food and oil cake meal as animal poultry feed. A large number of diseases attack groundnut in India (Ganesan and Sekar, 2004a).

Fungi cause majorities and several of them are yield reducers in certain region and seasons (Bowyer, 1999). Among the soil borne fungal diseases of groundnut, Web blight, caused by *Rhizoctonia solani* is the most common disease (Dubey, 2000).

Majority of work done on plant disease biocontrol relate to soil borne diseases using either bacteria or fungal antagonists (Montealegre *et al*., 2003; Askar and Rashad, 2010; Pandya and Saraf, 2010). Among bacteria, *Pseudomonas* and *Bacillus* spp. are widely used. However, the use of antagonistic fungi, especially *Trichoderma* and *Gliocladium* spp. has been more extensive than their bacterial counterparts (Harman, 2000; Ganesan, 2004; Harman, 2006; Neha and Dawande, 2010). Bacteria isolated from the rhizosphere and belonging to a wide variety of genera have the potential to suppress diseases caused by a diversity of soil borne plant pathogens. But the information available on the antagonistic effect of rhizobacteria against *R. solani* is very scanty.

Rhizoctonia solani Kuhn (teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk is a widespread and an ecologically diverse soil-borne fungus, causing different types of diseases in many plant species. It causes root rot, stem rot, fruit and seed decay, damping-off, foliar blight, stem canker and crown rot in various crops (Guleria *et al*., 2007).

Understanding the mechanism of action is important because it gives much useful information in determining the maintenance, enhancement and implementation of biological control. Biological control agents interact with phytopathogens directly or indirectly to reduce the population of pathogens or reduction in the ability of the pathogens to cause disease. In general, mechanisms implicated in antagonism towards the biological control of phytopathogenic fungi includes, A) Direct mechanism: parasitism, antibiosis, competition for nutrients or space, production of enzymes and inactivation of pathogen enzymes. B) Indirect mechanism: tolerance to stress through enhanced root and plant

development induced systemic resistance and solubilization and sequestration of inorganic nutrients (Liu, *et al*., 1995; Chet, *et al*., 1998 Altomare *et al*., 1999; Harman, 2000; Viswanathan and Samiyappan, 2002; Ganesan *et al*., 2003; Ganesan, 2004; Ganesan and Sekar, 2004a; 2004b; Gohel *et al*., 2006; Harman, 2006; Pal and Gardener, 2006).
In the present work, isolation and identification of native antagonists against web blight causing pathogen (*Rhizoctonia solani*) and comparing their antagonism with biocontrol agents obtained from different type culture collection and study the mechanism of the antagonism against the pathogens were studied**.**

2. Materials and methods

2.1 Isolation of pathogen from groundnut field

Groundnut (*Arachis hypogaea* L.) plants showing web blight symptoms were collected from Pallapatty village crop field, Tamil Nadu, India (77°81′- 78° -2′E longitude and 9°5′- 10°5′N latitude). The pathogenicity of the isolated pathogen (*R. solani*) was tested as described by Singh and Thapliyal, (1998).

2.2 Isolation and maintenance of biocontrol agents

Isolation of bacterial biocontrol agents was made according to the method of Khot *et al*., (1996). They were screened for their antagonistic activity against the pathogens by dual culture method, isolates which showed significant antagonistic activity was identified by using methods described in Bergey's Manual of Systematic Bacteriology (1984).
Isolation of fungal biocontrol agents was made from the pathogens infested field soil. Fungal species growing on the ungerminated sclerotia were isolated, screened for antagonistic activity by dual culture method, identified and maintained on Potato Dextrose Agar (PDA).
The following biocontrol agents used in the present study were obtained from Indian Type Culture Collection (ITCC), New Delhi and Microbial Type Culture Collection (MTCC), Chandigarh. *Trichoderma harzianum* (ITCC-4572), *T. koningii* (MTCC-2385), *T. viride* (MTCC-800), *T. pseudokoningii* (MTCC-3011), *T. hamatum* (MTCC-2577), *T. reesii* (MTCC-798), *Bacillus megaterium* (MTCC-453), *B. pumilus* (MTCC-170) and *B. subtilis* (MTCC-121).

3. Antagonistic activity of biocontrol agents

3.1 Dual culture method

The antagonistic ability of the bacterial biocontrol agents was tested by dual culture technique. The antagonism between selected fungal antagonists and the pathogen *R. solani* was tested following the method suggested by Bell *et al*., (1982). They were ranked according to modified Bell's ranking scale. R_1 = complete overgrowth, R_2 = 75% over growth, R_3 = 50% over growth, R_4 = locked at the point of contact, R_5 = pathogen over growing antagonist. The percentage of inhibition was calculated using the following formula: Percentage of inhibition = $A1 - A_2 / A_1 \times 100$, Where A1 = Area covered by the pathogen in control, A2 = Area covered by pathogen in dual culture.

4. Light microscopic and Scanning Electron Microscopic (SEM) observations

For light microscopic observations, the mycelium was aseptically removed from the site of interaction and mounted on microscopic slides using lactophenol cotton blue stain. Slides

were analyzed and photographed under photomicroscope (Nikon-20). For Scanning Electron Microscopic (SEM) studies, samples were mounted on the specimen stubs using fevicol adhesive. Small samples were mounted directly on Scotsch double adhesive tape, and were coated with gold to a thickness of 100 A^o using Hitachi Vacuum Evaporator, Model HUS 5GB. Coated samples were analyzed in a Hitachi Scanning Electron Microscope model S-450 operated at 15kv, and photographed.

5. Volatile activity

To study the effect of volatile metabolites of biocontrol agents on *R. solani*, paired plate technique was followed (Dennis and Webster, 1971). Three replicates were placed for each treatment. Growth of the pathogen was recorded on the 3rd day and on 5th day after incubation and the percentage of inhibition was calculated using the formula, Percentage of inhibition = A_1- A_2 / A_1 X 100.
Where A_1 = Area covered by the pathogen in control, A_2 = Area covered by pathogen in paired petriplate.

6. Non-volatile activity

Non-volatile activities of bacterial and fungal biocontrol agents were tested as described by Jariwala *et al*., (1991) with slight modification. Loop full of bacterial biocontrol agents were inoculated in Nutrient broth and incubated in a shaker (120 rpm) for 48hrs at room temperature (28° ± 2°). After the incubation, cultures were centrifuged at 5000rpm for 15min. and the supernatant was used for antibiotic activity.
In the case of fungal antagonists, 1ml of spore suspension (1 x 105 cfu / ml) was inoculated in Potato Dextrose (PD) broth and incubated at room temperature for 1 week. The fungal mat and the spores were removed by filtration through double layer filter paper followed by centrifugation at 5000rpm for 15min. the supernatant was used for antibiotic activity. Culture filtrates were added to PD Agar medium at 25%, 50%, 75% and 100% concentration, the pH was adjusted to 6.8 ± 0.2. Then the medium was sterilized and poured in the sterile Petri plates.
Three day old actively growing *R. solani* cultures were removed from the edge of the colony using 4mm diameter cork borer and placed at the center of these culture medium and the plates were incubated at room temperature. Three replicates were maintained for each concentration. Plates containing PDA medium with pathogens alone served as control. Radial growth of the fungal colony was measured on 3rd day and 5th day after incubation. Percentage of inhibition was calculated using the formula described earlier.

7. Enzymatic activity

Two sets of Erlenmeyer flasks (250ml) containing 50 ml of czapek dox broth containing carboxy methyl cellulose (CMC) (inducer of β-1- 4 endoglucanase) and minimal medium supplemented with chitin (inducer of chitinase), were inoculated with fungal and bacterial biocontrol agents respectively, Bacterial cultures were incubated on rotary shaker (120rpm) at (28° ± 2°C) for 24hrs. Fungal cultures were incubated on rotary shaker (120rpm) at (28° ± 2°C) for 1week. After incubation period, cultures were filtered using Whatman No.1 filter paper and filtrates were centrifuged at 5000rpm for 15 min. Supernatants were used as crude preparations for enzyme assays.

7.1 Chitinase activity

Enzymatic hydrolysis of colloidal chitin was assayed following the release of free N-Acetyleglucosamine (NAG) from colloidal chitin by spectrometric method (Ohtakara, 1988) and by clearing zone assay method (Frandberg and Schnurer, 1998).

7.2 Clearing zone assay

The clearing zone assay was performed on chitin agar containing 0.15% chitin, 1.5% agar, and 0.02% Sodium azide (Na N_3) in 50Mm potassium phosphate buffer (pH - 6.1). Crude enzymes samples (0.25ml) were added to wells (diameter 5-mm) made in the agar medium. The plates were incubated in a humidity chamber at 28° ± 2°C for 24hr. and the rate of clearing zones were measured.

7.3 Spectrometric method

Colloidal chitin was prepared from raw chitin, five grams of chitin powder was homogenized in 100ml of concentrated hydrochloric acid and left at 20°C. for 10 min. The suspension was poured into cold water under agitation and left to settle. The precipitate was washed with water and dried.

The reaction mixture containing 1 ml of 0.5% colloidal chitin, 2 ml of Mcllvaines's buffer (equal volume of 0.2M disodium hydrogen phosphate and 0.1M of citric acid, pH 4.0) and 1ml of culture filtrate, was incubated for 20 min. at 37° C in a shaker (120rpm) and the reaction was stopped by boiling for 3 min. After centrifugation of this mixture (2000rpm for 30 min.) 1.5 ml of supernatant fluid was mixed with 2 ml of potassium ferricyanide reagent (0.05% potassium ferricyanide in 0.5 M sodium carbonate) and heated in boiling water bath for 15 min. The amount of N- acetyl glucosamine released was estimated by absorbance of reaction mixture at 420 nm. One unit of enzyme activity was defined as release of 1 micro mol N-acetyleglucosamine (IU) /ml of culture filtrate /min.

7.4 β - 1-4- endoglucanase activity

β-1-4-endoglucanase attacks the 1-4-β-glucosidic linkages of cellulose molecule randomly. Enzymatic hydrolysis of carboxy methylcellulose (CMC) was assayed by dinitro salicyclic acid (DNS) method (Miller, 1959). The enzyme activity was expressed as release of 1 micromole glucose (IU) / ml of culture filtrate /min.

8. Result and discussion

Pathogen *Rhizoctonia solani* was isolated from web blight symptom showing infected groundnut plants and identified according to their colony characters and pathogenecity (Plate I). Fifteen bacterial biocontrol agents were isolated from soil. Among the isolates, 5 showed antagonistic activity against the pathogen. These isolates were identified as *Bacillus polymyxa*, *B. licheniformis*, *B. speriacus, B. thuringiensis* and *Pseudomonas pudita* based on different biochemical tests (Krieg, 1984; Sneath, 1984). These isolates were pure cultured and maintained on Nutrient Agar medium. Ten fungal antagonists were isolated and screened against *R. solani*, among these only 2 isolates showed significant reduction in the growth of the pathogen. They were identified as *Trichoderma longibrachiatum* and *T. virens* = *Gliocladium virens* (Plate II-3).

Several workers also found the successful control of *R. solani* in *invitro* condition using biocontrol agents (Dubey, 1998; Desai and Schlosser, 1999; Bunker and Mathur, 2001;

Ganesan and Sekar, 2004b; Bienkowski *et al*., 2010). In the present work to understand the biocontrol of pathogens by antagonists, light and scanning electron microscopic observations, involvement of volatile and non-volatile metabolites and secretion of enzymes (chitinase and β-1-4-glucanase) have been made (Table 1, 2a & 2b, 3a & 3b, Fig. 1a & 1b, Plate I & II).

In dual culture method, after third day of incubation all the biocontrol agents inhibited the pathogen to varying degree. Among the bacterial biocontrol agents, Maximum level of growth reduction of *R. solani* was noted in *B. subtilis* (42.53%) followed by *B. polymyxa* (39.13%) and *B. licheniformis* (30.36%). Lowest level of inhibition was found with *B. pumilus* (11.84%). Percentage of inhibition varied with biocontrol agents used. Among the fungal biocontrol agents, 78.76% of inhibition was recorded with *T. virens* followed by *T. hamatum* (77.64%) and *T. harzianum* (72.24%). Minimum level of inhibition was noticed with *T. reesi* (33.06% (Table 1). Further incubation of plates showed reduction in number of sclerotial production by the pathogen, when compared with control. They were ranked according to modified Bell's ranking scale (Table 1).

S. No	Bacterial Biocontrol agents	Radial growth of *R . solani* (mm)*	% inhi-bition	Fungal Biocontrol agents	Radial growth of *R. solani* (mm)*	% inhi-bition
1	Control	74.53 ± 0.28	-	Control	74.70 ± 0.15	-
2	*B. subtilis*	42.83 ± 0.27	42.53	*T. harzianum*	20.73 ± 0.73	72.24^{R1}
3	*B. megaterium*	64.90 ± 1.21	12.92	*T. hamatum*	16.70 ± 0.24	77.64^{R1}
4	*B. pumilus*	65.70 ± 0.31	11.84	*T. viride*	38.63 ± 0.37	48.28^{R2}
5	*B. thuringiensis*	62.93 ± 0.40	15.56	*T. koningii*	26.93 ± 0.53	63.94^{R3}
6	*B. licheniformis*	51.90 ± 0.73	30.36	*T. pseukoningii*	53.73 ± 0.63	28.07^{R4}
7	*B. speriacus*	64.60 ± 0.34	13.32	*T. reesii*	50.00 ± 0.34	33.06^{R5}
8	*B. polymyxa*	45.36 ± 1.83	39.13	*T. longibrachiatum*	25.20 ± 0.57	66.26^{R2}
9	*P. putida*	65.33 ± 0.60	12.34	*T. virens*	15.86 ± 0.23	78.76^{R1}

* = Each value is a mean of triplicate; ± = Std error; R_1- R_5 = Bell's ranging

Table 1. Interaction of biocontrol agents against *R. solani* – Dual culture method

Light and scanning electron microscopic observations of antagonists and pathogenic organisms in dual culture indicate that the principle mechanism of fungal antagonism is due to mycoparasitism. Antagonistic hyphae coil the pathogenic hyphae. Pathogenic organism showed severe vacuolation followed by coagulation, shrinkage of cytoplasm and finally lysis of cells. In some cases the tip of the pathogenic hyphae showed bulbous and tapering end. Bacterial antagonists showed attachment with the pathogenic hyphae and lysis. Pathogenic mycelium on the clearing zone showed swelling of hyphal tips, cells were found to be bulbous, swollen with shrunken and granulated cytoplasm (Plate II).

Under Scanning Electron Microscope, the fungal antagonists showed coiling, and lysis of pathogens mycelium. With the bacterial biocontrol agents, the affected pathogenic mycelium showed attachment, lysis and disintegration (Plate II). During antibiosis, both volatile and nonvolatile secondary metabolities have been implicated in restricting the vegetative growth of pathogenic fungi. Volatile activity of *T. harzianum* showed 100% inhibition of *R. solani*, where as *T. viride* showed 93.44% of inhibition against *R. solani*. *B. speriacus* and *B. polymyxa* showed 87.30% and 94.08% of inhibition. *B. megaterium* and *B.*

licheniformis showed 95.7% and 94.41% of inhibition and *P. putida* showed 88.5% inhibition of *R. solani* mycelial growth (Table 2a & 2b). In general the inhibition percentage was higher on the 3rd day of incubation than on 5th day of incubation. No changes in the colony characteristics of fungal and bacterial antagonists were observed.

S. No	Biocontrol agents	Mycelial Length on 3rd day (mm)*	% inhibition	Mycelial Length on 5th day (mm)*	% inhibition
1	Control	30.96 ± 0.26	-	39.83 ± 0.15	-
2	*B. subtilis*	1.93 ± 0.23	93.76	3.53 ± 0.46	91.13
3	*B. megaterium*	1.33 ± 0.33	95.70	5.53 ± 0.41	86.11
4	*B. pumilus*	6.8 ± 0.20	78.03	14.10 ± 0.40	64.59
5	*B. thuringiensis*	11.66 ± 0.23	62.33	15.53 ± 0.27	61.00
6	*B. licheniformis*	1.73 ± 0.40	94.41	2.56 ± 0.83	93.57
7	*B. speriacus*	3.93 ± 0.37	87.30	6.85 ± 0.45	82.80
8	*B. polymyxa*	1.83 ± 0.16	94.08	4.96 ± 0.18	87.54
9	*P. putida*	3.56 ± 0.23	88.50	8.56 ± 0.34	78.50

* = Each value is a mean of triplicate ± = Std error

Table 2a. Volatile activity of bacterial biocontrol agents against *R. solani*

S. No	Biocontrol agents	Mycelial Length on 3rd day (mm)*	% inhibition	Mycelial Length on 5th day (mm)*	% inhibition
1	Control	31.10 ± 0.75	-	43.66 ± 0.17	-
2	*T. harzianum*	-	100	0	100
3	*T. hamatum*	7.00 ± 0.40	77.49	10.66 ± 0.17	75.58
4	*T. viride*	-	100	2.86 ± 0.24	93.44
5	*T. koningii*	3.13 ± 0.27	89.93	10.93 ± 0.23	74.96
6	*T. pseudokoningii*	19.46 ± 0.60	37.42	32.96 ± 0.32	24.50
7	*T. reesii*	24.90 ± 0.45	12.91	39.10 ± 0.46	10.44
8	*T. longibrachiatum*	1.00 ± 0.00	96.78	4.46 ± 0.29	89.78
9	*T. virens*	2.56 ± 0.21	91.76	5.06 ± 0.17	88.41

* = Each value is a mean of triplicate ± = Std error

Table 2b. Volatile activity of fungal biocontrol agents against *R. solani*

These results indicate that antagonistic organisms produce volatile compounds having antibiotic activity. Non-volatile activity of the antagonistic organisms against pathogens revealed significant reduction of pathogenic growth. The maximum level of inhibition of *R. solani* (100%) was obtained from *T. harzianum* at 100% conc. of culture filtrates. Among the bacterial antagonists *B. subtilis* produced 90.31% of inhibition of *R. solani* mycelium at 100% concentration of culture filtrates. However, decreased concentrations were less inhibitory to the growth of *R. solani* (Fig. 1a & 1b).

Isolates of *Trichoderma* excrete some growth inhibitory substances. Of these, alkyl pyrons, isonitriles, polyketides, peptaibols diketopiperazines, sesquiterpenes, and steroids have frequently been associated with biocontrol activity (Howell, 1998; Sivasithamparam and Ghisalberti, 1998). Production of heat labile antifungal substances by *Bacillus* to control different fungal pathogens was reported by several workers (Cubeta *et al*., 1985; Podile *et al*., 1987; Dileepkumar *et al*., 1988). Cyanides were also considered as volatile metobolites produced by bacterial biocontrol agents (Laha *et al*., 1996; Rangheshwaran and Prasad, 2000).

Biocontrol agents are known to produce various enzymes like β-1-3 glucanase, cellulase (β, 1-4,gluganase), chitinase and proteases, which are involved in the antagonistic activity against phytopathogenic fungi (Frandberk and Schnurer, 1998; Singh *et al*., 1999; Berg *et al*., 2000; Ramamoorthy and Samiyappan, 2001). In the present study *T. harzianum* and *T. koningii* showed maximum chitin utilization in clearing zone assay. In the case of bacterial antagonists, except *B. pumilus* all the other antagonists showed high level of chitin degradation (Table 3a & 3b).

S. No	Biocontrol agents	Clearing zone assay of Chitinase	Chitinase activity IU/ml	β -1-4 Glucanase activity IU/ml
1	*B. subtilis*	+ + +	14.10	58.80
2	*B. megaterium*	+ + +	11.47	58.00
3	*B. pumilus*	+ +	9.73	70.80
4	*B. thuringiensis*	+ + +	19.81	71.60
5	*B. licheniformis*	+ + +	12.98	58.80
6	*B. speriacus*	+ + +	16.27	30.00
7	*B. polymyxa*	+ + +	17.29	87.20
8	*P. putida*	+ + +	15.06	71.20

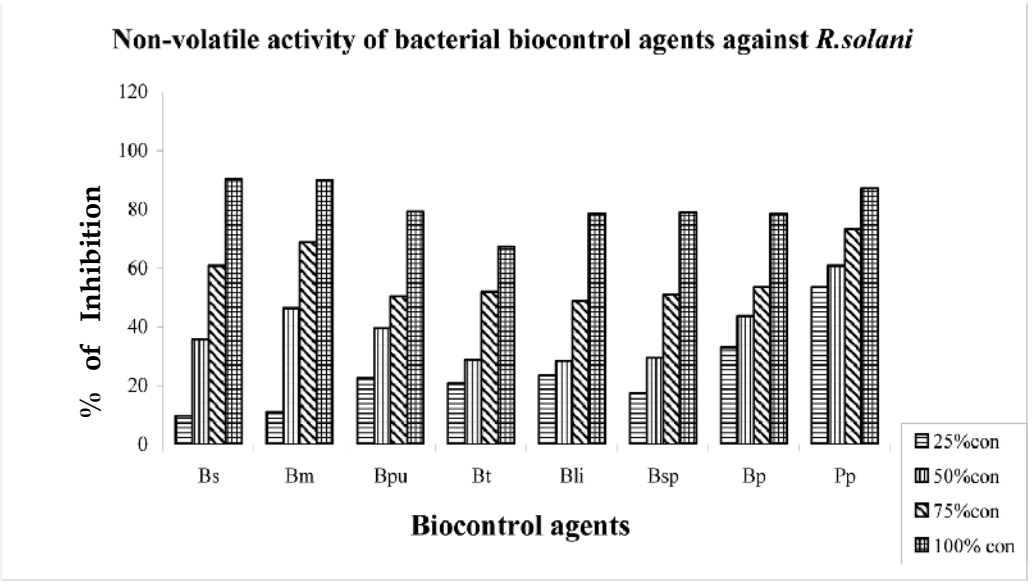
+ + + = Higher level of clearing zone + + = Moderate level

Table 3a. Enzymatic activity of bacterial biocontrol agents

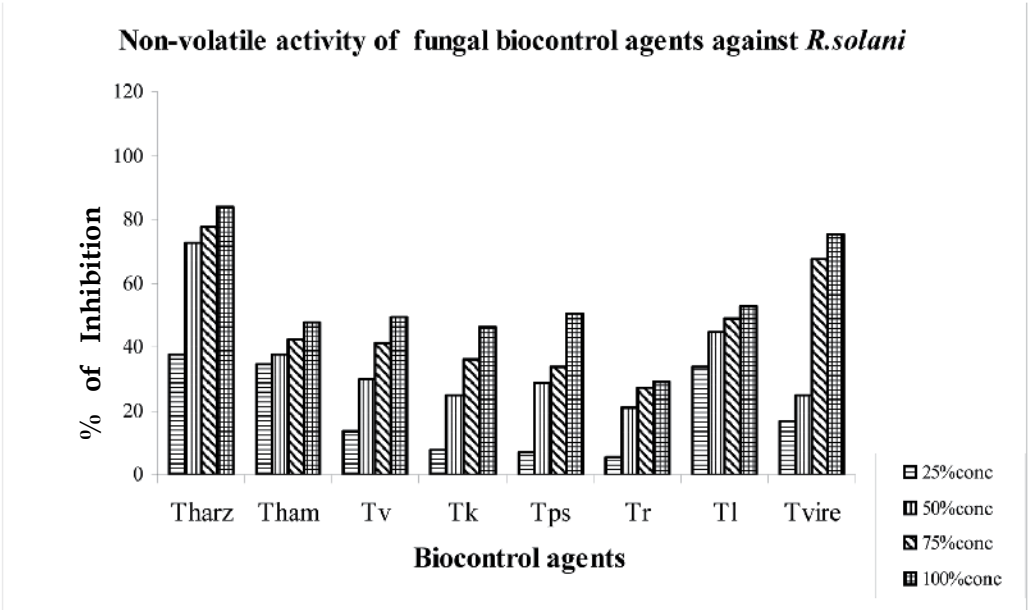
S. No.	Biocontrol Agents	Clearing zone assay of Chitinase	Chitinase Activity IU/ml	β-1-4Glucanase Activity IU/ml
1.	*T. harzianum*	+ + +	5.51	7.60
2.	*T. hamatum*	+ +	2.32	4.80
3.	*T. viride*	-	0.12	33.20
4.	*T. koningii*	+ + +	4.00	60.40
5.	*T. pseudokoningii*	+	3.80	34.40
6.	*T. reesii*	-	0.33	46.80
7.	*T. longibrachiatum*	+ +	0.85	6.40
8.	*T. virens*	+ +	2.50	36.80

+ + + = Higher level of clearing zone; + + = Moderate level; + = Low level
- = No clearing zone

Table 3b. Enzymatic activity of fungal biocontrol agents


Non-volatile activity of bacterial biocontrol agents against R.solani
% of Inhibition
120
100
80
60
40
20
0
Bs
Bm
Bpu
Bt
Bli
Bsp
Bp
Pp
Biocontrol agents
25%con
50%con
75%con
100% con

(a)


Non-volatile activity of fungal biocontrol agents against R.solani
% of Inhibition
120
100
80
60
40
20
0
Tharz
Tham
Tv
Tk
Tps
Tr
Tl
Tvire
Biocontrol agents
25%conc
50%conc
75%conc
100%conc

(b)

Fig. 1.

Maximum level of chitinase enzyme production was observed in *B. thuringiensis* (19.81 IU/ml). Among the fungal antagonists *T. harzianum* showed maximum activity (3.80 IU/ml). In the CMC amended medium maximum level of β-1.4-endoglucanase activity was observed in *B. polymyxa* (87.2 IU /ml). Among the fungal antagonist maximum level of enzyme activity was observed in *T. koningii* (60.41 IU /ml) (Table 3a & 3b). The levels of chitinase and glucanase increase dramatically as soon as a pathogen attack occurs (Ferraris *et al.,* 1987). Both these enzymes are responsible for disrupting the fungal cell wall and/or prevention of hyphal growth (Vaidya et al., 2001; Gohel *et al*., 2004). There was great concern regarding screening techniques for biocontrol strains of *Trichoderma*; the first and quickest ones were screens for antibiotic production and/or mycoparasitism in petridish assays (Harman, 2006).

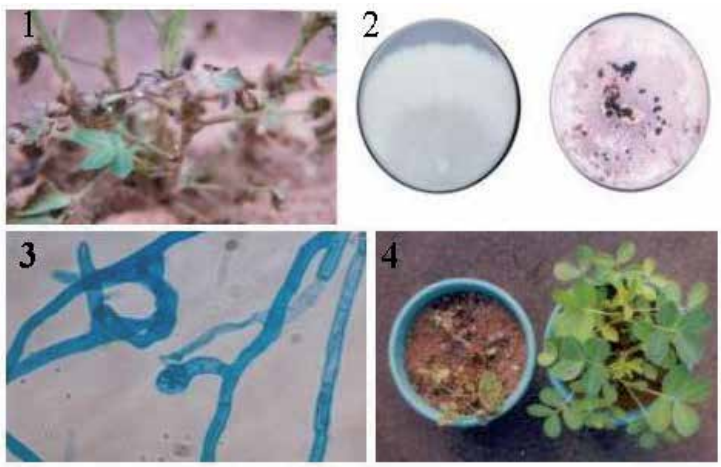


Plate I. Growth characters and pathogenicity of *Rhizoctonia solani*
1. Groundnut plants showing web blight disease
2. White hyaline mycelium of *R. solani* (on 3rd day of incubation) (Left) with irregular dark brown sclerotia on 7th day of incubation (Right)
3. Light microscopic picture of *R. solani* mycelium (400X)
4. Web blight symptom on groundnut plant in pot culture condition (left), healthy plants (Right)

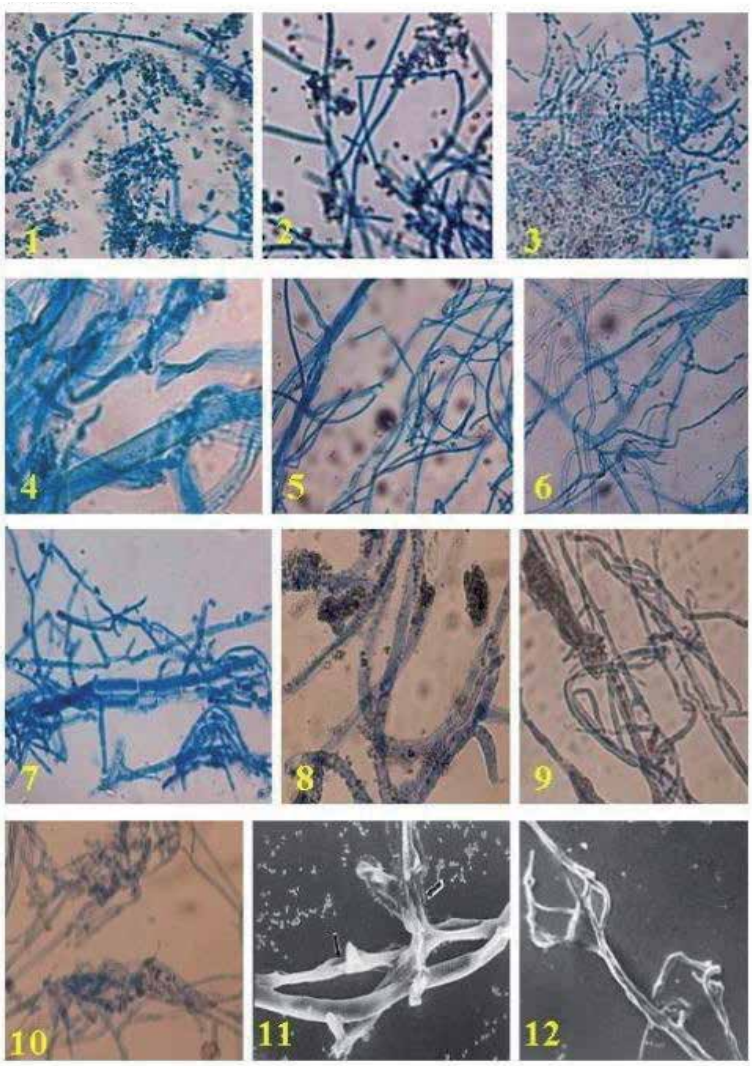


Plate II. Interaction between biocontrol agents and pathogens
Light Microscopic observation
1. Sporulated mycelium of *Trichoderma harzianum* (200X)
2. Sporulated mycelium of *T. hamatum* (200X)
3. Sporulated mycelium of *T. virens* (200X)
4. Coiling of *T. harzianum* hyphae on *R. solani* mycelium (400X)
5 & 6. Coiling and intermingling of *T. hamatum* mycelium with *R. solani* mycelium (200X).
7. Lysis and defragment of *R solani* mycelium due to *T. virens* (200X)
8. Light micrographic picture of interaction between *B. polymyxa* and *R. solani*- showing attachment of *B. polymyxa* cells on *R. solani* mycelium (200X)
9. Interaction between *B. megaterium* and *R. solani*- clumping of *R. solani* mycelium (200X)
10. Interaction between *Pseudomonas putida* and *R. solani* showing defragmented mycelium

of *R. solani* (200 X)

Scanning Electron Microscopic observations

11. Interaction between *T. harzianum* and *R. solani*- showing coiling and lysis of its host mycelium (800 X)
12. Interaction between *B. polymyxa* and *R. solani* showing abnormal structure of host mycelium (120 X).

9. Conclusion

The work indicates that both fungal and bacterial biocontrol agents ware effective against *R. solani.* These biocontrol agents inhibit the pathogen by antibiosis (volatile or non-volatile) or by enzyme production or by parasitism or by combinations of these methods.

10. References

Altomare, C., W.A; Norvell, T. Bjorkman & G.E. Harman 1999. Solubilization of phosphates and micronutrients by the plant growth promoting and biocontrol fungus *Trichoderma harzianum* Rifai. 1295 - 22. *Appl Environ Microbiol*, 65: 2926 - 2933.

Askar, A.A & Rasnae, Y.A. 2010. Arbuscular mycorrhizal fungi: a biocontrol agent's agaist common bean *Fusarium* root rot disease. *Plant Pathol. J*, 9(1): 31-38.

Bell, D.K.; H.D. Wells & C.R. Markham, 1982. *In vitro* antagonism of *Trichoderma* species against six fungal plant pathogens. *Phytopathology*, 72(4): 379–382.

Berg, G.B; S. Kurze, A. Buchner, E.M. Wellington & K. Smalla. 2000. Successful strategy for the selection of new strawberry associated rhizobacteria antagonistic to *Verticillium* wilt. *Can J Microbiol*, 46: 1128 – 1137.

Bienkowski1, D; A. Stewart1, R.E. Falloon; M. Braithwaite; L.L. Loguercio & E. Hicks, 2010. A disease assay for *Rhizoctonia solani* on potato (*Solanum tuberosum*). *New Zealand Plant Protection 63: 133-137.*

Bowyer, P. 1999. Plant disease caused by fungi phytopathogenicity, In: Molecular Fungal Biology, (Eds., R.P. Oliver and M. Schweizar), Cambridge University Press, Cambridge.

Bunkar, R.R & K. Mathur. 2001. Antagonism of local biocontrol agents to *Rhizactonia solani* inducing dry root rot of chilli. *J Mycol Pl Pathol*, 31(1): 50 – 53.

Chet, I.; N. Benhamou & S. Haran. 1998. Mycoparasitism and lytic enzymes. In: *Trichoderma* and *Gliocladium*. Vol. 2. (Eds., G.E. Harman and C.P. Kubicek), Taylor & Francis, London, pp. 153-172.

Cubeta, M.A; G.C. Harman & J.B. Sinclair. 1985. Interactions between *Bacillus subtilis* and *Phomopsis* spp. associated with soybean seeds. *Plant Dis.*, 69(6): 506-509.

Demirci *et al.,* 2009. Biological control of *Rhizoctonia solani* on potato by *Verticillium biguttatum. Afr. J. Biotechnol*. 8 (11): 2503-2507

Dennis, C & J. Webster. 1971. Antagonistic properties of species group of *Trichoderma.* II. Production of volatile antibiotics. *Trans Brit Mycol Soc,* 57: 41 - 48.

Desai, S & E. Schlosser. 1999. Parasitism of *Sclerotium rolfsii* by *Trichoderma*. *Indian Phytopath*., 52(1): 47 - 50.

Dileepkumar, B.S; A.R. Podile & H.C. Dube, 1988. Antagonistic activity of *Bacillus subtilis* towards *Rhizopus nigricans. J Biol Control*, 2(1): 42-45.

Dubey, S.C. 1998. Evaluation of different fungal antagonist, plant extract and oil cakes against *Thanatephorus cucumeris* (*Rhizoctonia solani*) causing banded blight of rice. *J Mycol Pl Pathol,* 28(3): 266 - 269.

Dubey, S.C. 2000. Biological management of web blight of Groundnut (*Rhizoctonia solani*). *J Mycol Pl Pathol,* 30(1): 89 - 90.

Frandberg, E & J. Schnurer, 1998. Antifungal activity of chitinolytic bacteria isolated from airtight stored cereal grain. *Can J Microbiol,* 44: 121–127.

Ferraris L; G.I. Abbattista & A. Matta. 1987. Activation of glycosidases as a consequence of infection stress in *Fusarium* wilt of tomato. *J Phytopathol*, 118: 317-325.

Ganesan, S & R. Sekar. 2004a. Biocontrol mechanism of Groundnut (*Arachis hypogaea* L.) Diseases- *Trichoderma* system. In: *Biotechnological Applications in Environment and Agriculture*, (Eds., G.R. Pathade and P.K. Goel), ABD Pub. Jaipur, India, pp. 312-327.

Ganesan, S & R. Sekar. 2004b. Biocontrol mechanism of *Trichoderma harzianum* (ITCC-4572) on groundnut web blight disease caused by *Rhizoctonia solani*. *Journal of Theoretical and Experimental Biology,* 1: 43-47.

Ganesan, S. 2004. Studies on the Biocontrol of Soilborne Plant Pathogens, Ph.D., Thesis, Madurai Kamaraj University, India.

Ganesan, S; P. Manimaran; K. Ramesh & R. Sekar. 2003. Biocontrol of onion basal rot disease caused by *Fusarium oxysporam*. f.sp. *cepae*, In: Proceeding of International conference of SAARC Countries: Biotechnology in Agriculture, Industry and Environment, (Ed., A.M. Desmukh), Microbiologist Society, Maharashtra, India. pp.119-124.

Ghisalberti, E.L & K. Sivasithamparam, 1991. Antifungal antibiotics produced by *Trichoderma* spp, *Soil Biol Biochem,* 23: 1011-1020.

Gohel, V; C. Megha; P. Vays & H.S. Chhatpar. 2004. Strain improvement of chitinolytic enzyme producing isolate *Pantoea dispersa* for enhancing its biocontrol potential against fungal plant pathogens. *Ann Microbiol*, 54:503-515.

Guleria, S; R. Aggarwal; T.S. Thind & T.R. Sharma, 2007. Morphological and pathological variability in rice isolates of *Rhizoctonia solani* and molecular analysis of their genetic variability. Journal Phytopathology 155: 654-661.

Harman, G.E. 2000. Myths and Dogmas of Biocontrol. *Plant Dis.,* 84(4): 377- 393.

Harman, G.E. 2006. Overview of mechanisms and uses of *Trichoderma* spp., *Phytopathology*, 96(2): 190-194.

Howell, C.R. 1998. The role of antibiosis in biocontrol. In: *Trichoderma and Gliocladium,* Vol 2, (Eds., G.E, Harman and C.P. Kubicek), Taylor and Francies, London. pp.173-184

Jariwala, S., Vinaykumari & Bharat Rai. 1991. Antagonistic activity of some fungi against *Alternaria solani* and *Drechlera oryzae*. *Acta Botanica Indica*, 19(2): 217 - 223.

Khot, G.G; P. Tauro & K.R. Dadarwal, 1996. Rhizobacteria from chickpea (*Cicer arietinum* L.) rhizosphere effective in wilt control and promote nodulation. *Indian J Microbiol*., 36: 217-222.

Krieg, N.R. 1984. Gram-negative aerobic rods and cocci. In: *Bergey's manual of systematic bacteriology,* 8th ed., Vol I, Williams & Wilkins, Baltimore. Pp.140-218.

Laha, G.S; J.P. Verma & R.P. Singh. 1996. Effectiveness of Fluorescent *Pseudomonads* in the management of sclerotial wilt of cotton. *Indian Phytopath.*, 49(1):3-8.

Liu, L; J.W. Kloepper & S. Tuzun, 1995. Induction of systemic resistance in cucumber against bacterial angular leaf spot by plant growth promoting rhizobacteria. *Phytopathology*, 85: 843 - 847.

Montealegre. J; Rodrigo Reyes; Luz María Pérez; Rodrigo Herrera & Polyana Silva, 2003. Selection of bioantagonistic bacteria to be used in biological control of *Rhizoctonia solani* in tomato, *Electronic Journal of Biotechnology*., 6 (2): 115-127

Miller, G. L.1959. Use of dinitrosalcylic acid reagent for the determination of reducing sugars. *Anal Chem*., 3: 426.

Neha Dev & A.Y. Dawande, 2010. Biocontrol of soil borne plant pathogen *Rhiozoctonia solani* using *Trichoderma* spp. and *Pseudomonas fluorescens*. *Asiatic J. Biotech. Res.* 2010; 01: 39-44

Ohtakara, A. 1988. Chitinase and β-N-acetyl hexosaminidase from *Pycnoporus cinnabarainus*. *Method Enzymol*., 168: 467-468.

Pal, K. K. & B. McSpadden Gardener, 2006. Biological Control of Plant Pathogens. *The Plant Health Instructor* DOI: 10.1094/PHI-A-2006-1117-02.

Pandya, U & Saraf, M, 2010. Application of Fungi as a Biocontrol Agent and their Biofertilizer Potential in Agriculture, *J. Adv. Dev. Res.,* 1(1): 90-99.

Podile, A.R.; G.S. Prasad & H.C. Dube. 1987. Partial characterization of antagonistic principle of *Bacillus subtilis* AF-1. *J Biol Control*., 1(1): 60-65.

Ramamoorthy, V & R. Samiyappan, 2001. Induction of defense- related genes in *Pseudomonas* fluorescens treated chilli plants in response to infection by *Colletotrichum capsici*. *J Mycol Pl Pathol*., 31(2): 146 - 155.

Rangeshwaran, R & R.D. Prasad. 2000. Biological control of *sclerotium* rot of sunflower. *Indian Phytopath.,* 53(4): 444- 449.

Singh, P.P; Y.C. Shin; C.S. Park & Y.R. Chung. 1999. Biological control of *Fusarium* wilt of cucumber by chitinolytic bacteria. *Phytopathology*, 89(1): 92-99.

Singh, U & P.N. Thapliyal. 1998. Effect of inoculum density, host cultivars and seed treatment on the seed and seedling rot of soybean caused by *Sclerotium rolfsii*. *Indian Phytopath.*, 51(3): 244–246.

Sivasithamparam, K & E.L. Ghisalberti. 1998. Secondary metabolism in *Trichoderma* and *Gliocladium*. In: *Trilchoderma* and *Gliocladium*, Vol I., (Eds., C.P. Kubicek and G.E. Harman), Taylor and Francis, London. pp.139-191.

Sneath, P.H.A. 1984. Endospore-forming gram-positive rods and cocci. In: Bergey's manual of systematic bacteriology, 8th ed., Vol I, Williams and Wilkins, Baltimore. pp.1104-1140.

Vaidya, R.J; I.M. Shah; P.R. Vyas & H.S. Chhatpar. 2001. Production of chitinase and its optimization from a novel isolate *Alcaligenes xylosoxydans*: potential in antifungal biocontrol. *World J Microbiol Biotechnol,* 17: 691-696.

Viswanathan, R & R. Samiyappan. 2002. Induced systemic Resistance by flouresent *pseudomonads* against red rot disease of sugarcane caused by *Collectotrichum falcatum. Crop Prot.,* 21: 1-10.

8

Optimization of the Strategy for Recombinant Baculovirus Infection of Suspended Insect Cells

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1. Introduction

1.1 Significance of baculovirus-insect cell culture system

The application of baculoviruses for insect pest management is traced back to the 19th century. One of the first reported attempts to use such a virus on an operational scale is the introduction of a multinucleocapsid nucleopolyhedroviruses (MNPV) into the populations of the nun moth, Lymantria monacha in Germany in 1892. Natural agents were used for controlling the insects in ancient China. For expample, pharoah's ants in barns were used to combat destructive insects. Chemical insecticides have been widely used in agriculture due to their strong toxicity to insects. Massive and long term use of chemical insecticides should be avioded because they not only are harmful to human health, but also contaminate water and soil. Viral pesticides are continuously studied and developed and the advantages of using baculoviruses in pest management programs are generally recognized. The important attribute of baculoviruses for pest control is their host-specificity. In most cases, only a few insect species from the same family or the same genus are susceptible to a given virus (Ignoffo, 1968). No member of the baculovirus family has ever been isolated from a host other than an arthropod. In addition, viral insecticides do not create resistance problems in either target or non-target species and they do not show cross-resistance with chemical compounds. A further advantage of using viruses is that they do not create residue problems. They neither accumulate in food chains, nor interact with other pesticide residues. In short, they do not create the problems associated with the use of many chemical insecticides (Primentel et al., 1980). Insect viruses are ideally suited for integration with most other plant protection measures used in integrated pest management (IPM) programs.

In spite of the obvious advantages of viral insecticides over chemical pesticides, the former are scantly used. It has been estimated that baculoviruses are capable of replacing 80% of the chemical insecticides currently used in agricultural applications. Nevertheless, *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus (HaSNPV) has been adopted for mass production as a viral pesticide and has been widely used to control the insect pests in China (Zhang et al., 1995) and in other countries (Jones, 1994) as well. Since 1993 the first HaSNPV pesticide was registered in China. It is estimated that in the last ten years, the annual output

of HaSNPV was about 200-300 tons, enough to treat about 100,000 ha of cotton. H. zea single nucleocapsid NPV (HzSNPV) was registered as one of the first commercial baculovirus pesticides (Viron-H, Biocontrol-VHZ, Elcar™) in the 1970s and has been extensively used to control the cotton bollworm in the USA and other countries (Shieh, 1989). HaSNPV has been successfully used to manage heliothine pests, such H. armigera and H. assulta, that are resistant to chemical insecticides and Bt toxin (Zhang, 1989).

The significance of the baculovirus-insect cell culture system has become increasingly evident since many methods have been developed to construct recombinant baculoviruses (Davies, 1994). On this account, there are incentives and advantages for studying this system. Firstly, baculovirus expression systems frequently give high level expression of proteins with full biologicals activity. This combination of highly desirable features is responsible for the widespread popularity of baculovirus expression systems and therefore insect cell cultures offer an attractive for manufacturing of pharmaceuticals, biologicals, etc. Secondly, this system has an additional interest in application of baculoviruses as biological insecticide. Considerable effort has been made to enhance the insecticidal potency of baculoviruses by introducing foreign genes, such as hormones or toxins, to the viral genome (Wood & Granados, 1991; Bonning & Hammock, 1992).

Baculoviruses have proven to be an efficient agent for control of insect pests (Bonning & Hammock, 1992). In addition to this, they can be used efficiently for the production of recombinant proteins by genetic engineering manipulation (Mariorella et al., 1988; Davies, 1994; Kitts, 1996). There is a strong interest in the development of large-scale processes for bio-pesticide production based on the cultivation of insect cells and subsequent infection with baculoviruses. This requires the design and optimization of bioreactors of relatively large volume, and the optimization of their operation strategy. Mathematical modeling has been an important tool in this task (de Gooijer et al., 1989; 1992; Kumar & Shuler, 1995; Power & Nielsen, 1996).

1.2 Bacloviruses

Baculoviruses comprise a large group of viral pathogens of arthropods, particularly of insects of the orders Lepidoptera, Hymenoptera and Diptera. Over 600 species of insects were susptible to baculoviruses. Viral isolates usually infect only a single or a few related species of insects.

The family Baculoviridae consists of a single genus, Baculovirus, which is divided into two subgroups based on their morphology. Subgroup a viruses, the nucleopolyhedroviruses (NPVs), produce virions which have either a single nucleocapsid per envelope (single-nucleocapsid nuclear polyhedrosis virus, SNPV) or one to many nucleocapsids per envelope (multinucleocapsid nucleopolyhedroviruses, MNPV). Occlusion bodies (OB), i.e. occluded viruses (OVs), called polyhedra due to their crystalline shape appearance in the light microscope, which protect the virions from rapid environmental decay, form in the nucleus of NPV-infected cells. Many enveloped nucleocapsids (virions) are embedded in each OB. Subgroup B viruses, the granulosis viruses (GVs), contain only one nucleocapsid per envelope and asingle virion per OB where it is called Granula (Blissard, 1996). The main characteristic of baculoviruses of both subgroups A and B is that crystalline OBs containing occluded virus particles form in the nuclei of infected cells. The major protein of OBs, called polyhedrin or granulin, accounts for approximately 95% of the protein of OBs. Baculoviruses from subgroups A and B are large, enveloped viruses with a double-stranded

circular DNA genome ranging in size from 80 to 200 kilobase pairs (kbp). They are named baculoviruses due to the rod-shaped nucleocapsids.

1.2.1 In vivo infection and replication of baculoviruses

The multiplication of baculoviruses in vivo is a complex and highly ordered process. The replication of AgMNPV in midgut epithelial cells is divided into three phases. In phase I (1 to 4 hr p.i.), the infection starts with the food contaminated with occluded viruses and their dissolution in the insect midgut due to the action of the highly alkaline digestive juices (pH 9.5 to 11.5) and possibly some enzymatic degradation also. The released virions are able to penetrate the peritrophic membrane and enter the epithelial cells of the midgut by fusion with the microvillus membrane and are transported into cell nucleus where uncoating of the viral genome occurs. Phase II (8 to 12 hr p.i.) represents the replicative stage where virogenic stroma give rise to enveloped nucleocapsids. Other nucleocapsids are released into cytoplasm by means of vesicles originating from the nuclear envelope. In final phase III ((14 to 24 hr p.i.) generally virus-free OBs are produced. In addition, nucleocapsids are released from the vesicles and they acquire an envelope by budding through the plasma membrane and the basal lamina. The budded nonoccluded virus (NOV or BV) possesses a peplomer structure and is responsible for secondary infection within the host insect blood.
The extracellular nonoccluded virus may infect blood cells and other tissues, such as trachea and fat body, where the infection process is repeated. Alternatively, blood cells may invade these tissues and transmit the infection by cell-to-cell contact (Keddie et al., 1989). Early after infection of cells of these tissue, NOVs are produced to vector the infection systemically. Later after infection, NOV production ceases and is followed by the assembly of nucleocapsids in the nucleus into de novo systhesized membranes and occlusion of these virions (OVs) into polyhedra. Nuclei of infected cells from the various tissues become filled with polyhedra. Thus, two infectious froms of the virus (NOVs and OVs), which are phenotypically different but genetically identical, are produced during baculovirus infection.

1.2.2 In vitro infection and replication of baculoviruses

The replication of baculoviruses in cell culture (in vitro) is similsr to the events occurring in vivo. However, in contrast to OV, which enters cells by direct fusion at the plasma membrane, NOV (BV) enters cells by endocytosis. After the uptake of the virion into an endocytic vesicle, membrane fusion must occur before releasing the nucleocapsid into the cytoplasm. The nucleocapsids are transported to the nucleus where they are uncoated and the viral DNA is transcribed and replicated. During this period syntheses of host macromoleculars are shut down with concomitant rearrangement of the cytoskeleton, such as disappearance of condensed chromatin. Prgeny DNA is filled into nucleocapsids. At this point, the two viral types begin to emerge in the infection process. Those virions that are destined to become occluded (OVs) remain in the nucleus, and they gain an envelope. In contrast, NOVs escape the nucleus possibly by budding through the inner nuclear membrane and migrate to the plasma membrane (Raghow & Grace, 1974; Hirumi et al., 1975; Knudson & Harrap, 1976). By the time they reach the plasma membrane, they are unenveloped nucleocapsids, having lost nuclear membrane remnants in transit. These nucleocapsids bud from plasma membrane into the medium, acquiring an envelope with apikes and resulting in virions composed of single nucleocapsids per envelope. The process

of budding starts at 10-12 h p.i. and completes around 24 h p.i., and the NOV production is discontinued after the appearance of polyhedra (Volkman, et al., 1976). The assembled virions are then, in turn, occluded into newly-emerging polyhedra. Finally, the cell and nucleus lyses, releasing the polyhedra that represent about 30% of the biomass of infected cells and this is the objective of the production of recombinant proteins using this system. In terms of the timing of infection process, the infection cycle of wild-type baculovirus can be divided into three phases: early (0 to 6 h p.i.), late (8 to 18 h p.i.), and very late (20 to 72 h p.i.) (Miller, 1988). The late phase is characterized by the synthesis of NOVs being capable of budding from the surface. The very late phase is characterized by the embedding of virions in occlusion bodies.

The cytopathic effect that accompanies viral replication is clearly observed with a light microscope. Initial signs of infections in the microscope are a general rounding of the cells, a swelling of the nucleus with a concomitant disappearance of condensed chromatin and the emergence of a defined stippled area, i.e. the virogenic stroma. These signs are evidences to determine which cells are infected when making viral titer of samples.

1.3 Factors affecting baculovirus-insect cell culture system

In vitro infection of insect cells with baculoviruses is increasingly considered as a viable means for the production of biopesticides, recombinant veterinary vaccines and other recombinant products (Atkinson et al., 1990). Over the last two decades, much effort has been focused on identifying factors that affect the productivities of such expression systems, including the cell line (Wickham et al., 1995), medium (Caron et al., 1990), oxygen supply (Gotoh et al., 2002), reactor design (Rice et al., 1993; Zhang et al., 1993) and infection strategy, which involves the multiplicity of infection (MOI), time of infection (TOI), initial cell density (ICD) and medium replacement (Carinhas et al., 2009; Hu and Bentley, 2001; Radford et al., 1997; Rodas et al., 2005; Yamaji et al., 1999; Zhang et al., 2005).

Batch processes usually employ intermediate to high MOIs in the exponential phase of growth, which result in synchronous infection of the insect cells and optimal yields of recombinant products or of polyhedra as bioinsecticides (Kumar et al., 1995). A recent study regarding the production of budded viruses (BVs) using a high MOI demonstrated that medium replacement at the time of infection considerably improved BV production, which increased with the cell concentration and reached a virus titer of 2.6×10^{10} infectious particles (IPs) per mL in cultures of 3.5×10^{6} Sf9-infected cells per mL (Carinhas et al., 2009). However, the use of such complex mixtures would involve higher operation costs and lead to variability in the process performance (Carinhas et al., 2009). In the case of high MOIs and higher cell densities, the technology for BV or recombinant protein production becomes more complex. However, the use of a low MOI has been proposed for three major reasons (Enden et al., 2005; Maranga et al., 2003; Wong et al., 1996; Zhang et al., 2005) as follows: (1) at the industrial production scale, the virus inoculum has to come from a certified and costly master bank, and use of a low MOI will increase the lifespan of the master bank; (2) if a low MOI is used, it is not necessary to replace spent medium with virus stock added into the bioreactor, which potentially interferes with the process performance; (3) if a low MOI is used, the problem of the "passage effect" will be minimized. The main consideration for this approach is that inoculation of the viral stock can be carried out directly from a well-characterized master bank into a single scaled-up bioreactor. Furthermore, it has been reported that the maximum product titers were lower for a high MOI than for a low MOI after infection in the early exponential growth phase (Hu & Bentley, 2001; Zhang et al.,

2005). Among all the factors that affect the productivities of insect cell-recombinant baculovirus expression systems, such as the cell line, medium, oxygen supply, bioreactor design and infection strategy involving the MOI, TOI, ICD and medium replacement, the medium, oxygen supply and bioreactor design are relatively fixed for a given expression system, but the infection strategy is relatively uncertain and can be manipulated. More specifically, the interplay among the MOI related to the number of IPs per cell, TOI related to the cell state and cell cycle distribution and ICD affecting the cell concentration at the TOI is of the utmost importance for the design of efficient infection strategies. To date, the relationships among the MOI, TOI and ICD have not been understood at the theoretical level. Quantitatively, orthogonal designs have been widely used to investigate the factors that influence each other in many fields, but the literature related to investigations of the MOI, TOI and ICD for insect cell-recombinant expression systems using orthogonal designs is scarce. To avoid the complexity of the technology associated with large scale productions, high MOIs and medium replacement are not considered to be factors that can be investigated by orthogonal designs. The purpose of the present study was to investigate the quantitative relationships among the TOI, ICD and low MOI and optimize the infection strategy for such expression systems using an orthogonal design.

2. Materials and methods

2.1 Stocks of cells

Heliothis zea HzAM1 cells were provided by Dr. Hualin Wang from the Wuhan Institute of Virology, Chinese Academy of Sciences (Wang et al., 2008). The cells were maintained at 27°C in 25-cm^2 T-flasks in Grace's medium, comprising Grace's insect medium supplemented with 1% Yeastolate Ultrafiltrate (Gibco), 1.5% chemically defined Lipid Concentrate (Gibco) and 5% fetal bovine serum (Gibco) (unpublished in English). The cells were routinely subcultured every 3–4 days.

2.2 Cell culture

HzAM1 cells were inoculated into 100-mL glass Erlenmeyer flasks containing 20 mL of medium supplemented with 0.2% Pluronic F-68 (Gibco), and cultured with shaking at 80 rpm. Suspension cultures of HzAM1 cells were collected daily for cell counting in a hemocytometer and cell viability determination by Trypan Blue staining exclusion using a 0.4% (w/v) solution (Sigma).

2.3 Virus stock and bioassay

A recombinant HaSNPV incorporating the green fluorescent protein (GFP) gene (HaBacHZ8-eGPF-PH) was constructed and kindly provided by Dr. Hualin Wang from the Wuhan Institute of Virology, Chinese Academy of Sciences (Jing et al., 2008; Pan et al., 2007). HzAM1 cells infected with this recombinant virus expressed GFP and were easy to detect under a fluorescence light microscope (BX-51; Olympus) (Cha et al., 1997). For propagation of HaSNPV, HzAM1 cells at a density of 2×10^6 cells mL^{-1} in T-flasks were infected with the recombinant baculovirus at MOIs of 2–5 and harvested at 72 h after infection. The infectivity of BVs was measured using the end-point dilution method, and expressed as the 50% tissue culture infective dose ($TCID_{50}$) (Nielsen et al., 1992; Reed & Muench, 1938). The $TCID_{50}$ was calculated according to Spearman and Karber (Finney, 1978). Samples were centrifuged at 10,000 × *g* for 1 min, and the supernatants and cell pellets were stored at –70°C and 4°C,

respectively, until analysis. For counts of occluded viruses (OVs), the pellets were treated with an equal volume of 2% SDS solution for 1 h at room temperature. Duplicate samples were homogenized and the numbers of OVs or polyhedra were counted in the central square of a hemocytometer under a light microscope (BX-51; Olympus).

2.4 Experimental protocol

Shaking cultures were performed in duplicate in 100-mL shake-flasks containing 20 mL of cell suspension solution with shaking at 80 rpm. The temperature was maintained at 27°C. Controls comprised cell cultures without virus infection. For the HzAM1 cell-recombinant virus system, the MOI, TOI and ICD in the shaking cultures were evaluated using an orthogonal design. The ICD was defined as the cell density when the cells were inoculated into the cultures, and not when the cells were infected. The TOI was defined as the time of infection. The TOIs investigated in the present study were the early, middle and late exponential phases, and the time periods of the cultures differed for different ICDs (Kioukia et al., 1995). The variable assignments and level settings in the orthogonal array design are listed in Table 1. The variable assignments, level settings and experimental arrangements in the orthogonal array design are shown in Tables 2 and 3. A total of nine experiments are

Levels	Factors		
	MOI ($TCID_{50}.cell^{-1}$) (A)	ICD (10^5 $cells.mL^{-1}$) (B)	TOI (h) (C)
1	0.01	1.0	Early exponential phase
2	0.1	3.0	Middle exponential phase
3	1.0	2.0	Late exponential phase

Table 1. Variable assignments for the MOI, ICD and TOI and the level settings

Experiment number \ Factors	MOI ($TCID_{50}.cell^{-1}$) (A)	ICD (10^5 $cells.mL^{-1}$) (B)	TOI (h) (C)	Maximum titer of BV (10^5 $TCID_{50}.mL^{-1}$)
1	1	1	1	2.00
2	1	2	2	28.00
3	1	3	3	9.28
4	2	1	2	20.00
5	2	2	3	92.8
6	2	3	1	200.00
7	3	1	3	6.32
8	3	2	1	6.32
9	3	3	2	13.30
K_1(10^5 $TCID_{50}.mL^{-1}$)	13.09	9.44	69.44	
K_2(10^5 $TCID_{50}.mL^{-1}$)	104.27	42.37	20.43	
K_3(10^5 $TCID_{50}.mL^{-1}$)	8.65	74.19	36.13	
R(10^5 $TCID_{50}.mL^{-1}$)	95.62	64.75	49.01	

Table 2. Effects of the MOI, ICD and TOI on the BV titer

Factors / Experiment number	MOI ($TCID_{50}$.cell^{-1}) (A)	ICD (10^5 cells.mL^{-1}) (B)	TOI (h) (C)	Maximum OV concentration (10^4 OV.mL^{-1})
1	1	1	1	110
2	1	2	2	120
3	1	3	3	125
4	2	1	2	145
5	2	2	3	160
6	2	3	1	190
7	3	1	3	125
8	3	2	1	142
9	3	3	2	160
K_1' (10^4 OVs.mL^{-1})	118	127	147	
K_2' (10^4 OVs.mL^{-1})	165	141	142	
K_3' (10^4 OVs.mL^{-1})	142	158	137	
R′ (10^4 OVs.mL^{-1})	47	32	11	

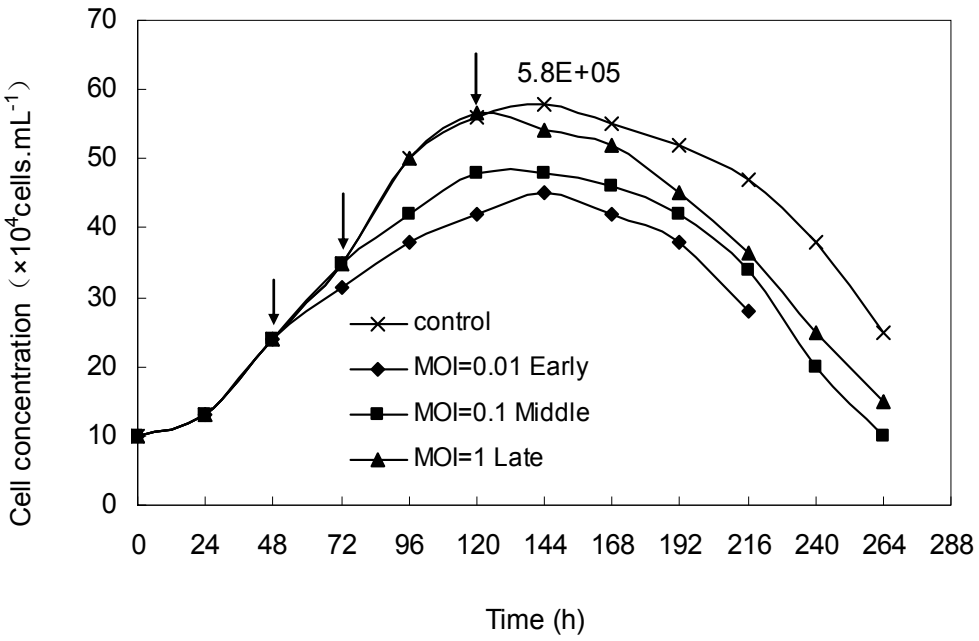
Table 3. Effects of the MOI, ICD and TOI on the OV concentration

listed in the trial columns. The numbers 1, 2 and 3 under the various column numbers in the tables represent the level settings in the experiments. The K_m or K_m' (m=1–3) values and the R or R′ values in the last four rows of Tables 2 and 3 are the average responses, i.e., BVs or OVs with variable m, and the differences between the maximum and minimum values with variable m, respectively. The K_m or K_m' values were used to estimate the effects of the variable and the R or R′ values were used to evaluate the sensitivity of the variable to the BV titer or OV concentration.

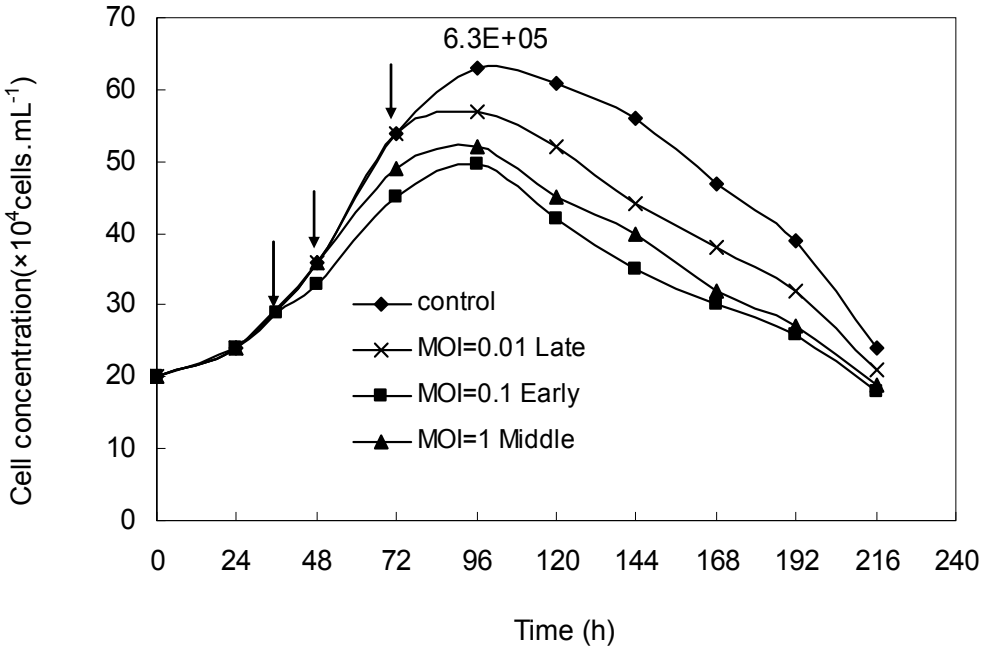
3. Results

After implementing the nine experimental trials designed according to the $L_9(3^4)$ orthogonal array shown in Table 2, with sampling for measurements of the BV titer and OV concentration every 12 h and the cell density every 24 h, the output responses for each experimental trial were calculated (Tables 2 and 3). As shown in the last column of Table 2, the maximum BV titer of 2.00×10^7 $TCID_{50}$ mL^{-1}, equivalent to 9.2×10^9 IPs mL^{-1} (1 $TCID_{50}$ mL^{-1} = 460 IPs mL^{-1}, based on flowcytometry analysis and the end-point dilution method; data not shown), was obtained for the condition of $A_2B_3C_1$. Furthermore, the optimal conditions for that experiment were also $A_2B_3C_1$, as evaluated by analyzing the K_m values, i.e., the average responses at each of the three levels. These results indicated that the optimal conditions for BV production in the suspended HzAM1 cell-recombinant virus system were inoculation of the suspended cells at an ICD of 2.0×10^5 cells mL^{-1} and a MOI of 0.1 when the cells were at the early exponential growth phase. As shown in the last column of Table 3, the maximum OV concentration of 1.9×10^6 OVs mL^{-1} was obtained for the condition of $A_2B_3C_1$. Furthermore, the optimal conditions for that experiment were also $A_2B_3C_1$, as evaluated by analyzing the K_m' values. Consequently, the optimal conditions for OV production were the same as those for BV production in the suspended HzAM1 cell-recombinant virus system. These findings are logically reasonable and indicate that BVs and OVs are closely related, representing only two different virion phenotypes.

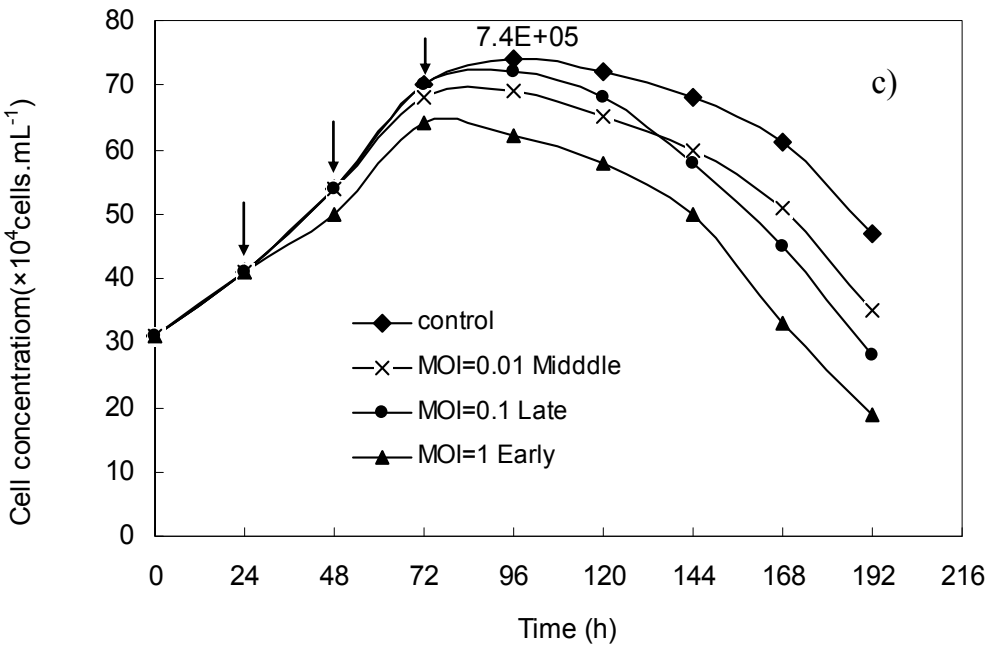
Fig. 1 shows the evolution of the unstained cell concentration for the ICDs of 1.0×10^5, 2.0×10^5 and 3.0×10^5 cells mL^{-1}, and in a control experiment. The data indicated that the maximum unstained cell concentration, decrease in growth rate upon infection, time of onset of a decrease in the unstained cell concentration and the decrease drop rate were dependent on not only the ICDs, but also the TOIs and MOIs (Fig. 1a–c). Since the large change in the BV concentration requires logarithmic presentation of the data and the primary data were obtained as the negative logarithm of the $TCID_{50}$ [-log($TCID_{50}$)], and the error of the method is estimated in the logarithmic scale and not easily translated into linear units, the evolution of the number of BVs was calculated in the form of -log($TCID_{50}$) (Zhang et al., 2005). Fig. 2 shows the evolution of the -log($TCID_{50}$) for the ICDs of 1.0×10^5, 2.0×10^5 and 3.0×10^5 cells mL^{-1}. The errors of all the sample analyses ranged from 0% to 5% and are not indicated in Fig. 2. The maximum value of the -log($TCID_{50}$) was 6.0 (Fig. 2b, compare with Fig. 2a and c) and the corresponding BV value was 2.0×10^7 $TCID_{50}$ mL^{-1} in the case of an ICD of 2.0×10^5 cells mL^{-1}, a MOI of 0.10 and a TOI at the early exponential phase. These represent the best results obtained for the HzAM1 cell-recombinant baculovirus system, based on comparisons with previously reported data (McIntosh et al., 2001; Wang et al., 2008). Fig. 3 shows the evolution of the amount of OVs for the ICDs of 1.0×10^5, 2.0×10^5 and 3.0×10^5 cells mL^{-1}. The errors of all the sample analyses ranged from 0% to 5% and are not indicated in Fig. 3. The maximum OV concentration of 1.9×10^6 OVs mL^{-1} compared with those in Fig. 3a and c was obtained for an ICD of 2.0×10^5 cells mL^{-1}, a MOI of 0.10 and a TOI at the early exponential phase.



(a)

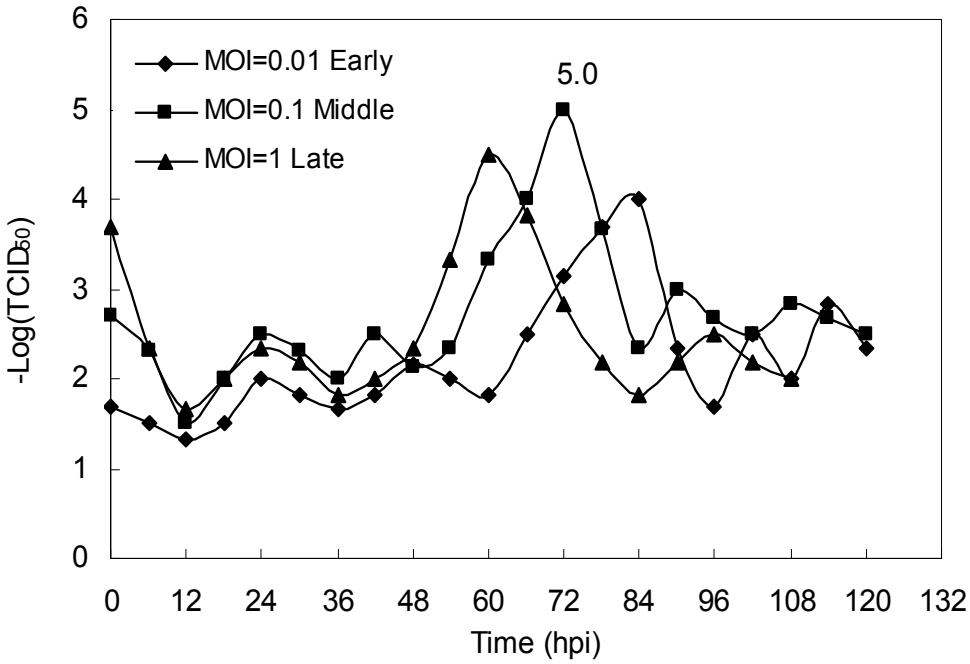


(b)

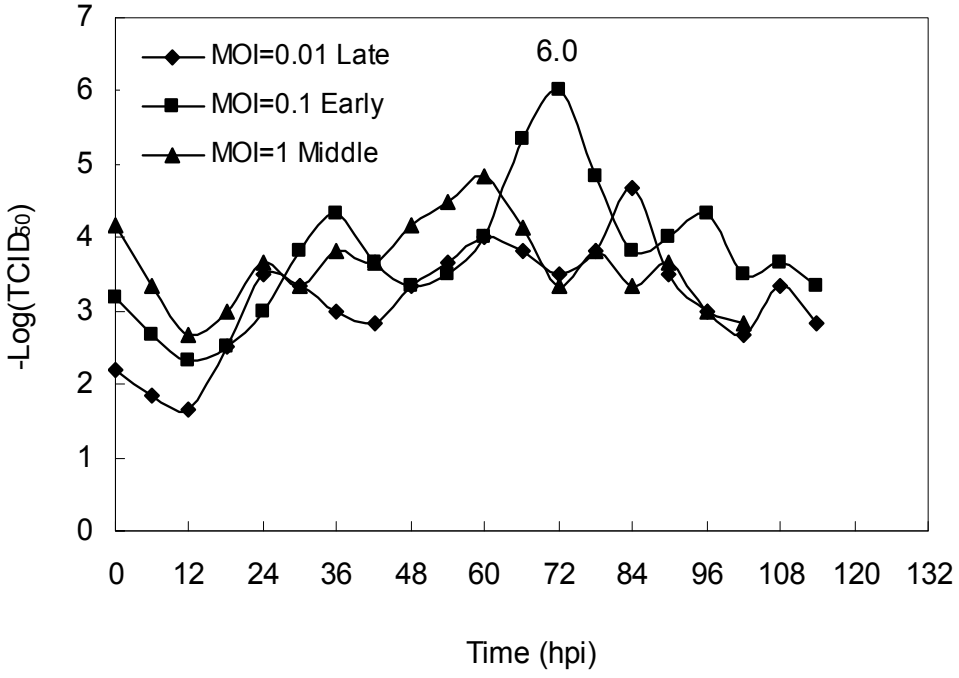


(c)

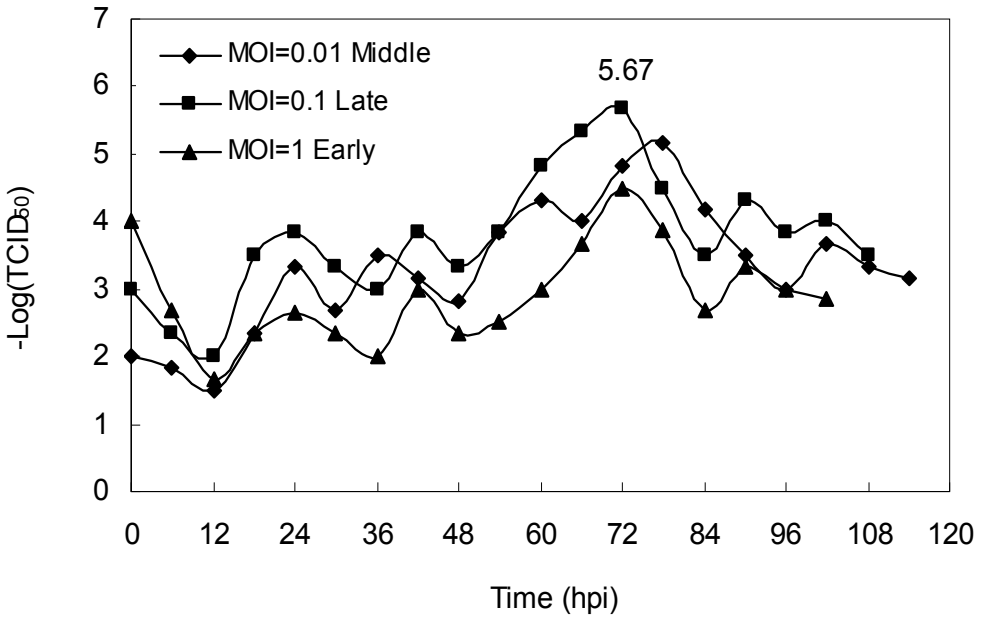
Fig. 1. Evolution of the unstained cell concentration for the ICDs of 1.0×10^5 cells mL^{-1} (a), 2.0×10^5 cells mL^{-1} (b) and 3.0×10^5 cells mL^{-1} (c) and in a control experiment. Arrows indicate the times when the viruses were added to the cultures.


MOI=0.01 Early
MOI=0.1 Middle
MOI=1 Late
5.0
-Log(TCID50)
Time (hpi)
0
1
2
3
4
5
6
0 12 24 36 48 60 72 84 96 108 120 132

(a)

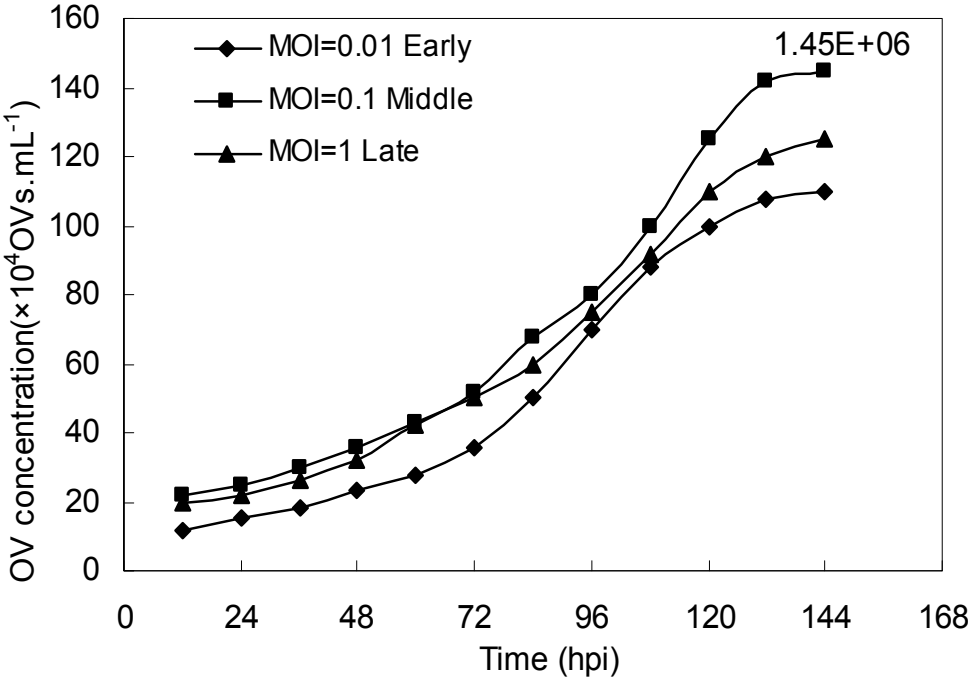

MOI=0.01 Late
MOI=0.1 Early
MOI=1 Middle
6.0
-Log(TCID50)
Time (hpi)
0
1
2
3
4
5
6
7
0 12 24 36 48 60 72 84 96 108 120 132

(b)

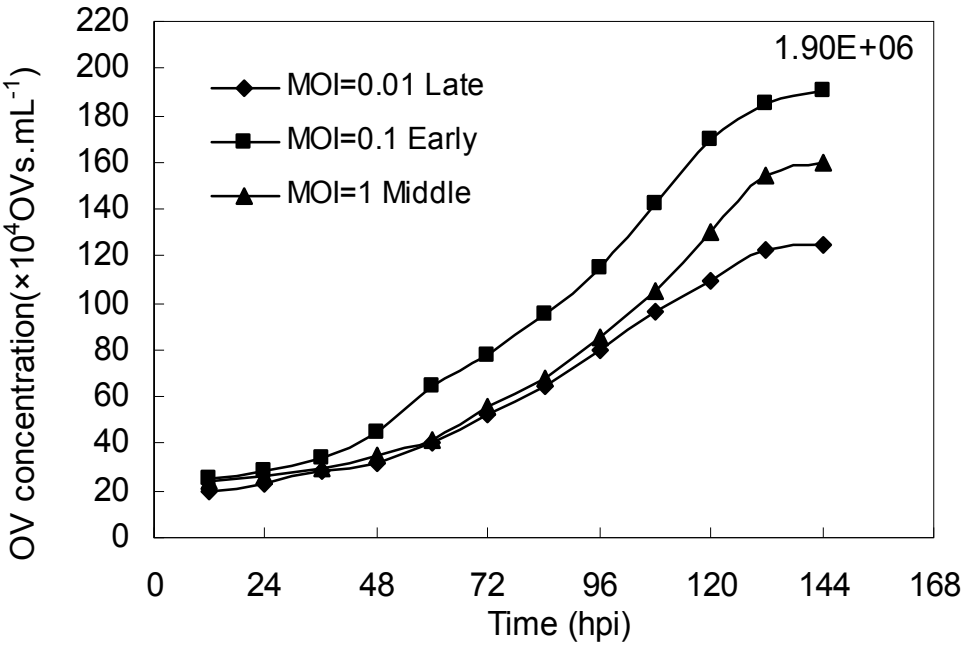


(c)

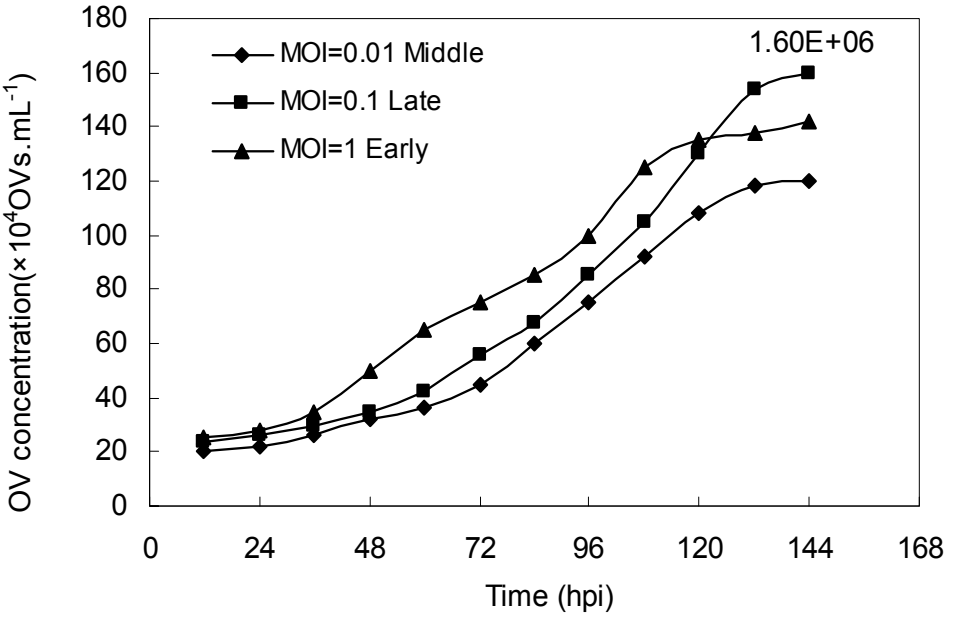
Fig. 2. Evolution of the minus logarithm of the $TCID_{50}$ for the ICDs of 1.0×10^5 cells mL^{-1} (a), 2.0×10^5 cells mL^{-1} (b) and 3.0×10^5 cells mL^{-1} (c). The errors of all the sample analyses ranged from 0% to 5% and are not indicated in the panels.



(a)



(b)



(c)

Fig. 3. Evolution of the amount of OVs for the ICDs of 1.0×10^5 cells mL^{-1} (a), 2.0×10^5 cells mL^{-1} (b) and 3.0×10^5 cells mL^{-1} (c). The errors of all the sample analyses ranged from 0% to 5% and are not indicated in the panels.

4. Discussion

4.1. Optimization of the MOI for BV and OV productions

The effects of the MOI on the BV titer and OV concentration at three different levels as factor A are shown in Tables 2 and 3. It can be seen from Table 2 that the Km (m=1–3) values corresponding to the MOIs were 1.309×10^6, 1.0427×10^7 and 8.65×10^5 $TCID_{50}$ mL^{-1}, which represent the average BV response values at the three MOIs of 1.0, 0.10 and 0.01, respectively, and reflect the effects of the MOI on BV production. The maximum value, K_2, was 1.0427×10^7 $TCID_{50}$ mL^{-1} at a MOI of 0.10. The difference between the maximum value K_2 and the minimum value K_3, i.e., the R value corresponding to the MOI, was 9.56×10^6 $TCID_{50}$ mL^{-1}, which is higher than the other R values of 6.48×10^6 and 4.90×10^6 $TCID_{50}$ mL^{-1} corresponding to the ICD and TOI, respectively. These findings indicate that the sensitivity of the MOI to the BV titer is highest when a low MOI is used. In the same way, it can be seen from Table 3 that the Km' (m=1–3) values corresponding to the MOIs were 1.18×10^6, 1.65×10^6 and 1.42×10^6 OVs mL^{-1}, which represent the average OV response values at the three MOIs of 1.0, 0.10 and 0.01, respectively, and reflect the effects of the MOI on OV production. The maximum value, K_2', i.e., the optimal condition, was achieved at a MOI of 0.10, indicating that the optimal MOI for OV production was the same as that for BV production. The difference between the maximum value K_2' and the minimum value K_1', i.e., the R' value corresponding to the MOI, was 4.7×10^5 OVs mL^{-1}, which is higher than the other R values of 3.2×10^5 and 1.1×10^5 OVs mL^{-1} corresponding to the ICD and TOI, respectively. These findings indicate that the sensitivity of the MOI to the OV concentration is highest when a low MOI is used. Therefore, it is concluded that the MOI has relatively significant influences on the BV titer and OV concentration when a low MOI is used. Furthermore, it can be seen from the data for the 9 experiments shown in the last columns of Tables 2 and 3 that both the maximum BV titer and maximum OV concentration were produced at A_2, i.e., a MOI of 0.10.

4.2 Optimization of the ICD for BV and OV productions

The effects of the ICD on the BV titer and OV concentration at three different levels as factor B are shown in Tables 2 and 3. It can be seen from Table 2 that the Km (m=1–3) values corresponding to the ICDs were 9.44×10^5, 4.24×10^6 and 7.42×10^6 $TCID_{50}$ mL^{-1}, which represent the average BV response values at the three levels of ICD, respectively, and reflect the effects of the ICD on BV production. The maximum value, K_3, was 7.419×10^6 $TCID_{50}$ mL^{-1} at the ICD of 2.0×10^5 cells mL^{-1}. The difference between the maximum value K_3 and the minimum value K_1, i.e., the R value corresponding to the ICD, was 6.48×10^6 $TCID_{50}$ mL^{-1}, which is higher than 4.90×10^6 $TCID_{50}$ mL^{-1} corresponding to the TOI, but smaller than 9.56×10^6 $TCID_{50}$ mL^{-1} corresponding to the MOI. These findings indicated that the sensitivity of the ICD to the BV titer is not high (as described above). In addition, it can be seen from Table 3 that the Km' (m=1–3) values corresponding to the ICDs were 1.27×10^6, 1.41×10^6 and 1.58×10^6 OVs mL^{-1}, which represent the average OV response values at the three levels of ICD, respectively, and reflect the effects of the ICD on OV production. The maximum value, K_3', i.e., the optimal condition, was achieved at an ICD of 2.0×10^5 cells mL^{-1}, indicating that the optimal ICD for OV production was the same as that for BV production. Furthermore, it can be seen from the data for the nine experiments shown in the last columns of Tables 2 and 3 that both the maximum BV titer and maximum OV concentration were produced at B_3, i.e., an ICD of 2.0×10^5 cells mL^{-1}.

4.3 Optimization of the TOI for BV and OV productions

The effects of the TOI on the BV titer and OV concentration at three different levels as factor C are shown in Tables 2 and 3. It can be seen from Table 2 that the Km (m=1–3) values corresponding to the TOIs were 6.94×10^6, 2.04×10^6 and 3.61×10^6 $TCID_{50}$ mL^{-1}, which represent the average BV response values at the three levels of TOI, respectively, and reflect the effects of the TOI on BV production. The maximum value, K_1, was 6.94×10^6 $TCID_{50}$ mL^{-1} at the early exponential phase. The difference between the maximum value K_1 and the minimum value K_2, i.e., the R value corresponding to the TOI, was 4.9×10^6 $TCID_{50}$ mL^{-1}, which is the smallest among the three R values (as described above), indicating that the TOI has a weak influence on BV production when a low MOI is used. In addition, it can be seen from Table 3 that the Km' (m=1–3) values corresponding to the TOIs were 1.47×10^6, 1.42×10^6 and 1.37×10^6 OVs mL^{-1}, which represent the average OV response values at the three TOIs, respectively, and reflect the effects of the TOI on OV production. The maximum value, K_1', i.e., the optimal condition, was achieved at the early exponential phase, indicating that the optimal TOI for OV production was the same as that for BV production. Furthermore, it can be seen from the data for the 9 experiments shown in the last columns of Tables 2 and 3 that both the maximum BV titer and maximum OV concentration were produced at C_1, i.e., at the early exponential phase.

4.4 Effects of the MOI, ICD and TOI on BV and OV productions

The effects of the MOI, ICD and TOI on BV and OV productions can be seen from the R and R' values (Tables 2 and 3), which were used to evaluate the sensitivity of each variable to the BV titer or OV concentration. The R values corresponding to the MOI, ICD and TOI were 9.56×10^6, 6.48×10^6 and 4.90×10^6 $TCID_{50}$ mL^{-1}, respectively, indicating that the MOI has the most significant influence on BV production, followed by the ICD and finally the TOI in the HzAM1 cell-recombinant baculovirus system when a low MOI is used. Furthermore, the R' values corresponding to the MOI, ICD and TOI were 4.7×10^5, 3.2×10^5 and 1.1×10^5 OVs mL^{-1}, respectively, demonstrating similar effects of the MOI, ICD and TOI on OV production compared with BV production. As stated above, the optimal conditions for both BV production and OV production in the HzAM1 cell-recombinant baculovirus system were a MOI of 0.10, an ICD of 2.0×10^5 cell mL^{-1} and a TOI at the early exponential phase.

With respect to the MOIs of 1.0, 0.10 and 0.01, most of the HzAM1 cells were likely to be infected at a MOI of 1.0 at the early exponential phase because more viruses were added to the culture compared with the MOIs of 0.10 and 0.01, and as a result, there were insufficient uninfected cells to propagate for infection at later time points with viruses generated during the 'primary infection process' (Kioukia et al., 1995; Zhang et al., 2005). As shown in Fig. 1c, the unstained cell concentration showed a sharp decrease at 72 h after the inoculation when the cells were infected at a MOI of 1.0 at 24 h, i.e., the early exponential phase. Therefore, the numbers of BVs and OVs produced would exhibit lesser increases in the secondary and tertiary infections when a MOI of 1.0 was used at the early exponential phase as shown in Figs. 2c and 3c. Conversely, if the HzAM1 cells were infected at a MOI of 0.01 at the early exponential phase, fewer cells would be infected and the number of viruses generated in the primary infection process would be lower. It is possible that most of the uninfected cells and their progeny would not be infected before the cells entered into the stationary phase or death phase as shown in Fig. 1a, indicating that these cells would not play a role in virus replication because of their weak physiological state. Therefore, the maximum BV and OV productions were not obtained when the cells were infected at MOIs of 0.01 (Figs. 2a and 3a) and 1.0 (Figs.

2c and 3c) at the early exponential phase. However, the number of cells infected was likely to be suitable for later infection cycles when the MOI was 0.1, which resulted in the maximum productions of BVs (Fig. 2a–c) and OVs (Fig. 3a–c). The evolution of unstained cells (Fig. 1) and the dynamics of BVs (Fig. 2) and OVs (Fig. 3) at a MOI of 0.1 were very complicated and similar to those in our previous studies (Enden et al., 2005; Zhang et al., 2005), and are thus not elaborated upon in this paper. With respect to the three ICDs, when the ICD was 1×10^5 cells mL^{-1}, the remaining uninfected cells would be too few to propagate for infection in later infection cycles compared with the ICDs of 2×10^5 and 3×10^5 cells mL^{-1}. However, when the ICD was 3×10^5 cells mL^{-1}, the remaining uninfected cells would be too many to propagate for infection in later infection cycles compared with the ICDs of 1×10^5 and 2×10^5 cells mL^{-1}. These extra cells would be likely to proliferate vigorously in the early exponential phase, and as a result, the nutrient components in the culture would be consumed too early and too rapidly for subsequent infection cycles. This means that the physiological environment in the culture would deteriorate and have no benefit for the uninfected cells and their progeny to be infected with BVs during the later infection process. Therefore, the cells at the ICDs of 1×10^5 cells mL^{-1} (see Figs. 2a and 3a) and 3×10^5 cells mL^{-1} (see Figs. 2c and 3c) infected at a MOI of 0.1 would not reach the maximum amounts of BVs and OVs, whereas the cells infected at an ICD of 2×10^5 cells mL^{-1} would reach the maximum amounts of BVs and OVs (see Figs. 2b and 3b). With respect to the TOI (early, middle or late exponential growth phase), the cells infected at a low MOI at the early exponential growth phase achieved a longer period of culture with the added viruses and their progeny viruses (BVs) to create more infection cycles before the cells entered the stationary phase or death phase compared with the cells infected at the middle and late exponential growth phases. This is probably an important reason why better effects on the amounts of BV and OV productions were observed when infection was carried out at the early exponential growth phase (see Figs. 2b and 3b). The interplay among the MOI, TOI and ICD in the HzAM1 cell-recombinant baculovirus system has not been understood at the theoretical level. Currently, our research is focusing on the relationships of the cell cycle phases and cell cycle distributions with virus infection using a synchronization method and flow cytometry, with further attempts to understand the relationships between the amounts of viruses produced and the cell states when infected.

In conclusion, to avoid the complexity of the technology associated with large scale production, high MOIs and medium replacement were not considered as factors to be investigated in our studies. An orthogonal design was applied to select the optimum conditions, in which the effects of the MOI, ICD and TOI on the productions of BVs and OVs were evaluated in the HzAM1 cell-recombinant baculovirus system. The results indicated that among the MOI, TOI and ICD, the MOI had relatively significant influences on the amounts of BVs and OVs produced at a low MOI. The optimal conditions for both BV and OV productions in the HzAM1 cell-recombinant baculovirus system were a MOI of 0.10, an ICD of 2.0×10^5 cells mL^{-1} and a TOI at the early exponential phase. These findings are logically reasonable and indicate that BVs and OVs are closely related, representing only two different virion phenotypes. Virus titers as high as 2.0×10^7 $TCID_{50}$ mL^{-1}, equivalent to 9.2×10^9 IPs mL^{-1}, were obtained without medium replacement at a low MOI, which represent the best results obtained for the HzAM1 cell-recombinant baculovirus system, based on comparisons with previously reported data (McIntosh et al., 2001; Wang et al., 2008). Overall, the present data demonstrate that high titers can be obtained at a low MOI and that the technology required for this bioprocess was shortened by an efficient orthogonal design. Furthermore, the interplay among the MOI, TOI and ICD was elucidated.

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6. References

Atkinson, A.E.; Weitzman, M.D.; Obosi, L.; Beadle, D.J. & King, L.A. (1990). Baculovirus as vectors for foreign gene expression in insect cells. *Pesticide. Sci*. Vol. 28, No. 2, (August 1989), pp. 215-224, ISSN 0031-613X

Blissard, G.W. (1996). Baculovirus-insect cell interactions. *Cytotechnology: International Journal of Cell culture and Biology,*, Vol. 20, (1996), pp. 73-93, ISSN 0920-9069

Bonning, B.C. & Hammock, B.D. (1992). Development and potential of genetically engineered viral insecticides. *Biotechnology and genetic engineering reviews,* Vol. 10, (1992), pp. 455-489, ISSN 026-8725

Carinhas, N.; Bernal, V.; Yokomizo, A.Y.; Carrondo, M.J.; Oliveira, R. & Alves, P.M. (2009). Baculovirus production for gene therapy: the role of cell density, multiplicity of infection and medium exchange. *Appl. Microbiol. Biotechnol.* Vol. 81, No. 6, (October 2008), pp. 1041-1049, ISSN 0175-7598

Caron, A.W.; Archambault, J. & Massie, B. (1990). High-level recombinant protein production in bioreactors using the baculovirus-insect cell expression system. *Biotechnol. Bioeng.* Vol. 36, No. 11, (December 1990) pp. 1133-1140, ISSN 0006-3592

Cha, H.J.; Gotoh, T. & Bentley, W.E. (1997). Simplification of titer determination for recombinant baculovirus by green fluorescent protein marker. *Biotechniques* Vol. 23, No. 5, (1997), pp. 782-786, ISSN 0736-6205

Davies, A.H. (1994). Current methods for manipulating baculoviruses. *Bio/Technology.* Vol. 12, (1994), pp. 47-50, ISSN 1087-0156

de Gooijer, C.D.; van Lier, F.L.J.; van den End, E.J.; Vlak, J.M. & Tramper, J. (1989). A model for baculovirus production with continuous insect cell cultures. *Appl. Microbiol. Biotechnol.* Vol. 30, No. 5, (1989), pp. 497-501, ISSN 0175-7598

de Gooijer, C.D.; Koken, R.H.M.; van Lier, F.L.J.; Kool, M.; Vlak, J.M. & Tramper, J. (1992). A structured dynamic model for the baculovirus infection process in insect-cell reactor configuration. *Biotechnol. Bioeng.* Vol. 40, No. 4, (August 1992), pp. 537-548, ISSN 0006-3592

Enden, G.; Zhang, Y.H. & Merchuk, J.C. (2005). A model of the dynamics of insect cell infection at low multiplicity of infection. *J. Theor. Biol.* Vol. 237, No. 3, (December 2005), pp. 257-264, ISSN 0022-5193

Ernst, W.J.; Grabherr, R.M. & Katinger, H.W.D. (1994). Direct cloning into the Autographa californica nuclear polyhedrosis virus for generation of recombinant baculoviruses. *Nuclei Acids Res.* Vol. 22, No. 14, (1994), pp. 2855-2856, ISSN 0305-1048

Gotoh, T.K.; Miyazaki, Y.H.; Chiba, K.H. & Kikuchi, K.I. (2002). Significant increase in recombinant protein production of a virus-infected Sf-9 insect cell culture of low MOI under low dissolved oxygen conditions. *J. Biosci. Bioeng.* Vol. 94, No. 5, (August 2002), pp. 426-433, ISSN 1389-1723

Hirumi, H.; Hirumi, K. & Mclntosh, A.H. (1975). Mrophogenesis of a nuclear polyhedrosis virus of the alfafa looper in a continuous cabbage looper cell line. *Ann. N.Y. Acad. Sci.* Vol. 226, (November 1975), pp. 302-326, ISSN 0077-8923

Hu, Y.C. & Bentley, W.E. (2001). Effect of MOI ratio on the composition and yield of chimeric infectious bursal disease virus-like particles by baculovirus co-infection: deterministic predictions and experimental results. *Biotechnol. Bioeng.* Vol. 75, No. 1, (October 2001), pp. 104-109, ISSN 0006-3592

Ignoffo, C.M. (1968). Specificity of insect viruses. Bull. Entomol. *Soc. Am.* Vol. 14, No. 4, (1968), pp. 265-276, ISSN 0013-8754

Jing, T.; Li, X.; Song, J.H.; Ling, C.Y. & Chen, X.W. (2008). Baculovirus per os infectivity factors are involved in HearNPV ODVs infection of HzAM1 cells in vitro. *Virol. Sin.* Vol. 23, No. 1, (February 2008), pp. 25-30, ISSN 1674-0769

Jones, K.A. 1994. Use of baculovirus for cotton pest control. In Insect pests of cotton (Matthews, G.A.; Tunstall, J.P., eds), CAB International, Wallingford, UK, （November 1994）pp. 477-504, ISBN 0-85198-724-9

Keddie, G.A.; Aponte, G.W. & Volkman, L.E. (1989). The pathway of infection of Autographa californica nuclear polyhedrosis virus in an insect host. *Science* Vol. 243, No. 4899, (March 1989), pp. 1728-1730, ISSN 0036-8075

Kitts, P.A. (1996). Construction of baculovirus recombinants. In: Vlak, J.M.; de Gooijer, C.D.; Tramper, J. & Miltenburger, H.G. (eds) International journal of cell culture and biology: cytotechnology. Vol. 20 (pp. 111-123) Kluwer Academic Publishers, Netherlands.

Kioukia, N.; Nienow, A.W.; Emery, A.N. & AI-Rubeai, M. (1995). Physiological and environmental factors affecting the growth of insect cells and infection with baculovirus. *J. Biotechnol.* Vol. 38, (October 1994), pp. 243-251, ISSN 0168-1656

Knudon, D.L. & Harrap, K.A. (1976). Replication of a nuclear polyhedrosis virus in a continuous cell culture of Spodoptera frugiperda: microscopy study of the sequence of events of the virus infectiion. *J. Virol.* Vol. 17, No. 1, (January 1976), pp. 254-268, ISSN 0022-538X

Kumar, A. & Shuer, M.L. (1995). Model of a split-flow airlift bioreactor for attachment-dependent, baculovirus-infected insect cells. *Biotechnol. Prog.* Vol. 11, No. 4, (August 1995), pp. 412-419, ISSN 1520-6033

Maranga, L.; Brazao, T.F. & Carrondo, M.J.T. (2003). Virus-like particle production at low multiplicities of infection with the baculovirus insect cell system. *Biotech. Bioeng.* Vol. 84, (October 2003), pp. 245-253, ISSN 0006-3592

Mariorella, B.; Inlow, D.; Shauger, A. & Harano, D. (1988). Large scale insect cell culture for recombinant production. *Biotechnology* Vol. 6, (1988), pp. 1406-1410, ISSN 1087-0156

McIntosh, A.H.; Grasela, J.J.; Goodman, C.L. & Ignoffo, C.M. (2001). Growth of a clonal cell line of *Helicoverpa zea* (Lepidoptera: Noctuidae) in suspension culture and replication of its homologous baculovirus HzSNPV. *Appl. Entomol. Zool.* Vol. 36, (April 2001), pp. 349–352, ISSN 0003-6862

Nielsen, L.K.; Smyth, C.K. & Greenfield, P.F. (1992). Accuracy of the endpoint assay for virus titration. *Cytotechnology* Vol. 8, No. 3, (1992), pp.231-236, ISSN 0920-9069

Pan, X.Y.; Long, G.; Wang, R.R.; Hou, S.W.; Wang, H.Y.; Zheng, Y.T.; Sun, X.L.; Westenberg, M.; Deng, F.; Wang, H.L.; Vlak, J.M. & Hu Z. (2007). Deletion of a *Helicoverpa armigera* nucleopolyhedrovirus gene encoding a virion structural protein (ORF107) increases the budded virion titre and reduces in vivo infectivity. *J. Gen. Virol.* Vol. 88, (2007), pp. 3307-3316, ISSN 0022-1317

Power, J.F.; Reid, S.; Greenfield, P.F. & Nielsen, L.K. (1996). The kinetics of baculovirus adsorption to insect cells in suspension culture. *Cytotechnology* Vol. 21, No. 2, (1996), pp. 155-163, ISSN 0920-9069

Primentel, D. & Andow, D. (1980). Environmental and social costs of pesticides: a preliiminary assessment. *OIKOS.* Vol. 34, No. 2, (1980), pp. 126-140, ISSN 1600-0706

Radford, K.M.; Cavegn, C.; Bertrand, M.; Bernard, A.R.; Reid, S. & Greenfield, P.F. (1997). The indirect effects of multiplicity of infection on baculovirus expressed proteins in insect cells: secreted and non-secreted products. *Cytotechnology* Vol. 24, No. 1, (1997), pp. 73-81, ISSN 0920-9069

Raghow, R. & Grace, T.D. (1974). Studies on a nuclear polyhedrosis virus in Bombyx mori cells in vitro I. Multiplication kinetics and ultrastructural studies. *J. Ultrastruct. Res.* Vol. 47, No. 3, (1974), pp. 384-399, ISSN 0022-5320

Reed, L. & Muench, H. (1938). A simple method for estimating fifty percent endpoints. *Am. J. Epidemiol.* Vol. 27, No. 3, (May 1938), pp. 493–497, ISSN 0002-9262

Rice, J.W.; Rankl, N.B.; Gurganus, T.M.; Marr, C.M.; Barna, J.B.; Waiters, M.M. & Burns, D.J. (1993). A comparison of large-scale Sf 9 insect cell growth and protein production: stirred vessel vs airlift. *Biotechniques.* Vol. 15, No. 6, (1993), pp. 1952-1959, ISSN 0736-6205

Rodas, V.M.; Marques, F.H.; Honda, M.T.; Soares, D.M.; Jorge, S.A.C.; Antoniazzi, M.M.; Medugno, C.; Castro, M.E.B.; Ribeiro, B.M.; Souza, M.L.; Tonso, A. & Pereira, C.A. (2005). Cell culture derived AgMNPV bioinsecticide: biological constraints and bioprocess issues. Cytotechnology Vol. 48, No. 1-3, (2005), pp. 27–39, ISSN 0920-9069

Shieh, T.R. 1989. Industrial production of viral pesticides. *Adv. Virus Res. Vol.* 36, (1989) pp. 315-343, ISSN 0065-3527

Volkman, L.E.; Summers, M.D. & Hsieh, C.H. (1976). Occluded and nonoccluded nuclear polyhedrosis virus grown in Trichoplusia ni: neutralization, comparativve infectivity, and in vitro grown studies. *J. Virol.* Vol. 19, No. 3, (September 1976), pp. 820-832, ISSN 0022-538X

Wang, M.L.; Tan, Y.; Yin, F.F.; Deng, F.; Vlak, J.M.; Hu, Z.H. & Wang, H.L. (2008). The F protein of *Helicoverpa armigera* single nucleopolyhedrovirus can be substituted functionally with its homologue from Spodoptera exigua multiple nucleopolyhedrovirus. *J. Gen. Virol.* Vol. 89, (2008), pp. 791–798, ISSN 0022-1317

Wickham, T.J.; Nemerow, G.R.; Wood, H.A. & Shuler M.L. (1995). Comparison of different cell lines for the production of recombinant baculovirus proteins. *Methods Mol. Biol.* Vol. 39, (1995), pp. 385-395, ISSN 1064-3746

Wong, K.T.K.; Peter, C.H.; Greenfield, P.F.; Reid, S. & Nielsen, L.K. (1996). Low multiplicity infection of insect cells with a recombinant baculovirus: The cell yield concept. *Biotechnol. Bioeng.* Vol. 49, No. 6, (March 1996), pp. 659–666, ISSN 1097-0290

Wood, H.A. & Granados, R.R. (1991). Genetically engineered baculoviruses as agents for pest control. *Ann. Rev. Microbiol.* Vol. 45, (October 1991), pp. 69-87, ISSN 0066-4227

Yamaji, H.K.; Tagai, S.I. & Fukuda, H.K. (1999). Optimal production of recombinant protein by the baculovirus-insect cell system in shake-flask culture with medium replacement. *J. Biosci. Bioeng.* Vol. 87, No. 5, (January 1999), pp. 636-641, ISSN 1389-1723

Zhang, J.; Kalogerakis, N.; Behie, L.A. & Iatrou K. (1993). A two-stage bioreactor system for the production of recombinant proteins using a genetically engineered baculovirus/ insect cell system. Biotechnol. Bioeng. Vol. 42, (July 1993), pp. 357-366, ISSN 1097-0290

Zhang, G.Y. 1989. Commercial viral insecticide-Heliothis armigera viral insecticide in China. *The IPM Practitioner* Vol. 11, (1989) pp. 13

Zhang, G.Y.; Sun, X.L.; Zhang, Z.X.; Zhang, Z.F. & Wan, F.F. 1995. Production and effectiveness of the new formulation of Helicoverpa virus pesticide-emulsifiable suspension. *Virologica Sinica* Vol. 10, (1995) pp. 242-247, ISSN 1674-0769

Zhang, Y.H.; Enden, G. & Merchuk, J.C. (2005). Insect cells-baculovirus system: Factors affecting growth and low MOI infection. *Biochem. Eng. J.* Vol. 27, No. 1, (December 2005), pp. 8-16, ISSN 1369-703X

Part 2

Pesticides Biomarkers

9

Pesticide Biomarkers

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1. Introduction

Biomarkers were originally identified in the field of human medicine and were first promoted for use in ecotoxicology in the early 1990s. The simplest and most often-used definition of a biomarker is the one devised by David Peakall: "a biological response to chemicals that give a measure of exposure and sometimes, also of toxic effect". However, the term biomarker and the related term bioindicator have been defined and redefined by many different researchers and institutions (Peakall, 1994).

The National Academy of Science, in 1987, defined a biomarker as "a xenobiotically induced variation in cellular or biochemical components or processes, structures, or function that is measurable in a biological system" (Huggett et al., 1992). The term is most often employed to refer to molecular, physiological, and organismal responses to contaminant exposure that can be quantified in organisms inhabiting or captured from natural systems. A response that is limited to laboratory studies falls outside the generally held concept of a biomarker.

The use of biomarkers has great potential to complement the current methods used to determine the presence and potential impact of environmental pollutants. However, chemical analysis is expensive and is applicable only to a small percentage of environmental contaminants. Acceptable and critical contaminant levels have been established for only a few compounds, so determination of only the concentration does not really provide any information on the ecological hazards. In addition, chemical concentrations do not account for the complexity of the systems involved and provide little meaningful information on the possible effects of the contamination on the organisms. It is difficult to predict such effects based on chemical concentration alone due to variable environmental factors such as pH, temperature, and moisture. These factors impact the contaminant form, its movement through the environment and its ultimate uptake by organisms, thereby impacting the ultimate toxicity. To address these issues, chemical contaminant concentration analysis has been supplemented with the use of both acute and chronic biomonitoring in which live organisms are subjected, in a laboratory setting, to varying amounts of contaminants and observed for toxic effects. The primary disadvantage of these tests lies with the established trend to use only a few types of organisms that are easily handled and maintained in the laboratory. This limits the validity of the application of information obtained from disparate species.

Many scientists recommend that biomarkers be used as an additional ecotoxicology assessment method based on the concept that contaminant-based changes at molecular,

cellular, and genetic levels occur in response to the stress caused by the action of human-introduced contaminants, thereby giving advanced warning of future ecosystem damage. Biomarker study results cannot always confirm the presence of an exact chemical but instead give an indication of the presence of contamination by a class of chemicals. This finding provides evidence that further, and more expensive, chemical analyses are warranted (Peakall, 1994; van Gestel and Brummelen, 1996).

Biomarkers or biological markers are molecular-, biochemical-, or cellular-level indicators in either wild populations taken from contaminated habitats or in organisms experimentally exposed to pollutants that indicate that such organisms have been exposed to toxic chemicals and the magnitude of the organism's response to the contaminant. Biological markers measured in wild animals can directly contribute to the detection, quantification, and understanding of the significance of the exposure to chemicals in the environment. These measurements in environmental species may also help to assess the potential for human exposure to environmental pollutants and to predict human health risks (Shugart, 2005).

Another practical use for biomarkers is the detection and quantification of prior or ongoing exposures to specific chemicals; biomarkers have been successfully used in biological monitoring programs in industry but have only recently been used to monitor environmental exposures. Medical researchers are seeking biomarkers that can be employed to i) detect early stages of a disease to enhance successful intervention; ii) determine the effectiveness of intervention strategies; and iii) detect cells at risk from a toxicant. Finally, research is ongoing, particularly in the field of genetics, to find inherited biomarkers of susceptibility that can be used for the detection and protection of sensitive populations (Klaassen, 2008).

The specificity of biomarkers to chemicals varies greatly. Both specific and nonspecific biomarkers have their place in environmental assessment. A nonspecific biomarker can demonstrate that a pollutant is present in a meaningful concentration but does not indicate which particular chemical is present. Based on such information, a more detailed chemical investigation could be justified. In contrast, specific biomarkers indicate that a specific chemical is present but give no information on the presence of other chemicals (Shugart, 2005).

A list of criteria for the evaluation of biomarkers should include the following: a) Biological specificity. It is important to know to which species or classes a biomarker is relevant. The inhibition of the enzyme acetylcholinesterase (AChE) by organophosphate (OP) and carbamate (CAR) pesticides can be applied throughout the animal kingdom, whereas the induction of vitellogenin is confined to those vertebrates that lay eggs; b) Clarity of interpretation. One should be able to clearly distinguish whether a stress is natural or anthropogenic. It is valuable to know the mechanism of response to the chemical in assessing this point; c) Time of response. The temporal expression of different biomarkers can vary widely from instantaneous to years. Depending on the type of study, a slow or a rapid manifestation may be desirable; d) Permanence of response. It is important to know how long the response lasts. If it is transient, it may be easily missed. The inhibition of AChE, especially in blood, is a transient response, and thus it is necessary to know when the exposure occurred to assess the importance of the degree of inhibition. In contrast, the inhibition of the enzyme amino levulinic acid dehydratase by lead is only slowly reversed; e) Reliability. This criterion can be considered in two ways: 1) environmental influences that modulate an organism's response to a chemical, and 2) inherent variations in the biological response to a given exposure. To have a reliable biomarker, it is important to know the extent of all variations; f) Methodological considerations. Important considerations include the precision (analytical reproducibility of the method), cost, and ease of analysis. Although

many reliable assays have been developed, there is a need for standardization, similar to that used in analytical chemistry, so that the results from different laboratories can be comparable; g) Relative sensitivity. It is important that the biomarker be sensitive when compared to other endpoints, such as mortality or reproductive impairment, and it is important to know the relative sensitivity of this comparison; h) Validation in the field. For a biomarker to be useful in environmental assessment, it must be validated in the field. Organisms in the field are subjected to a wide range of variables that are usually accounted for or controlled in laboratory experimentation; i) Linkage to higher-level effects. A biomarker is more useful if there is clear linkage to an effect at higher levels of organization. Studies on invertebrates have been particularly fruitful, as population changes among such species occur more rapidly than in higher species (Shugart, 2005).

Biomarkers have an advantage over chemical analysis in that they can demonstrate whether or not an organism has been meaningfully exposed. For some classes of persistent organic chemicals, such as the organochlorines, current detection limits are as low as parts per trillion. Thus, these man-made chemicals can be detected in almost all samples, but the physiological significance is rarely known. With biomarkers, it is possible to determine whether the physiology of the organism is significantly different from normal. If it is, then the organism is considered to be meaningfully exposed. Equally important, if the physiology is not significantly different, then the organism is considered not to be meaningfully exposed, even if the chemical(s) can be detected. The ability to determine whether or not an organism is meaningfully exposed is important in deciding whether regulatory action should be taken or in determining whether or not remedial action has been successful.

Biomarkers are generally divided into three categories as markers of exposure, effect, and susceptibility. Each of these types of biomarkers is described below:

Biomarkers of exposure are measures of internal substances and thus reflect various manifestations of the internal doses that result from exposure. Markers of interest include those that provide measures of the i) total internal dose (such as the blood, urine, or breath level of a chemical); ii) dose to a target organ (which may be in the form of a macromolecular adduct formed between the chemical or its metabolite and the organ tissue); or iii) biologically effective dose (which can only be measured if the mechanism of disease induction is known in sufficient detail to suggest what entities might represent the biological effect).

Biomarkers of effect are any changes in a biological system that reflects qualitative or quantitative impairment resulting from exposure. While a distinction is made between biomarkers of exposure and biomarkers of effect, in practice the two areas overlap. For example, DNA adducts may be biomarkers of exposure, but if they occur at specific sites known to induce mutations leading to cancer, the adducts may also be biomarkers of effect.

Biomarkers of susceptibility. Indicators of individual or population differences that influence the response to environmental agents are called "biomarkers of susceptibility." These indicators might include such characteristics as an enhanced metabolic capacity for converting a chemical to its reactive, more toxic, metabolites or differences in the number of receptor sites that are critical for a specific response. An example is the inherited deficiency in the enzyme α-1-antitypsin, which is associated with an increased susceptibility to the development of emphysema. New assays developed by researchers in the field of toxicogenomics allow for the detection of genetic polymorphisms that can affect the susceptibility to pollutant exposure. Such markers can be quite valuable in providing

information that can contribute to the protection of susceptible populations. Knowledge of the mechanisms of susceptibility can be important in designing the therapy for a disease. However, the use of such markers is fraught with legal and ethical problems, as the identification of persons with enhanced susceptibility to adverse health effects from exposure to chemicals could lead to discrimination against those persons in obtaining jobs and insurance (Henderson, 2005).

1.1 Biomarkers and pesticides

Pesticides are one group of toxic compounds linked to human use that have a profound effect on aquatic life and water quality. Pesticides are substances used to control pests such as insects, water weeds, and plant diseases. Naturally occurring pesticides have been used for centuries, but the widespread production and use of modern synthetic pesticides did not begin until the 1940s. When pesticides enter aquatic systems, the environmental costs can be high. Unintentional pesticide-related fish kills occur. Some of these kills have been large, involving thousands of fishes as well as frogs, turtles, mussels, water birds, and other wildlife. Fish and other wildlife species, including rare and endangered ones, such as the peregrine falcon, bald eagle, and osprey, have been victims of pesticide poisoning. Pesticide use is one of many factors contributing to the decline of fish and other aquatic species (Helfrich et al., 2009). The initial efforts to monitor pesticide exposure in organisms focused on the major plasma esterases in humans, as such targets can be inhibited and modified by some pesticides (Black et al., 1999; Peeples et al., 2005), although investigators now indicate than the identification and characterization of other biomarkers is necessary (Kim et al., 2010).

2. Biomarkers of pesticide exposure in aquatic organisms and human populations

2.1 Esterase inhibition as a biomarker of pesticide exposure in aquatic organisms

Recently, several studies have evaluated AChE and butyrylcholinesterase (BChE) activities as biomarkers of the exposure to OP in different aquatic organisms from contaminated areas in several countries. Tlili et al. (2010) found an inhibition of AChE activity in the bivalve *Donax trunculus* from a polluted site (Radès Méliane) compared to that from a reference site (Sidi Jehmi) in the Gulf of Tunis (Tunisia). Bernal-Hernández et al. (2010) showed than AChE activity was 65% lower in another bivalve, *Crassostrea corteziensis*, from Boca de Camichín than in control oysters, in a subtropical Mexican Pacific estuary, suggesting the presence of OP and CAR pesticides in these aquatic environments. In a similar study performed in Argentina, Attademo et al. (2011) reported that BChE activity was lower in a native frog, *Leptodactylus chaquensis*, from rice fields where pesticides such as methamidophos (OP), cypermethrin (pyrethroid) and endosulfan (organochloride) were used, as compared to those from a reference site. In contrast, Printes et al. (2011) did not observe an association of cholinesterase (ChE) activity in *Chironomus xanthus* with exposure to sediments containing pesticides from Monjolinho River (Southeast Brazil); the authors suggested that the selected biomarker was not sensitive and specific enough to detect the effects of pesticide contamination at the levels measured in the study area. As in aquatic organisms, several studies around the world have evaluated the exposure to OP by measuring cholinesterase activity.

2.2 Esterase inhibition as a biomarker of pesticide exposure in human populations

The inhibition of AChE activity has been observed in subsistence farmers from rural communities of Campeche, Mexico (Rendón von Osten et al., 2004), and in young children working on Mexican tobacco plantations in Nayarit, Mexico (Gamlin et al., 2007); BChE activity was also shown to be inhibited in Tunisian agricultural workers (Araoud et al., 2010).

However, OP exposure has been assessed using a blood cholinesterase test in which a reduction on AChE or BChE activity indicates exposure to OP (Ellman et al., 1961). BChE assays are used for the early and acute effects of exposure to OP because AChE is less sensitive (Wilson et al., 1996). However, BChE has limited utility due to its 11-day half-life in plasma (Richards et al., 2000), as opposed to AChE membrane-bound protein on red blood cells, which has a lifespan of 120 days. AChE inhibition has been used as one standard method to detect OP exposure (Holmstedt, 1959), but this assessment has disadvantages, as intra- and inter-subject variabilities are about 10% in the same person and about 10-40% among subjects (Lotti, 1995), respectively. The integrated use of several biomarkers, such as cholinesterases and others, may be necessary for biomonitoring programs to diagnose pesticide exposure in wild populations (Attademo et al., 2011). Thus, it was proposed that other biomarkers should be identified and characterized, such as the esterase identified as acetyl peptide hydrolase (APH), which was inhibited by some OP (Quistad et al., 2005). Similarly, Noort et al. (2009) suggested that the affinity of OP for albumin could provide a mechanism for a more complete assessment of OP pesticide exposure. In addition, a mass spectrometry method to identify exposure to several pesticides (dichlorvos, chlorpyrifos oxon and aldicarb) based on the identification of pesticide adducts on the active site (i.e., serine) of human BChE has been proposed by Li et al. (2010a).

2.3 Analytical determinations

Biomonitoring helps to identify new exposures to chemicals, trends in exposure, the distribution of chemicals in the population and particularly vulnerable groups, and it is a tool for scientists as well as for policy makers (Angerer et al., 2007). Many authors have monitored the levels of pesticides as persistent organic pollutants (POPs) in ecological studies. Zapata-Pérez et al. (2007) reported the presence of HCHs, DDTs and chlordanes in ariidae *Ariopsis felis* (Linnnaeus, 1766) in three ecosystems in the Southern Gulf of Mexico, and contaminants were higher in Laguna de Terminos than in Celestun and Dzilam. In a different study, p,p´-DDE, toxaphene, total chlordanes, dieldrin, dacthal, endosulfan, gamma-HCH and methoxychlor were detected in fish from the Colorado River and its tributaries (Hinck et al., 2007). The direct measurement of pesticides is a challenge; for example, OP quantification is a challenge due to its rapid metabolism in organisms and its breakdown in the environment, thus the estimations are rough or involve the identification of OP metabolites or degradation products (Barr et al., 2005).

Garabrant et al. (2009) reported a negative relation between urinary 3,5,6-tricholo-2-pyridinol (TCPy) and BChE in workers occupationally exposed to chlorpyrifos. As approximately 75% of OP yield dialkylphosphates (DAPs), gas chromatography coupled with mass spectrometry (GC-MS) has been employed to detect these primary metabolites in urine (Barr et al., 2005). Cocker et al. (2002) reported than the DAPs found in urine from workers potentially exposed to OP were lower and were unlikely to cause a significant reduction of AChE. One study of immigrant Latino farm workers reported that DAPs were not associated with hazardous work conditions (Grzywacz et al., 2010). Another study in

Latino farmers (mostly from Mexico) in eastern North Carolina, in which determinations of DAPs were conducted using urine, blood and saliva samples taken several times monthly, showed variability in the DAPs frequencies, and the authors indicate the importance of longitudinal studies in such populations (Arcury et al., 2009). Other studies with agricultural workers from Mexico have evaluated the presence of DAPs in urine (Lacasaña et al., 2010; Recio et al., 2001, 2005).

Recently, Tsatsakis et al. (2010) proposed a new, simple and fast method to evaluate DAPs in human head hair by GC-MS, in which the metabolites were detected in individuals with occupational exposure. A sensor that provided a rapid, clinically accurate and quantitative tool for TCP detection showed great promise for testing a metabolite biomarker (3,5,6-trichloropyridinol) in humans exposed to pesticides (Zou et al., 2010).

Sunyer et al. (2010) reported the presence of p,p´-DDE, hexachlorobenzene and beta-hexachlorocyclohexane in the serum of pregnant women in the first trimester from a general population in Catalonia, Spain. Similarly, the presence of p,p´-DDE was observed in pregnant women in Greenland (Inuit, Kharkiv and Warsaw mothers) by Wojtyniak et al. (2010). In a similar study, Chevrier et al. (2011) observed quantifiable levels of azatrine or atrazine mercapturate and dealkyllated and hydroxylated traizine metabolites in the urine of pregnant women from the Britany region. Pesticide exposure has been associated with illnesses such as diabetes and pre-diabetes; heptachlor epoxide, oxychlordane, intermediates for p,p´-DDT, beta-hexachlorocyclohexane, p,p´-DDE and trans-nonachlor were related to diabetes in the National Health and Nutrition Examination Survey (NHANES) (Everrett and Matheson, 2010). In patients with exocrine pancreatic cancer, the years worked in agriculture did not associate with the p,p´-DDT, p,p´-DDE, hexachlorobenzene or β–hexachlorocyclohexane detected by GC with electron capture detection (Bosch de Basea et al., 2010).

3. Biomarkers of effect

3.1 Biomarkers of effect in aquatic organisms

The use of molecular biomarkers in aquatic organisms is essential to address the broad spectrum of industrial, agricultural, commercial and domestic chemicals that are entering the environment, especially the aquatic environment, and being taken up into the tissues of aquatic organisms. The process of ecological risk assessment is continually developing in ecotoxicological studies to address changing needs and diverse toxicological issues. Risk assessment methods are designed to provide a quantitative estimate of the probability of an adverse effect occurring as a consequence of environmental pollution of a diverse mixture of chemical pollutants, which can also act synergistically (Valavanidis & Vlachogianni, 2010).

Biomarkers of effect and exposure may often be combined into a single biomarker (Barrett et al., 1997). For the purposes of this section, it should be noted that many markers may be used in one or two categories simultaneously.

Among the various types of biomarkers of note in ecotoxicological studies are the following: cytochrome P450 activity (an indicator of the exposure and effect of organic contaminants, such as PAHs, PCBs, and pesticides), the inhibition of AChE activity (a biomarker of the exposure and effect of OP and CAR), metallothionein synthesis in hepatic and other tissues (exposure to the metals Zn, Cu, Cd, Hg, and Fe and some pesticides), and antioxidant enzymes such as superoxide dismutase, catalase, glutathione transferase (exposure to ROS, free radicals, and pollutants causing oxidative stress and lipid peroxidation, such as oxidants, pesticides, and metals).

There are molecular, cellular and whole-animal biomarkers that can be measured in samples of body fluids, cells or tissues. Some biomarkers are specific to a certain set of pollutants, and others change in response to both pollutants and natural factors, causing oxidative stress or adverse effects on biological metabolism. Some biomarkers have prognostic value and can provide an early warning, while others offer specificity, sensitivity or the ability to be applied to a wide range of organisms.

3.1.1 Acetylcholinesterase

AChE is an enzyme responsible for hydrolyzing the neurotransmitter acetylcholine into choline and acetic acid. This enzyme is located in the membranes of vertebrates and invertebrates. The inhibition of AChE is linked directly with the mechanism of toxic action of organophosphorous and carbamade insecticides; the inhibition of this enzyme has also been used to indicate the exposure and effects of other contaminants (cadmium, lead and copper) in fishes, marine bivalves and other organisms (Fulton & Key, 2001; Monserrat et al., 2002; Binelli et al., 2006; Bernal-Hernández et al., 2010; Leite et al., 2010).

Significant depressions of AChE activities in brain and liver tissues of *Oreochromis niloticus* following single and multiple exposures of chlorpyrifos and carbosulfan in the laboratory were reported by Chandrasekara & Pathiratne (2005). Similar results were reported in oysters exposed to dichlorvos (Bernal Hernandez et al., 2010). It has been shown that crude oil in amounts equivalent to sediment concentration inhibits AChE activity in the homogenate of brain fishes (Rodriguez-Fuentes & Gold-Bouchot, 2000). Minier et al. (2000) reported that muscle AChE of flounder from polluted sites with high levels of PAHs was inhibited by 40%. Also, a reduction of 40% of brain AChE was observed in *Mullus barbatus* from three polluted sites in Salento Apulia (Italy) and was related with the presence of great variety of compounds (PAHs, heavy metals and pesticides) in the sediment (Lionetto et al., 2003). Chitmanat et al. (2008) reported low AChE activity in snails (Sinotaia ingallsiana) from Ping River (Thailand), related with the presence of pesticides. Also, other studies have shown seasonal variation of AChE activity in mussel gills, and their changes were related to the periods of pesticide use in the polluted areas (Valavanidis & Vlachogianni, 2010).

3.1.2 Antioxidant enzymes

Biological systems generate endogenous reactive oxygen species (ROS) and other oxidants during their metabolism. Pesticides with redox potential can produce increasing amounts of ROS in marine species in polluted sites. Biological systems are detoxified from ROS by enzymatic and non-enzymatic antioxidant defenses that are ubiquitous in the tissues of most animal species. The enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, reductase and glutathione S-transferase (GST). The measurement of antioxidant enzymes and lipid peroxidation in aquatic organisms can be used as sensitive biomarkers for the biomonitoring of polluted marine areas containing contaminants, such as pesticides, heavy metals, PAHs, and TCDD, which can generate ROS (Livingstone, 2001; Vlachogianni et al., 2007; Di Giulio & Hinton, 2008).

3.1.3 Heat shock proteins (HSPs)

The exposure of living beings to sub-lethal levels of environmental pollution has been shown to trigger several defense mechanisms at the cellular and molecular levels. There is a cellular accumulation of stress proteins, which mainly act as molecular chaperones (Bauman

et al., 1993; Feder & Hofmann, 1999). Among stress proteins, the HSP70 group has been studied as being regularly over-expressed in response to a wide variety of natural or anthropogenic aggressors (alcohols, oxidative stress, radiations, heavy metals, arsenic, pesticides and others) (Delaney & Klesius, 2004). The role of HSPs during stress is related to a cytoprotective function, as these proteins can act to prevent and repair protein damage (Ananthan et al., 1986). Recently, it was shown that elevated HSP70 is critical in the protection of sea brim cells against chemical-induced apoptosis (Deane et al., 2001). HSP levels have been shown to be modulated in fish cells and tissues upon exposure to an array of stressors (Iwama et al., 1998). Studies performed in low vertebrates are already numerous, and the expression of stress proteins in different fish species in response to various stressors has been investigated by many authors (Iwama et al., 1998, 1999). For instance, several HSPs have been detected after the exposure of various kinds of fish cells to heat shock, arsenate and several metal ions (Misra et al., 1989; Currie et al., 1999, 2000). The accumulation of these HSPs has been linked to the intensity of stress; these proteins have been regarded as a suitable biomarker in assessing reactions of biota to environmental and physiological stressors (Hightower, 1991; Sanders, 1990, 1993).

3.1.4 Metallothioneins (MTs)

Metallothioneins are low-molecular-weight peptides, high in the amino acid cysteine (which contains a thiol group, -SH), that are found mainly in the cytosol, lysosomes and nucleus. MTs are also considered to be stress proteins because they protect cells against excessive metal uptake (Bauman et al., 1993) by virtue of their high proportion of -SH groups, which sequester the metallic ions (Kagi & Schaver, 1988; Klaassen et al., 1999). They are found in many aquatic invertebrates and species of fishes. The overexpression of MTs has been studied in different fish species, and their use as a biomarker for monitoring metal pollution in the environment has been proposed (Carbonell et al., 1998; Hamilton & Mehrle, 1986). Investigations have indicated that simple tissue residue measurements of metals would provide the same information as MTs and would be a better indicator of exposure and effect (Perkins et al., 1996). MT protein determination has a strong correlation with lipid peroxidation in trout chronically exposed to zinc and copper (Farag et al., 1995). Schlenk et al. (1997) examined the effects of low-level arsenic exposure and demonstrated dose-dependent increases in MT expression in channel catfish. In aquatic invertebrates, the development of procedures for the study of MTs is relatively recent. A few studies have shown that digestive glands and gills have the highest concentrations of MTs in aquatic invertebrates (Geffard et al., 2001, 2002; Ceratto et al., 2002; Bernal-Hernández et al., 2010).

3.1.5 CYP1A

Cytochrome P450 monooxygenases (CYPs) are a multi-gene family of enzymes that play a key role in the biotransformation of pollutants, such as dioxins, pesticides, PCBs and PAHs. One of the most common and highly conserved is the CYP1A subfamily. The CYP1A biomarker is widely used as a biomarker of effect both in vertebrates and invertebrates for environmental biomonitoring, especially in marine bivalves and fish (Valavanidis & Vlachogianni, 2010; Di Giulio & Hinton, 2008). The induction of CYP1A is triggered via the cytosolic aryl hydrocarbon (Ah) receptor due to exposure to pollutants, such as polychlorinated biphenyls (PCBs), dioxins, and numerous polycyclic aromatic hydrocarbons (PAHs). Studies have shown relationships between CYP1A expression and reproductive alterations following exposure to PCBs or 2,3,7,8-tetrachlorodibenzo-dioxin (TCDD) (Cook

et al., 1997; Teraoka et al., 2003). CYP1A activity is typically measured using the substrate ethoxyresorufin, which is o-deethylated by ethoxyresorufin-O-deethylase (EROD) to a fluorescent product, resorufin, which can be easily measured. Because EROD activities are generally measured using liver homogenates that also tend to accumulate numerous CYP1A substrates, activity may be inhibited by residual substrates or metals (Valavanidis & Vlachogianni, 2010; Di Giulio & Hinton, 2008).

3.2 Biomarkers of effect in humans

Biomarkers of effect in the blood of humans have been related to changes in hemoglobin synthesis upon the exposure to pesticides, as is the case with porphyrins. A large number of organochlorine compounds affect hemoglobin synthesis and result in an accumulation of highly carboxylated porphyrins, which may be detected in the liver, blood, urine or feces (Gil Hernandez, 2000). These have been detected in urine samples from various populations, including workers exposed to hexachlorobenzene and octachlorostyrene (Selden et al., 1999) and a Spanish population environmentally exposed to hexachlorobenzene (Herrero et al., 1999). However, there have been more recent works where there is no alteration in the excretion of urinary porphyrins upon exposure to organochlorines, like the study conducted in a population environmentally exposed to hexachlorobenzene (Sunyer et al., 2002) and in neonates born to exposed mothers (Ozalla et al., 2002).

Regarding the nervous system, it is important to consider that neurochemical measures for the detection of neurotoxicity are limited by the inaccessibility of the target tissue, and hence the identification and characterization of neurotoxicity is dependent on finding parameters in peripheral tissues that reflect the behavior of parameters in the nervous system (Costa & Manzo, 1995). One specific biomarker of neurotoxicity is the inhibition of AChE by anticholinesterase pesticides (OP and CAR). AChE activity is present in many tissues, but its inhibition is usually determined in blood samples (whole blood or plasma) and brain (Gil Hernandez, 2000). This biomarker has been widely used in occupationally exposed populations, such as Egyptian cotton field workers (Farahat et al., 2011), workers occupationally exposed during the manufacture of chlorpyrifos (Garabrant et al., 2009), young children working on Mexican tobacco plantations exposed to OP and CAR pesticides (Gamlin et al., 2007) and farm workers occupationally exposed to agricultural chemicals (Panemangalore et al., 1999), among others.

The effects of certain xenobiotics on the immune system can cause disturbances in normal functioning, decreased resistance to infections or tumors, autoimmune responses and even hypersensitivity reactions (Van Loveren et al., 1995; Kimber, 1995).

Among the assays recommended as biomarkers of immunotoxicity in humans include the following: lymphocyte count, the study of antibody-mediated immunity (Ig in serum), phenotypic analysis of lymphocytes (flow cytometry), the study of cellular immunity, measurements of autoantibodies and markers of inflammatory response, and measurement of nonspecific immunity (Gil Hernandez, 2000). Directly associated with pesticide exposure, adverse effects have been reported in the development of the immune response in two-year-old children living in agricultural areas, who had high levels of Th2, which associated with asthma and wheezing (Duramad et al., 2006).

Another field is the development of adverse effects such as tumors, which are related with xenobiotics and are associated with the aberrant expression of genes encoding proteins involved in cell growth, such as growth factors and oncoproteins. These biomarkers have been studied in plasma or serum samples using ELISA, RIA or immunoblotting and have

also been detected in urine or bronchoalveolar fluid (Gil Hernandez, 2000). In a specific case, it was observed that HER-2/neu oncoprotein is overexpressed in patients with extensive-stage small cell lung cancer and is associated with decreased survival, and it was also observed that pesticide exposure seemed to be related to HER-2/neu overexpression in the study population (Potti et al., 2003). Similarly, an interaction between organophosphate pesticide exposure and PON1 activity on thyroid function in a population of floriculture workers from Mexico was observed (Lacasaña et al., 2010).

Oxidative stress and DNA damage have been proposed as mechanisms linking pesticide exposure to health effects such as cancer and neurological disease (Kisby et al., 2009). In response to oxidative stress, adaptive mechanisms are triggered by protective systems and are commonly quantified in plasma, including the oxidized glutathione (GSSG)/glutathione (GSH) ratio and the activities of glutathione reductase, catalase, superoxide dismutase and peroxidase. Macromolecules that may be affected include lipids, proteins and nucleic acids (Gil Hernandez, 2000). In this regard, a study showed that exposure to OP produces oxidative membrane damage to the erythrocytes of individuals with pathologic complications (Sharma et al., 2010), and a separate study showed an association of oxidative damage with exposure to OP in the blood of horticultural farmers (Atherton et al., 2009). A similar result was observed in a pilot study of pesticide applicators and farm workers working in the fruit orchards of Oregon (Kisby et al., 2009).

More sophisticated techniques allow for the detection of covalent interactions between xenobiotics and proteins and other macromolecules. Many reactive metabolites originating from organic compounds form adducts with proteins or DNA. These can be used as markers of the damage from the exposure to pesticides that causes an increase in the carcinogenic process (Gil Hernandez, 2000). Biological monitoring is done by detection with 32P radiotracers or by immunoassays (with specific antiserum against DNA adducts). Measurements are performed on blood, urine or homogenates of tissues that are obtained from biopsies.

Specifically, adducts have been detected on tyrosine 411 of human albumin exposed to dichlorvos (Li et al., 2010a), and adducts have also been identified at Ser 198 of human BChE upon exposure to carbofuran (Li et al., 2009) and on the DNA-adduct 8-hydroxy-2-deoxy-guanosine (8-OHdG) in the plasma of farm workers (Tope & Panemangalore, 2007), among others.

Pesticides have been considered as potential mutagens because they contain ingredients capable of causing changes in DNA. Therefore, in addition to the determination of adducts, there have been several studies on cytogenetic damage to assess the potential, especially for agricultural workers, for chromosomal aberrations (CA), micronuclei (MN) and sister chromatid exchange (SCE); it has also been possible to determine changes that occur in the kinetics of cell proliferation, which can be observed and evaluated during mitosis. The alkaline single-cell electrophoresis or comet assay (EC) has been designed to assess damage and DNA repair both in vitro and in vivo (Martínez-Valenzuela & Gómez-Arroyo, 2007), but the results are controversial because there are several factors that may cause differences, such as the chemical group to which the pesticide belongs, the technical formulation and active ingredient (which is the product), the type of exposure (chronic or acute), the specific time of exposure for the individual, the manner of contact (direct or indirect), the amount used, the exposure to mixtures, the climate and season of the year, and the person's age, among other factors (Martínez-Valenzuela & Gómez-Arrollo, 2007).

CA, cytological changes, which affect the number or structure of chromosomes that constitute the karyotype of the species, can be observed by light microscopy. These changes correspond to breaks and rearrangements in the same or between different chromosomes (Martínez-Valenzuela & Gómez-Arroyo, 2007).
MN involves the expression at the interface of acentric fragments that do not have centromeres, that are not included in the daughter nuclei during cell division, and that do not interact with mitotic spindle fibers in anaphase; such fragments are surrounded by nuclear membranes and appear as small nuclei. When the damage occurs in the centromere, an imbalance in the distribution of the chromosomes is produced, disturbing the normal kinetics of anaphase and causing envelopment by a nuclear envelope. Assays of this type of damage may be performed using epithelial cells of the urinary bladder and oral and nasal mucosa (Stich & Rosin, 1984; Rosin & Gilbert, 1990) or peripheral blood (Lee et al., 2002; Clare et al., 2006).
SCE occurs during the synthesis phase and represents symmetrical exchanges between homologous loci of replication products, occurring without DNA loss or changes in chromosome morphology (Norppa, 2004). Although not considered to be mutations, sister chromatid exchanges have been noted to increase in frequency when cells are exposed to known mutagenic and carcinogenic agents. Tests of such disturbances are used in the biological monitoring of individuals exposed to potential or known genotoxic agents (Lambert et al., 1982; Cavallo et al., 2006).
Known as single-cell alkaline electrophoresis, EC is a fast, simple, visual and sensitive biomarker used to measure and analyze breaks in DNA. This assay detects intracellular differences and damage to the repair processes of cells (Speit & Hartmann, 2006). The comet assay consists of quantifying the damage induced in the DNA of cells that are embedded in agarose, lysed and then subjected to electrophoresis in alkaline pH, which ensures that fragments of chromosomes are directed toward the anode and are revealed as the tail of a comet upon staining with a fluorescent dye (Tice et al., 2000). The extent of DNA migration depends on the number of breaks produced by the agent in question (Garaj-Vrhovac & Zeljezic, 2001), so each cell has the appearance of a comet with a head and tail under bright fluorescence, while undamaged cells appear as intact nuclei without tails (Møller, 2006).
Cytogenetic biomarkers have been widely used in populations occupationally exposed to pesticides, including European farmers (Pastor et al., 2003), female workers exposed to pesticides in banana plantations in Costa Rica (Ramírez & Cuenca, 2002), Mexican retailers (Rojas-García et al., 2011), workers involved in the pesticide manufacturing industry in Pakistan (Bhalli et al., 2006), domestic users of OP (Lieberman et al., 1998), and populations residing in pesticide-contaminated regions in Göksu Delta (Ergene et al., 2007), among many others.
However, it is important to consider that studies of pesticide exposure and genotoxic effects must take into account the reliability of damage from exposure, the strength of the studies, the similarity of the control group and the protocols used for genotoxicity (Bull et al., 2006).

4. Susceptibility biomarkers

Phenotypic and genotypic variation between individuals is a fundamental characteristic of living beings. With the revealing of the human genome, more "susceptibility" genes will be discovered, and it is likely that the etiology of many diseases or health outcomes will be shown to be related to a combination of genetics and environment. Simple blood tests may

ultimately be developed that allow an individual to learn whether he or she may be particularly susceptible to specific environmental pollutants (Klaassen, 2008). In the case of pesticides, there is growing recognition that genetic factors can account for individual susceptibility to a range of responses.

Pesticide adverse effects result from the complex interactions between internal (genes, age, sex, species, pathological and physiological status) and external factors (temperature, diet, lifestyle and others). The adverse health effects of pesticides are in most cases related with the toxicokinetics of those compounds; variabilities in the absorption, distribution, biotransformation and excretion of pesticides may play essential roles in the modulation of the internal dose and the effects (Figure 1).

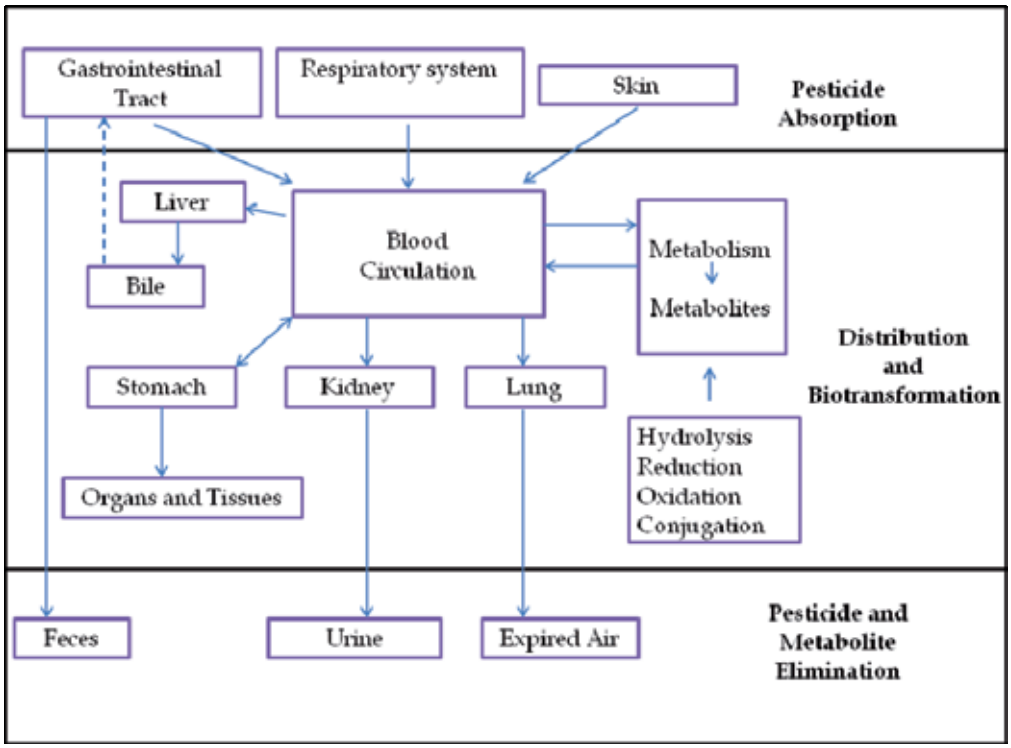

Fig. 1. General pesticide pathways in an organism

Some pesticides are bioactivated by metabolic enzymes in organisms and are converted into toxic compounds. In addition, there are also enzymes that participate in the detoxification of pesticides. It is important to evaluate the bioactivation/inactivation ratio in individuals to produce a more complete scenario of pesticide susceptibility.

In this regard, pesticides are metabolized by a variety of cytosolic and microsomal enzymes. Biotransformation of pesticides is a fast process that involves different families of enzymes, such as hydrolases, oxidases, reductases, and conjugation enzymes. There is evidence that the genes coding for the different enzymes that biotransform pesticides have genetic variations in human populations. Changes in the biotransformation ability of these compounds can have an impact on the toxicokinetics and thus the toxicity of these pollutants.

4.1 Cytochrome P450 (CYP450)

CYP450 is a family of hemoproteins that catalyze monooxygenation reactions (Santiago et al., 2002); as a result of these reactions, P450 accelerates the body's elimination of many drugs and toxic compounds, but it is also responsible for the activation of toxins or pre-carcinogens (Donato, 2004). All known P450s are named according to common criteria and are grouped into families and subfamilies based on similarities in the encoding DNA sequences (Donato, 2004). In humans, 18 families and 43 subfamilies of CYP450 have been identified (Nelson, 2002). Families 1, 2 and 3 are made up of enzymes responsible for the biotransformation of xenobiotics, while other families are involved in the biosynthesis and metabolism of endogenous compounds (Donato, 2004). The P450s are widely distributed throughout the body, but the liver is the organ with the highest expression of these enzymes. Its expression is regulated by genetic, pathophysiological and environmental factors (Donato, 2004).

Of the cytochrome P450 members, CYP2D6 is the one with the greatest genetic influence (Espíritu, 2008). The gene that encodes it is located near two pseudogenes on chromosome 22q13.1, CYP2D7P and CYP2D8P (Grimán et al., 2009). CYP2D6 is involved in the metabolism of many drugs, particularly those that work in the central nervous system and cardiovascular system, but it also catalyzes the oxidative biotransformation of organophosphorus pesticides such as parathion and diazinon (Schaeffeler et al. 2003). CYP2D6 has variations in its gene sequence, called polymorphisms, that may or may not change its amino acid sequence. For each CYP isoenzyme, there are several genetic polymorphisms, some of which are critical in the metabolism of drugs and environmental pollutants such as pesticides (Espíritu, 2008). The diversity of polymorphisms in this gene produces four phenotypes known as poor (PM), intermediate (MI), rapid or extensive (EM) and ultrarapid metabolizers (UM); these phenotypes are associated with variable responses to drugs, adverse reactions to drugs upon increasing the concentrations of drugs in the PM, or treatment failure as a result of the degradation of drugs in UM (Schaeffeler et al., 2003). In the case of pesticides, it is expected that these phenotypes are related to differences in the bioactivation abilities and differences in the toxicities of these xenobiotics. There are about 20 polymorphisms related to the phenotype MP; 95% correspond to the alleles * 3, * 4, * 5 and * 6, with the most frequent allele as * 4, characterized by a base substitution G1934A in the splicing site between introns and exons three and four; a truncated protein results from this mutation (Grimán et al., 2009). CYP1A2 and 2B6 have also been reported as possible metabolic biomarkers of susceptibility to OP-induced toxic effects at actual human exposure levels (Buratti et al., 2005).

Of note, organochlorine pesticides (OCP) and polymorphisms of xenobiotic metabolizing enzymes are reported to be associated with a possible risk of prostate cancer. OCPs are endocrine disruptors (ED) that may act by disrupting the physiologic function of endogenous hormones and therefore possibly increase prostate cancer risk. CYP1A1 metabolizes several carcinogens and estrogens, and polymorphisms of this gene have been reported to be associated with prostate cancer risk. Kumar et al. (2010) studied 70 newly diagnosed prostate cancer patients and 61 age-matched healthy male controls. OCP levels in blood were determined, and CYP1A1 polymorphisms were analyzed. Significantly higher levels of β-HCH, γ-HCH and p,p'-DDE were found in cases as compared to controls (p-values=0.04, 0.008, and 0.01, respectively). Higher levels of γ-HCH were observed in advanced stages of prostate cancer cases (<or=T(2) vs. >or=T(3)) (p=0.04). Dieldrin was found to be significantly higher in cases with initial stages (p=0.03). However, there was no

observed correlation between prostate cancer and CYP1A1 polymorphisms. Hence, higher level of OCPs, especially β-HCH, γ-HCH and p,p'-DDE, might be associated with prostate cancer risk.

4.2 Glutathione S-transferase (GSTs)

The glutathione S transferases are classified into eight families (alpha, kappa, mu, pi, sigma, theta, zeta and omega) based on the amino acid sequence, immunogenic properties and physiological role. These enzymes may be localized in the cytoplasm or in the endoplasmic reticulum of cells and are present in almost all tissues, but their greatest expressions occur in liver, kidney, intestine, testis and lung (Klaassen, 2008). GSTs catalyze the nucleophilic conjugation of different biologically active and potentially carcinogenic compounds that can be further biotransformed to mercapturic acids (Ortiz et al., 2001; Oude et al., 1998). Among the GST substrates are a variety of pesticides, and GST seems to play an important role in the elimination and hence in the detoxification of these compounds. GSTs include a superfamily of highly polymorphic genes (Stanulla et al., 2000). In humans, polymorphisms of GST associated with a decreased activity (GTSM1, GSTM3, GSTM4, GSTP1, GSTT1 and GSTZ1) have been identified (Fernández, 2005).

The most important polymorphisms are those on the locus GTSM1, which has four variant alleles: GSTM1 * A, GSTM1 * B, GSTM1 * 0 o C and GSTM1 null. The first three have no apparent differences in catalytic activity. The variant null, or 0, is a partial deletion of the gene that leads to a total loss of enzyme activity (Oude et al., 1998). This null polymorphism is present in 30-60% in the general population and has been associated with a risk of developing lung cancer in Asian, Caucasian and Latino populations (Ortega, 2007; Fernandez, 2005). Also, several studies have demonstrated a direct association of this polymorphism with increased risks of bladder, gastric, colorectal and skin cancers (Lan et al., 2000; Stucker et al., 2002).

In a study conducted on agricultural workers of the Punjab region of northwestern India, the GSTT1 gene deletion and simultaneous deletions of GSTM1 and GSTT1 genes were related with increasing DNA damage evaluated using an alkaline comet assay (Abhishek et al., 2010).

4.3 Human serum paraoxonases (PONs)

Human serum paraoxonases are a multigene family comprised of PON1, PON2 and PON3. Among the PONs, PON1 (EC 3.1.8.1) is the most studied family member. PON1 is a 355-amino-acid calcium-dependent esterase predominantly synthesized in the liver and closely associated with high-density lipoproteins (HDL). Even though PON1 has no known physiological substrate and no clear biological function, it is known that PON1 is capable of hydrolyzing certain toxic metabolites of OP, such as paraoxon, diazoxon, and chlorpyrifos oxon, as well as nerve agents, such as soman and sarin. This hydrolyzation significantly influences the detoxification of the toxic compounds and hence can modify their toxicities. Besides being capable of hydrolyzing OP, PON1 has been shown to hydrolyze phenyl acetate, an aromatic carboxyl ester, and hence is involved in the metabolism of drugs and xenobiotics. Furthermore, PON1 hydrolyzes some naturally occurring lactone metabolites and estrogen esters.

The concentration of PON1 in human plasma varies among individuals. PON1 activity levels are determined by a combination of complex genetic interactions and environmental-

dietary factors, leading to a 40-fold variation in PON1 in single individuals. In addition, the PON1 gene shows several polymorphisms in the promoter and coding regions, which explain, at least in part, the large variations in activity and concentration among individuals.

4.3.1 Polymorphisms in the coding region

Two common polymorphisms in the coding region of PON1 have been reported, at positions 55 and 192. The polymorphism at position 55 (Leu/Met) has been related with different PON1 activities, with the 55M isoenzyme having lower enzymatic activity than the 55L isoenzyme (Blatter-Garin et al., 1997). It has been proposed that this is due in part to linkage disequilibrium with the -108C allele (Brophy et al., 2001) and also to an increase in the stability of the 55L isoenzyme (Leviev et al., 2000). More attention has been paid to the 192 polymorphism because the two allozymes differ considerably in their affinities and catalytic activities with a number of substrates (Draganov et al., 2004). This polymorphism (192 Glu/Arg) has two isoforms that hydrolyze phenylacetate at similar rates (La Du et al., 1986), but their paraoxon and diazoxon hydrolysis rates are different. Linkage disequilibrium between PON1 55 and PON1 192 has been reported (Blatter-Garin et al., 1997; Brophy et al., 2000; Rojas-García et al., 2005).

It has been suggested that serum paraoxonase is mostly involved in the detoxification of a variety of OP compounds in mammalian species. One study showed that species lacking paraoxonase are more susceptible to OP toxicity (Mackness et al., 1998). This finding supports the hypothesis that serum paraoxonase is the main enzyme responsible for the deactivation of OP compounds, thereby reducing the risk to the toxic effect of the corresponding oxons.

Experiments have investigated the toxicity of OP in PON1 knockout (PON1-/-) mice, which have no serum paraoxonase or diazoxonase activity and a very low activity toward chlorpyrifos oxon. PON1 knockout mice have dramatically increased sensitivities to chlorpyrifos oxon and diazoxon and slightly increased sensitivities to chlorpyrifos and diazinon, but they do not show increased sensitivity to paraoxon (Costa et al., 2003). Studies in animals show that injection of partially purified PON1 into rats increases their resistance to paraoxon. Additionally, injection of purified rabbit PON1 into mice 4 h prior to exposure to chlorpyrifos dramatically increases their resistance to chlorpyrifos and its oxon. These experiments support the hypothesis that high levels of plasma paraoxonase could protect against exposure to chlorpyrifos (Furlong et al., 2005). Further studies have investigated whether the administration of exogenous PON1 restores plasma PON1 levels in PON1-/- mice and whether these PON1 levels in plasma provide protection against OP toxicity. Human PON1Q192 or PON1R192 were injected intravenously into PON1-/- mice, and the effects of OP on brain and diaphragm AChE were determined. Both isoforms (PON1R192 and PON1Q192) were protective to chlorpyrifos oxon and diazoxon, but neither human PON1 isoform protected against the toxicity of paraoxon (Costa et al., 2003).

Some studies in the literature evaluate the role of PON1 as a susceptibility biomarker to adverse effects of OP. Most of them analyze the PON1 polymorphisms and their relationship with the neurological effects caused by OP. In this regard, Haley et al. (1999) reported that ill veterans with neurological symptom complexes were more likely to have the R allele than to be homozygous for Q. Additionally, Sirivarasai et al. (2007) found a relationship between the PON1 polymorphism and cholinesterase activities in an OP-exposed population. In agreement with this, there are reports showing that PON1 genotypes

are associated with exposure-related changes in paraoxonase, arylesterase and acetylcholinesterase activities and with abnormal electroencephalography patterns at sub-threshold pesticide exposure (Browne et al., 2006). Similarly, Mackness et al. (2003) investigated the relationship between PON1 genetic polymorphisms and PON1 activity in farmers who reported chronic symptoms related to OP exposure, concretely in sheep dipping (cases) and controls. Individuals showing toxic effects were found to be more likely to have the R192 and L55 alleles than the controls, and the combination of R and L genotypes was associated with lower PON1 activity toward diazoxon. Additionally, the farmers reporting chronic symptoms due to OP exposure had a higher proportion of the PON1 192R polymorphism, which is associated with lower rates of diazoxon hydrolysis, as compared to controls. Their symptoms may be explained by a lower ability to detoxify diazoxon.

The role of PON1 genotypes in OP toxicity has also been investigated in OP-poisoned patients. For this purpose, Akgür et al. (2003) evaluated the effect of PON1 on the outcome of acute OP intoxication and the effect of this on PON1. The frequency of the PON192Q allele was significantly higher in patients than controls, which suggests that differences in PON1 activity and the PON1 55 and 192 polymorphisms are important risk factors in the susceptibility to acute OP poisoning. In addition, in another work conducted on acute OP insecticide poisoning cases, a correlation between the stimulation of PON1 and butyrylcholinesterase activity was found, but this correlation was lower than that in cases with chronic exposure to OP insecticides. The authors suggest that in both chronic and acute OP exposures, both PON1 levels and phenotypes must be taken into consideration.

The effects of PON1 genotypes on male reproductive outcomes related to OP exposure have been poorly investigated. In one study, Padungtod et al. (1999) studied the allele frequency of PON1 192 and its relationship with semen quality and hormone profile in Chinese pesticide-factory workers and in controls. Both unexposed 192QQ and exposed 192QR showed significantly lower sperm concentrations than the reference group. In addition, exposed individuals carrying at least one R allele had significantly higher serum LH (luteinizing hormone) levels than the control group. Similarly, a cross-sectional study of farmers with Mayan ascendancy from southeastern Mexico chronically exposed to pesticides (mostly OP) was performed by Pérez-Herrera et al. (2008). Exposure to OP was associated with *in situ* nick-translation-positive cells and sperm viability in homozygote 192RR subjects. Furthermore, dose-effect relationships were observed between OP exposure for three months before sampling and both sperm quality parameters and nick-translation-positive cells in 192RR farmers. The authors suggest that PON1Q192R polymorphisms could modulate the OP-mediated toxicity on spermatogenic cells.

In a study conducted by Singh et al. (2011), several related aspects were evaluated: (a) the prevalence of two common PON1 polymorphisms, (b) the activity of PON1 and acetylcholinesterase enzymes, and (c) the influence of PON1 genotypes and phenotypes variation on DNA damage in workers exposed to OP. A total of 230 subjects were examined, including 115 workers exposed to OP and an equal number of normal healthy controls. The results revealed that PON1 activities toward paraoxon (179.19±39.36 *vs*. 241.52±42.32 nmol/min/ml in controls) and phenylacetate (112.74±17.37 *vs*. 134.28±25.49 µmol/min/ml in controls) were significantly lower in workers than in control subjects ($p<0.001$). No significant differences were observed in the distributions of genotypes and allelic frequencies of PON1(192)QR (Gln/Arg) and PON1(55)LM (Leu/Met) in workers and control subjects ($p>0.05$). The PON1 activity toward paraoxonase was found to be

significantly higher in the R/R (Arg/Arg) genotype than the Q/R (Gln/Arg) genotype and was lowest in Q/Q (Gln/Gln) genotype in both workers and control subjects ($p<0.001$). For PON1(55)LM (Leu/Met), PON1 activity toward paraoxonase was observed to be higher in individuals with the L/L (Leu/Leu) genotype and was lowest in individuals with the M/M (Met/Met) genotype in both groups ($p<0.001$). No influence of the PON1 genotype or phenotype was seen on the activity of acetylcholinesterase or arylesterase. The DNA damage was observed to be significantly higher in workers than in control subjects ($p<0.05$). Furthermore, the individuals who showed least paraoxonase activity, i.e., those with (Q/Q [Gln/Gln] and M/M [Met/Met]) genotypes, showed significantly higher DNA damage compared to other isoforms in workers exposed to OP ($p<0.05$). These results indicate that individuals with PON1 Q/Q and M/M genotypes are more susceptible to genotoxicity. The study concluded that there were wide variations in enzyme activities and DNA damage due to polymorphisms in the PON1 gene that might have important roles in the identification of individual risk factors in workers occupationally exposed to OP.

In addition, there have been studies where OP pesticides and adverse pregnancy outcomes were analyzed. In this regard, Wolff et al. (2007) measured biomarkers of maternal exposure to DDE, PCB, and OP metabolites in pregnancy among exposed mothers, as well as maternal paraoxonase (PON1), BChE, and PON1Q192R gene variants. They found that infant birth lengths were shorter for mothers with the PON192RR genotype compared with PON192QQ, and head circumference was inversely associated with maternal PON1 activity. A relationship between prenatal environmental biomarkers and birth outcomes modulated by PON1 and maternal weight was suggested.

4.3.2 Studies with two or more enzymes as susceptibility biomarkers

Hernández et al. (2005) conducted a study in 135 pesticide applicators (sprayers), and the authors investigated changes in erythrocyte delta-aminolevulinic acid dehydratase (ALA-D) after exposure to different pesticides, including OP and paraquat. AChE was used as a reference biomarker. The effects of the combined polymorphisms of enzymes involved in the detoxification of pesticides (PON1, benzoylcholinesterase (BeChE), and glutathione S-transferase (GSTM1 and GSTT1)) on the levels of the target erythrocyte enzymes were also studied as biomarkers of individual susceptibility. Sprayers presented significantly lower levels of ALA-D and AChE than controls (41.3% and 14.5%, respectively) at the high exposure period. When all biomarkers of individual susceptibility to pesticides were considered at the same time, the GSTT1 null allele determined higher ALA-D and AChE activities at the period of high exposure to pesticides. The PON1 R allele in turn determined lower AChE activity at the low exposure period. Null genotypes for both GST subclasses (GSTM1 and GSTT1) were found to be unique independent predictors of pesticide-related symptomology. Interestingly, sprayers were consistently underrepresented among carriers of "unfavorable" BeChE variants. The conclusions of the study were that ALA-D appears to be an important biological indicator of pesticide exposure and that PON1 and GSTT1 are relevant determinants of susceptibility to chronic pesticide poisoning.

A study conducted by Tsatsakis et al. (2009) investigated the correlation of CYP1A1 and PON1 enzymes with the incidence of various medical examination findings in a Greek rural population professionally exposed to a variety of pesticides. The medical history of 492 individuals, randomly selected from the total population of 42,000, was acquired by interviews, and their genotypes were determined for CYP1A1*2A, PON1 M/L and PON1 Q/R polymorphisms. The assessment of the population's pesticide exposure was verified by

analytical methods. Analysis of the genetic data showed that the allele frequencies of PON1 R, M and CYP1A1*2A alleles were 0.243, 0.39 and 0.107, respectively. The CYP1A1*2A polymorphism was found to have a significant association with chronic obstructive pneumonopathy (p=0.045), peripheral circulatory problems (trend p=0.042), arteritis (p=0.022), allergies (trend p=0.046), hemorrhoids (trend p=0.026), allergic dermatitis (p=0.0016) and miscarriages (p=0.012). The PON1 Q/R polymorphism was found to have a significant association with hypertension (p=0.046) and chronic constipation (p=0.028), whereas the L/M polymorphism was associated with diabetes (p=0.036), arteritis (trend p=0.022) and hemorrhoids (trend p=0.027). These results demonstrate an association between the CYP1A1/PON1 polymorphisms and several medical examination findings, indicating the possible involvement of the human detoxification system in the health effects of a rural population exposed professionally to pesticides.

Greater appreciation of the mechanisms and extent of individual variation in the susceptibility among humans can improve the protection of susceptible populations and better relate findings in animals to the characterization of risk in humans.

By definition, biomarkers do not directly provide information concerning impacts on the higher levels of organization that ecotoxicology ultimately endeavors to discern. Nevertheless, biomarkers can provide important ancillary tools for revealing contaminant exposure and potential impacts of ecological importance.

The development and use of biomarkers in ecotoxicology is motivated by several factors. These factors include the inherent instabilities of many contaminants (which complicate measures of exposure by direct tissue residue analysis), the relative biological sensitivities of many biomarkers, and the chemical specificity of some biomarkers on underlying mechanisms of toxin action. Additionally, the variables associated with these levels are often relatively insensitive to chemicals, such as some pesticides and other stressors, take long periods of time to manifest, and have difficult or imprecise methods of analysis. Thus, biomarkers can provide sensitive early warning signals of incipient ecological damage. However, biomarkers do not provide adequate standard data in the context of the ecological assessment of contaminant effects. At this time and for the foreseeable future, such assessments generally involve a "weight of evidence approach", coalescing information obtained from analyses, toxicity tests, biomarkers, and ecological indicators, which are sometimes referred to as bioindicators.

5. References

Abhishek, S.; Kaur, N.; Kaur, S.; Lata, M.; Sharma, J.K. & Sharma, A. (2010). Association of GSTM1 and GSTT1 gene deletions with susceptibility to DNA damage in the pesticide-exposed workers of Punjab. *Rejuvenation Research*, Vol.13, No.2-3, (April 2010), pp. 281-284, ISSN 1549-1684

Akgür, S.A.; Oztürk, P.; Solak, I.; Moral, A.R. & Ege, B. (2003). Human serum paraoxonase (PON1) activity in acute organophosphorous insecticide poisoning. *Forensic Science International*, Vol.133, No.1-2, (May 2003), pp. 136-140, ISSN 0379-0738

Ananthan, J.; Goldberg A.L. & Voellmy, R. (1986). Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science*, Vol.232, No.4749, (April 1986), pp. 522-524, ISSN 0036-8075

Angerer, J.; Ewers, U. & Wilhelm, M. (2007). Human biomonitoring: state of the art. *International Journal of Hygiene and Environmental Health*, Vol.210, No.3-4 (May 2007), pp. 201-228, ISSN 1438-4639

Araoud M.; Neffeti F.; Douki W.; Najjar M.F & Kenani A. (2010). Paraoxonase 1 correlates with butyrylcholinesterase and gamma glutamyl transferase in workers chronically exposed to pesticides. *Journal of Occupational Healt,* Vol.52, No.6, (October 2010), pp. 383-388, ISSN 1348-9585

Arcury, T.A.; Grzywacz, J.G.; Chen, H.; Vallejos, Q.M.; Galván, L.; Whalley, L.E.; Isom, S.; Barr D.B. & Quandt, S.A. (2009). Variation across the agricultural season in organophosphorus pesticide urinary metabolite levels for Latino farmworkers in eastern North Carolina: project design and descriptive results. *American Journal of Industrial Med*icine, Vol.52, No.7, (July 2009), pp. 539-50, ISSN 1097-0274

Atherton, K.M.; Williams, F.M.; Egea, F.J.; Glass, R.; Rushton, S.; Blain, P.G. & Mutch E. (2009). DNA damage in horticultural farmers: a pilot study showing an association with organophosphate pesticide exposure. *Biomarkers: biochemical indicators of exposure, response, and susceptibility to chemicals,* Vol.14, No.7, (October 2009), pp. 443-451, ISSN 1366-5804

Attademo, A.M.; Cabanga-Zenklusen, M.; Lajmanovich, R.C.; Peltzer, P.M.; Junges, C. & Bassó A. (2011). B-esterase activities and blood cel morphology in the frog Leptodactylus chaquensis (Amphibia: Leptodactylidae) on rice agroecosystems from Santa Fe Province (Argentina). *Ecotoxicology,* Vol.20, No.1, (January 2010), pp. 274-82, ISSN 093-9292

Barr, D.B.; Allen, R.; Olsson, A.O.; Bravo, R.; Caltabiano, L.M.; Montesano, A.; Nguyen, J.; Udunka, S.; Walden, D.; Walker, R.D.; Weerasekera, G.; Whitehead, R.D. Jr.; Schober, S.E. & Needham, L.L. (2005). Concentrations of selective metabolites of organophosphorus pesticides in the United States population. *Environmental Researchs*, Vol.99, No.3, (November 2005), pp. 314-26, ISSN 0013-9351

Barrett, J.C.; Vainio, H.; Peakall, D. & Goldstein, B.D. (1997). Twelfth meeting of the scientific group on methodologies for the safety evaluation of chemicals: susceptibility to environmental hazards. *Environmental Health Perspectives*, Vol.105, No.4, (November 1997), pp. 699–737, ISSN 0091-6765

Bauman, J.W.; Liu J. & Klaassen, C. (1993). Production of metallothioneins and heat shock proteins in response to metals. *Fundamental and Applied Toxicology*, Vol.21, No.1, (July 1993), pp. 15-22, ISSN 0272-0590

Bernal-Hernández, Y.Y.; Medina-Díaz, I.M.; Robledo-Marenco, M.L.; Velázquez-Fernández, J.B.; Girón-Pérez, M.I.; Ortega-Cervantes, L.; Maldonado-Vázquez, W.A. & Rojas-García, A.E. (2010). Acetylcholinesterase and metallothionein in oysters (*Crassostrea corteziensis*) from a subtropical Mexican Pacific estuary. *Ecotoxicology*, Vol.19, No.4, (January 2010), pp. 819-825, ISSN 0963-9292

Bhalli, J.A.; Khan, Q.M.; Haq, M.A.; Khalid, A.M. & Nasim, A. (2006). Cytogenetic analysis of Pakistani individuals occupationally exposed to pesticides in a pesticide production industry. *Mutagenesis,* Vol.21, No.2, (March 2006), pp. 143-148, ISSN 0267-8357

Binelli, A.; Ricciard, F.; Riva, C. & Provini, A. (2006). Integrated use of biomarkers and bioaccumulation data in zebra mussel (*Dreissena polymorpha*) for site-specific quality assessment. *Biomarker*. Vol.11, No.5, (September 2006), pp. 428–448, ISSN 13665804

Black, R.M.; Harrison, J.M. & Read, R.W. (1999). The interaction of sarin and soman with plasma proteins: the identification of a novel phosphorylation site. *Archives Toxicology,* Vol.73, No.2, (March 1999), pp. 123-6, ISSN 1432-0738

Blatter-Garin, M.C.; James, R.W.; Dussoix, P.; Blanché, H.; Passa, P.; Froguel P. & Ruiz, J. (1997). Paraoxonase polymorphism Met-Leu54 is associated with modified serum

concentrations of the enzyme. A possible link between the paraoxonase gene and increased risk of cardiovascular disease in diabetes. *The Journal of Clinical Investigation*, Vol.99, No.1, (January 1997), pp. 62–66

Bosch de Basea, M.; Alguacil, J.; Puigdomènech, E.; Gasull, M.; Garrido, J.A. & López, T. (2010). Relationships between occupational history and serum concentrations of organochlorine compounds in exocrine pancreatic cancer. *Occupational Environmental Medicine* Vol.68, No.5, (November 2010), pp. 332-338, ISSN 1470-7926

Brophy, V.H.; Jampsa, R.L.; Clendenning, J.B.; Mckinstry, L.A. & Furlong, C.E. (2001). Effects of 5´ regulatory-region polymorphisms on paraoxonase-gene (PON1) expression. *American Journal of Human Genetics,* Vol. 68, No.1, (June 2001), pp. 1428-1436, ISSN 0002-9297

Brophy, V.H.; Jarvik, G.P.; Richter, R.J.; Rozek, L.S.; Schellenberg, G.D. & Furlong, C.E. (2000). Analysis of paraoxonase (PON1) L55M status requires both genotype and phenotype. *Pharmacogenetics,* Vol.10, No.5, (July 2000), pp. 453-460, ISSN 1744-6872

Browne, W.R. & Feringa, B.L. (2006). Making molecular machines work. *Nature Nanotechnology*. Vol.1, No.1, (July 2008), pp. 25-35, ISSN 1748-3387

Bull, S.; Fletcher, K.; Boobis, A.R. & Battershill, J.M. (2006). Evidence for genotoxicity of pesticides in pesticide applicators: a review. *Mutagenesis,* Vol.21, No.2, (March 2006), pp. 93-103, ISSN 0267-8357

Buratti, F.M.; D'Aniello, A.; Volpe, M.T.; Meneguz, A. & Testai, E. (2005). Malathion bioactivation in the human liver: the contribution of different cytochrome p450 isoforms. *Drug Metabolism Disposition,* Vol.33, No.3, (March 2005), pp. 295-302, ISSN 0090-9556

Carbonell, G.; Martinez-Pereida, J.A. & Tarazona, J.V. (1998). Mobilization of essential metals during and after short-term lethal cadmium exposure in rainbow trout, *Oncorhynchus mykiss*. *Ecotoxicology and Environmental Restoration*, Vol.1, No.2, (July 1998), pp. 85-91

Cavallo, D.; Cinzia, L.U.; Carelli, G.; Iavicoli, I.; Ciervo, A.; Perniconi, B.; Rondinone, B.; Gismondi, M. & IavicoLi, S. (2006). Occupational exposure in airport personnel characterization and evaluation of genotoxic and oxidative effects. *Toxicology*, Vol.223, No.1-2, (April 2006), pp. 26-35, ISSN 0300-483X

Ceratto, N.; Dondero, F.; van de Loo, J.W.; Burlando, B. & Viarengo, A. (2002). Cloning and sequencing of a novel metallothionein gene in Mytilus galloprovincialis Lam. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology,* Vol.131, No.3, (March 2002), pp. 217–222, ISSN 1532-0456

Chandrasekera, H.U & Pathiratne, A. (2005). Acetylcholinesterase inhibition and haematological alterations induced in common carp, (*Cyprinus carpio*) following exposure to low concentrations of Trichlorfon. *Journal of Aquaculture Research*, Vol.36, No.36, pp. 146-150, ISSN 1355-557X

Chevrier, C.; Limon, G.; Monfort, C.; Rouget, F.; Garlantézec, R.; Petit, C.; Durand, G. & Cordier, S. (2011). Urinary Biomarkers of Prenatal Atrazine Exposure and Adverse Birth Outcomes in the PELAGIE Birth Cohort. *Environmental Health Perspective,* (March 2011), pp. 1-32, ISSN 0091-6765

Chitmanat, C.N.; Prakobsin, P.; Chaibu, P. & Traichaiyaporn, S. (2008). The use of acetylcholinesterase inhibition in river snails (*Sinotaia ingallsiana*) to determine the pesticide contamination in the Upper Ping River. *International Journal of Agriculture & Biology*, Vol.10, No.6, (July 2008), pp. 658–660, ISSN 1560–8530

Clare, M.G.; Lorenzon, G.; Akhurst, L.C.; Marzin, D.; van Delft, J.; Montero, R.; Botta, A.; Bertens, A.; Cinelli, S.; Thybaud, V. & Lorge, E. (2006). SFTG international collaborative study on in vitro micronucleus test II. Using human lymphocytes. *Mutation Research*, Vol.607, No.1, (June 2006), pp. 37-60, ISSN 0027-5107

Cocker, J.; Mason, H.J.; Garfitt, S.J. & Jones, K. (2002). Biological monitoring of exposure to organophosphate pesticides. *Toxicology Lett*ers, Vol.134, No.1-3, (August 2002), pp. 97-103, ISSN 0378-4274

Cook, P.M., Zabel, E.W. & Peterson, R.E. (1997). The TCDD toxicity equivalence approach for characterizing risks for early life-stage mortality in trout. In *Chemically Induced Alterations in Functional Development and Reproduction of Fishes*, Rolland, R.M.; Gilbertson, M. & Peterson, R. E. Editors, pp. 9–27, SETAC Press, Pensacola, FL, ISBN 1880611198, Racino, Wisconsin

Costa, L.G. & Manzo, L. (1995). Biochemical markers of neurotoxicity: research strategies and epidemiological applications. *Toxicology letters,* Vol.77, No.1-77, (May 1995), pp. 137-144, ISSN 0378-4274

Costa, L.G.; Richter, R.J.; Li, W.F.; Cole, T.; Guizzetti, M. & Furlong, C.E. (2003). Paraoxonase (PON 1) as a biomarker of susceptibility for organophosphate toxicity. *Biomarkers: biochemical indicators of exposure, response, and susceptibility to chemicals*. Vol.8, No.1, (January 2010), pp. 1-12, ISSN 1354-750X

Currie, S.; Moyes, C.D. & Tufts, B.L. (2000). The effects of heat shock and acclimation temperature on Hsp70 and Hsp30 mRNA expression in rainbow trout: In vivo and in vitro comparisons. *Journal of Fish Biology*. Vol.56, No.2, (February 2000), pp. 398-408, ISSN 1095-8649

Currie, S.; Tufts, B.L. & Moyes, C.D. (1999). Influence of bioenergetic stress on heat shock protein gene expression in nucleated red blood cells of fish. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology.* Vol.276, No.4, (December 1998), pp. 990-996, ISSN 0363-6119

Deane, E.E; Li, J. &. Woo, N.Y.S. (2001). Hormonal status and phagocytic activity in sea bream infected with vibriosis. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. Vol.129, No.2-3, (June 2001), pp. 687-693, ISSN 1096-4959

Delaney, M.A. & Klesius, P.H. (2004). Hypoxic conditions induce Hsp70 production in blood, brain and head kidney of juvenile Nile tilapia *Oreochromis niloticus* (L.). *Aquaculture*. Vol.236, No.1-4, (June 2004), pp. 633-644, ISSN 0044-8486

Di Giulio R.T. & Hinton D.E. (2008). *The Toxicology of Fishes*. Taylor & Francis, London, ISBN 139780415248686, Boca Raton

Donato, M. (2004). Qué es el Citocromo P-450 y cómo funciona. *Instituto de España, Real Academia Nacional de Farmacia, Realigraf Madrid, Spain.* (08.2009), Available from: http://www.analesranf.com/index.php/mono/article/viewFile/515/533

Draganov, D.I. & La Du, B.N. (2004). Pharmacogenetics of paraoxonases: a brief review. *Nauny-Schmiedeberg's Archives Pharmacology,* Vol.369, No.1, (October 2004), pp. 78-88, ISSN 0028-1298

Duramad, P.; Harley, K.; Lipsett, M.; Bradman, A.; Eskenazi, B.; Holland, N.T & Tager, I.B. (2006). Early Environmental Exposures and Intracellular Th1/Th2 Cytokine Profilesin 24-Month-Old Children Living in an Agricultural Area. *Environmental Health Perspectives*, Vol.114, No.12, (December 2006), pp. 1916-1922, ISSN 0091-6765

Ellman, G.L.; Courtney, K.D.; Andres, V.Jr. & Feather-Stone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology,* Vol.7, (July, 1961), pp. 88-95, ISSN 0006-2952

Ergene, S.; Celik, A.; Cavaş, T. & Kaya, F. (2007). Genotoxic biomonitoring study of population residing in pesticide contaminated regions in Goksu Delta: micronucleus, chromosomal aberrations and sister chromatid exchanges. *Environment international,* Vol.33, No.7, (May 2007), pp. 877-885, ISSN 0160-4120

Espíritu, P. (2008). Los polimorfismos genéticos del Citocromo P450 y su relevancia en el metabolismo de xenobióticos. Infármate. 08.2009, Available from http://www.infarmate.org/pdfs/infarmate21/metabolismo.pdf.

Everett, J. C. & Matheson, M. E. (2010). Biomarkers of pesticide exposure and diabetes in the 1999–2004 National Health and Nutrition Examination Survey. *Environment International,* Vol.36, (June 2009), pp. 398-401, ISSN 01604120

Farag, A.M.; Stansbury, M.A.; Hogstrund, C.; MacConnell, E. & Bergman, H. (1995). The physiological impairment of free-ranging brown trout exposed to metals in the Clarke Fork River, Montana. *Canadian Journal of Fisheries and Aquatic Sciences*, Vol.52, pp. 2038–2050, ISSN 0706-652X

Farahat, F.M.; Ellison, C.A.; Bonner, M.R.; McGarrigle, B.P.; Crane, A.L.; Fenske, R.A.; Lasarev, M.R.; Rohlman, D.S.; Anger, W.K.; Lein, P.J. & Olson, J.R. (2011). Biomarkers of Chlorpyrifos Exposure and Effect in Egyptian Cotton Field Workers. *Environmental Health Perspectives,* [Epub ahead of print], (January 2011), ISSN 0091-6765

Feder, M.E. & Hofmann, G.E. (1999). Heat shock proteins, molecular chaperones and stress response: Evolutionary and ecological physiology. *Annual Review of Physiology,* Vol.61, No.1, (March 1999), pp. 243-282, ISNN 0066-4278

Fernández, M. (2005). Fundamentos de Farmacología Básica y Clínica. Primera edición. Ed. Ramón Arece, ISBN, 9788480046893, Madrid Spain

Fulton, M.H. & Key, P.B. (2001). Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environmental Toxicology and Chemistry,* Vol.20, No.1, (January 2001), pp.37–45, ISSN 1552-8618

Furlong, C.E.; Cole, T.B.; Jarvik, G.P.; Pettan-Brewer, C.; Geiss, G.K.; Richter, R.J.; Shih, D.M.; Tward, A.D.; Lusis, A.J. & Costa, L.G. (2005). Role of paraoxonase (PON1) status in pesticide sensitivity: genetic and temporal determinants. *Neurotoxicology,* Vol.26, No.4, pp. 651-659, (August 2008), ISSN 0161-813X

Gamlin, J.; Diaz Romo, P. & Hesketh, T. (2007). Exposure of young children working on Mexican tobacco plantations to organophosphorous and carbamic pesticides, indicated by cholinesterase depression. *Child: Care Health and Development,* Vol.33, No.3, (May 2007), pp. 246-248, ISSN 0305-1862

Garabrant, D.H.; Aylward, L.L.; Berent, S.; Chen, Q.; Timchalk, C.; Burns, C.J.; Hays, S.M. & Albers, J.W. (2009). Cholinesterase inhibition in chlorpyrifos workers: Characterization of biomarkers of exposure and response in relation to urinary TCPy. *Journal of Exposure Analysis and Environmental Epidemiology*, Vol.19, No.7 (November 2009), pp. 634-42, ISSN 1559-064X

Garaj-Vrhovac, V. & Zeljezic, D. (2001). Cytogenetic monitoring of croatian population occupationally exposed to a complex mixture of pesticides. *Toxicology,* Vol.165, No.2-3, (August 2001), pp. 153-62, ISSN 0300-483X

Garin, M.C.; James, R.W.; Dussoix, P.; Blanche, H.; Passa, P.; Froguel, P. & Ruiz, J. (1997). Paraoxonase polymorphism Met–Leu is associated with modified serum concentrations of the enzyme a possible link between the paraoxonase gene and increased risk of cardiovascular disease in diabetes. *Journal of Clinical Investigation*, Vol.99, No.1, (January 2003), pp. 62-66, ISSN 0021-9738

Geffard, A; Amiard, J.C. & Amiard-Triquet, C. (2002). Use of metallothionein in gills from oysters (*Crassostrea gigas*) as a biomarker: seasonal and intersite fluctuations. *Biomarkers,* Vol.7, No.2, (March-April 2002), pp. 123–137, ISSN 1366-5804

Geffard, A; Amiard-Triquet, C; Amiard, J.C. & Mouneyrac, C. (2001). Temporal variations of metallothionein and metal concentrations in the digestive gland of oysters Crassostrea gigas from a clean and a metal-rich site. *Biomarkers,* Vol.6, No.2 (1 March 2001), pp. 91–107, ISSN 1366-5804

Gil Hernández, F. (2000). The role of biomarkers in human toxicology. *Revista de Toxicología,* Vol.17, No.1, pp. 19-26, ISSN 0212-7113

Grimán, P.; Morán, Y.; Camargo, M. & Chiurillo, M. (2009). Caracterización de variantes alélicas de Citocromo CYP2D6 en la población de la región centroccidental de Venezuela. *Acta Biológica Colombiana*, Vol.14, No.1, (2009), pp. 195-202, ISSN 1900-1649

Grzywacz, J.G.; Quandt, S.A.;Vallejos, Q.M.; Whalley, L.E.; Chen, H.; Isom, S.; Barr, D.B. & Arcury, T.A. (2010). Job demands and pesticide exposure among immigrant Latino farmworkers. *Journal of Occupational Health Psychology*, Vol.15, No.3, (July 2010), pp. 252-66, ISSN 1076-8998

Haley, R.W.; Billecke, S. & La Du, B.N. (1999). Association of low PON1 type Q (Type A) arylesterase activity with neurologic symptom complexes in Gulf War veterans. *Toxicology and Applied Pharmacology,* Vol.157, No.3, (June 1999), pp. 227-233, ISSN 0041-008X

Hamilton, S.J. & Mehrle, P.M. (1986). Metallothionein in fish: Review of its importance in assessing stress from metal contaminants. *Transactions of the American Fisheries Society,* Vol.115, No.4, (January 2011), pp. 596-609, ISSN 0002-8487

Hamilton, S.J. & Mehrle, P.M. (1986). Metallothionein in fish: review of its importance in assessing stress from metal contaminants. *Transactions of the American Fisheries Society,* Vol.115, No.4, pp. 596-609, ISSN 0002-8487

Helfrich, L.A.; Weigmann, D.L. & Stinson, E.R. (2009). Pesticides and Aquatic Animals: A Guide to Reducing Impacts on Aquatic Systems. Communications and Marketing, College of Agriculture and Life Sciences, Virginia Polytechnic Institute and State University. 08.2009, Available from http://pubs.ext.vt.edu/420/420-013/420-013_pdf.pdf

Henderson, R. (2005). Biomarker Human Health, In: Enciclopedia of Toxicology, Wexler P., (Editor in Chief), pp. 290-294, Elselvier, ISBN 0-12-745354-7, New York

Hernández, A. F.; López, O.; Rodrigo, L.; Gil, F.; Pena, G.; Serrano, J.L.; Parrón, T.; Álvarez, J.C.; Lorente, J.A. & Pla, A. (2005). Changes in erythrocyte enzymes in human long term exposed to pesticides influence of several markers of individual susceptibility. *Toxicology Letters,* Vol.159, No.1, (October 2005), pp. 13–21, ISSN 0378-4274

Herrero, C.; Ozalla, D.; Sala, M.; Otero, R.; Santiago-Silva, M.; Lecha, M.; To-Figueras, J.; Deulofeu, R.; Mascaró, J.M.; Grimalt, J. & Sunyer, J. (1999). Urinary porphyrin excretion in a human population highly exposed to hexachlorobenzene. *Archives of Dermatology,* Vol.135, No.4, (April 1999), pp. 400-404, ISSN 0003-987X

Hightower, L.E. (1991). Heat shock, stress proteins, chaperones, and proteotoxicity. *Cell,* Vol.66, No.2, (July 1991), pp 191-197, ISSN 0092-8674

Hinck, J.E.; Blazer, V.S.; Denslow, N.D.; Echols, K.R.; Gross, T.S.; May, T.W.; Anderson, P.J.; Coyle, J.J. & Tillitt, D.E. (2007). Chemical contaminants, health indicators, and reproductive biomarker responses in fish from the Colorado River and its tributaries. *Science of the Total Environmental*, Vol.378, No.3, (Jun 2007), pp. 376-402, ISSN 0048-9697

Holmstedt, B. (1959). Pharmacology of organophosphorus cholinesterase inhibitors. *Pharmacology Reviews,* Vol.11, (September 1959), pp. 567-688, ISSN 1521-0081

Huggett, R.J.; Kimerle, R.A.; Mehrle, P.M. & Bergman, H.L. (Editors) (1992). *Biomarkers: Biochemical, Physiological and Histological Markers of Anthropogenic Strees.* A special publication of the Society of Environmental Toxicology and Chemistry. Lewis Publishers, Boca Raton, FL, 1992, ISBN 087371-505-5

Iwama, G.K; Thomas, P.T; Forsyth, R.B. & Vijayan M.M. (1998). Heat shock proteins expression in fish. *Reviews in Fish Biology and Fisheries*, Vol.8, No.1, (October 1997), pp. 35-56, ISSN 0960-3166

Iwama, G.K; Vijayan, M; Forsyth, R.B. & Ackerman, P. (1999). Heat shock proteins and physiological stress in fish. *American Zoologist,* Vol.39, No.6, (January 1999), pp. 901-909, ISSN 0003-1569

Kagi, J.H. & Schaffer, A. (1988). Biochemistry of metallothionein. *Biochemistry,* Vol.27, No.23, (November 1988), pp. 8509-8515, ISSN 0006-2960

Kim, J.H.; Stevens, R.C.; MacCoss, M.J.; Goodlett, D.R.; Scherl, A.; Richter, R.J.; Suzuki, S.M. & Furlong, C. (2010). Identification and characterization of biomarkers of organophosphorus exposures in humans. *Advances in Experimental Medicine and Biology,* Vol.660, (2010), pp. 61-71, ISSN 0065-2598

Kimber, I. (1995). Biomarkers of immunotoxicity in man. *Human & Experimental Toxicology,* Vol.14, No.1, (January 1995), pp. 148-149, ISSN 0960-3271

Kisby, G.E.; Muniz, J.F.; Scherer, J.; Lasarev, M.R.; Koshy, M.; Kow, Y.W. & McCauley, L. (2009). Oxidative stress and DNA damage in agricultural workers. *Journal of Agromedicine,* Vol.14, No.2, (May 2009), pp. 206-214, ISSN 1545-0813

Klaassen, C.D. (2008). Cazarett and Doull's Toxicology the Basic Science of Poisons, (Seventh Edition), McGraw-Hill, ISBN 0071470514, New York

Klaassen, C.D.; Liu, J. & Choudhuri, S. (1999). Metallothionein: an intracellular protein to protect against cadmium toxicity. *Annual review of pharmacology and toxicology,* Vol.39, (May 1999), pp. 267-294, ISSN 0362-1642

Kumar, V.; Yadav, C.S.; Singh, S.; Goel, S.; Ahmed, R.S.; Gupta, S.; Grover, R.K. & Banerjee, B.D. (2010). CYP1A1 polymorphism and organochlorine pesticides levels in the etiology of prostate cancer. *Chemosphere*, Vol.81, No.4, (September 2010), pp. 464-468, ISSN 0045-6535

La Du, B.N.; Piko, J.I.; Eckerson, H.W.; Vincent-Viry, M. & Siestm, G. (1986). An improved method for phenotyping individuals for the human serum paraoxonase arylesterase polymorphism. *Annales de Biologie Clinique*, Vol.44, No.4, pp. 369-372, ISSN 0003-3898

Lacasaña, M.; López-Flores, I.; Rodríguez-Barranco, M.; Aguilar-Garduño, C.; Blanco-Muñoz, J.; Pérez-Méndez, O.; Gamboa, R.; Gonzalez-Alzaga, B.; Bassol, S. & Cebrian, M.E. (2010). Interaction between organophosphate pesticide exposure and PON1 activity on thyroid function. *Toxicology and Applied Pharmacology,* Vol.249, No.1, (August 2010), pp. 16-24, ISSN 1096-0333

Lambert, B.; Lindbland, A.; Holmberg, L. & Francesconi, D. (1982). The use of sister chromatid exchange to monitorhuman populations for exposure to toxicologically harmful agents, In: *Sister chromatid exchanges,* S. Wolff, Editors, pp. 149-182, Avery A. Sandberg, ISBN 0845124013, Wiley Nueva York

Lan, Q.; He, X.; Costa, D.J.; Tian, L.; Rothman, N.; Hu, G. & Mumford, J.L. (2000). Indoor coal combustión emissions, GSTM1 and GSTT1 genotypes, and lung cáncer risk: A case-control study in Xuan Wei, China. *Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* Vol.9, No.6, (July 2000), pp. 605-608, ISSN 1065-9965

Lee, T.K.; Allison, R.R.; O'Brien, K.F.; Naves, J.L.; Karlsson, U.L. & Wiley, A.L. (2002). Persistence of micronuclei in lymphocytes of cancer patients after radiotherapy. *Radiation research,* Vol.157, No.6, (May 2005), pp. 678-684, ISSN 0033-7587

Leite, P.Z.; Margarido, T.C.; de Lima, D.; Rossa-Feres, D. C. & de Almeida, E.A. (2010). Esterase inhibzition in tadpoles of Scinax fuscovarius (Anura, Hylidae) as a biomarker for exposure to organophosphate pesticides. *Environmental Science and Pollution Research,* Vol.17, No.8, (March 2010), pp.1411-1421, ISSN 0944-1344

Leviev, I. & James, R.W. (2000). Promoter polymorphisms of human paraoxonase PON1 gene and serum paraoxonase activities and concentrations. *Arteriosclerosis, Thrombosis, and Vascular Biology Arterioscler,* Vol.20, No.2, (February 2000), pp. 516–521, ISSN 1079-5642

Li, B.; Ricordel I.; Schopfer, L.M.; Baud, F.; Mégarbane, B.; Nachon, F.; Masson, P. & Lockridge, O. (2010a). Detection of adduct on tyrosine 411 of albumin in humans poisoned by dichlorvos. *Toxicological sciences: an official journal of the Society of Toxicology,* Vol.116, No.1, (April 2010), pp. 23-31, ISSN 1096-0929

Li, B.; Ricordel, I.; Schopfer, L.M.; Baud, F.; Mégarbane, B.; Masson, P. & Lockridge, O. (2010b). Dichlorvos, chlorpyrifos oxon and Aldicarb adducts of butyrylcholinesterase, detected by mass spectrometry in human plasma following deliberate overdose. *Journal of Applied Toxicology, Vol*.30, No.6, (August, 2010), pp. 559-65, ISSN 1099-1263

Li, H.; Ricordel, I.; Tong, L.; Schopfer, L.M.; Baud, F.; Mégarbane, B.; Maury, E.; Masson, P. & Lockridge, O. (2009). Carbofuran poisoning detected by mass spectrometry of butyrylcholinesterase adduct in human serum. *Journal of Applied Toxicology,* Vol.29, No.2, (October, 2008), pp. 149-155, ISSN 1099-1263

Lieberman, A.D.; Craven, M.R.; Lewis, H.A. & Nemenzo, J.H. (1998). Genotoxicity from domestic use of organophosphate pesticides. *Journal of Occupational and Environmental Medicine,* Vol.40, No.11, (November 1998), pp. 954-957, ISSN 1076-2752

Lionetto, M.G.; Caricato, R.; Giorda , M.E; Pascariello, M.F.; Mari sci, L. & Schetti , T. (2003). Integrated use of biomarkers (acetylcholinesterase and antioxidant enzymes activities) in Mytilus galloprovincialis and Mullus barbatus in an Italian coastal marine area. Marine Pollution Bulletin, Vo.46, No.3, (March, 2003), pp. 324-330, ISSN 0025-326X

Livingstone, D.R. (2001). Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Marine Pollution Bulletin*, Vol.42, No.8, (August 2001) pp. 656-666, ISSN 0025-326X

Lotti, M. (1995). Cholinesterase inhibition: complexities in interpretation. *Clinical Chemistry*, Vol.41, No.12 (1995), pp. 1814-1818, ISSN 0009-9147

Mackness, B.; Durrington, P.; Povey, A.; Thomson, S.; Dippnall, M.; Mackness, M.; Smith, T. & Cherry, N. (2003). Paraoxonase and susceptibility to organophosphorus

poisoning in farmers dipping sheep. *Pharmacogenetics,* Vol.13, No.2, (February 2003), pp. 81-88, ISSN 1744-6872

Mackness, B.; Durrington, P.N. & Mackness, M.I. (1998). Review: Human Serum Paraoxonase. *General Pharmacology*, Vol.31, No.3, (September 1998), pp. 329-336, ISSN 1537-1891

Martínez-Valenzuela, C. & Gómez-Arrollo, S. (2007). Riesgo genotóxico por exposición a plaguicidas en trabajadores agrícolas. *Revista Internacional de Contaminacion Ambiental,* Vol.23, No.4, (2007), pp. 185-200, ISSN 0188-4999

Minier, C.; Levy, F.; Rabel, D.; Bocquene, G.; Godefroy, D.; Burgeot T. & Leboulenger, F. (2000). Flounder health status in the Seine bay. A multimarkers study. *Marine Environmental Research,* Vol.50, No.1-5, (December 1999), pp. 373-377, ISSN 0141-1136

Misra, S.; Zafarullah, M.; Price-Haughey, J. & Gedamu, L. (1989). Analysis of stress induced gene expression in fish cell lines exposed to heavy metals and heat shock. *Biochimica et Biophysica Acta,* Vol.1007, No.3, (April 1989), pp. 325-333, ISSN 0167-4781

Møller, P. (2006).The alkaline comet assay: towards validation in biomonitoring of DNA damaging exposures. *Basic & clinical pharmacology & toxicology,* Vol.98, No.4, (April 2006), pp. 336-345, ISSN 1742-7835

Monserrat, J.M.; Bianchini, A.; Bainy, A.C. (2002). Kinetic and toxicological characteristics of acetylcholinesterase from the gills of oysters (Crassostrea rhizophorae) and other aquatic species. *Marine Environmental Research,* Vol.54, No.3-5, (May 2002), pp.781–785, ISSN 0141-1136

Nelson, D. (2002). *Cytochrome P450s in humans*. 08.2009, Available from: http://drnelson.utmem.edu/P450lect.html.

Noort, D.; Hulst, A.G.; van Zuylen, A.; van Rijssel, E. & van der Schans, M.J. (2009). Covalent binding of organophosphorothioates to albumin: a new perspective for OP-pesticide biomonitoring?. *Archives of Toxicology,* Vol.83, No.11, (Nov, 2009), pp. 1031-1036, ISSN 1432-0738

Norppa, H. (2004). Cytogenetic biomarkers and genetic polymorphisms. *Toxicology Letters,* Vol.149, No.1-3, (April 2004), pp. 309-334, ISSN 0378-4274

Ortega, C.L. (2007). Determinación del polimorfismo de la glutatión S transferasa M1 (GSTM1) en individuos fumadores. Tesis de licenciatura. Universidad Autónoma de Nayarit, México

Ortíz, E.; Lo Bello, M. & García L. (2001). Glutation S-transferasa P1-1 humana y glutatión reducido; estudio termodinámico y su interacción. (Fecha). Available from: http://campus.usal.es/~quimfis/resumen/Emilia_Ortiz.pdf

Oude Ophuis, M.B.; van Lieshout, E.M.; Roelofs, H.M.; Peters, W.H. & Manni J.J. (1998). Glutathione S-transferase M1 and T1 and cytochrome P4501A1 polymorphisms in relation to the risk for benign and malignant head and neck lesions. *Cancer*, Vol.82, No.5, (March 1995), pp. 936-943, ISSN 0008-543X

Ozalla, D.; Herrero, C.; Ribas-Fitó, N.; To-Figueras, J.; Toll, A.; Sala, M.; Grimalt, J.; Basagaña, X.; Lecha, M. & Sunyer J. (2002). Evaluation of Urinary Porphyrin Excretion in Neonates Born to Mothers Exposed to Airborne Hexachlorobenzene. *Environmental Health Perspectives,* Vol.110, No.2, (February 2002), pp. 205-209, ISSN 0091-6765

Padungtod, C.; Niu, T.; Wang, Z.; Savitz, D.A.; Christiani, D.C.; Ryan, L.M. & Xu, X. (1999). Paraoxonase polymorphism and its effect on male reproductive outcomes among Chinese pesticide factory workers. *American Journal of Industrial Medicine,* Vol.36, No.3, (September 1999), pp. 379-387, ISSN 0271-3586

Panemangalore, M.; Dowla, H.A. & Byers, M.E. (1999). Occupational exposure to agricultural chemicals: effect on the activities of some enzymes in the blood of farm workers. *International Archives of Occupational and Environmental Health,* Vol.72, No.2, (April 1999), pp. 84-88, ISSN 0340-0131

Pastor, S.; Creus, A.; Parron, T.; Cebulska-Wasilewska, A.; Siffel, C.; Piperakis, S. & Marcos, R. (2003). Biomonitoring of four European populations occupationally exposed to pesticides: use of micronuclei as biomarkers. *Mutagenesis*, Vol.18, No.3, (April 2003), pp. 249–258, ISSN 0267-8357

Peakall, D.B. (1994). The role of biomarker in environmental assessment. *Ecotoxicology,* Vol.3, No.3, (September 1994), pp. 157-160, ISSN 0963-9292

Peeples, E.S.; Schopfer, L.M.; Duysen, E.G.; Spaulding, R.; Voelker, T.; Thompson, C.M. & Lockridge, O. (2005). Albumin, a new biomarker or organophosphorus toxicant exposure, identified by mass spectrometry. *Toxicology Science,* Vol.83, No.2, (February, 2005), pp. 303-312, ISSN 1096-0929

Pérez-Herrera, N.; Polanco-Minaya, H.; Salazar-Arredondo, E.; Solís-Heredia, M. J.; Hernandez-Ochoa, I.; Rojas-García, E.; Alvarado-Mejia, J.; Borja-Aburto, V. H. & Quintanilla-Vega, B. (2008). PON1Q192R genetic polymorphism modifies organophosphorous pesticide effects on semen quality and DNA integrity in agricultural workers from southern Mexico. *Toxicology and Applied Pharmacology,* Vol.230, No.2, (July 2008), pp. 261-268, ISSN 0041-008X

Perkins, E.J.; Griffith, B.; Hobbs, M.; Gollon, J.; Wolford, L. & Schlenk, D. (1996). Sexual differences in mortality and sublethal stress in channel catfish following a 10 week exposure to copper sulfate. *Aquatic Toxicology*, Vol.37, No.4, (April 1997), pp. 327–339, ISSN 0166-445X

Potti, A.; KishorGanti, A.; Sholes, K.; Langness, E.; Koka, V.; Horvarth, L. & Koch, M. (2003). Effect of pesticide exposure on HER-2/neu overexpression seen in patients with extensive stage small cell lung carcinoma. *Clinical Cancer Research,* Vol.9, No.13, (October 2003), pp. 4872–4876, ISSN 1078-0432

Printes, L.B.; Fernandes, M.N. & Espíndola, E.L. (2011). Laboratory measurements of biomarkers and individual performances in Chironomus xanthus to evaluate pesticide contamination of sediments in a river of southeastern Brazil. *Ecotoxicology Environmental Safety,* Vol.74, No.3, (March, 2011), pp. 424-30, ISSN 0147-6513

Quistad, G.B.; Klintenberg, R. & Casida, J.E. (2005). Blood acylpeptide hydrolase activity is a sensitive marker for exposure to some organophosphate toxicants. *Toxicology Sci*ence, Vol.86, No.2, (August, 2005), pp. 291-299, ISSN 1096-0929

Ramírez, V. & Cuenca, P. (2002). [DNA damage in female workers exposed to pesticides in banana plantations at Limon, Costa Rica]. *Revista de Biologia Tropical,* Vol.50, No.2, (September 2002), pp. 507-518, ISSN 0034-7744

Recio, R.; Ocampo-Gómez, G.; Morán-Martínez, J.; Borja-Aburto, V.; López-Cervante, M.; Uribe, M.; Torres-Sánchez, L. & Cebrián M.E. (2005). Pesticide exposure alters follicle-stimulating hormone levels in Mexican agricultural workers. *Environmental Health Perspectives,* Vol.113, No.9, (September 2005), pp. 1160-1163

Recio, R.; Robbins, W.A.; Borja-Aburto, V.; Morán-Martínez, J.; Froines, J.R.; Hernández, R.M. & Cebrián, M.E. (2001). Organophosphorous pesticide exposure increases the frequency of sperm sex null aneuploidy. *Environmental Health Perspectives,* Vol.109, No.12, (December 2001), pp. 1237-1240

Rendón von Osten, J.; Epomex, C.; Tinoco-Ojanguren, R.; Soares, A.M. & Guilhermino, L. (2004). Effect of pesticide exposure on acetylcholinesterase activity in subsistence

farmers from Campeche, Mexico. *Archives of Environmental Health*, Vol.59, No.8, (August 2004), pp. 418-425, ISSN 0003-9896

Richards, P.G.; Johnson, M.K. & Ray, D.E. (2000). Identification of Acylpeptide Hydrolase as a Sensitive Site for Reaction with Organophosphorus Compounds and a Potential Target for Cognitive Enhancing Drugs. *Molecular Pharmacology*, Vol.58, No.3, (September 2000), pp. 577-83, ISSN 1521-0111

Rodriguez-Fuentes & Gold-Bouchot, G. (2000). Environmental monitoring using acetylcholinesterase inhibition in vitro. A case study in two Mexican lagoons. *Marine Environmental Research,* Vol.50, No.1-5 (July 2000), pp. 357-360, ISSN 0141-1136

Rojas-Garcia, A.E.; Medina-Diaz, I.M.; Robledo-Marenco, M.L.; Barron-Vivanco, 1 B.S.; Giron- Perez, M.I.; Velazquez-Fernandez, J.B.; Gonzalez-Arias, C.A.; Albores-Medina, A.; Quintanilla-Vega, B.; Ostrosky-Wegman, P.; Rojas-Garcia, M.C.; Perez-Herrera, N.E. & Lopez-Flores, J.F. (2011). Hematological, Biochemical Effects, and Self reported Symptoms in Pesticide Retailers. *Journal of Occupational and Environmental Medicine*, Vol.53, No.5, (March 2011), pp. 517-521, ISSN 1076-2752

Rojas-García, A.E.; Solís-Heredia, M.J.; Piña-Guzmán, B.; Vega, L.; López-Carrillo, L. & Quintanilla-Vega, B. (2005). Genetic polymorphisms and activity of PON1 in a Mexican population. *Toxicology and Applied Pharmacology,* Vol.205, No.3, (June 2005), pp. 282-289, ISSN 0041-008X

Rosin, M.P. & Gilbert, A.M. (1990). Modulation of genotoxiceffects in humans. *Progress in clinical and biological research,* Vol.340E, (January 1990), pp. 351-359, ISSN 0361-7742

Sanders, B.M. (1993). Stress proteins in aquatic organisms: An environmental perspective. *Critical Reviews in Toxicology*, Vol.23, No.1. pp. 49-75, ISSN 1040-8444

Sanders, B.M., 1990. Stress Proteins: Potential as Multitiered Biomarkers. In: Biomarkers of Environmental Contamination, McCarthy, J.F. and L.R. Shugart, (Eds.). Lewis, Boca Raton, pp: 165-191

Santiago, C.; Bandrés, F. & Gómez-Gallego, F. (2002). Polimorfismos de Citocromo p450: Papel como marcador biológico. *Medicina del Trabajo*, Vol.11, No.3, (Mayo-Junio 2002), pp. 130-140

Schaeffeler, E.; Schwab, M.; Eichelbaum, M. & Zanger U. (2003). CYP2D6 Genotyping Strategy Based on Gene Copy Number Determination by TaqMan Real-Time PCR. *Human Mutation,* Vol.22, No.6, (November 2003), pp. 476-485, ISSN 1098-1004

Schlenk, D; Wolford, L; Chelus, M; Steevens, J; & Chan, K.M. (1997). Effect of arsenite, arsenate, and the herbicide, monosodium methylarsonate (MSMA) on hepatic metallothionein expression and lipid peroxidation in channel catfish. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology,* Vol.118, No.2, (October 1997), pp. 177-183, ISSN: 1532-0456

Selden, A.I.; Floderus, Y.; Bodin, L.S.; Westberg, H.B. & Thunell, S. (1999). Porphyrin status in aluminum foundry workers exposed to hexachlorobenzene and octachlorostyrene. *Archives of environmental health,* Vol.54, No.4, (August 1999), pp. 248-253, ISSN 0003-9896

Sharma, B.; Rai, D.K.; Rai, P.K.; Rizvi, S.I. & Watal, G. (2010). Determination of erythrocyte fragility as a marker of pesticide-induced membrane oxidative damage. *Methods in molecular biology,* Vol.594, (January 2010), pp. 123-128, ISSN 1064-3745 Shugart, L.R. (2005). Biomarker Environmental, In: *Enciclopedia of Toxicology*, Wexler P., (Editor in Chief), pp 287-290, Elselvier, ISBN, 0-12-745354-7, New York

Shugart L.R, (2005). Biomarker Environmental, In: Enciclopedia of Toxicology, Wexler P., (Editor in Chief), pp. 287-290, Elselvier, ISBN 0-12-745354-7, New York

Singh, S.; Kumar, V; Thakur, S.; Banerjee, B.D.; Rautela, R.S.; Grover, S.S.; Rawat, D.S.; Pasha, S.T.; Jain, S.K.; Ichhpujani, R.L. & Rai, A. (2011). Paraoxonase-1 genetic polymorphisms and susceptibility to DNA damage in workers occupationally exposed to organophosphate pesticides. *Toxicology and Applied Pharmacology,* Vol.252, No.2, (February 2011), pp. 130-137, ISSN 0041-0084

Sirivarasai, J.; Kaojarern, S.; Yoovathaworn, K. & Sura, T. (2007). Paraoxonase (PON1) polymorphism and activity as the determinants of sensitivity to organophosphates in human. *Chemical Biological Interactions*, Vol.168, No.3, (July 2007), pp. 184-192, ISSN 0009-2797

Speit, G. & Hartmann, A. (2006).The comet assay: a sensitive genotoxicity test for the detection of DNA damage and repair. *Methods in molecular biology,* Vol.314, (May 2006), pp. 275-286, ISSN 1064-3745

Stanulla, M.; Schrappe, M.; Brechlin, A.M.; Zimmermann, M. & Welte, K. (2000). Polymorphisms within glutathione S-transferases genes (GSTM1, GSTT1, GSTP1) and risk of relapse in childhood B-cell precursor acute lymphoblastic leukemia: a case control study. *Blood,* Vol.95, No.4, (October 2000), pp. 1222-1228, ISSN 0006-4971

Stich, H.F. & Rosin, M.P. (1984). Micronuclei in exfoliated human cells as a tool for studies in cancer risk and cancer intervention. *Cancer letters,* Vol.22, No.3, (April 1984), pp. 241-253, ISSN 0304-3835

Stücker, I.; Hirvonen, A.; De Waziers, I.; Cabelguenne, A.; Mitrunen, K.; Cennée, S.; Koum-Besson, E.; Hémon, D.; Beaune, P. & Loriot, M.A. (2002). Genetic polymorphisms of glutathione S-transferases as a modulator of lung cancer susceptibility. *Carcinogenesis*, Vol.23, No.9, (May 2002), pp. 1475-1481, ISSN 0143-4434

Sunyer, J.; Garcia-Esteban, R.; Alvarez, M.; Guxens, M.; Goñi, F.; Basterrechea, M.; Vrijheid, M.; Guerra, S. & Antó, J.M. (2010). DDE in mothers' blood during pregnancy and lower respiratory tract infections in their infants. *Epidemiology,* Vol.21, No.5, (September 2010), pp. 729-35, ISSN 1531-5487

Sunyer, J.; Herrero, C.; Ozalla, D.; Sala, M.; Ribas-Fito, N.; Grimalt, J. & Basagaña, X. (2002). Serum organochlorines and urinary porphyrin pattern in a population highly exposed to hexachlorobenzene. *Environmental health: a global access science source,* Vol.19, No.1, (December 2002), pp. 1, ISSN 1476-069X

Teraoka, H; Dong, W; Tsujimoto, Y; Iwasa, H; Endoh, D; Ueno, N; Stegeman, J.J; Peterson, R.E. & Hiraga, T. (2003). Induction of cytochrome P450 1A is required for circulation failure and edema by 2,3,7,8–tetrachlorodibenzo-p-dioxin in zebrafish. *Biochemical and Biophysical Research Communications*, Vol.304, No.2, (May 2003), pp. 223-228, ISSN 0006-291X.

Tice, R.R.; Agurell, E.; Anderson, D.;Burlinson, B.; Hartmann, A.; Kobayashi, H.; Miyamae, Y.; Rojas, E.; Ryu, J.C. & Sasaki, Y.F. (2000). Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environmental and molecular mutagenesis,* Vol.35, No.3, (March 2000), pp. 206-221, ISSN 0893-6692

Tlili, S.; Métais, I.; Boussetta, H. & Mouneyrac C. (2010). Linking changes at sub-individual and population levels in Donax trunculus: assessment of marine stress. *Chemosphere,* Vol.81, No.6, (October 2010), pp. 692-700, ISSN 0045-6535

Tope, A.M. & Panemangalore, M. (2007). Assessment of oxidative stress due to exposure to pesticides in plasma and urine of traditional limited-resource farm workers: formation of the DNA-adduct 8-hydroxy-2-deoxy-guanosine (8-OHdG). *Journal of Environmental Science and Health. Part. B, Pesticides, food contaminants, and agricultural wastes,* Vol.42, No.2, (March 2007), pp. 151-155, ISSN 0360-1234

Tsatsakis, A.M. Barbounis, M.G.; Kavalakis, M.; Kokkinakis M.; Terzi, I. & Tzatzarakis, M.N. (2010). Determination of dialkyl phosphates in human hair for the biomonitoring of exposure to organophosphate pesticides. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences,* Vol.878, No.17-18, (May 2010), pp. 1246-52, ISSN 1570-0232

Tsatsakis, A.M.; Zafiropoulos, A.; Tzatzarakis, M.N.; Tzanakakis, G.N. & Kafatos, A. (2009). Relation of PON1 and CYP1A1 genetic polymorphisms to clinical findings in a cross-sectional study of a Greek rural population professionally exposed to pesticides. *Toxicology Letters*, Vol.186, No.1, (April 2009), pp. 66-72, ISSN 0378-4274

Valavanidis, A. & Vlachogianni, T. (2010). "Integrated Biomarkers in Aquatic Organisms as a Tool for Biomonitoring Environmental Pollution and Improved Ecological Risk Assessment". 02.2010, Available from chem-tox-ecotox.org/wp/wp-content/.../01-January-20101.pdf

van Gestel, C.A.M & van Brummelen, T.C. (1996). Incorporation of the biomarker concept in ecotoxicology call for a redefinition of terms. *Ecotoxicology*, Vol.5, No.4, (August 1996), pp. 217-225, ISSN 0963-9292

van Loveren, H.; Steerenberg, P.A. & Vos, J.G. (1995). Early detection of immunotoxicity: from animal studies to human biomonitoring. *Toxicology Letters*, Vol.77, No.1-3, (May 1995), pp. 73-80, ISSN 0378-4274

Vlachogianni, T.; Dassenakis, M.; Scoullos, M.J. & Valavanidis, A. (2007). Integrated use of biomarkers (superoxide dismuatse, catalase and lipid peroxidation) in mussels Mytilus galloprovincialis for assessing heavy metals' pollution in coastal areas from the Saronikos Gulf of Greece. *Marine Pollution Bulletin*, Vol.54, No.9, (September 2007), pp. 1361-1371, ISSN 0025-326X.

Wilson, B.W.; Hooper, M.J.; Hansen, M.E. & Nieberg P.S. (1999). *Reactivation of organophosphorus inhibited AChE with oximes.* In: Organophosphates: Chemistry, Fate, and Effects (Chamberss JE, Levi PE, eds.), Academic Press, ISBN 0121673456, San Diego, CA

Wojtyniak, B.J.; Rabczenko, D.; Jönsson, B.A.; Zvezday, V.; Pedersen, H.S.; Rylander, L.; Toft, G.; Ludwicki, J.K.; Góralczyk, K.; Lesovaya, A.; Hagmar, L. & Bonde, J.P.; INUENDO research group. (2010). Association of maternal serum concentrations of 2,2', 4,4'5,5'-hexachlorobiphenyl (CB-153) and 1,1-dichloro-2,2-bis (p-chlorophenyl)-ethylene (p,p'-DDE) levels with birth weight, gestational age and preterm births in Inuit and European populations. *Environmental Health,* Vol.9, No.5, (September 2010), pp. 1-10

Wolff, M.S.; Engel, S.; Berkowitz, G.; Teitelbaum, S.; Siskind, J.; Barr, D.B. & Wetmur, J. (2007). Prenatal pesticide and PCB exposures and birth outcomes. *Pediatric Researcher,* Vol.61, No.2, (February 2007), pp. 243-250, ISSN 0031-3998

Zapata-Pérez, O.; Ceja-Moreno, V.; Olmos, M.R.; Pérez, M.T.; Río-García, M.D.; Yarto, M.; Mendoza-Cantú, A.; Ize-Lema, A.I.; Gavilán-García, A.; Felipe, S.T. & Gold-Bouchot, G. (2007). Ecotoxicological effects of POPs on ariidae Ariopsis felis (Linnaeus, 1766) from three coastal ecosystems in the Southern Gulf of Mexico and Yucatan Peninsula. *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering,* Vol.42, No.10, (August 2007), pp. 1513-20, ISSN 1532-4117.

Zou, Z.; Du, D.; Wang, J.; Smith, J.N.; Timchalk, C.; Li, Y. & Lin, Y. (2010). Quantum dot based immunochromatographic fluorescent biosensor for biomonitoring trichloropyridinol, a biomarker of exposure to chlorpyrifos. *Analitical Chemistry,* Vol.82, No.12, (June 2010), pp. 5125-33

10

Biological Markers of Human Exposure to Pesticides

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1. Introduction

Pesticides are used extensively throughout the world and, in recent years, their use has increased considerably. Large amounts of these chemicals are released into the environment and many of them affect non-target organisms, being a potential hazard to human health. Pesticide exposure is ubiquitous, due not only to agricultural pesticide use and contamination of foods, but also to the extensive use of these products in and around residences. Hence, all people are inevitably exposed to these toxics through environmental contamination or occupational use. The general population is exposed to the residue of pesticides, including physical and biological degradation products in air, water and food. Occupational exposure occurring at all stages of pesticide formulation, manufacture and application involves exposure to complex mixtures of different types of these chemicals.

Pesticides are responsible for several adverse effects on human health, and they represent a potential risk to human. Several studies revealed that the risk of neurodegenerative diseases, particularly Parkinson's and Alzheimer disease, as well as the increase in endocrine, immune and neuropsychological disorders are among the harmful effects of these compounds on human health (Stephens et al., 1995; Parron et al., 1996; Mathur et al., 2002; Baldi et al., 2003; Hernandez et al., 2003; Salvi et al., 2003; Kamel & Hoppin, 2004). Other reports indicated that pesticides possess a potential genotoxicity in occupationally exposed populations and they induced some types of cancers (Anwar, 1997; Mathur et al., 2002; Hernandez et al., 2003; Clary & Ritz, 2003; Rusiecki et al., 2004; Bolognesi, 2003).

Defending against such toxic compounds requires sensitive and specific detection of them and their biological effects. Thus, the biomonitoring is a useful tool for assessing exposure to pesticides and for the evaluation of potential health risks, because of the various routes of exposure involved and the possible combination of occupational and non-occupational exposures. The biological monitoring methods are becoming available for a greater variety and number of pesticides, and biomonitoring data are being used to validate questionnaires as meaningful indicators of exposure. However, these biomonitoring data are difficult to interpret, and currently there are no guidelines for their interpretation. The lack of human pharmacokinetic data for many pesticides impedes efforts to estimate external exposure or target organ dose. A major problem in interpreting biomonitoring studies is estimating the degree of exposure (Bolognesi, 2003; Remor et al., 2009). Moreover, the relationship between internal exposure and health effects was often unknown.

In recent years, increasing attention has been given to the development of biomarkers of human exposures to pesticides. These biological markers are used to detect the effects of pesticides before adverse clinical health effect occur. In human, biomarkers must be present in easily and ethically obtainable tissues such as blood or urine. Biomarkers are usually divided in three categories: biomarkers of exposure, of effect and of susceptibility.
Biological markers of exposure can be used to confirm and assess the exposure of populations to a particular substance. These biomarkers provide information on the potential or external dose, internal or absorbed dose and biologically effective dose of pesticide, and they can predict a change in risk of that disease, but it cannot predict a toxic effect (Benford et al., 2000). However, biomarkers of effect can be used to document either preclinical alterations or adverse health effects elicited by the external exposure and the absorption of a compound. These biomarkers might reflect an early stage in the development of a disease, and therefore may be predictive of eventual disease (Benford et al., 2000). Genotypes responsible for interindividual differences in enzymes involved bioactivation and detoxification of pesticides are recognised as biomarkers of susceptibility to pesticide toxicity. The individual susceptibility, caused by polymorphic key enzymes, plays a critical role in the assessment of exposure to pesticides.
This review will focus and describe selected aspects of the biomarkers used for the assessment of human exposure to pesticides.

2. Biomarkers

The term biomarker is used in a broad sense to include almost any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical or biological (WHO, 1993).
Biological markers for monitoring pesticide exposures are typically divided into three broad categories: Biomarkers of exposure, effect and biomarkers of susceptibility (Figure 1).

2.1 Biomarkers of exposure

The biomarkers of exposure constitute an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism (WHO, 1993).
Knowledge of exposure levels is a first step in risk-evaluation process, and can be acquired by measuring the dose of pesticide entering the body. Hence, biological indicator of exposure or dose is the measurement of chemical agents or their metabolites either in tissues, secreta, excreta, exhaled air, or any combination of them, in order to evaluate exposure and health risk compared to an appropriate reference (Aprea et al., 2002). These biomarkers can predict a change in risk of that disease, but it cannot predict a toxic effect (Benford et al., 2000).
The biomarkers of exposure can be divided into three groups: potential dose or external dose, internal or absorbed dose, and biologically effective dose.
Because human exposure to pesticides is multi-media and multi-route and varies with the usage of pesticides, environmental monitoring of exposure which determines the potential dose, must account for all media and routes in order to accurately calculate individual exposures.
Biomarkers of internal dose integrate all pathways of exposure by estimating the amount of pesticide that is absorbed into the body via measurements of the pesticide, its metabolite, or its reaction product in biological media.

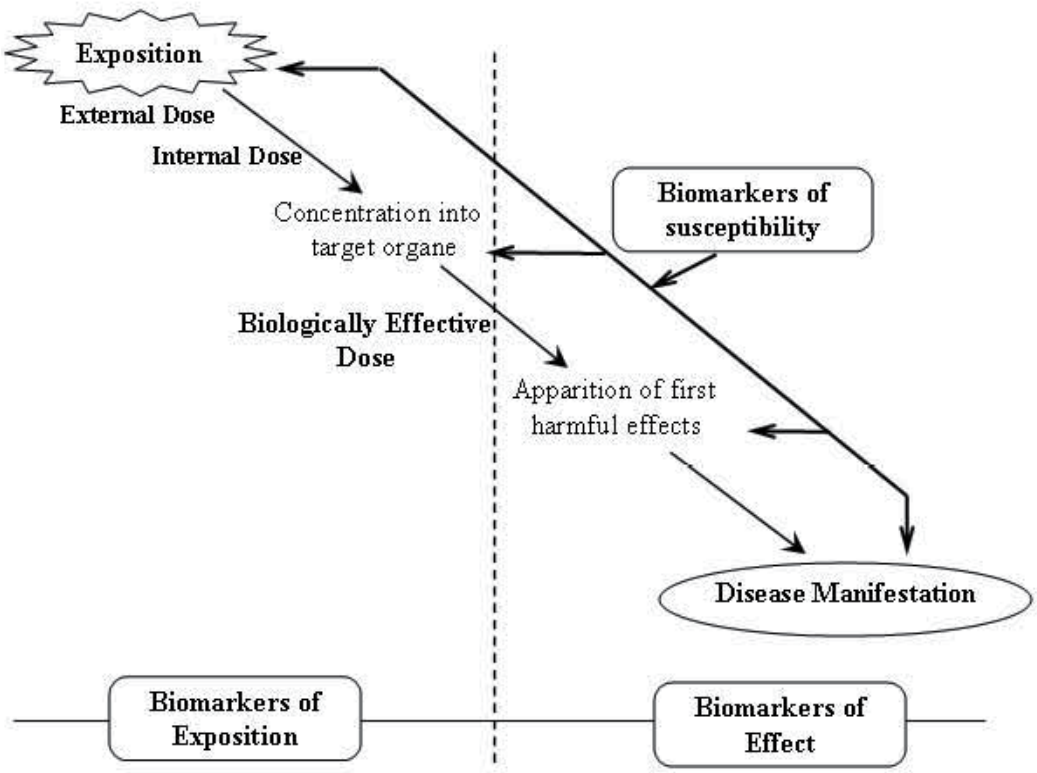

Fig. 1. Categories of biological markers of toxicity

The biologically effective dose is the amount of a toxicant that has interacted with a target site and altered a physiological function (Aprea et al., 2002).
Biological indicators of exposure can usually be measured by different analytical methods. These methods all use some form of chromatography, but the detection systems range from simple UV absorbance detection to sophisticated mass spectrometric analyses (Frias et al., 2001; Smith et al., 2002; Barr et al., 2002; Aprea et al., 2002; Bouwman et al., 2006; Russo et al., 2002; Lacassie et al., 2001a). They have been used to measure pesticides and/or their metabolites in a variety of matrices including urine, serum, breast milk, adipose tissus and postpartum meconium.
This followed section will focus the main biological indicators of exposure to insecticides.

2.1.1 Intact compounds

2.1.1.1 Organochlorine compounds

Organochlorine (OC) pesticides are a broad class of pesticides that were widely used as insecticides in the 1950 and 1960s. Their use was subsequently discontinued in many countries due to persistent contamination of the environment. Determination of intact OC compounds is a valuable method to monitor short- and long-term exposures to these pesticides (Table1). Exposure to OC pesticides has been investigated by several studies, in occupationally exposed subjects and in the general population by measuring intact compounds in blood, urine, adipose tissue and human milk (Frias et al., 2001; Barr et al.,

2002; Heudorf et al., 2003; Thomas et al., 2006; Bouwman et al., 2006; Ennaceur et al., 2008; Tsatsakis et al., 2009; Turci et al., 2010). Dichlorodiphenyltrichloroethane (DDT), dieldrin, hexachlorocyclohexane isomers (HCHs) and heptachlor-epoxide are the pesticide residues most frequently found in biological fluids of the general population.

2.1.1.2 Organophosphorous compounds

Measurement of unchanged organophosphorous (OP) pesticides in blood, and/or urine, or in gastric content has often been performed to confirm exposure in acute poisoning cases (Maroni et al., 2000a; Lacassie et al., 2001a,b; Aprea et al., 2002; Inoue et al., 2007). In fatalities, unchanged compounds may be measured in central nervous system and other tissues (Aprea et al., 2002). However, this method is unfavourable for biological monitoring of occupational exposure. At the dose levels usually found in exposed workers, OP compounds disappear quickly from blood and are rapidly excreted in urine at concentrations usually too low to be detected (Maroni et al., 2000a).

2.1.1.3 Carbamate compounds

Measurement of unmodified carbamate (CB) pesticides in blood and/or urine has often been performed to confirm exposure in acute poisoning cases (Table1), in order to identify the agent responsible for the intoxication (Lee et al., 1999; Lacassie et al., 2001b). In fatal cases, intact compounds may be measured in various organs. In cases of occupational exposure, unmodified compounds is rarely measured since the metabolic pathway of these substances is very complex, and brings forth a number of polarized molecules, which are more water-soluble than parent compounds (Maroni et al., 2000b; Aprea et al., 2002).

2.1.1.4 Pyrethroids compounds

Synthetic pyrethroids are a group of insecticides largely used in agriculture and public health because of their relatively low toxicity to man and mammalian species at the usual application rates, and because of their short environmental persistence. Occupational exposure to pyrethroids may be assessed by measuring intact compounds or their metabolites in urine. As a result of their rapid metabolism, determination of blood concentrations can only be used to reveal recent high level exposures (Table 1). Because of their rapid elimination, unmodified compounds are less sensitive indicators of exposure than metabolites, although certainly more specific (Aprea et al., 2002). In field workers exposed through the dermal route, urinary excretion of intact compounds and metabolites peaked 36 hours after exposure.
Table1 summarized some examples of intact insecticide compounds measured in human biological samples.

Pesticides	Exposure characteristics	Sample	Analytical methods	References
Organochlorine compounds				
α- et β-endosulfan	Acute intoxication cases	Serum	GC/MS*	Lacassie et al., 2001b
Lindane, Aldrin, Vinclozolin, p,p'-DDT, o,p'-DDT	Women living agricultural area	Serum	GC/ECD* GC/MS/MS*	Frias et al., 2001
β-HCH, p,p'-DDT, o,p'-DDT	General population	Serum	GC/MS*	Turci et al., 2006

Pesticides	Exposure characteristics	Sample	Analytical methods	References
β-HCH, p,p'-DDT	General adult population	Serum	GC/MS*	Thomas et al., 2006
DDT, HCH, dieldrin	General population	Breast milk	GC/ECD*	Ennaceur et al., 2008
α-HCH, p,p'-DDT, lindane	Population professionally exposed to pesticides	Serum	GC/MS*	Tsatsakis et al., 2009
Organophosphorous compounds				
Parathion-ethyl	Poisoning cases	Serum	GC/MS*	Lacassie et al., 2001a,b
Fenitrothion, diazinon, acephate	Poisoning cases	Serum	LC/MS*	Inoue et al., 2007
Diazinon, malathion, chlorpyriphos	Population professionally exposed to pesticides	Serum	GC/MS*	Tsatsakis et al., 2009
18 OP detected 20 OP detected	Patients with various causes of death (without pathologies or with cancer pathologies)	Kidney, Liver, adipose tissues	GC/MS*	Russo et al., 2002
Carbamates compounds				
Furathiocarb	Fatal poisoning case	Gastric contents	GC/MS*	Lee et al., 1999
Carbofuran, aldicarb	Poisoning cases	Serum	LC/MS*	Lacassie et al., 2001b
Carbofuran Carbendazime	Agricultural workers	Serum	LC/MS/MS*	Araoud et al., 2010
Pyrethroid compounds				
Bifenthrin	Poisoning cases	Serum	GC/MS*	Lacassie et al., 2001b
Cyfluthrin, Cypermethrin, Permethrin, Deltamethrin	General population	Breast milk	GC/ECD* GC/MS*	Bouwman et al., 2006

**: GC/MS: gas chromatography coupled to mass spectrometry; GC/ECD: gas chromatography coupled to electron-capture detector;*
GC/MS/MS: gas chromatography tandem mass spectrometry; LC/MS/MS: liquid chromatography tandem mass spectrometry.

Table 1. Examples of intact insecticide compounds measured in human biological samples

2.1.2 Pesticide metabolites

2.1.2.1 OC metabolites

Biological monitoring of OC can be carried out by determination of their metabolites in biological samples (Frias et al., 2001; Aprea et al., 2002; Bouwman et al., 2006; Ennaceur et al., 2008; Tsatsakis et al., 2009). Table2 lists the main OC pesticides and their metabolites, for which data on the use of biological indicators of internal dose have been found in literature.

Compounds	Metabolites	Biological samples
Dichlorodiphenyl trichloroethane (DDT)	Mainly p,p'-dichlorodiphenyl dichloroethylene (p,p'-DDE)	Adipose tissue, blood, breast milk, urine
Aldrin	Dieldin	Blood, serum, fatty tissue, milk
Endrin	Anti-12-hydroxy-endrin	Urine
Heptachlore	Heptachlore-epoxide	Milk, serum, adipose tissue, urine

Table 2. Metabolites of main OC pesticides used for biological monitoring of human exposure to OC

2.1.2.2 OP metabolites

In the human body, the main metabolic reaction, common to all OP, is hydrolysis of the ester bond, with the production of alkylphosphate derivatives and chemical residues specific for each compound. Most OP compounds are metabolized yielding six dialkylphosphates (DAP), as terminal products, which are excreted in urine. These six metabolites are: dimethylphosphate (DMP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethylphosphate (DEP), diethylthiophosphate (DETP), and diethyldithiophosphate (DEDTP) (Table3). These DAP were among biomarkers of OP exposure used in epidemiology of children's environmental health (Wessels et al., 2003). They are suitable for biological monitoring of exposure to OP, and the determination of the alkylphosphate metabolites in urine is the most practical and widely used method to estimate the internal dose of most OP pesticides used worldwide (Angerer et al., 2007; Bouchard et al., 2006; Margariti & Tsatsakis., 2009; Dulaurent et al., 2006). However, it is necessary to understand the kinetics of metabolite excretion to know the optimum time for urine collection. Besides, not all OP are metabolized to a measurable level of DAP (Wessels et al., 2003).

In addition to DAP, some pesticide-specific metabolites of OP frequently measured in urine for biological monitoring of human population exposed to OP pesticides, such as para-nitrophenol (PNP) and trichloro-pyridinol (TCP) (Hryhorczuk et al., 2002; Aprea et al., 2002, Maroni et al., 2000a) are listed in Table3. In general, they are approved as useful indices of recent exposure and used for risk assessment of OP pesticides as a biological indicator of exposure (Aprea et al., 2002).

2.1.2.3 CB metabolites

Biological monitoring of CB pesticides can be carried out by determination of their metabolites in biological samples. Specific metabolites of main carbamate compounds, measured in urine for biomonitoring of human population exposed to these pesticides are summarised in Table4.

Metabolites	Main parent compounds
Alkylphosphates:	
Dimethylphosphate (DMP)	Malathion, dichlorvos, dimethoate, , temephos fenchlorphos, mevinphos.
Dimethylthiophosphate (DMTP)	Azinphos-methyl, dimethoate, fenchlorphos, fenitrothion, malathion.
Dimethyldithiophosphate (DMDTP)	Azinphos-methyl, dimethoate, malathion.
Diethylphosphate (DEP)	Thion, disulfoton, parathion, phorate, terbufos quinalphos, demeton, diazinon, dichlofenthion,
Diethylthiophosphate (DETP)	Diazinon, demethon, parathion, phorate, quinalphos
Diethyldithiophosphate (DEDTP)	Disulfoton, phorate
Para-nitrophenol (PNP)	Parathion Parathion-methyl
3,5,6-trichloro-pyridinol (TCP)	Chlorpyrifos Chlorpyrifos-methyl
3-methyl-4-nitrophenol (MNP)	Fenitrothion
Mono- and Di-carboxylic phosphorus acids	Malathion
Aminomethyl-phosphonic acid	Glyphosate

Table 3. Main metabolites of OP, measured in urine for human biomonitoring.

Metabolites	Parent compounds
Carbendazim, methyl (5-hydoxy-1Hbenzimidazol-2-yl) Methyl (4-hydoxy-1H-benzimidazol-2-yl)	Benomyl
1-naphthol	Carbaryl
2-dimethylamino-4-hydroxy-5,6-dimethylpyrimidine 2-methylamino-4-hydroxy-5,6-dimethylpyrimidine	Pirimicarb
2-hydroxyphenyl N-methylcarbamate 2-isopropoxyphenol	Propoxur
Carbofuranphenol	Carbofuran Benfuracarb Carbosulfan Furathiocarb

Table 4. Specific metabolites of main carbamate compounds measured in human urine.

2.1.2.4 Pyrethroids metabolites

Pyrethroids are more modern insecticides which are more neurotoxic to insects and less neurotoxic to humans than organophosphates. This led to lower amounts which have to be applied and to lower exposure of the general population. The most important pyrethroids permethrin, cyfluthrin, cypermethrin and deltamethrin, are immediately hydrolysed in the human body. Several methods exist for the measurement of synthetic pyrethroid metabolites in human urine. (Leng et al., 1997; Aprea et al. 1997; Maroni et al., 2000c; Smith et al., 2002; Schettgen et al., 2002; Leng et al., 2006; Fortin et al.,2008). The metabolites 3-

phenoxybenzoic acid (3-PBA), 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA), 4-fluoro-3-phenoxybenzoic acid (F-PBA) and 3-(2,2-Dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid (DBCA), which are excreted in urine are commonly used as human biomonitoring parameters (Table5).

Substance	**Metabolite in urine**
Allethrin, bioallethrin Phenothrin Pyrethrum Resmethrin Tetramethrin	*trans*-Chrysanthemumdicarboxylic acid
Cyfluthrin Cypermethrin Permethrin	*cis*- and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (*cis* and *trans*-DCCA)
Deltamethrin	*cis*-3-(2,2-Dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid (*cis*-DBCA)
Cypermethrin Deltamethrin Permethrin	3-Phenoxybenzoic acid (3-PBA)
Cyfluthrin	4-Fluoro-3-phenoxybenzoic acid (F-PBA)

Table 5. Pyrethroid insecticides and their corresponding metabolites

2.2 Biomarkers of effect

Biomarker of effect is a measurable biochemical, physiological, behavioural or other alteration within an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease (WHO, 1993). Such markers include several specific markers for target tissues and should reflect early biochemical modifications that precede structural or functional damage. Thus, knowledge of the mechanism(s) that lead to ultimate toxicity is necessary or at least extremely important to develop specific and useful biomarkers. Many biomarkers of effect are used in everyday practice to assist in clinical diagnosis, but for preventive purposes an ideal biomarker of effect is one that measures change that is still reversible. Nevertheless, certain biomarkers of non-reversible effects may still be very useful in epidemiological studies or provide the opportunity for early clinical intervention.

A limited range of tissues is available for routine analysis of these biomarkers. The more accessible tissues are therefore used as surrogates for the known or putative target tissues. In some instances biomarkers of effect are not mechanistically related to chemically induced lesions, but may represent concomitant, independent changes (WHO, 1993).

2.2.1 Biological markers of OC effects

Animal studies have shown that the earliest biological modification after exposure to OC pesticides is a dose-dependent induction of the activity of certain microsomal enzymes. This effect can be measured by indirect methods, such as the determination of 6-β-hydroxycortisol and D-glucaric acid excretion in urine, or the measurement of blood half-life of the test drugs. Enzyme induction has been documented in workers with repeated exposure to aldrin/dieldrin, endrin, lindane and DDT. However, these tests are not specific

for OC exposures because several other xenobiotics (e.g. alcohol) or drugs (e.g. barbiturates) have inductive effects on the liver microsomal enzymes. The only specific test for the biological monitoring of human exposure to OC pesticides is the measurement of the intact compounds or their metabolites in biological samples. Since hexachlorobenzene can cause chemical porphyria in man, the change in urinary excretion of porphyrins has been proposed as an early biomarker of effect (Maroni et al., 2000d).

2.2.2 Inhibition of cholinesterase activity

Measurements of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities have been used as primary biomarkers to take necessary measures in the field of occupational as well as emergency medicine in cases of clinical poisoning and accidental OP and/or CB exposure (Hernandez et al., 2005; Souza et al., 2005; Safi et al., 2005; Stefanidou et al., 2009; Ng et al., 2009; Simoniello et al., 2010; Ueyama et al., 2010). Blood cholinesterases have been widely used for monitoring exposure to OP and CB pesticides. Strong associations were reported between exposure to these compounds and symptoms of chronic pesticide toxicity, and cholinesterase activities were significantly reduced in exposed populations (Hernandez et al., 2004, 2005; Remor et al., 2009).

The AChE is enzyme instantly performs the hydrolytic cleavage of acetylcholine, the chemical mediator responsible for the physiological transmission of nerve action potential. The main mechanism of action of OP and CB compounds is inhibition of cholinesterase activity. Different OP pesticides may inhibit AChE or BChE to a different degree (Costa et al., 2005). Nearly all OP insecticides cause toxic effects in humans through the inhibition of AChE in the nervous system. When OP insecticides are present, AChE is phosphorylated and is no longer able to break down acetylcholine into choline and acetic acid. The resulting accumulation of endogenous acetylcholine is responsible for the typical signs and symptoms (muscarine-like effects and nicotine-like effects) occurring after acute poisoning (cholinergic over-stimulation syndrome) (Maroni et al., 2000d).

BChE, also known as pseudo-cholinesterase, is found in plasma. The physiological function of this enzyme in blood is yet to be discovered (Costa et al., 2005). Depression of the BChE enzyme activity is not necessarily associated with symptoms of anti-cholinergic toxicity, and large depressions in BChE have been noted in the absence of any effect on erythrocyte AChE (Soltaninejad et al., 2007). The BChE is still a useful biomarker to predict and prevent health hazards of workers chronically and occupationally exposed to pesticides (He, 1999; Ranjbar et al., 2002; Rastogi et al., 2008; Araoud et al., 2011). The plasma cholinesterase might be a more sensitive indicator of exposure to some OP compounds such as malathion, diazinon and dichlorvos (He, 1999; Costa et al., 2005; Hernandez et al., 2006; Jintana et., 2009). According Jintana et al. (2009), the effect of OP exposure on cholinesterase activity was found predominantly in BChE. One of the possible reasons is that potential inhibition of AChE and BChE varies widely among the different OP compounds. Moreover, some OP inhibits BChE more strongly than AChE. The inhibition of BChE is highly correlated with intensity and duration of higher exposure to a large group of OP and carbamate pesticides. Ancientness of exposure as well as type of pesticides used were also implicated in a significant variation of BChE activity and can be considered as risk factors of exposure to pesticides (Araoud et al., 2011). However, BChE inhibition does not mirror the biological effects of OP in the nervous system (He, 1999). On the other hand, AChE is better than BChE for the assessment of chronic exposure to OPs, since a cumulative inhibition is observed due to its lower recovery rate compared to that of BChE (Kamel & Hoppin, 2004).

2.2.3 Inhibition of neuropathy target esterase activity

In addition to cholinergic effects, certain OP can cause another type of neurotoxicity, a central peripheral distal sensory-motor axonopathy, known as organophosphate-induced-delayed-polyneuropathy (OPIDP). OPIDP is not related to inhibition to AChE but rather is associated with phosphorylation of another esterase denominated neuropathy target esterase (NTE) in the nervous system (Costa et al., 2005). In humans, NTE is present in the nervous tissue, liver, lymphocytes, platelets and other tissues. Its physiological function, if any, is still unknown (Maroni et al., 2000a). Moreover, limited information is available on the degree of inter- and intra-individual variation (Costa et al., 2005).

The initial biochemical reaction is represented by the phosphorylation of NTE, while the second step is the transformation of the phosphorylated target into an 'aged' form. The ageing reaction depends on the chemistry of OP pesticides and may only occur with phosphate, phosphonates and phosphoramidates. Compounds such as, sulphonates and carbamates are not able to age and, if they are linked to NTE before an axonopathic OP compound is administered, they block the receptor preventing the development of the neuropathy (Maroni et al., 2000a). For the OP compounds causing delayed polyneuropathy, measurement of the activity of NTE in lymphocytes appears to offer a useful marker for biological monitoring and for predicting purposes (Costa & Manzo, 1995).

2.2.4 β-Glucuronidase

β-Glucuronidase (BG) was isolated from microsomal, Golgi, lysosome, and eventually released into the serum fraction (Ueyama et al., 2010). It has been previously reported that BG activity could be considered as a novel biomarker of anticholinesterase pesticides exposure. This enzyme is stabilized within the luminal site of the microsomal membrane by a complex with egasyn, one of the carboxylesterase isoenzymes (Hernandez et al., 2004; Ueyama et al., 2010). Exposure to OP or CB insecticides is followed by the cleavage of the egasyn-glucuronidase complex, leading to a rapid increase in plasma BG activity (Hernandez et al., 2004). The mechanism involved was not fully understood at that time. A massive increase in plasma BG was noted in rats within a few hours after paraoxon or parathion-treatment (Ueyama et al., 2010). In human, a significant increase in blood BG activity was reported in acute OP poisoning cases (Soltaninejad et al., 2007). These authors suggested that BG is very sensitive biomarker at high exposure to OP. Recently, Ueyama et al. (2010) explained that plasma BG activity, in a group of chronically OP-exposed farmers, was significantly increased compared to that in controls. Hence, they indicated that the measurement of BG activity is a practical approach to detect low-level occupational exposure, at which no BChE inhibition is observed. Blood BG can be a more sensitive biomarker of OP exposure than the inhibition of AChE and BChE activities in rats as well as humans (Ueyama et al., 2010).

2.2.5 Cytogenetic biomarkers

Cytogenetic markers such as chromosomal aberrations (CA), sister chromatid exchange (SCE), micronuclei (MN) and single cell gel electrophoresis SCGE or Comet assay have been extensively used for the detection of early biological effects of DNA-damaging agents. Regarding pesticide exposure, a positive association between occupational exposure to complex pesticide mixtures and the presence of CA, SCE and MN, has been detected in the majority of reports, providing suggestive evidence of genotoxic effects induced by pesticides (Bolognesi, 2003; Pastor et al., 2003; Das et al., 2007; Remor et al., 2009).

The Comet assay has been used to determine the extent of DNA damage in lymphocytes from farmers with occupational exposure to a variety of pesticides (Angerer et al., 2007). Occupational exposure to pesticides resulted in a significant increase in DNA and chromosome damages in blood cells (Paz-y-Mino et al., 2004). Studies of environmental pesticide exposure, using Comet assays of leukocytes, showed a positive correlation between DNA damage and dichlorodiphenyl dichloroethylene (DDE), dichlorodiphenyl dichloroethane (DDD), and dichlorodiphenyl trichloroethane (DDT) levels. However, exposure to deltamethrin did not induce DNA damage (Valverde & Rojas, 2009). But, according Villarini et al. (1998), the in vitro genotoxicity of deltamethrin evaluated by assessing the ability of the insecticide to damage DNA, using the SCGE or SCE assay and MN test in human peripheral blood leukocytes, revealed that this pyrethroid insecticide has a weak genotoxic activity and is not completely devoid of long-term effects as a consequence of its interaction with DNA (Villarini et al., 1998). Generally, since workers are frequently exposed to complex mixtures of pesticides, it is difficult to attribute the genotoxic damage to any particular chemical class or compound.

2.2.6 δ-aminolevulinic acid dehydratase

Changes in erythrocyte δ-aminolevulinic acid dehydratase (ALA-D), an erythrocyte enzyme, have been reported after exposure to different pesticides both in vitro and in vivo (Hernandez et al., 2005; Remor et al., 2009). The inhibition of ALA-D (40%) shortly after the administration of paraquat has been attributed to the generation of oxidative stress (Noriega et al., 2002, as cited in Hernandez et al., 2005). The results of the study of Hernandez et al. (2005), indicate that exposure to pesticides in an intensive farming setting could lead to a reduction in erythrocyte ALA-D. The decrease of ALA-D observed may partly be due to non-competitive binding of pesticides to the enzyme, since these chemicals are neither substrates nor competitors of substrates. However, the vicinal sulfhydryl of ALA-D may have been modified by the exposure leading to inhibition of enzyme activity (Hernandez et al., 2005). The relevance of this inhibition is unknown, although a failure of heme synthesis could account for the haemogram changes previously reported in the context of long-term exposure to pesticides (Parron et al., 1996). Therefore, the ALA-D may become an important sensitive biomarker that can be used together with AChE and/or BChE for the assessment of long-term health risks of workers exposed to pesticides.

2.2.7 Other biomarkers

Several additional biochemical and haematological parameters present in human biological fluids were used as biomarkers to detect early effects of pesticides before adverse clinical effects occur. Parron et al. (1996), reported decrease in the mean corpuscular haemoglobin concentration (MCHC) and in the mean platelet volume (MPV) in respectively, 38% and 15% of greenhouse sprayers chronically exposed to pesticides. The main alterations found in the total and differential white blood cell count were the increase of monocytes in 5% of workers and of eosinophiles in 4% of these sprayes (Parron et al., 1996).

Studies on human toxicity of pesticides had also focused on biological parameters related to organ functions. The biochemical dysfunctions could reflect either hepatic or renal cytotoxicity. A subtle nephrotoxic changes in workers occupationally exposed to pesticides was reported, because of their higher levels of serum creatinine and/or blood urea (Hernandez et al., 2006). Altered liver enzyme activities, such as serum alanine

aminotransferase (ALT) and aspartate aminotransferase (AST), have been reported among pesticide workers exposed to OP alone or in combination with OC or other pesticides (Anwar, 1997; Altuntas et al., 2003; Khan et al., 2008). The presence of the pesticide in the body affects the metabolism of tryptophan leading to hyperglycemia and affect the ALT, AST, gamma glutamyl transferase (GGT) and lactate dehydrogenase (LDH) activities (Tsatsakis et al., 2009). An increase in triglycerides levels, GGT activity, and inorganic phosphorus levels was reported in 17%, 8% and 7% respectively, of a cohort of pesticide sprayers (Parron et al., 1996). Friedman et al. (2003) reported elevations in creatine kinase (CK) in patients more than 10 years after acute exposure to anticholinesterases.

2.3 Biomarkers of susceptibility

Biomarkers of susceptibility constitute an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance (WHO, 1993). They are of special interest as they are genetically determined and may predispose to an increased risk in the case of exposure to pesticides (Hernandez et al., 2003). These biological markers indicate which factors may increase or decrease an individual's risk of developing a toxic response following exposure to pesticides. The use of these biomarkers, reflecting genetically linked or acquired susceptibility to pesticides or their metabolites, provides an opportunity for the recognition and protection of sensitive individuals (WHO, 1993).

Pesticides undergo bioactivation and detoxification processes that can be affected by genetic polymorphisms in biotransformation enzymes. Genetic variations in such enzymes as well as in the enzymes that are targeted by pesticides can greatly influence their toxicity and would, therefore, render an individual more or less susceptible to adverse effects of these compounds (Costa et al., 2005). Pesticide metabolism studies conducted with human provide valuable information as to metabolic pathways and the enzymes involved in these pathways, and can aid in the identification of individuals that may have increased risk after exposure to pesticides (Rose et al., 2005).

Individual susceptibility has been reported to play a critical role in the assessment of exposure to pesticides, because of at the same exposure level individual susceptibility determines whether or not clinical symptoms or even intoxication appear. This individual susceptibility is caused by polymorphic key enzymes like esterases and transferases as well as their synergisms. Most studies have focused on the influence of isolated enzyme activities on the toxicokinetics of the foreign substance; however, there is little data on the combined effects of a number of polymorphic enzymes mainly involved in the detoxification of pesticides (Lewalter & Leng, 1999; Hernandez et al., 2005). Inheritance of the unfavourable versions of the different polymorphic genes has been associated with an increased activation or reduced detoxification and elimination of pesticides, and could entail an increased susceptibility to pesticides (Bolognesi, 2003). Although all the pathways for pesticide detoxification are not fully understood, the process involves three main systems: the cytochrome P450 enzymes, glutathione S-transferases, and the esterases system that metabolizes insecticides.

2.3.1 Cytochrome P450 enzymes

Many pesticides are converted to active intermediates via reactions catalyzed by cytochrome P450 (CYP450). Variations in P450 enzymes have considerable in vivo effects on the

sensitivity of humans to chemical toxicity. Therefore, some humans may be at increased risk of toxicity due to their P450 enzymes profiles (Mutch & Williams, 2006). CYP450 enzymes metabolize some carbamate and nicotinoid insecticides (Hodgson, 2003). However, these enzymes catalyse mainly the oxidative desulfuration of organothiophosphates to the corresponding oxons (Figure2) and also mediate detoxification of several OP. Both bioactivation and detoxification of OP are mediated by multiple CYP450, some of which present polymorphisms that can confer differences in catalytic activity or level of expression of various substrates (Costa et al., 2005). CYP1A2 and members of CYP2 and CYP3 families are involved in activation as well as detoxification some of OP such as parathion, which is converted to paraoxon by the combination of CYP2C8, CYP3A4/5, CYP2D6 and CYP1A2 (Nebert, 2005). The functional expression of CYP450 in human liver will influence formation of the oxon and its systemic concentration. Therefore, the individuals' profile of participatory P450 will more accurately predict susceptibility to OP toxicity, and such information will be useful to complement risk assessment of OP exposures (Mutch & Williams, 2006). A number of pesticides may modify the cancer risk through the altered CYP2E1 enzyme activity (Bolognesi, 2003). However, the significant increase in DNA damage found in population of pesticide sprayers was not significantly influenced by genetic polymorphisms of the CYP1A1 gene (Paz-y-Mino et al., 2004).

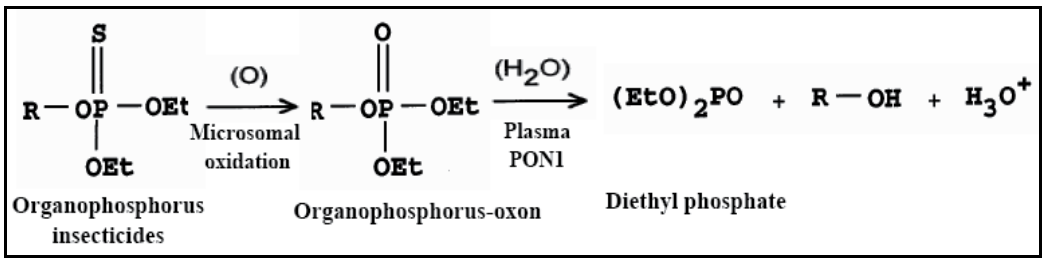

Fig. 2. The cytochrome P450/PON1 pathway for the bioactivation and subsequent detoxication of several organophosphorus insecticides.

2.3.2 Esterase

2.3.2.1 Cholinesterase

Cholinesterase variants are known to be responsible for the individual susceptibility regarding the occurrence and severity of the related symptom. A large number of polymorphisms have been described for BChE target for OP. In addition to the wild-type allele, there are at least 39 identified genetic variants with nucleotide alterations in the coding regions (Lockridge & Masson, 2000). Most of these variants, which are usually grouped in four categories (silent, K-variant, atypical and fluoride), are silent, i.e. they have 0 or less than 10 % of normal activity. Limited evidence in animals and humans suggests that individuals with genetic variants of BChE that have reduced activity may be more sensitive to OP toxicity (Lockridge & Masson, 2000). A genetic variant of human AChE has been described, which is not associated with alterations in enzyme activity (Bartels et al., 1993, as cited in Hernandez et al., 2005). However, based on work in AChE knockout mice, it has been suggested that silent AChE alleles may exist in humans, and may be associated with increased sensitivity to OP toxicity (Costa et al., 2005).

2.3.2.2 Carboxylesterases

The carboxylesterases (CbE), are members of the serine hydrolase superfamily of esterases that metabolize ester-containing xenobiotics such as pyrethroids, and they are implicated in the detoxification of a number of OP insecticides. These hepatic enzymes are expressed abundantly in the mammalian liver and utilized by insects to gain resistance against insecticides (Ross et al., 2006; Zhou et al, 2007).

Individual susceptibility plays a major role in pyrethroid induced adverse effects like skin paresthesia. For pyrethroids, marker of susceptibility is not well known yet. The determination of the carboxylesterase activity in human isolated lymphocytes was a first step in the search of a marker of pyrethroid susceptibility in man (Leng et al., 1999). The study of Ross et al., (2006) has demonstrated that hCbE-1 and hCbE-2 are human pyrethroid-hydrolyzing CbE and that these enzymes will be useful biomarkers of susceptibility in populations that are occupationally and environmentally exposed to these xenobiotics.

2.3.2.3 Paraoxonase

Paraoxonase1 (PON1) is a member, of family of proteins that includes PON2 and PON3. This enzyme, which takes its name from its most studied substrate, paraoxon, is capable of hydrolyzing the oxygen analogs of a number of commonly used OP (Figure2) as well aromatic esters and carbamate insecticides (Hernandez et al., 2004; Costa et al., 2005). Variations in PON1 activity may contribute to interindividual variations in susceptibility, and this detoxifying enzyme was considered as one important biomarker of susceptibility to long term exposure to pesticides (Leng et al., 1999; Hernandez et al., 2003; Costa et al., 2005; Sirivarasai et al., 2007; Zhou et al, 2007; Perez-Herrera et al., 2008). This enzyme exhibits substrate-dependent genetic polymorphisms, both in coding and promoter regions, leading to changes in PON1 activity and level. The Q192R substitution has been shown to be responsible for the substrate-dependent activity polymorphism. The PON1 192R isoform hydrolyzes paraoxon more rapidly than the PON1 192Q isoform, whereas the PON1 192Q isoform hydrolyzes diazoxon, soman and sarin more rapidly than the PON1 192R (Costa et al., 2005; Sirivarasai et al., 2007). The polymorphism at position 55 (L55M) does not affect catalytic activity, but has been associated with plasma PON1 protein levels, with PON1 55M being associated with low plasma PON1. The polymorphism at position -108 (T/C), in the promoter region of PON1, is the major contributor to differences in the level of PON1 expression, and appears to have the major effect on the levels of PON1 found in plasma of individuals.

Although PON1 activity remains stable over time within a given individual, it varies 10 to 40 fold between individuals. PON1 genotype is an important determinant of a farmworker's susceptibility to chronic pesticide poisoning, as PON1Q allele is an independent predictor of chronic toxicity (odds ratio 2.9; 95% confidence interval: 1.7-6.7) (Lee et al., 2003). Since the genetic polymorphisms of PON1, at position 192 and -108, infer different catalytic activity and levels expression, it is reasonable to assume that some individuals in the population will exhibit a significantly increased sensitivity to OP exposure (Costa et al., 2005). It has been also reported that the health status of individuals after exposure to OP pesticides is related to the polymorphism of PON1. Lee et al. (2003) have examined the association between PON1 genotype and symptoms of chronic pesticide toxicity in pesticide-exposed workers. A significantly higher proportion of subjects with the slow genotype reported two or more symptoms of chronic toxicity compared to those with fast PON1 activity. Moreover,

the genetic variants of this pesticide metabolizing enzyme may confer a predisposition factor to Parkinson disease, and thus, is considered candidate gene for association studies. An interaction, between OP exposure and PON1Q192R polymorphism, was found on adverse effects on sperm DNA integrity in agricultural workers, and these individuals featuring the 192RR genotype were more susceptible to develop reproductive toxic effects by OP exposure (Perez-Herrera et al., 2008). According Chia et al. (2009), PON1 polymorphisms differed among ethnic groups, implying that ethnicity could be an important surrogate for identifying susceptible groups in case of OP exposure.
All these reports pinpoint the potential usefulness of PON1 genotyping as a biological indicator of susceptibility to long term exposure to OP. Thus, genotyping individuals for PON1 polymorphisms may provide a method for the identification of individuals with the high risk of OP poisoning.

2.3.3 Glutathione S-transferases

This family of enzymes presents genetic polymorphisms in human populations responsible for the glutathione conjugation of various reactive species of many chemicals including pesticides (Bolognesi, 2003; Schroeder, 2005). Several glutathione S-transferases (GST) polymorphisms have been identified. The GSTP1 polymorphisms, affecting substrate selectivity and stability, may increase the conversion of a pesticide to a toxic metabolite and hence, can increase the toxicity of some substrates. These enzymes have been investigated as risk factors for and non-Hodgkin's lymphoma in individuals exposed to pesticides (Schroeder, 2005). The significant differences in GSTP1 genotype frequencies shown in patients with Parkinson's disease exposed to pesticides, might explain susceptibility to Parkinson's disease after pesticide exposure (Menegon et al., 1998). The polymorphisms of GST mu and theta (GSTM1 and GSTT1) were studied as biomarkers of individual susceptibility in professional applicator of pesticides. Null genotype for both GST subclasses (GSTM1 and GSTT1) was found to be the unique independent predictor of pesticide-related symptomatology. Moreover, GSTT1 was a relevant determinant of susceptibility to chronic pesticide poisoning (Hernandez et al., 2005). Liu et al. (2006) revealed that metabolic GSTP1 gene may modulate DNA damage in pesticide-exposed fruits growers. The GST may play a role in the detoxification of certain OP, particularly those with a methyl group in the alkyl chain. Indeed, the potential significance of human GST polymorphisms as determinants of individual difference in human susceptibility of OP has not been well investigated (Costa et al., 2005).

2.3.4 Gene–gene interactions

Enzymes involved in pesticide metabolism have overlapping substrate specificities, and the lack of one-to-one specificity may buffer effects of any single metabolism enzyme variant by providing an alternate catalyst for metabolic transformation. Likewise, effects of "high-risk" polymorphisms affecting a single metabolic step may be diminished by "low-risk" variants acting at subsequent steps, such that consequences of rapid phase I activation may be lessened by rapid phase II detoxification. Allelic variants of CYP1A1 and PON1 have been studied as susceptibility factors in pesticides toxic responses. Tsatsakis et al. (2009) has reported an association between the CYP1A1/PON1 polymorphisms and various clinico-pathological findings, in rural population professionally exposed to pesticides. According to Schroeder (2005), PON1 and BChE enzyme variants, associated with altered activity and

acute OP toxicity, are strong candidate susceptibility factors for pesticides and non-Hodgkin's lymphoma (Schroeder, 2005). Therefore, it may be necessary to evaluate joint effects of multiple functional gene polymorphisms to detect an effect on pesticide metabolism and impaired health.

3. Conclusion

The biological monitoring is becoming an increasingly important element of field studies designed to assess the risk from pesticide exposure for preventive purposes. The use of biomarkers as an integrated measure of exposure and/or effects is increasing as a result of difficulties in exposure source identification and demands of more integrated data for risk assessment. This is inevitable in environmental health, with often unknown and very mixed exposures, but also occupational health implies mixed exposures and several routes of exposure, impossible to detect by conventional environmental monitoring. Biomonitoring data will be valuable in making associations with health effects and risk of adverse outcome. Therefore, the biological markers must provide the critical link between chemical exposure, internal dose and health impairment, and are of value in assessment of risk. However, there is a need to identify and validate for each organ system those characteristic parameter(s) that are indicative of induced dysfunction, clinical toxicity or pathological change, as well as to establish the specificity and sensitivity of each biomarker and its method of measurement. Thus, clinical issues connected with acute poisoning, identification of long-term effects, the development and validation of new and effective biomarkers of human exposure, effect and susceptibility, and study of interaction mechanisms are areas in which continuing research is warranted in order to improve the quantitative risk assessment to protect human health.

4. References

Angerer, J.; Ewers, U. & Wilhelm, M. (2007). Human Biomonitoring: State of the art. International *Journal of Hygiene and Environmental Health*, Vol. 210, pp. 201-228.

Anwar, W.A. (1997). Biomarkers of Human Exposure to Pesticides. *Environmental Health Perspective,* Vol. 105, pp. 801-806.

Aprea, C.; Colosio, C.; Mammone, T.; Minoia, C. & Maroni M. (2002). Biological Monitoring of Pesticide Exposure: A Review of Analytical Methods. *Journal of Chromatography B*, Vol. 769, pp. 191-219.

Aprea, C.; Stridori, A. & Sciarra G. (1997). Analytical Method for the Determination of Urinary 3-Phenoxybenzoic Acid in Subjects Occupationally Exposed to Pyrethroid Insecticides. *Journal of Chromatography B*, Vol. 695, pp. 227-236.

Araoud, M.; Douki, W.; Najjar, M.F. & Kenani, A. (2010). Simple Analytical Method for Determination of Pesticide Residues in Human Serum by Liquid Chromatography Tandem Mass Spectrometry. *Journal of Environmental Science and Health, Part B*, Vol. 45, pp. 242-248.

Araoud, M.; Neffeti, F.; Douki, W.; Ben Hfaiedh, H.; Akrout, M.; Najjar, M.F. & Kenani, A. (2011). Factors Influencing Plasma Butyrylcholinesterase Activity in Agricultural Workers. *Annales de Biologie Clinique*, Vol.69, pp. 159-166.

Baldi, I.; Lebailly, P.; Mohammed-Brahim, B.; Letenneur, L.; Dartigues, J.F. & Brochard, P. (2003). Neurodegenerative Diseases and Exposure to Pesticides in the Elderly. A*merican Journal of Epidemiology*, Vol. 157, pp. 409-414.

Barr, D.B. & Needham, L.L. (2002). Analytical Methods for Biological Monitoring of Exposure to Pesticides. *Journal of Chromatography B,* Vol. 778, pp. 5-29.

Bartels, C.F.; Zelinski, T. & Lockridge, O. (1993). Mutation at Codon 322 in the Human Acetylcholinesterase (AChE) Gene Accounts for YT Group Polymorphism. *American Journal of Human Genetics*, Vol.52, pp. 928-936.

Benford, D.J.; Hanley, A. B.; Bottrill, K.; Oehlschlager, S.; Balls, M.; Branca, F.; Castegnaro, J.J.; Descotes, J.; Hemminiki, K.; Lindsay, D. & Schilter, B. (2000). Biomarkers as Predictive Tools in Toxicity Testing. *ATLA*, Vol. 28, pp. 119-131.

Bolognesi, C. (2003). Genotoxicity of pesticides: A Review of Human Biomonitoring Studies. *Mutation Research*, Vol. 543, pp. 251-272.

Bouchard, M.; Carrier, G.; Brunet, R.C.; Dumas, P. & Noisel, N. (2006). Biological Monitoring of Exposure to Organophosphorus Insecticides in a Group of Horticultural Greenhouse Workers. *Annals of Occupational Hygiene*, Vol. 50, pp. 505-515.

Bouwman, H.; Sereda, B. & Meinhardt, H.M. (2006). Simultaneous Presence of DDT and Pyrethroid Residues in Human Breast Milk From a Malaria Endemic Area in South Africa. *Environmental pollution*, Vol. 144, pp. 902-917.

Chia, S.E.; Safiyya, M.A.; Yap, P.H.E.; Linda, G.; Ong, Y.B. & Chia, K.S. (2009). Distribution of PON1 Polymorphisms PON1Q192R and PON1L55M among Chinese, Malay and Indian males in Singapore and Possible Susceptibility to Organophosphate Exposure. *Neurotoxicology*, Vol. 30, pp. 214-219.

Clary, T. & Ritz, B. (2003). Pancreatic Cancer Mortality and Organochlorine Pesticide Exposure in California, 1989-1996. *American Journal of Industrial Medicine*, Vol. 43, 306-313.

Costa, L.G. & Manzo, L.C. (1995). Biochemical Markers of Neurotoxicity: Research Strategies and Epidemiological Applications. *Toxicology Letters*, Vol. 77, pp. 137-144.

Costa, L.G. (1996). Biomarker Research in Neurotoxicology: The Role of Mechanistic Studies to Bridge the Gap between the Laboratory and Epidemiological Investigations. *Environmental Health Perspectives*, Vol. 104, pp. 55-67.

Costa, L.G.; Cole, T.B.; Vitalone, A. & Furlong, C.E. (2005). Measurement of Paraoxonase Status as a Potential Biomarker of Susceptibility to Organophosphate Toxicity. *Clinica Chimica Acta*, Vol. 352, pp. 37-47.

Das, P.P.; Shaik, A.P. & Jamil, K. (2007). Genotoxicity Induced by Pesticide Mixtures: In-vitro Studies on Human Peripheral Blood Lymphocytes. *Toxicology and Industrial Health*, Vol. 7; pp. 449-458.

Dulaurent, S.; Saint-Marcoux, F.; Marquet, P. & Lachâtre, G. (2006). Simultaneous Determination of Six Dialkylphosphates in Urine by Liquid Chromatography Tandem Mass Spectrometry. *Journal of Chromatography B*, Vol. 831, pp. 223-229.

Ennaceur, S.; Gandoura, N. & Driss, M.R. (2008). Distribution of Polychlorinated Biphenyls and Organochlorine Pesticides in Human Breast Milk From Various Locations in Tunisia: Levels of Contamination, Influencing Factors, and Infant Risk Assessment. *Environmental Research*, Vol. 108, pp. 86-93.

Fortin, M.C.; Carrier, G. & Bouchard, M. (2008). Concentrations Versus Amounts of Biomarkers in Urine: A Comparison of Approaches to Assess Pyrethroid Exposure. *Environmental Health*, Vol. 7, pp. 55-67.

Frias, M.M.; Frenich, A.G.; Martinez Vidal, J.L.; Sanchez, M.M.; Olea, F. & Olea, N. (2001). Analyses of Lindane, Vinclozolin, Aldrin, p,p′-DDE, o,p′-DDT and p,p′-DDT in

Human Serum Using Gas Chromatography with Electron Capture Detection and Tandem Mass Spectrometry. *Journal of Chromatography B*, Vol. 760, pp. 1-15.

Garaj-Vrhovac, V. & Zeljezic, D. (2000). Evaluation of DNA Damage in Workers Occupationally Exposed to Pesticides Using Single-Cell Gel Electrophoresis (SCGE) Assay: Pesticide Genotoxicity Revealed by Comet Assay. *Mutation Research*, Vol. 469, pp. 279-285.

He, F. (1999). Biological Monitoring of Exposure to Pesticides: Current Issues. *Toxicology Letter*, Vol. 108, pp. 277-283.

He, F.; Chen, S.; Tang, X.; Ganw, T.B. & Wen, B. (2002). Biological Monitoring of Combined Exposure to Organophosphates and Pyrethroids. *Toxicology Letter*, Vol.134, pp. 119-124.

Hernandez, A.F.; Gomez, M.A.; Pena. G.; Gil, F.; Rodrigo, L.; Villanueva, E. & Pla, A. (2004). Effect of Longterm Exposure to Pesticides on Plasma Esterases From Plastic Greenhouse Workers. *Journal of Toxicology Environmental Health A*, Vol. 67, pp. 1095-1108.

Hernandez, A.F.; Gomez, M.A.; Perez, V.; Garcia-Lario, J.V.; Pena, G.; Gil, F.; Lopez, O.; Rodrigo, L.; Pino, G; & Pla, A. (2006). Influence of Exposure to Pesticides on Serum Components and Enzyme Activities of Cytotoxicity Among Intensive Agriculture Farmers. *Environmental Research*, Vol. 102, pp. 70-76.

Hernandez, A.F.; Lopez, O.; Rodrigo, L.; Gil, F.; Pena, G.; Serrano, J.L.; Parron, T.; Alvarez, J.C.; Lorente, J.A. & Pla, A. (2005). Changes in Erythrocyte Enzymes in Humans Long-term Exposed to pesticides. Influence of Several Markers of Individual Susceptibility. *Toxicology Letters*, Vol. 159, pp. 13-21.

Hernandez, A.F.; Mackness, B.: Rodrigo, L.; Lopez, O.; Pla, A.; Gil, F.; Durrington, P.N.; Pena, G.; Parron, T.; Serrano, J.L.; & Mackness, M.I. (2003). Paraoxonase Activity and Genetic Polymorphisms in Greenhouse Workers with Long Term Pesticide Exposure. *Human and Experimental Toxicology*, Vol.22, pp. 565-574.

Heudorf, U.; Angerer, J. & Drexler, H. (2003). Current Internal Exposure to Pesticides in Children and Adolescents in Germany: Blood Plasma Levels of Pentachlorophenol (PCP), Lindane (γ-HCH), and Dichloro(diphenyl)ethylene (DDE), a Biostable Metabolite of Dichloro(diphenyl) trichloroethane (DDT). *International Journal of Hygiene and Environmental Health*, Vol. 206, pp. 485-491.

Hodgson, E. (2003). In Vitro Human Phase I Metabolism of Xenobiotics I: Pesticides and Related Compounds Used in Agriculture and Public Health, May 2003. *Journal of Biochemical and Molecular Toxicology*, Vol. 17, pp. 201-206.

Hryhorczuk, D.O.; Moomey, M.; Burton, A.; Runkle, K.; Chen, E.; Saxer, T; Slightom, J.; Dimos, J.; McCann K. & Barr, D. (2002). Urinary p-Nitrophenol As a Biomarker of Household Exposure to Methyl Parathion. *Environmental Health Perspectives*, Vol. 110, pp. 1041-1046.

Inoue, S.; Saito, T.; Maseb, H.; Suzuki, Y.; Takazawa, K.; Yamamotoa, I. & Inokuchi, S. (2007). Rapid Simultaneous Determination for Organophosphorus Pesticides in Human Serum by LC-MS. *Journal of Pharmaceutical and Biomedical Analysis*, Vol. 44, pp. 258-264.

Jintana, S.; Sming, K.; Krongtong, Y. & Thanyachai S. (2009). Cholinesterase Activity, Pesticide Exposure and Health Impact in a Population Exposed to

Organophosphates. *International Archives of Occupational and Environmental Health*, Vol. 82, pp. 833-842.

Kamel, F. & Hoppin, J.A. (2004). Association of Pesticide Exposure with Neurologic Dysfunction and Disease. *Environmental Health Perspectives*, Vol. 112, pp. 950-958.

Khan, D.A.; Bhatti, M.M.; Khan, F.A.; Naqvi, S.T. & Karam, A. (2008). Adverse Effects of Pesticides Residues on Biochemical Markers in Pakistani Tobacco Farmers. *International Journal of Clinical and Experimental Medicine*, Vol. 1, pp. 274-282.

Lacassie, E.; Dreyfuss, M.F.; Gaulier, J.M.; Marquet, P.; Daguet, J.L. & Lachâtre, G. (2001a). Multiresidue Determination Method for Organophosphorus Pesticides in Serum and Whole Blood by Gas Chromatography-Mass-Selective Detection. *Journal of Chromatography B*, Vol. 759, pp. 109-116.

Lacassie, E.; Marquet, P.; Gaulier, J.M.; Dreyfuss, M.F. & Lachâtre, G. (2001b). Sensitive and Specific Multiresidue Methods for Determination of Pesticides of Various Classes in Clinical and Forensic toxicology. *Forensic Science International*, Vol. 121, pp. 116-125.

Lee, B.W.; London, L.; Paulauskis, J.; Myers, J. & Christiani, D.C. (2003). Association Between Human Paraoxonase Gene Polymorphism and Chronic Symptoms in Pesticide-Exposed Workers. *Journal of Occupational and Environmental Medicine*, Vol. 45, pp. 118-122.

Lee, C.H.; Kamijima, M.; Kim, H.; Shibata, E.; Ueyama, J.; Suzuki, T.; Takagi, K.; Saito, I.; Gotoh, M.; Hibi, H; Naito, H. & Nakajima, T. (2007). 8-Hydroxydeoxyguanosine Levels in Human Leukocyte and Urine According to Exposure to Organophosphorus Pesticides and Paraoxonase 1 Genotype. *International Archives of Occupational and Environmental Health*, Vol. 80, pp. 217-227.

Lee, S.K.; Ameno, K.; In, S.W.; Yang, W.K.; Koo; K.S.; Yoo, Y.C.; Kubota, T.; Ameno, S. & Ijiri, I. (1999). Acute Fatal Poisoning Cases due to Furathiocarb. *Forensic Science International*. Vol. 101, pp. 65-70.

Leng, G.; Gries, W. & Selim, S. (2006). Biomarker of Pyrethrum Exposure. *Toxicology Letters,* Vol.162, pp. 195-201.

Leng, G.; Kiihn, K.H. & Idel, H. (1997). Biological Monitoring of Pyrethroids in Blood and Pyrethroid Metabolites in Urine: Applications and Limitations. *The Science of the Total Environment*, Vol. 199, pp. 173-181.

Leng, G.; Lewalter, J.; Rohrig, B. & Idel, H. (1999). The Influence of Individual Susceptibility in Pyrethroid Exposure. *Toxicology Letters*, Vol. 107, pp. 123-130.

Lewalter, J. & Leng, G. (1999). Consideration of Individual Susceptibility in Adverse Pesticide Effects. *Toxicology Letters,* Vol.107, pp. 131-144.

Liu, Y.J.; Huang, P.L.; Chang, Y.F.; Chen, Y.H.; Chiou, Y.H.; Xu, Z.L. & Wong, R.H. (2006). GSTP1 Genetic Polymorphism is Associated with a Higher Risk of DNA Damage in Pesticide-Exposed Fruit Growers. *Cancer Epidemiology Biomarkers and Prevention*, Vol. 15, pp. 659-666.

Lockridge, O. & Masson, P. (2000). Pesticides and Susceptible Populations: People with Butyrylcholinesterase Genetic Variants May be at Risk. *Neurotoxicology*, Vol. 21, pp.113-126.

Margariti, M.G. & Tsatsakis, A.M. (2009). Analysis of Dialkyl Phosphate Metabolites in Hair Using Gas Chromatography-Mass Spectrometry: a Biomarker of Chronic Exposure to Organophosphate Pesticides. *Biomarkers*, Vol. 14, pp. 137-147.

Maroni, M.; Colosio, C.; Ferioli, A. & Fait, A. (2000a). Organophosphorous Pesticides. *Toxicology*, Vol. 143, pp. 9-37.

Maroni, M.; Colosio, C.; Ferioli, A. & Fait, A. (2000b). Carbamate Pesticides. *Toxicology*, Vol. 143, pp. 39-46.

Maroni, M.; Colosio, C.; Ferioli, A. & Fait, A. (2000c). Synthetic Pyrethroids. *Toxicology*, Vol. 143, pp. 53-59.

Maroni, M.; Colosio, C.; Ferioli, A. & Fait, A. (2000d). Organochlorine Pesticides. *Toxicology*, Vol. 143, pp. 61-75.

Mathur, V.; Bhatnagar, P.; Sharma, R.G.; Acharya, V.; Sexana, R. (2002). Breast Cancer Incidence and Exposure to Pesticides Among Women Originating from Jaipur. *Environment International*, Vol. 28, pp. 331-336.

Menegon, A.; Board, P.G.; Blackburn, A.C.; Mellick, G.D. & Le Couteur, D.G. (1998). Parkinson's Disease, Pesticides, and Glutathione Transferase Polymorphisms. *Lancet*, Vol.352, pp. 1344-1346.

Mutch, E. & Williams, M. (2006). Diazinon, Chlorpyrifos and Parathion are Metabolised by Multiple Cytochromes P450 in Human Liver. *Toxicology*, Vol. 224, 22–32.

Nebert, D.W. (2005). Role of Host Susceptibility to Toxicity and Cancer Caused by Pesticides: Cytochromes P450. *Journal of Biochemical and Molecular Toxicology*, Vol. 19, pp. 184-186.

Ng, V.; Koh, D.; Wee, A. & Chia, S.E. (2009). Salivary Acetylcholinesterase as a Biomarker for Organophosphate Exposure. *Occupational Medicine*, Vol. 59, 120-122.

Noriega, G.O.; Gonzales, S.; Tomaro, M.L. & Batle, A.M. (2002). Paraquat-Generated Oxidative Stress in Rat Liver Induces Hemeoxygenase-1 and Aminolevulinic Acid Synthase. *Free Radical Research*, Vol. 36, pp. 633-639.

Parron, T.; Hernandez, A.F.; Pla, A. & Villanueva, E. (1996). Clinical and Biochemical Changes in Greenhouse Sprayers Chronically Exposure to Pesticides. *Human and Experimental Toxicology*, Vol. 15, pp. 957-963.

Pastor, S.; Creus, A.; Parron, T.; Wasilewska, A.C.; Siffel, C.; Piperakis, S. & Marcos, R. (2003). Biomonitoring of Four European Populations Occupationally Exposed to Pesticides: Use of Micronuclei as Biomarkers. *Mutagenesis*, Vol. 18, pp. 249-258.

Paz-y-Mino, C.; Arevalo, M.; Sanchez, M.E. & Leone, P.E. (2004). Chromosome and DNA Damage Analysis in Individuals Occupationally Exposed to Pesticides with Relation to Genetic Polymorphism for CYP 1A1Gene in Ecuador. *Mutation Research*, Vol. 562, pp. 77-89.

Perez-Herrera, N.; Polanco-Minaya; H., Salazar-Arredondo; E., Solis-Heredia M.J.; Hernandez-Ochoa I.; Rojas-Garcia E.; Alvarado-Mejia J.; Borja-Aburto, V.H. & Quintanilla-Vega, B. (2008). PON1Q192R Genetic Polymorphism Modifies Organophosphorous Pesticide Effects on Semen Quality and DNA Integrity in Agricultural Workers from Southern Mexico. *Toxicology and Applied Pharmacology*, Vol. 230, pp. 261-268.

Ranjbar, A.; Pasalar, P. & Abdollahi, M. (2002). Induction of Oxidative Stress and Acetylcholinesterase Inhibition Organophosphorous Pesticide Manufacturing Workers. *Human and Experimental Toxicology*, Vol. 21, pp. 179-182.

Rastogi, S.K.; Singh, V.K.; Kesavachandram, C.; Yoti, J.; Siddiqui, M.K.J.; Mathur, N.; Bharti, R.S. (2008). Monitoring of Plasma Butyrylcholinesterase Activity and

Haematological Parameters in Pesticides Sprayers. *Indian Journal of Occupational and Environmental Medicine*. Vol 12, pp. 29-32.

Remor, A.P.; Totti, C.C.; Moreira, D.A.; Dutra, G.P.; Heuser, V.D. & Boeira, J.M. (2009). Occupational Exposure of Farm Workers to Pesticides: Biochemical Parameters and Evaluation of Genotoxicity. *Environment International*, Vol. 35, 273-278.

Rose, R.L.; Tang, J.; Choi, J.; Cao, Y.; Usmani, A.; Cherrington, N. & Hodgson E. (2005). Pesticide Metabolism in Humans Including Polymorphisms. *Scandinavian Journal of Work Environment and Health*, Vol. 31, pp. 156-163.

Ross, M.K.; Borazjani, A.; Edwards, C.C. & Potter, P.M. (2006). Hydrolytic Metabolism of Pyrethroids by Human and Other Mammalian Carboxylesterases. *Biochemical Pharmacology*, Vol. 71, pp. 657- 669.

Rusiecki, J.A.; Roos, A.D.; Lee, W.J.; Dosemeci, M.; Lubin, J.H.; Hoppin, J.A.; Blair, A.; & Alavanja, M.C.R. (2004). Cancer Incidence Among Pesticide Applicators Exposed to Atrazine in the Agricultural Health Study. J. Natl. Cancer. Inst., Vol. 96, pp. 1375-1382.

Russo, M.V.; Campanella, L. & Avino, P. (2002). Determination of Organophosphorus Pesticide Residues in Human Tissues by Capillary Gas Chromatography-Negative Chemical Ionization Mass Spectrometry Analysis. *Journal of Chromatography B*, Vol. 780, pp. 431-441.

Safi, J.M.; Abu Mourad, T.A. & Yassin, M.M. (2005). Hematological Biomarkers in Farm Workers Exposed to Organophosphorus Pesticides in the Gaza Strip. *Archives Environmental and Occupational and Health*, Vol. 60, pp. 235-241.

Salvi, R.M.; Lara, D.R.; Ghisol, E.S.; Portela, L.V.; Dias, R.D. & Souza, D.O. (2003). Neuropsychiatric Evaluation in Subjects Chronically Exposed to Organophosphate Pesticides. *Toxicological Sciences*, Vol. 72, pp. 267-271.

Schettgen, T.; Koch, H.M.; Drexler, H. & Angerer, J. (2002). New Gas Chromatographic–Mass Spectrometric Method for the Determination of Urinary Pyrethroid Metabolites in Environmental Medicine. *Journal of Chromatography B*, Vol.778, pp. 121-130.

Schroeder, J.C. (2005). Metabolic Susceptibility to Agricultural Pesticides and Non-Hodgkin's Lymphoma. *Scandinavian Journal of Work Environment and Health*, Vol. 31, pp. 26-32.

Simoniello, M.F.; Kleinsorge, E.C.; Scagnetti, J.A.; Mastandrea, C.; Grigolato, R.A.; Paonessa, A.M. & Carballo MA. (2010). Biomarkers of Cellular Reaction to Pesticide Exposure in a Rural Population. *Biomarkers*, Vol. 15, pp. 52-60.

Sirivarasai J, Kaojarern S, Yoovathaworn K, Sura T. (2007). Paraoxonase (PON1) polymorphism and activity as the determinants of sensitivity to organophosphates in human subjects. *Chemico-Biological Interactions*, Vol. 168, pp. 184-192.

Smith, P.A.; Thompson, M.J. & Edwards, J.W. (2002). Estimating Occupational Exposure to the Pyrethroid Termiticide Bifenthrin by Measuring Metabolites in Urine. *Journal of Chromatography B*, Vol. 778, pp. 113-120.

Soltaninejad, K.; Shadnia, S.; Afkhami-Taghipour, M.; Saljooghi, R.; Mohammadirad, A. & Abdollahi, M. (2007). Blood ß-glucuronidase as a Suitable Biomarker at Acute Exposure of Severe Organophosphorus Poisoning in Human. *Human and Experimental Toxicology*, Vol. 26, pp. 963-966.

Souza, M.S.; Magnarelli, G.G.; Rovedatti, M.G; Cruz, S.S. & De D'Angelo, A.M. (2005). Prenatal Exposure to Pesticides: Analysis of Human Placental Acetylcholinesterase, Glutathione S-Transferase and Catalase as Biomarkers of Effect. *Biomarkers*, Vol.10, pp. 376-89.

Stefanidou, M.; Athanaselis, S. & Spiliopoulou, H. (2009). Butyrylcholinesterase: Biomarker for Exposure to Organophosphorus Insecticides. *Internal Medicine Journal*. Vol.39, pp. 57-60.

Stephens, R.; Spurgeon, A.; Calvert, I.A.; Beach, J.; Levy, L.S.; Berry, H. & Harrington, J.M. (1995). Neuropsychological Effects of Longterm Exposure to Organophosphates in Sheep Dip. *The Lancet*, Vol. 345, pp.1135-1139.

Thomas, G.O.; Wilkinson, M.; Hodson, S. & Jones, K.C. (2006). Organohalogen Chemicals in Human Blood from the United Kingdom. *Environmental Pollution*, Vol. 141, pp. 30-41.

Tsatsakis, A.M.; Zafiropoulos, A.; Tzatzarakis, M.N.; Tzanakakis, G.N. & Kafatos A. (2009). Relation of PON1 and CYP1A1 Genetic Polymorphisms to Clinical Findings in a Cross-Sectional Study of a Greek Rural Population Professionally Exposed to Pesticides. *Toxicology Letters*, Vol. 186, pp. 66-72.

Turci, R.; Balducci, C.; Brambilla, G.; Colosio, C.; Imbriani, M.; Mantovani, A.; Vellere, F. & Minoia, C. (2010). A Simple and Fast Method for the Determination of Selected Organohalogenated Compounds in Serum Samples From the General Population. *Toxicology Letters*, Vol. 192, pp. 66-71.

Ueyama, J.; Satoh, T.; Kondo, T.; Takagi, K.; Shibata, E.; Goto, M.; Kimata, A.; Saito, I.; Hasegawa, T.; Wakusawa, S. & Kamijima, M. (2010). β Glucuronidase Activity is a Sensitive Biomarker to Assess Low-level Organophosphorus Insecticide Exposure. *Toxicology Letters*, Vol. 193, pp. 115-119.

Valverde, M. & Rojas, E. (2009). Environmental and Occupational Biomonitoring Using the Comet Assay. *Mutation Research*, Vol. 681, pp. 93-109.

Villarini, M.; Moretti, M.; Pasquini, R.; Scassellati-Sforzolini, G.; Fatigoni, C.; Massimiliano, M.; Monarca, S. & Rodriguez, A.V. (1998). In Vitro Genotoxic Effects of the Insecticide Deltamethrin in Human Peripheral Blood Leukocytes: DNA Damage ('Comet'Assay) in Relation to the Induction of Sister-Chromatid Exchanges and Micronuclei. *Toxicology*, Vol. 130, pp. 129-139.

Wessels, D.; Barr, D.B. & Mendola, P. (2003). Use of Biomarkers to Indicate Exposure of Children to Organophosphate Pesticides: Implications for a Longitudinal Study of Children's Environmental Health. *Environmental Health Perspectives,* Vol.111, pp. 1939-1946.

WHO. (1993). Biomarkers and Risk Assessment: Concepts and Principles Environmental Health Criteria, No. 155. Geneva, Switzerland: World Health Organisation.

Zhou, Z.J.; Zheng, J.; Wu, Q.E. & Xie, F. (2007). Carboxylic Esterase and its Associations With Long-term Effects of Organophosphorus Pesticides. *Biomedical Environmental Sciences*, Vol. 20, pp. 284-290.

11

Pesticide Biomarkers in Terrestrial Invertebrates

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1. Introduction

Nowadays it is widely accepted that current agricultural practices cause a loss of biodiversity (Bianchi et al., 2006). Moreover, the introduction of vast areas of monocultives (e.g., biofuel crops) contributes to increase the risk for crop loss by pest infestation (Landis et al., 2008). Despite the introduction of integrated pest management (IPM) strategies in an attempt to reduce pesticide inputs, chemical control is still necessary to combat pests (Devine & Furlong, 2007). As an example, the figure 1 shows the evolution of pesticide

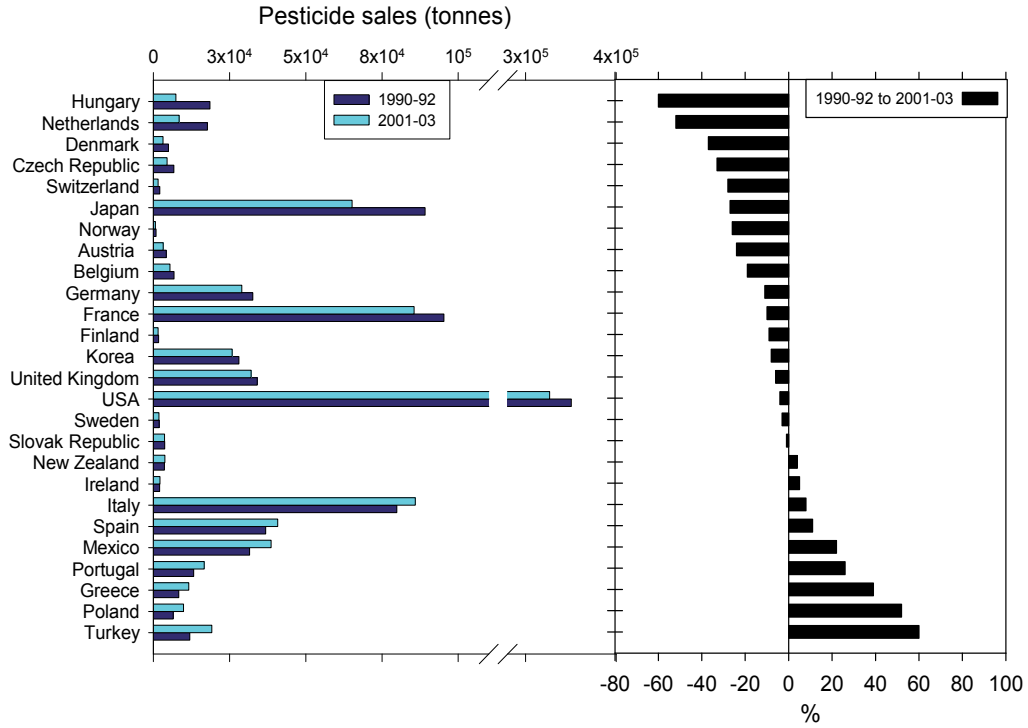


Fig. 1. Pesticide consumption worldwide. Data taken from OECD 2009 (http://dx.doi.org/10.1787/286683827028).

consumption in the last decade. Some European Union (EU) member states have experienced a notable increase (>20%) of pesticide use (OECD 2009). According to Eurostat (2007), five EU member states (France, Spain, Germany, Italy and the United Kingdom) account for the nearly 75% of the total plant protection products (PPPs) consumed in the EU. In the particular case of insecticides, Italy and Spain represented the 33% and 29%, respectively, of the insecticide consumption in 2003 (Eurostat, 2007). The organophosphate (OP) and, in a less extent, the carbamate (CM) insecticides are the most used chemical classes of PPPs in the EU (Fig. 2). Chlorpyrifos, parathion-methyl, dimethoate, imidacloprid, methomyl, fenthion, methiocarb, methidathion, chlorpyrifos-methyl and endosulfan represent the top-10 active substances in the European PPP market (Eurostat, 2007). Beside insecticides, OP and CM compounds are also present in the formulation of herbicides (21,722 and 2,144 tonnes of OP and thiocarbamate herbicides, respectively, in 2003) and fungicides (21,149 and 3,466 tonnes of dithiocarbamate and OP fungicides, respectively, in 2003) (Eurostat, 2007). Taken together, these data show that OP and CM agrochemicals are still two important groups of pesticides in the current agriculture despite the progressive increase in the use of synthetic pyrethroids (SPTs), among other new pesticide classes. Although data in figure 1 show a generalized global tendency in reducing the pesticide consumption, it does not seem the same scenario in emerging countries like India (Abhilash & Singh, 2009).

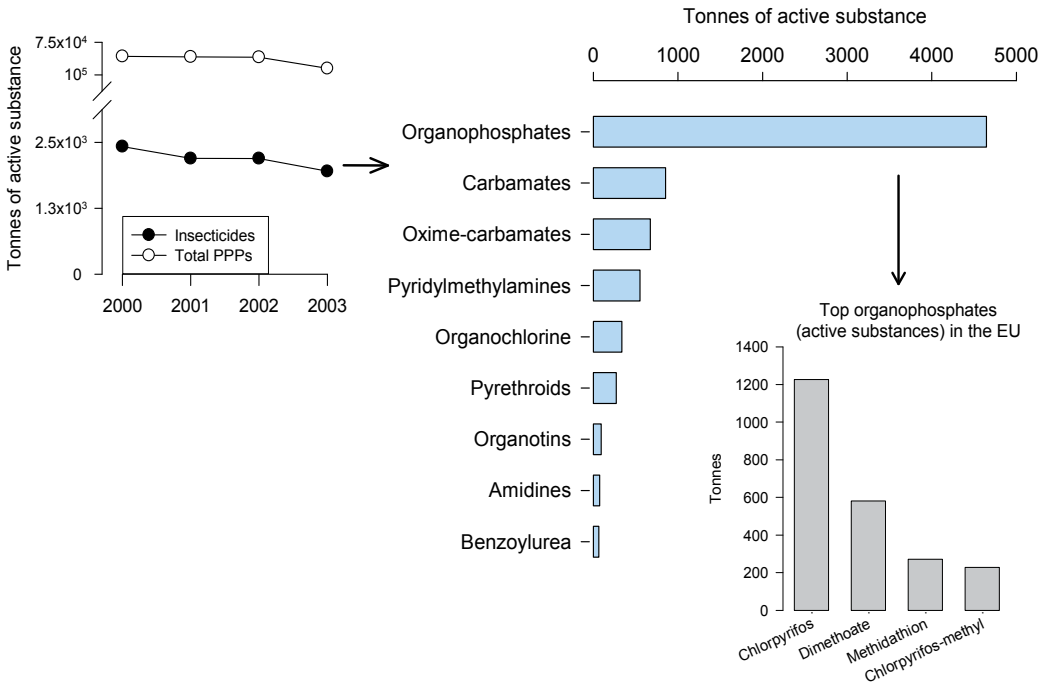


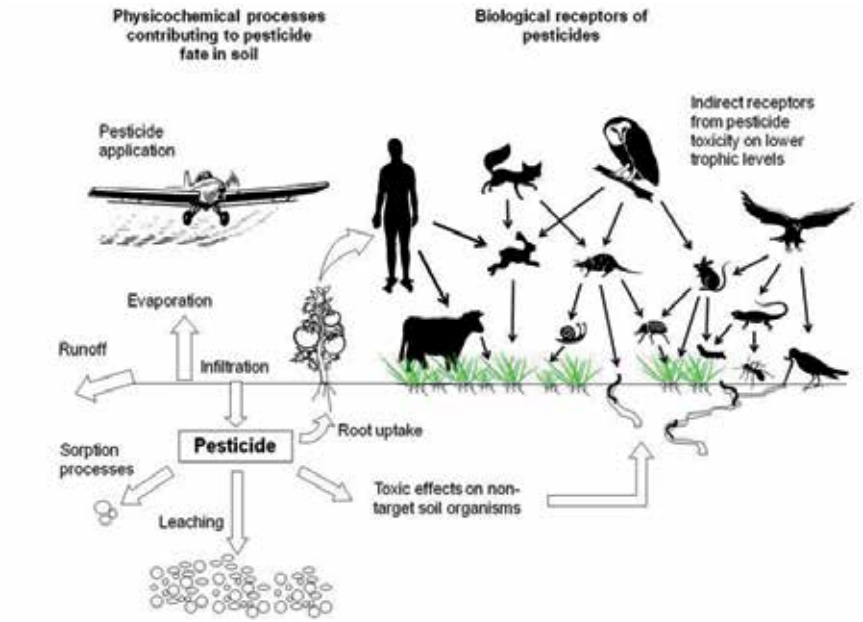
Fig. 2. Use of plant protection products (PPPs) and, particularly, insecticides in the European Union (EU) between 2000 and 2003. Horizontal bar chart shows the main chemical classes of insecticides used in the EU, whereas vertical bar chart shows the most used insecticide active substances (reference year 2003). Data taken from the Eurostat (2007).

Therefore, it would seem evident that pesticide consumption will not decrease substantially and globally in the next decade. Under this assumption, exposure and effect assessment of pesticides in the environment would be necessary for decision-making related to pesticide use and agroecosystem protection, even in a post-authorization phase.

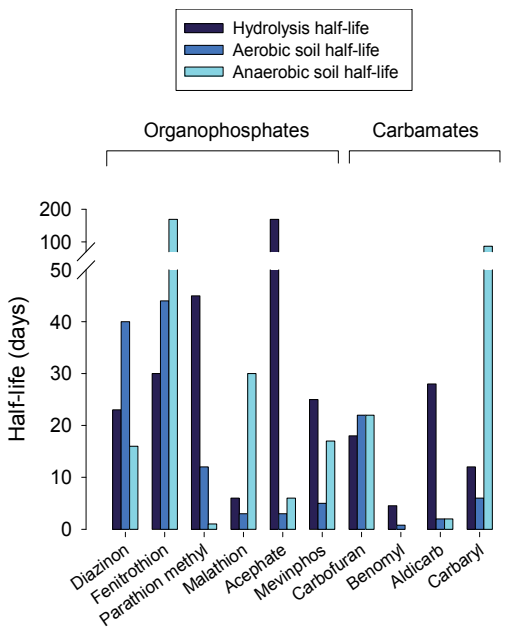
1.1 Why soil is an environmental compartment of (eco)toxicological concern?

The Soil Science Society of America defines soil quality as the "capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation" (Karlen et al., 1997). Therefore, soil degradation is the situation by which soil losses its agronomic and environmental qualities. Both natural phenomena and human activities cause soil degradation. For example, the EU has identified up to eight primary threats for soil quality: erosion, decline in organic matter, contamination, salinisation, compaction, loss of biodiversity, soil sealing, landslides and flooding (European Commission, 2002). Among these soil-deteriorating processes, contamination is probably the most important and dangerous phenomenon to humans because soil is a vital natural resource and, in turn, the man is a significant vector to soil contamination.

Transport of hazardous chemicals, agricultural pesticide applications, oil and fuel dumping, and discharge of industrial/urban wastes are the main human activities causing soil contamination (Mirsal, 2008). Moreover, soil is the environmental compartment where most of the pollutants released into the biosphere are accumulated (Köhne et al., 2009). Once in soil, a wide variety of physicochemical and biological processes, shown in the figure 3A, contribute jointly to the environmental transport and fate of contaminants (Cáceres et al., 2010). Soil acts therefore as a "filter" or "reactor" reducing pollutant leaching towards groundwater or leakage into atmosphere. However, soil contaminants represent a serious hazard to organisms living in both belowground and aboveground systems. The scientific literature is plenty of examples that illustrate the negative impact of agrochemicals on wildlife (Devine & Furlong, 2007; Newman et al., 2006). Agrochemicals take part in the population decline of amphibians (e.g., Mann et al., 2009) and pollinators (e.g., Potts et al., 2010; Spivak et al., 2011). Likewise, pesticides cause side-effects on natural populations of pest enemies (Devine & Furlong, 2007; Devotto et al., 2007) which can make the IPM strategies ineffective. Moreover, reduction of prey populations as a consequence of pesticide applications can lead to indirect effects on top predators (Flavia et al., 2010; Fleeger et al., 2003). Soil microorganisms are also affected by pesticide applications. For example, one of the main metabolites of the OP chlorpyrifos, i.e., 3,5,6-trichloro-2-pyridinol, displays antimicrobial properties. This metabolite inhibits the proliferation of soil microorganisms and, therefore, the subsequent metabolism of chlorpyrifos is reduced (Racke, 1993). The impact of agrochemicals on soil microorganisms can result in changes in the soil nutrient cycles and in the failure of microorganism-assisted bioremediation actions (Barker & Bryson, 2002; Gianfreda & Rao, 2008). Soil enzymes are another molecular target of pesticide inputs. Most of the soil enzyme activities are considered the direct expression of microorganism communities involved in nutrient cycles and they are therefore an indicator of soil fertility (e.g., Gianfreda & Rao, 2008). Many investigations have documented the effects of agrochemicals such as triazines, OPs or CMs on soil enzyme activities (reviewed in Gianfreda & Rao, 2008). Taken together, these studies suggest that control of pesticide residues in soil should be a priority strategy in those agroecosystems where pesticides are intensively used.



A)



B)

Fig. 3. A) Main physicochemical and biological processes contributing to pesticide fate and toxicity (conceptual scheme elaborated from Köhne et al. (2009). B) Hydrolysis and soil (under aerobic and anaerobic conditions) half-lives of selected organophosphate and cabarmate insecticides. Data taken from the Pesticide Action Network (PAN)–Pesticide Database (www.pesticideinfo.org) and from Cáceres et al. (2010).

1.2 Why invertebrate biomarkers can be useful in the assessment of soil pollution by pesticides?

In environmental toxicology, biomarkers are defined as molecular, biochemical, physiological or histological indicators of contaminant exposure or effects (van Gestel & van Brummelen, 1996). This definition frequently includes behavioral changes (e.g., Walker et al., 2001). Biomarkers have shown their ecotoxicological role as indirect measurements of bioavailability or toxicant's absorption when used in toxicity testing (Lanno et al., 2004), or as key elements in the understanding of the toxic mechanism underlying observed effects at whole-organism level (Forbes et al., 2006). They have also been useful to distinguish acute toxicity from long-term effects (Hagger et al., 2009).

Nowadays, there is an intense debate on the suitability and meaning of biomarkers in the environmental risk assessment of environmental contaminants. Traditionally, biomarkers tried to be early indicators of adverse effects at population or community levels (e.g., Peakall, 1992). In addition, a battery of biomarkers covering multiple levels of biological organization is recommended to distinguish reversible adaptive responses from irreversible toxic effects (Galloway et al., 2004; Gastaldi et al., 2007). However, the use of biomarkers for making ecologically relevant predictions is questioned (e.g., Chapman, 2002; Forbes et al., 2006). But, most of the researchers agree that biomarkers provide evidences on the molecular mechanisms operating to cause observed toxic effects on the whole individual.

Biomarker research has had an increasing development in aquatic toxicology. A survey of the scientific literature for biomarker studies indicates that its use has been scarcely investigated with terrestrial organisms, particularly invertebrates. The figure 4 is an attempt to illustrate this marked difference in the impact of biomarker research in terrestrial organisms. We searched the biomarker literature focused on ecotoxicological investigations involving the aquatic and terrestrial systems in the past 10 years using the *ISI Web of Knowledge* search engine. We filtered the searching with multiple keywords specific to aquatic and terrestrial systems. It is evident that the difference in the number of publications between biomarker studies involving the aquatic ecosystem and those performed in the terrestrial system increases progressively since 2000. Moreover, when we limited the searching to 'invertebrates' and 'pesticides', the number of studies addressed on biomarkers increased for aquatic invertebrates, whereas their use in terrestrial invertebrates seems merely anecdotic.

From the literature survey showed in the figure 4, the pertinent question is why biomarkers have not had a similar concern for terrestrial invertebrates if we consider that soil is the primary environmental media where pesticide are accumulated and transformed. Furthermore, pesticides such as OPs, CMs or SPTs generally display short half-lives (from days to a few months) in the environment, and high concentrations of pesticides (and their metabolites) in water are not the most frequent scenario (Cáceres et al., 2010; Gavrilescu, 2005; Katagi, 2004). Agrochemicals can reach the aquatic systems by direct application, runoff from pesticide-treated fields or wind-borne drift (Fig. 3A). However, the figure 3B shows that persistence of OP and CM pesticides in soil seems lower than that observed in water, although it is shown the water half-lives by hydrolysis solely and other degradation processes (photolysis or microbial breakdown) are not considered (Fig. 3B). Because agricultural pesticides are not intended to be used in water bodies and the persistence of pesticides in soil is relatively low, concentrations of agrochemicals in water systems would be assumed as very low otherwise intentional applications into water take place. One possible explanation to the limited studies on pesticide biomarkers in soil invertebrates

could be the heterogeneous and complex nature of the terrestrial environment that makes difficult to identify harmful effects on biota from pesticide exposure. Moreover, economic aspects could also account for a high research interest in aquatic invertebrate. Nevertheless, terrestrial invertebrates are key components of the soil system. For example, earthworms are considered soil engineers with a notable contribution to soil function and structure (Lavelle et al., 2004) as well as to plant growth and health (Scheu, 2003). Other terrestrial invertebrates such as honey bees or the natural enemies of pests play an unquestionable pivotal function in the agroecosystem.

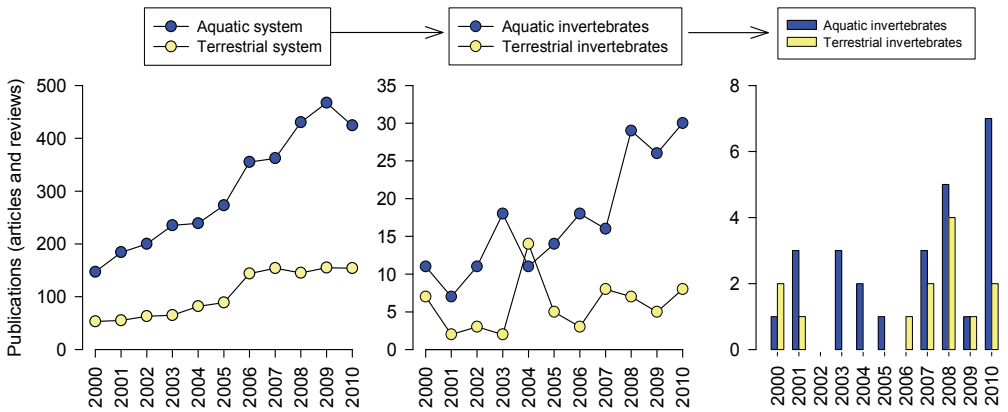


Fig. 4. Number of annual publications (limited to articles and reviews) involving biomarkers. Left graph shows the evolution of biomarker studies in the aquatic and terrestrial ecosystem. Centre graph shows those investigation limited to aquatic and terrestrial invertebrates, whereas the right graph illustrates the biomarker research in these groups of organisms when agrochemicals were the chemical stress. Searching was made using the ISI Web of Knowledge bibliographic search engine (www.accesowok.fecyt.es/login/).

To the question why biomarkers can be useful in the assessment of pesticide toxicity, we could find an answer in their valuable use in the understanding of the mechanistic basis of pesticide toxicity in non-mammal species. Most of the interpretations and conclusions drawn from biomarker responses measured in invertebrates are provided on the basis of the biomarker knowledge in mammals. However, the physiology and biochemistry of many terrestrial invertebrates are not well known, in particular those species that have not an economic or recreational interest. This is not the case of some pest species. For example, it is well known that carboxylesterases play a significant role in the mechanism of pesticide tolerance and resistance in the pest species (Hemingway et al., 2004; Oakeshott etal., 2005). These esterases modulate the toxicity of OPs, CMs and SPTs through or hydrolysis reactions with this agrochemicals (Wheelock et al., 2008). These chemico-biological interactions between esterases and pesticides have not been extensively investigated in non-target terrestrial invertebrates.

This chapter examines the current knowledge on biomarkers of pesticide exposure and effects in terrestrial invertebrates. Particular emphasis will be put in earthworms because of their ecological, toxicological and agronomic concern. Comparisons with related studies performed with aquatic organisms are unavoidable, and will enable us to know at what

extend the findings with aquatic organism biomarkers have been reproduced in soil organisms. Finally, we suggest some issues of methodological concern when biomarkers are used for monitoring pesticide effects or to provide mechanistic understandings on the toxic effects observed at the whole-organism level.

2. Biomarkers of pesticide exposure and effect

2.1 Cholinesterases and carboxylesterases

Esterases act on the ester bond. According to the International Union of Biochemistry they are included in the subgroup 3.1 of hydrolases. In environmental toxicology, acetylcholinesterases (AChE, EC 3.1.1.7), butyrylcholinesterases (BChE, EC 3.1.1.8) and carboxylesterases (CbE, EC 3.1.1.1) have had particular attention because of their role in pesticide toxicity and detoxification. The inhibition of AChE activity is the most used biomarker in the field monitoring of OP and CM impact. This is not surprising because the primary mechanism of acute toxicity of these agrochemicals is the inhibition of AChE activity at the nervous tissue (Thompson & Richardson, 2004). Some reviews provide a comprehensive analysis of this biomarker in the aquatic system (e.g., Domingues et al., 2010; Fulton & Key, 2001; Hyne & Maher, 2003; Jemec et al., 2009; Monserrat et al., 2007; Sanchez-Hernandez, 2001). Carbamate and OP pesticides interact with ChEs, and CbEs in a very similar way (Fig. 5). The carbonyl group of the CM reacts with the serine hydroxyl group at the active site of the esterase to yield a Michaelis-type complex. The carbamylated enzyme is unstable and the activity is rapidly reversed in the presence of water. In this reaction, the CM is chemically destroyed to form

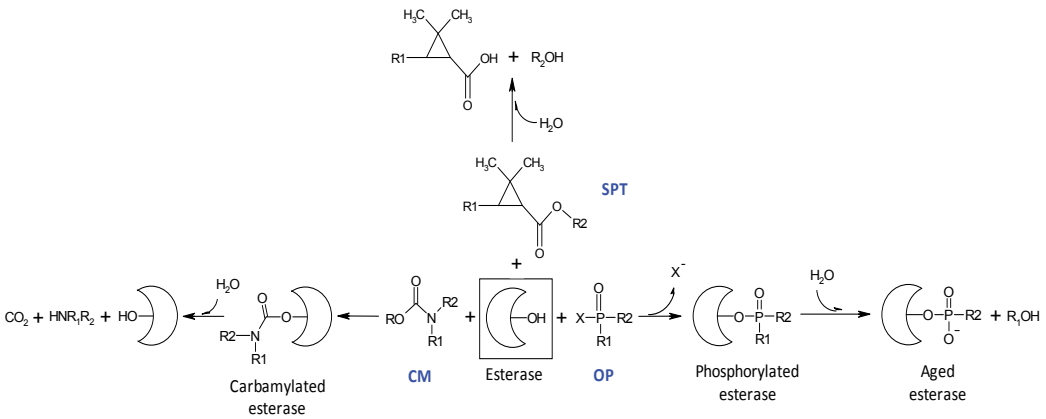


Fig. 5. Interactions of esterases (cholinesterases and carboxylesterases) with carbamates (CM), the oxon metabolites of organophosphates (OP) and synthetic pyrethroids (SPT). Inhibition of esterases by CMs yields a carbamylated complex which is unstable and the esterase activity is rapidly recovered in the presence of water. Organophosphates inhibit irreversibly the hydrolysis activity of ChEs and CbEs by the formation of a stable phosphorylated complex. Under this condition, restoration of the esterase activity requires the synthesis of new enzyme. Synthetic pyrethroids interact only with CbEs, and these esterases hydrolyze them to yield the corresponding alcohol and carboxylic acid. Scheme elaborated from Sogorb & Vilanova (2002) and Thompson & Richardson (2004).

CO_2 and an amine (Sogorb & Vilanova, 2002; Thompson & Richardson, 2004). Similarly, the phosphoryl moiety of the OPs reacts with the serine hydroxyl group of ChEs, or CbEs, to form a stable complex. At this point, the phosphorylated enzyme can undergo three main pathways: spontaneous reactivation, oxime-induced reactivation, or aging. Spontaneous reactivation occurs in the presence of water but it is a very slow reaction, and the enzyme does not recover its full normal activity. Chemical-induced reactivation employing oximes is a faster mechanism of activity restoration. Oximes are nucleophilic reagents able to attack the phosphoryl group bound to the active site of the esterase resulting in the recovery of the activity (Thompson & Richardson, 2004). If the enzyme remains long time inhibited, one alkyl group can release from the phosphyl-esterase complex (dealkylation) and leads the enzyme to a permanent inactivation, a situation known as aging (Fig. 5). At this stage, the esterase cannot be reactivated either spontaneously or using oximes. The type of esterase and the chemical nature of the inhibitor are determinants in the pathway that the phosphorylated enzyme will follow. These post-inhibitor pathways indirectly modulate the toxicity of the OP compound or even may contribute to the chemical interactions between pesticides (e.g., synergism and antagonism). In the case of SPTs, CbEs hydrolyze these agrochemicals to yield the corresponding alcohol and carboxylic acid (Fig. 5).

Cholinesterases and CbEs have been extensively investigated in many pest species because of their implications in pesticide resistance (Hemingway et al., 2004; Oakeshott et al., 2005). Conversely, little is known about these esterases in terrestrial invertebrates of ecological concern such as earthworms or pollinator species. Most of the available works with non-target invertebrate esterases are focused on enzymological aspects of these hydrolases. Laboratory studies have been aimed to examine three main features commonly associated to a good biochemical biomarker: 1) *in vitro* sensitivity to OP and CB insecticides, 2) recovery of ChE activity following pesticide exposure and 3) relationship between esterase inhibition and observed adverse effects at whole-individual level (e.g., growth, mortality or behavior changes).

Earthworms have been model organisms in these esterase investigations. Enzymological characterization of earthworm ChE activity has been extensively investigated in the past (Stenersen, 1980), and more recently, some laboratory studies have suggested the inclusion of CbE activity in the assessment of pesticide exposure and toxicity in these soil organisms (Collange et al., 2010; Sanchez-Hernandez & Wheelock, 2009). Many authors postulate that sensitivity of CbE activity to both OP and CM insecticides modulates the acute toxicity of these agrochemicals. The stoichiometric binding between CbEs and the insecticide (see Fig. 5) can lead to a reduction in the number of inhibitor molecules able to interact with nervous AChE. This assumption has led to compare the sensitivity of ChE and CbE activities to model OP and CM insecticides. Thus, CbE activity of aquatic organisms generally displays a higher *in vitro* and *in vivo* sensitivity to OPs than ChE activity (e.g. Barata et al., 2004; Kuster, 2005; Wogram et al., 2001). These observations are also reproduced in some terrestrial invertebrate groups. For example, earthworms exposed to chlorpyrifos-spiked soils showed a higher percentage of CbE inhibition than ChE activity, irrespective of the tissue used for esterase measurements (Collange et al., 2010; González Vejares et al., 2010). However, foot CbE activity in juvenile garden snails (*Helix aspersa*) was less sensitive to inhibition by dimethoate than foot AChE activity (Coeurdassier et al., 2002). It was suggested in this latter study that the lower sensitivity of foot CbE to the OP could mean a reduced ability of CbEs to protect AChE activity against OP inhibition. Similar results were observed by Laguerre et al. (2009) in the

terrestrial snail *Xeropicta derbentina*. They reported an IC50 (the concentration of pesticide causing a 50% reduction in the enzyme activity) value of 3.8 x 10^{-8} M for AChE activity against chlorpyrifos-oxon, whereas the IC50 was 3.2 x 10^{-6} M for CbE activity. However, when dichlorvos was the inhibitor, the degree of CbE inhibition was stronger than that of AChE (Laguerre et al., 2009).

In general, phosphorylated ChE and CbE activities in earthworms and snails display an extremely slow recovery rate (Coeurdassier et al. 2001, Rault et al. 2008, Collange et al. 2010). This limited capacity of returning to normal activity levels following OP exposure is a generalized phenomenon in earthworms (Table 1). Synthesis of new enzyme would be the most plausible explanation for this slow recovery of OP-inhibited esterase activity. Although spontaneous reactivation of the inhibited enzyme could also contribute to full recovery of the esterase activity, this is not true when the inhibitor is an OP compound (Rodríguez-Castellanos & Sanchez-Hernandez, 2007). A slow recovery rate enables to detect the OP exposure over a longer period of time after OP applications. This is a desirable feature for assessing anti-ChE exposure because of most of these pesticides show a low persistence in the environment (Fig. 3B).

Species	Biological material	Insecticide (concentration)	Time of exposure	Cholinesterase response	Reference
Eisenia fetida	Whole body	Chlorpyrifos (240 mg/kg dry wt)	2 days	No recovery of E2 (a carbaryl-resistant form of ChE) activity during 84 days of transferring earthworms to clean soil. E1 (a carbaryl-sensitive form of ChE) recovered its normal level of activity after 21 days followed OP exposure.	(Aamodt et al., 2007)
Aporrectodea caliginosa and *Allolobophora chlorotica*	Whole body	Parathion-ethyl (1 and 10 mg/kg dry wt)	14 days	No recovery of ChE activity of *A. caliginosa* exposed to both OP concentrations after 70 days of transferring earthworms to clean soil. Full recovery of ChE activity in *A. chlorotica* exposed	(Rault et al., 2008)

				to 1 mg kg^{-1} after 70 days of transferring earthworms to clean soil, but no recovery of ChE activity in the group exposed to 10 mg/kg.	
A. caliginosa	Whole body	Diazinon (60 mg/kg dry wt). Chlorpyrifos (28 mg/kg dry wt)	14 days	No recovery of ChE activity during the 14 days of OP exposure (inhibition percent > 85 %).	(Booth et al., 2000)
Lumbricus terrestris	Body wall muscle	Chlorpyrifos (3, 12 and 48 mg/kg)	2 days	No recovery of muscle ChE activity of *L. terrestris* exposed to 12 and 48 mg/kg dry wt after 35 days of transferring earthworms to clean soil.	(Collange et al., 2010)
L. terrestris	Pharynx, crop, gizzard, foregut and seminal vesicles	Chlorpyrifos (3, 12 and 48 mg/kg)	2 days	Carboxylesterase activity of gut tissues (measured with the substrate 4-NPV) did not recover its activity in earthworms exposed to 12 and 48 mg/kg.	(González Vejares et al., 2010)

Table 1. Recovery of cholinesterase (ChE) activity in several adult earthworm species following exposure to organophosphorus (OP)-spiked soils. This table was elaborated from data published as supplementary material in Collange et al. (2010).

One of the most desirable attribute of a biomarker when it is used in the environmental assessment of contaminants is to be a predictor of adverse effects at higher levels of biological organization (e.g., whole individual or population). However, many researchers have shown that such a link is hard to establish (Chapman, 2002; Forbes et al., 2006). In the cascade of biological responses occurring when the organism is exposed to environmental contaminants, it is expected that early responses occur at biochemical and molecular levels before than observed effects at higher level (changes in growth, reproduction or behavior). However, biological responses at the whole-individual level are often more sensitive or more easily detectable than biochemical biomarkers. For example, burrowing activity of earthworms was

a more sensitive endpoint to imidacloprid than ChE and GST activities (Capowiez et al., 2003). Comparisons of LOEC (lowest observed effect concentration) values between biochemical biomarkers and whole-organism responses measured in the terrestrial isopod *Porcellio scaber* evidenced that the biochemical biomarkers were not necessarily more sensitive to diazinon or imidacloprid exposures (Jemec et al., 2009). To a less extent, inhibition of ChE activity by pesticides has been related to tissue damage. The carbamate insecticides methomyl and methiocarb caused histopatological and ultrastructural alterations in the nervous tissue of the land snail *Eobania vermiculata* after 14 d of exposure to sublethal concentrations of these CMs (Essawy et al., 2008).

The interaction between ChE activity and pesticides has been explored with terrestrial non-target organisms other than earthworms and snails. Some studies have involved the use of ChE inhibition as an indicator of pesticide exposure in bees, isopods or spiders. Recently, there is a global concern in population decline of honey bees (Spivak et al., 2011). Among the multiple factors potentially responsible for this phenomenon, agricultural pesticide applications seem to contribute to this global bee's population decline (Gross, 2011). Nevertheless, little is known about the use of pesticide biomarkers in bees. Past studies have documented many biochemical aspects of bees CbEs. As with many other organisms, multiple CbE isozymes are generally found in the bee (Krieg & Marek, 1983), which play an important role in the metabolism of pesticides (Yu et al., 1984). Frohlich (1990) compared the hydrolytic activity of CbE in males and females of the solitary bee *Megachile rotundata* using multiple substrates. Bee sex had a significant impact on the variability of CbE activity. It was suggested that esterases of *M. rotundata* may be involved in the nest construction which would explain the higher levels of CbE activity in the females. This speculation suggests further exploration to examine whether anti-ChE pesticides are able to disrupt nest performance by inhibition of CbE activity because this enzyme is likely involved in the chemical process that leads to the formation of the brood cells (Frohlich, 1990). Isopods are another group of invertebrates that, despite of their growing concern in terrestrial ecotoxicology (Drobne, 1997), their esterases have been little studied in relation to pesticide contamination. Stanek et al. (2006) compared the inhibitory response of AChE activity in both adults and juveniles *P. scaber* exposed to diazinon-spiked food for two weeks. They found that AChE activity of juveniles was more sensitive to the OP than that of adults. Moreover, inhibition of AChE activity was linked to mortality of isopods, however other biological traits such as feeding activity or weight change did not vary with the diazinon exposure. The study by van Erp et al. (2002) is an example on the toxic effects of pesticides on a pest natural enemy. The wolf spider (*Lycosa hilaris*) is frequently found in the agroecosystem and it is a natural predator of many pest species. Cholinesterase activity was investigated in adults of this arachnid exposed to environmentally realistic concentrations of diazinon and chlorpyrifos. A ChE inhibition >80 % was associated to high mortality of male and female spiders in both laboratory and mesocosm trials, although females were more resistant to the toxic action by diazinon (van Erp et al., 2002).

Despite the widespread use of ChE inhibition as a sensitive indicator of OP and CM exposure, its use in terrestrial invertebrates sampled from, or caged in, the agroecosystem has been little explored. The soil-dwelling earthworm *A. caliginosa* has been used for monitoring OP exposure in the agroecosystem (Reinecke & Reinecke, 2007). Although ChE inhibition was recorded in the earthworms, it was not possible to make clear predictions at whole-individual level (e.g., changes in behaviour). Inhibition of ChE activity in earthworms and terrestrial snails has been satisfactorily used to distinguish the impact of multiple pest

control strategies in apple orchards (Denoyelle et al., 2007; Mazzia et al., 2011). These field studies only show that exposure to anti-ChE pesticides took place in the moment of specimens' collection, but information about detrimental effects at whole-individual level, indirect effects on other non-target organisms, or recovery of inhibited ChE is unknown. As argued by others, species selection, exposure design (e.g., *in situ* exposure, mesocosm), simulated pesticide applications, selection of tissues according to the mode of toxic action or detoxification pathways, among other factors, should be considered before planning a biomonitoring program for assessing environmental impact of post-authorized pesticides (Newman et al., 2006; Sanchez-Hernandez, 2010).

Esterases are generally considered indicators of toxicant's absorption. In addition, mammalian BChE and CbE activities are efficient bioscavengers of OPs reducing the impact of these compounds on brain AChE activity (Masson & Lockridge, 2010; Maxwell & Brecht, 2001; Wheelock et al., 2005). For example, Dettbarn et al. (1999) demonstrated that rat plasma CbE activity decreased the acute toxicity of paraoxon and, furthermore, a rapid recovery of both plasma and liver CbE activities following OP exposure contributed to a lack of toxicity. Beside the affinity of esterases for OP compounds, the number of enzyme molecules is also critical in the efficacy of this stoichiometric mechanism of detoxification. For example, Chanda et al. (1997) observed that liver CbE activity of female and male rats showed the same affinity for binding chlorpyrifos-oxon, however the liver of males had twice specific CbE activity than the liver of females. This variation in the CbE activity was a determinant factor in OP toxicity beside of CbE affinity for these pesticides. On the other hand, the interaction of these esterases with CM insecticides is a reversible inhibition that destroys chemically the parent compound (*see* Fig. 5). Taken together, these studies lead to postulate that BChE and CbE activities contribute significantly to modulate the acute toxicity of OPs and CMs. However, little is known about the detoxification role of BChE and CbE activities in terrestrial invertebrates.

2.2 Glutathione S-transferases and other related antioxidant enzymes.

Many agrochemicals such as OP insecticides are able to induce oxidative stress (Lukaszewicz-Hussain, 2010), a situation in which the production of reactive oxygen species (ROS) overcomes the cellular antioxidant mechanisms (molecular and enzymatic), leading to the oxidative damage of biomolecules (e.g., lipids, proteins or DNA). Glutathione level is one of the most used biomarker of pro-oxidant exposure in fish (van der Oost et al., 2003) and birds (Koivula & Eeva, 2010). In the detoxification of environmental contaminants, glutathione plays two main roles (van der Oost et al., 2003):

1. This tripeptide acts directly as a scavenger of ROS. In this interaction, the reduced glutathione (GSH) is oxidized to the disulfide form (GSSG). Thus, the GSH/GSSG ratio is a suitable biomarker of oxidative stress.
2. Glutathione is the cofactor of some enzymatic reactions involved in the metabolism of xenobiotics. For example, glutathione S-transferases (GSTs) use glutathione to form a conjugated metabolite with electrophilic intermediates that, in turn, are generated from the phase-I metabolism of xenobiotics. Similarly, hydrogen peroxide and other organic hydroperoxides are reduced to their corresponding alcohols by the action of glutathione peroxidases (GPx) which use glutathione as a cofactor. In this reaction GSH is oxidized to GSSG.

The GSSG formed during these detoxication pathways is reduced back to GSH by the glutathione reductase (GR), which is an essential enzyme in the GSH/GSSG balance. In summary, the GSH/GSSG ratio as well as the main enzymes involved in its redox homeostasis are proposed as sensitive exposure biomarkers of cellular oxidative stress (Koivula & Eeva, 2010; Maity et al., 2008; van der Oost et al., 2003). Few studies have been concerned with changes in glutathione concentration and glutathione-dependent enzymes in terrestrial invertebrates. Biomarkers of oxidative stress have been mainly explored in earthworms exposed to, or inhabiting in, metal-polluted environments. For example, earthworm GST activity is a noteworthy detoxication system (Stenersen, 1984), which is induced in earthworms exposed to organochlorine pesticides (Hans et al., 1993). However, no effects on this enzyme activity were observed in earthworms exposed to the OP fenitrothion (Booth & O'Halloran, 2001) or the CM carbaryl (Ribera et al., 2001). Herbicides also induce the GST activity of earthworms. For example, a strong induction of GST activity was found in *E. fetida* exposed for 24 and 48 h to fenoxaprop and metolachlor (Aly & Schröder, 2008). In the terrestrial isopod *P. scaber*, GST activity increased after two weeks of dietary exposure to 5 μg/g imidacloprid, but decreased in adults exposed to 25 μg/g of this neonicotinoid insecticide (Drobne et al., 2008). Despite this limited number of studies, it is not clear how the enzymatic (e.g., GST, GR, GPx, catalase, etc.) and molecular (e.g., GSH) antioxidant mechanisms work in terrestrial invertebrates exposed to pesticide-contaminated environments.

2.3 Behavioral changes as indicators of pesticide exposure

Behavior is the final integrated result of a diversity of physiological processes interacting with the surrounding abiotic and biotic components (Adkins-Regan & Weber 2002). In soil toxicity testing, body weight changes and reproduction rate are common sublethal toxicity endpoints. However, there is a growing concern to include new sublethal variables with ecological relevance such as behavior (Hellou, 2011). Many investigations have evidenced that pesticides are able to cause behavioral changes. Acephate (Moulton et al., 1996) and dichlorvos (McHenery et al., 1997) altered the ability of mussels to retract the mantle fringes and close the valves. The OP azamethiphos caused significant changes in the sheltered behavior of juvenile lobsters (*Homarus americanus*) (Abgrall et al., 2000). Similarly, the literature is plenty of examples describing perturbation or disruption of physiological systems directly involved in fish behavior (Scott & Sloman, 2004). Behavior is also a sensitive indicator of pesticide exposure in invertebrates. For example, the OP dimethoate caused significant changes in the locomotor activity of the collembolan *Folsomia candida* (Sorensen et al., 1995). Burrowing of *A. caliginosa* was examined in soil spiked with parathion-ethyl and this behaviour was more sensitive than ChE inhibition (Olveravelona et al., 2008). Similarly, a bioassay with the terrestrial isopod *Porcellio dilatatus* and dimethoate evidenced a significant relationship between ChE inhibition and locomotor impairment following 48 h of OP exposure (Engenheiro et al., 2005). Moreover, although such a correlation was lost within 10 d of pesticide exposure, locomotor variables (path length, average velocity, active time or stops per path) and AChE activity were still affected by the OP (1–60 μg/g soil).

According to the concept of a hierarchical cascade of biological responses to pollutants, sub-individual biomarkers should be linked to behavioral responses. The OP and CM pesticides are a good example to test this hypothesis. Their primary mechanism of acute toxicity is the

inhibition of the AChE activity at the nervous system and neuromuscular junction. A severe inhibition of this enzyme should cause behavior changes mediated by cholinergic synapses. A good example of such a relationship is the study by Beauvais and coworkers. A correlation between inhibition of brain AChE activity and decreasing of swimming speed was found in larval rainbow trout exposed for 24 and 48 h to carbaryl (Beauvais et al., 2001). In other related study, changes in the swimming speed or distance of larval rainbow trout exposed to malathion and diazinon significantly correlated with AChE inhibition (Beauvais et al., 2000). In the light of these studies, implementation of biochemical biomarkers directly implicated in behavior (e.g., AChE inhibition) could increase the toxicological meaning of behavior bioassays for assessing soil pollution.

The standardized avoidance behavior test with earthworms is an example of how behavior changes can be used easily as a screening toxicity test of soil pollution (ISO, 2005). The most common design to carry out the avoidance behavior test is a two-chamber system (ISO, 2005). This is a rectangular container which is divided in two equal compartments by a removable plastic separator. A control soil is placed in a compartment whereas the contaminated soil is placed in the other. Earthworms are then released in the middle of the rectangular container after remove the plastic split. Elapsed a period of exposure (normally 48 h), the plastic separator is inserted again in the middle of the container and individuals are counted in each soil compartment. The avoidance response is judged as positive when a percent of live earthworms higher than 80 % is found in the compartment containing the reference soil. This simple test can be of ecological concern because this escape behavior could alter the earthworm community of the soil or to change earthworm-induced physicochemical properties of soil. However, the meaning of the avoidance behavior test may be modified whether earthworms are released in the chamber containing the contaminated soil and after a fixed period of time, the separator is removed enabling to earthworms move toward the clean soil (Rodríguez-Castellanos & Sanchez-Hernandez, 2007). With this alternative approach, avoidance ability, locomotor activity and AChE inhibition can be evaluated all together and quantitative relationships may be established in relation to pesticide exposure; which is an important aspect not considered in the standardized avoidance behavior test. Attempts to increase the environmental realism of the avoidance behavior response test have been performed by others. For example, a vertical avoidance behavior test was proposed by Ellis et al. (2010) to be used with soil-dwelling earthworms. Inclusion of biomarkers directly related to pesticide toxicity (ChE inhibition) and detoxification (CYP-dependent monooxygenases, GST or CbEs) could be helpful in the understanding of the underlying mechanistic events that yield toxic-induced behavior responses or behavioral adaptive responses (Pereira et al., 2010).

3. Some methodological issues with biomarkers

3.1 Tissue-specific analysis

Selection of the target tissue or organ is critical for biomarker analysis. However, many studies have used the whole organism or pooled individuals for biomarker determinations. Moreover, when the body size is often not sufficiently large to perform accurate molecular and biochemical analysis, portions of the animal where is suspected a high concentration of the proteins of interest are used for biomarker measurements (Rault et al., 2007; Ribeiro et al., 1999). The biomarker literature is, however, plenty of examples that illustrate significant

tissue-specific variations in biomarker responses and sensitivity to environmental pollutants. For example, *E. fetida* has two different ChEs with a marked difference in OP and CM sensitivity (Aamodt et al., 2007; Stenersen, 1980). Likewise, when earthworm ChE and CbE activities are measured in a tissue-dependent way, there is a strong variation in the activity levels of these esterases (Rault et al., 2007; Sanchez-Hernandez & Wheelock, 2009). Our laboratory has determined the normal variation of ChE and CbE activities in the soil-dwelling earthworm *L. terrestris* (Fig. 6A). The highest levels of ChE activity were observed in the body wall muscle and the pharynx, the latter probably as a consequence of the nervous tissue (ganglions) dissected together with the pharynx (Fig. 6B). This esterase

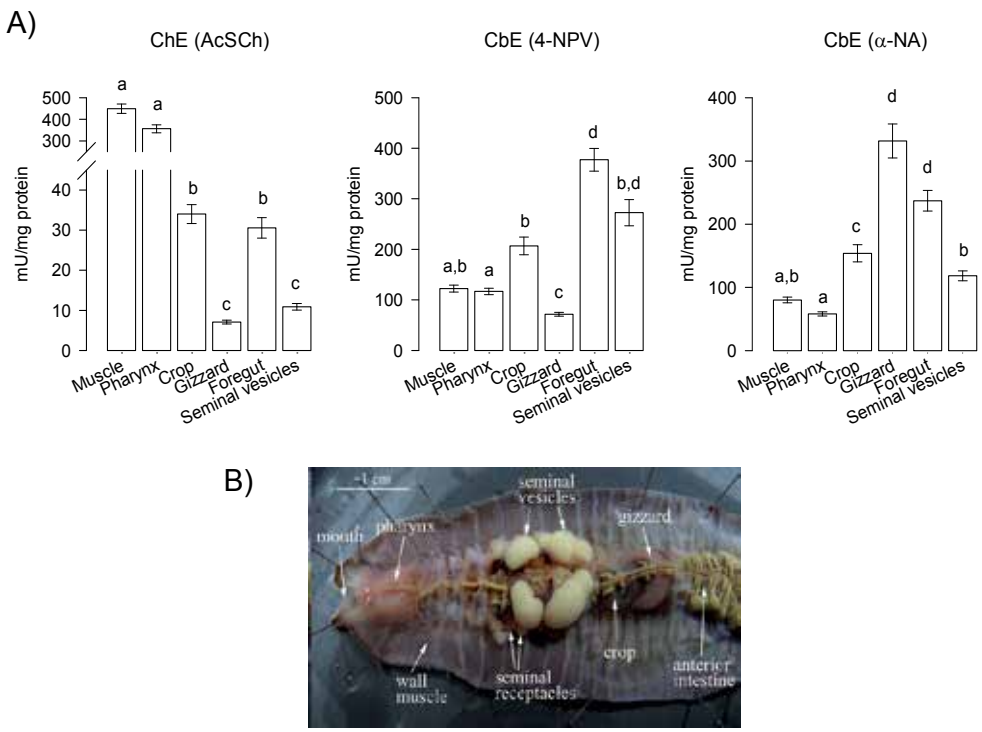


Fig. 6. A) Cholinesterase (ChE) and carboxylesterase (CbE) activities in multiple tissues of the earthworm *Lumbricus terrestris*. The substrate acetylthiocholine iodide (AcSCh) was used for ChE determination, whereas CbE activity was assayed using α-naphthyl acetate (α-NA) and 4-nitrophenyl valerate (4-NPV). B) Internal anatomy of *L. terrestris* showing some structures and organs of the gastrointestinal tract, and the reproductive system. Bars are the mean and the standard errors of 36 individuals. Different lower case letters denote significant differences (pairwise multiple comparison Dunn's test, $P<0.05$).
Data taken from the supplementary material provided in González Vejares et al. (2010) and Collange et al. (2010). Photograph of the internal anatomy of *L. terrestris* was kindly provided by Christopher Mazzia and previously published in Sanchez-Hernandez and Wheelock (2009).

activity showed a marked regional variation along the alimentary canal of *L. terrestris*. Carboxylesterase activity also displayed a tissue-specific variation of its hydrolytic activity towards two common substrates, i.e., α-naphthyl acetate (α-NA) and 4-nitrophenyl valerate (4-NPV). Although the hydrolysis of both substrates could be carried out by the same CbEs, the activities measured in the gizzard suggested the presence of multiple isozymes with a different substrate preference (Fig. 6A).

Beside this marked tissue-specific variation in esterase activity, its sensitivity to pesticide is also highly dependent on the tissue where esterases are expressed. For example, Sanchez-Hernandez & Wheelock (2009) found that the *in vitro* inhibition of CbE activity by chlorpyrifos-oxon varied with the tissue. Furthermore, the substrate used for CbE measurements evidenced multiple isozymes with marked differences in sensitivity to chlorpyrifos-oxon. In general, IC50 values for CbE activity using 4-NPV were lower than those reported with the use of 4-nitrophenyl acetate (4-NPA) (Sanchez-Hernandez & Wheelock, 2009).

These *in vitro* outcomes have been reproduced in microcosm trials (Collange et al., 2010; González Vejares et al., 2010). Earthworms (*L. terrestris*) exposed to chlorpyrifos-spiked soils for 2 days showed a tissue-specific variation in ChE and CbE inhibition, and the recovery pattern of the enzyme activities was also different dependent on the tissue and, in the case of CbE activity, the substrate used in the enzyme assay (Fig. 7). As with other organisms, the CbE activity was more sensitive to chlorpyrifos exposure than ChE activity, but not all tissues showed such a response. For example, gizzard ChE activity was more depressed that CbE activity (Fig. 7). Moreover, when α-NA was used as the substrate for CbE measurements, we found a significant increase of this esterase activity compared to controls. These microcosm studies clearly indicate that determination of esterase inhibition as a biomarker of pesticide exposure should be made in a tissue-specific way, instead of using the whole organism or portions containing multiple tissues. A similar conclusion can be drawn from other detoxifying enzymes such as cytochrome P450-dependent monooxygenases (Stenersen, 1984) or GST activity (LaCourse et al., 2009).

3.2 Substrate-specific analysis

In general, enzyme kinetic procedures (e.g., spectrophotometric assays) used in invertebrates are directly reproduced, or include slight modifications, from those validated for mammals. However, the biochemistry and physiology of terrestrial invertebrates such as earthworms or isopods are not well known as in mammals, and there is a serious risk of making erroneous conclusions about the toxic effects of environmental contaminants. Some biochemical biomarkers are commonly measured in aquatic and terrestrial invertebrates using protocols developed and optimized for mammals. For example, specific inhibitors for AChE (BW284C51) and BChE (tetraisopropyl pyrophosphoramide or iso-OMPA) activities or selective substrates (acetyl-β-(methyl)thiocholine for AChE or butyrylthiocholine for BChE) allow to distinguish both ChEs when co-exist in the same tissue or organ. Although this approach is suitable for mammalian ChE activities, when it is used with terrestrial invertebrates arises atypical or overlapping mammalian ChEs-type properties (Rault et al., 2007; Stenersen, 1980). For example, ChE activity of *E. andrei* was not sensitive to iso-OMPA when the esterase activity was assayed with butyrylthiocholine but was sensitive to the inhibition by BW284C51 (Caselli et al., 2006). However, Stenersen (1980) used

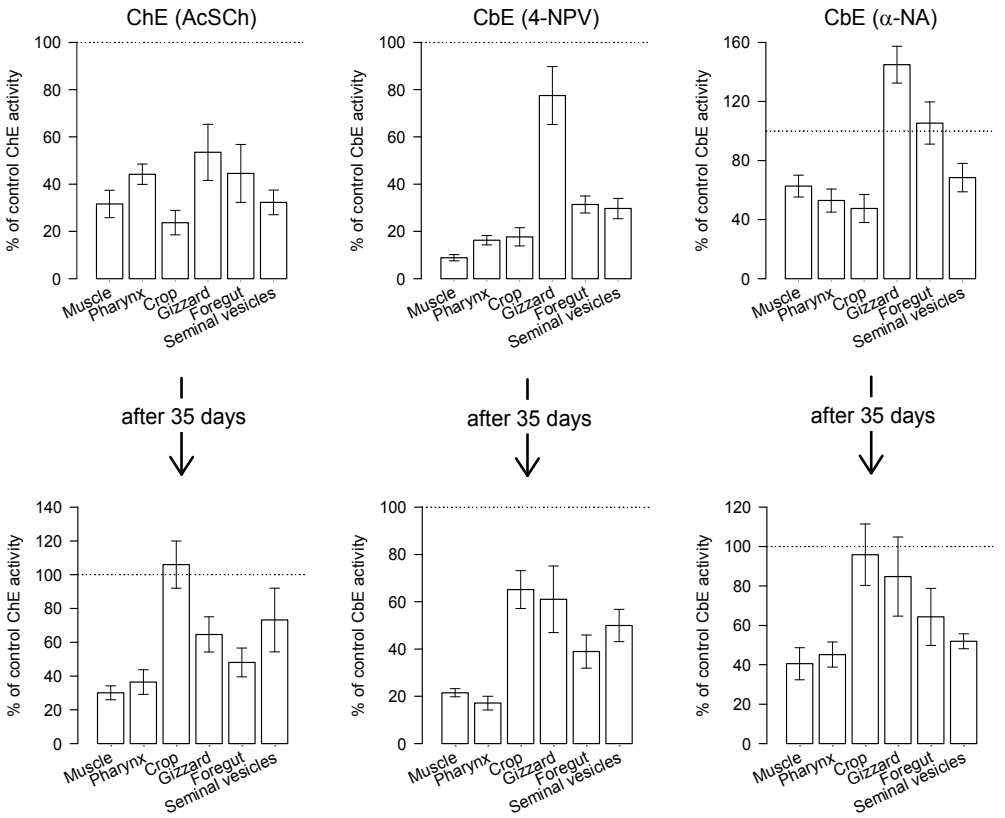


Fig. 7. Porcentages of remaining cholinesterase (ChE) and carboxylesterase (CbE) activities measured in multiple tissues of the earthworm *Lumbricus terrestris* following two days of exposure to soils spiked with 48 mg/kg dry wt chlorpyrifos, and after 35 days of transferring earthworms into clean soils. The substrate acetylthiocholine iodide (AcSCh) was used for ChE determination, whereas CbE activity was assayed using α-naphthyl acetate (α-NA) and 4-nitrophenyl valerate (4-NPV). Bars are the mean and the standard errors of 6 individuals. Data taken from the supplementary material provided in González Vejares et al. (2010) and Collange et al. (2010). Horizontal dotted lines indicate the mean esterase activity of controls set to 100%.

carbaryl as a specific inhibitor to differentiate two ChEs in *E. fetida*, a mammalian-type AChE activity named by the author as E1 and a mammalian-type BChE activity named E2 (carbaryl-resistant ChE). These earthworm ChE activities showed different recovery rates following exposure to chlorpyrifos (Aamodt et al., 2007), which indicate that determination of both ChEs should be performed individually to know the real impact of OP exposure on ChEs of this earthworm species.

The measurement of CbE activity is another example that illustrates why common substrates routinely used for enzymatic assays should be implemented in invertebrates with some reservations. Carboxylesterases comprise multiple isozymes whose number and activity depend on the tissue where they are present (Satoh & Hosokawa, 2006; Wheelock et al., 2008). These esterases display a broad range of substrate specificity (Wheelock et al.,

2005). Naphthyl and nitrophenyl esters are the usual substrates for CbE determinations, and some researchers recommend the use of multiple substrates when these esterases are used as biomarkers of pesticide exposure (Wheelock et al., 2005; Wheelock et al., 2008). However, most of the ecotoxicological studies use one or two substrates (usually α-NA or 4-NPA) to determine the CbE activity with the risk that these substrates be not efficiently hydrolyzed. On the other hand, because these substrates have not any known biological significance, it is difficult to understand the toxicological meaning of CbE activity and inhibition. In mammals, some authors have used more realistic substrates from an environmental and pharmacological viewpoint. Thus, liver and intestinal CbE activities were able to hydrolize efficiently type-I pyrethroids compared to type-II pyrethroids (Ross et al., 2006; Ross & Crow, 2007). In addition, the hydrolysis kinetic parameters usually obtained with SPT insecticides are different to those obtained with common substrates such as 4-NPA or 4-NPV. Indeed, it is suggested that different CbE isozymes are involved in the hydrolysis of pyrethroids and the nitrophenyl esters (Wheelock et al., 2003). Our studies with *L. terrestris* also show the occurrence of multiple CbE isozymes with marked differences in substrate specificity in the gastrointestinal, reproductive and muscle tissues (Fig. 6). More recently, we have detected pyrethroid hydrolysis by CbEs in the earthworm gut, which does not correlate with the CbE activity towards naphthyl or nitrophenyl esters (Sanchez-Hernandez, pers. comm.).

Glutathione *S*-transferases and cytochrome P450-dependent monooxygenases (CYPs) are two groups of detoxifying enzymes that participate in the biotransformation of liphophilic compounds (Hodgson, 2010). For example, GST activity catalyzes glutathione-aryltransfer or glutathione-alkyltransfer reactions of OP insecticides forming non-toxic conjugate metabolites (Jokanovic, 2001). In routine assays, GST activity is determined by a spectrophotometric assay in which the substrate 1-chloro-2,4-dinitrobenzene is conjugated with GSH to form a conjugated metabolite, i.e., 1-(S-glutathionyl)-2,4-dinitrobenzene, which is monitored at 340 nm (Habig et al., 1974). This is the most common spectrophotometric method to determine GST activity for biomonitoring purposes. Again, the occurrence of multiple forms of GST (cytosolic and microsomal) not only in mammals (Hayes et al., 2005) but also in earthworms (Aly & Schröder, 2008; LaCourse et al., 2009) suggests that more than one substrate should be used for exploring induction or inhibition of GST activity by pesticides. Ethoxyresorufin-O-deethylase (EROD) activity is the most common enzymatic assay to measure the induction of the cytochrome P4501A (CYP1A) isozyme (van der Oost et al., 2003; Whyte et al., 2000). This isozyme plays a pivotal role in the detoxication and bioactivation of pesticides. For example, CYP1A catalyzes the conversion of phosphorothioate- and phosphorodithioate-type OP pesticides into their highly toxic 'oxon' forms (Jokanovic, 2001). Earthworms present two CYP subfamilies, i.e., the polyaromatic hydrocarbon-inducible form (CYP1A) and the phenobarbital-inducible form (CYP2B) (Stenersen, 1984). However, there is a marked species-specific difference in the catalytic properties of CYPs and induction capability. For example, microsomes of *L. terrestris* midgut showed CYP activity when benzyloxyresorufin was used as substrate, but no dealkylation activity was detected towards other resorufin derivates such as methyloxy-, ethyloxy- or propyloxyresorufin (Bergholjt et al., 1991). Conversely, microsomes of whole *E. fetida* displayed CYP activity when benzyloxy- and pentoxyresorufin were used as substrates (Achazi et al., 1998). *Lumbricus rubellus* did not show detectable CYP1A activity using EROD as the catalytic assay even when earthworms were exposed to known inducers of CYP1A

activity (Brown et al., 2004). Taken together, these studies show that CYP activity is highly dependent on substrate and earthworm species. However, the role of this detoxifying multienzymatic system should be investigated in detail in pesticide-exposed earthworms to propose the most appropriate substrates to measure induction (or inhibition) of CYP450 activity.

3.3 Complementary methods

The chemical reactivation of the phosphorylated ChE activity using oximes is a workable methodology of assessment of wildlife exposure to OP insecticides in vertebrates. However, this approach has not had a comparable attention with terrestrial invertebrates. Some laboratory studies have proved that oximes are able to recover the activity of the OP-inhibited ChE activity in earthworms (Rodriguez and Sanchez-Hernandez 2007), snails (Laguerre et al. 2009) and honey bees (Polyzou et al. 1998). One of the main limitations of this method is the lack of oxime-induced reactivation when the esterase remains long time inhibited. However, this drawback could still be useful for detecting multiple and short-term exposures to OP insecticides because aged ChE and new synthetized ChE could be estimated with the use of oximes. For example, ChE activity of the earthworm *L. terrestris* was significantly reactivated with 2-PAM or obidoxime within one week following acute OP exposure, although such a chemically-induced recovery decreased with time as a consequence of ChE aging (Collange et al. 2010). Thus, comparison of ChE activity levels between OP-exposed and control earthworms in combination with oxime reactivation assays would enable to detect inhibition of newly synthesized enzyme if earthworms suffer an additional OP exposure event. Nonetheless, optimization of oxime-induced reactivation of phosphorylated ChE activity should be performed when we use a new species or a new tissue as target for ChE determination and reactivation.

4. Concluding remarks

Plant protection products are still necessary for combat pests. The massive use of pesticides leads to a set of environmental hazards on non-target organisms of ecological and agronomic concerns such as earthworms, pollinators or natural enemies of pests. Moreover, the occurrence of pesticide residues in soil can change microbial communities and soil enzyme activities involved in nutrient cycles. These effects can lead, in turn, to a loss of soil quality. In the predictive and retrospective (post-authorized) environmental risk assessment of PPPs, exposure and effect assessment of pesticide toxicity on non-target organisms is an essential step for decision-making related to pesticide use and agroecosystem protection. Besides toxicity and bioaccumulation bioassays, biomarkers are often used to provide mechanistic understandings on the toxic effects observed at the whole-organism level. Classical biochemical biomarkers have been used in terrestrial invertebrates, mainly earthworms, to assess exposure to OP and CM pesticides. Below it is emphasized a set of practical issues that would require further investigation to use biochemical biomarkers in the understanding of pesticide toxicity and tolerance in terrestrial invertebrates.

When possible, the analysis of biomarkers should be performed in a tissue-specific way because level, degree of response or persistence of the response is highly dependent on the tissue. Moreover, a tissue-specific analysis of the biomarkers can be helpful to understand local toxic effects of pesticides or possible mechanisms involved in the reduction of pesticide

uptake and detoxication (e.g., sensitivity and expression of pesticide-detoxifying enzymes in the gastrointestinal tract).

Linking biochemical biomarkers to behavior changes is a growing ecotoxicological topic that requires further studies aimed to examine adaptive behavior responses following pesticide exposure, or the impact of long-term and low-level pesticide exposure on the "behavior-biomarkers" interaction. Current behavior protocols such as the standardized avoidance behavior bioassay with earthworms (ISO, 2005) and other more ecologically relevant alternatives provide an excellent opportunity to link those biomarkers directly related to pesticide toxicity and metabolism with behavior.

When enzyme inhibition (e.g., CbEs or ChEs) or induction (e.g., GST or CYP1A) are used to assess pesticide toxicity and detoxication, appropriate substrates or multiple substrates are recommended because of multiple isozymes cco-existing in the target tissue or organ. Some studies discussed in this chapter have demonstrated that earthworm CbE activity (an esterase of notable importance in the metabolism of OP, CM and SPT pesticides) display multiple tissue-specific isozymes and, further, these isozymes respond (inhibition and recovery) differently to OP exposure.

When ChE activity is used as a biomarker of pesticide exposure, it is recommended the use of oximes (nucleophilic compounds able to restore the ChE activity following OP exposure). Some studies with earthworms have shown that phosphorylated ChE activity can be reversed *in vivo* and *in vitro* by pralidoxime and obidoxime. This methodology would allow to assess multiple OP exposures in the field or to examine the potential contribution of ChEs (AChE and BChE) as bioscavengers of OP pesticides.

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6. References

Aamodt, S., Konestabo, H. S., Sverdrup, L. E., Gudbrandsen, M., Reinecke, S. A., Reinecke, A. & Stenersen, J. (2007). Recovery of cholinesterase activity in the earthworm *Eisenia fetida* Savigny following exposure to chlorpyrifos. *Environmental Toxicology and Chemistry*, 26, 1963-1967.

Abgrall, P., Rangeley, R. W., Burridge, L. E. & Lawton, P. (2000). Sublethal effects of azamethiphos on shelter use by juvenile lobsters (*Homarus americanus*). *Aquaculture*, 181, 1-10.

Abhilash, P. C. & Singh, N. (2009). Pesticide use and application: An Indian scenario. *Journal of Hazardous Materials*, 165, 1-12.

Achazi, R. K., Flenner, C., Livingstone, D. R., Peters, L. D., Schaub, K. & Scheiwe, E. (1998). Cytochrome P450 and dependent activities in unexposed and PAH-exposed terrestrial annelids. *Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology*, 121, 339-350.

Aly, M. A. S. & Schröder, P. (2008). Effect of herbicides on glutathione S-transferases in the earthworm, *Eisenia fetida*. *Environmental Science and Pollution Research*, 15, 143-149.

Barata, C., Solayan, A. & Porte, C. (2004). Role of B-esterases in assessing toxicity of organophosphorus (chlorpyrifos, malathion) and carbamate (carbofuran) pesticides to *Daphnia magna*. *Aquatic Toxicology*, 66, 125-139.

Barker, A. V. & Bryson, G. M. (2002). Bioremediation of heavy metal and organic toxicants by composting. *TheScientificWorldJournal*, 2, 407-420.

Beauvais, S. L., Jones, S. B., Brewer, S. K. & Little, E. E. (2000). Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout (*Oncorhynchus mykiss*) and their correlation with behavioral measures. *Environmental Toxicology and Chemistry*, 19, 1875-1880.

Beauvais, S. L., Jones, S. B., Parris, J. T., Brewer, S. K. & Little, E. (2001). Cholinergic and behavioral neurotoxicity of carbaryl and cadmium to larval rainbow trout (*Oncorhynchus mykiss*). *Ecotoxicology and Environmental Safety*, 49, 84-90.

Bergholjt, A. G. R. V., Wenzel, E., Büld, J. & Netter, K. J. (1991). Isolation, partial purification, and characterization of the cytochrome 450-dependent monooxygenase system from the midgut of the earthworm *Lumbricus terrestris*. *Comparative Biochemistry and Physiology*, 100C, 389-396.

Bianchi, F. J. J. A., Booij, C. J. H. & Tscharntke, T. (2006). Sustainable pest regulation in agricultural landscapes: a review on landscape composition, biodiversity and natural pest control. *Proceedings of the Royal Society B: Biological Sciences*, 273, 1715-1727.

Booth, L. H., Hodge, S. & O'Halloran, K. (2000). Use of cholinesterase in *Aporrectodea caliginosa* (Oligochaeta; Lumbricidae) to detect organophosphate contamination: comparison of laboratory tests, mesocosms, and field studies. *Environmental Toxicology and Chemistry*, 19, 417-422.

Brown, P., Long, S., Spurgeon, D., Svendsen, C. & Hankard, P. (2004). Toxicological and biochemical responses of the earthworm to pyrene, a non-carcinogenic polycyclic aromatic hydrocarbon. *Chemosphere*, 57, 1675-1681.

Cáceres, T., Megharaj, M., Venkateswarlu, K., Sethunathan, N. & Naidu, R. (2010). Fenamiphos and related organophosphorus pesticides: environmental fate and toxicology. *Reviews of Environmental Contamination and Toxicology*, 205, 117-162.

Capowiez, Y., Rault, M., Mazzia, C. & Belzunces, L. (2003). Earthworm behaviour as a biomarker – a case study using imidacloprid. *Pedobiologia*, 47, 542-547.

Caselli, F., Gastaldi, L., Gambi, N. & Fabbri, E. (2006). In vitro characterization of cholinesterases in the earthworm Eisenia andrei. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 143, 416-421

Chapman, P. M. (2002). Integrating toxicology and ecology: putting the ''eco'' into ecotoxicology. *Marine Pollution Bulletin*, 44, 7-15.

Coeurdassier, M., Gomot-de Vaufleury, A., Saint-Denis, M., Ribera, D., Narbonne, J. F. & Badot, P. M. (2002). Effects of dimethoate on snail B-esterase and growth as a function of dose, time and exposure route in a laboratory bioassay. *Biomarkers*, 7, 138-150.

Collange, B., Wheelock, C. E., Rault, M., Mazzia, C., Capowiez, Y. & Sanchez-Hernandez, J. C. (2010). Inhibition, recovery and oxime-induced reactivation of muscle esterases following chlorpyrifos exposure in the earthworm Lumbricus terrestris. *Environmental Pollution*, 158, 2266-2272.

Denoyelle, R., Rault, M., Mazzia, C., Mascle, O. & Capowiez, Y. (2007). Cholinesterase activity as a biomarker of pesticide exposure in *Allolobophora chlorotica* earthworms living in apple orchards under different managements strategies. *Environmental Toxicology and Chemistry*, 26, 2644-2649.

Devine, G. J. & Furlong, M. J. (2007). Insecticide use: Contexts and ecological consequences. *Agriculture and Human Values*, 24, 281-306.

Devotto, L., Carrillo, R., Cisternas, E. & Gerding, M. (2007). Effects of lambda-cyhalothrin and *Beauveria bassiana* spores on abundance of Chilean soil surface predators, especially spiders and carabid beetles. *Pedobiologia*, 51, 65-73.

Domingues, I., Agra, A. R., Monaghan, K., Soares, A. M. V. M. & Nogueira, A. J. A. (2010). Cholinesterase and glutathione-S-transferase activities in freshwater invertebrates as biomarkers to assess pesticide contamination. *Environmental Toxicology and Chemistry*, 29, 5-18.

Drobne, D. (1997). Terrestrial isopods - a good choice for toxicity testing of pollutants in the terrestrial environment. *Environmental Toxicology and Chemistry*, 16, 1159-1164.

Drobne, D., Blazic, M., van Gestel, C.A.M., Leser, V., Zidar, P. Jemec, A. & Trebse, P. (2008). Toxicity of imidacloprid to the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea). *Chemosphere*, 71, 1326-1334.

Ellis, S. R., Hodson, M. E. & Wege, P. (2010). The soil-dwelling earthworm Allolobophora chlorotica modifies its burrowing behaviour in response to carbendazim applications. *Ecotoxicology and Environmental Safety*, 73, 1424-1428.

Engenheiro, E.L., Hankard, P.K., Sousa, J.P., Lemos, M.F., Weeks, J.M. & Soares, A.M.V.M. (2005). Influence of dimethoate on acetylcholinesterase activity and locomotor function in terrestrial isopods. *Environmental Toxicology and Chemistry*, 24, 603-609.

Essawy, A. E., Abdelmeguied, N. E., Radwan, M. A., Hamed, S. S. & Hegazy, A. E. (2008). Neuropathological effect of carbamate molluscicides on the land snail, Eobania vermiculata. *Cell Biology and Toxicology*, 25, 275-290.

Eurostat (2007). The use of plant protection products in the European Union. 2007 edition, European Commission, Luxembourg, European Union.

European Commission (2002). "Communication from the Commission to the Council, the European Parliament, the Economic and Social Committee and the Committee Of The Regions - Towards a Thematic Strategy for Soil Protection, COM, 179 final.

Flavia G., Bengtssonb, J., Berendse, F., Weisser, W.W., Emmersond, M., Morales, M.B., Ceryngier, P., Liirah, J., Tscharntke, T., Winqvist, C., Eggers, S., Bommarcob, R., Pärt, T., Bretagnolle, V., Plantegenest, M., Clement, L.W., Dennis, C., Palmer, C., Oñate, J.J., Guerrero, I. Hawrog, V., Aavikh, T. Thies, C., Flohre, A., Hänke, S., Fischer, C., Goedhart, P.W. & Inchausti, P. (2010). Persistent negative effects of pesticides on biodiversity and biological control potential on European farmland. *Basic and Applied Ecology*, 11, 97-105.

Fleeger, J., Carman, K. & Nisbet, R. (2003). Indirect effects of contaminants in aquatic ecosystems. *The Science of The Total Environment*, 317, 207-233.

Forbes, V. E., Palmqvist, A. & Bach, L. (2006). The use and misuse of biomarkers in ecotoxicology. *Environmental Toxicology and Chemistry*, 25, 272-280.

Frohlich, D. R. (1990). Substrate specificity of esterases in a solitary bee, *Megachile rotundata* (Hymenoptera: Megachilidae): variability in sex, age and life stage. *Biochemical Systematics and Ecology*, 18, 539-547.

Fulton, M. H. & Key, P. B. (2001). Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environmental Toxicology and Chemistry*, 20, 37-45.

Galloway, T. S., Brown, R. J., Browne, M. A., Dissanayake, A., Lowe, D., Jones, M. B. & Depledge, M. H. (2004). A multibiomarker approach to environmental assessment. *Environmental Science and Technology*, 38, 1723-1731.

Gastaldi, L., Ranzato, E., Capri, F., Hankard, P., Peres, G., Canesi, L., Viarengo, A. & Pons, G. (2007). Application of a biomarker battery for the evaluation of the sublethal effects of pollutants in the earthworm *Eisenia andrei*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 146, 398-405.

Gavrilescu, M. (2005). Fate of pesticides in the environment and its bioremediation. *Engineering in Life Science*, 5, 497-526

Gianfreda, L. & Rao, M. (2008). Interactions between xenobiotics and microbial and enzymatic soil activity. *Critical Reviews in Environmental Science and Technology*, 38, 269-310.

González Vejares, S., Sabat, P. & Sanchez-Hernandez, J. C. (2010). Tissue-specific inhibition and recovery of esterase activities in *Lumbricus terrestris* experimentally exposed to chlorpyrifos. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 151, 351-359.

Gross, M. (2011). New fears over bee declines. *Current Biology*, 21, R137-R139.

Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1974). Glutathione S-Transferases: the first enzymatic step in mercapturic acid formation. *The Journal of Biological Chemistry*, 249, 7130-7139.

Hagger, J. A., Galloway, T. S., Langston, W. J. & Jones, M. B. (2009). Application of biomarkers to assess the condition of European Marine Sites. *Environmental Pollution*, 157, 2003-2010.

Hans, R. K., Khan, M. A., Farooq, M. & Beg, M. U. (1993). Glutathione-S-transferase activity in an earthworm (*Pheretima posthuma*) exposed to three insecticides. *Soil Biology and Biochemistry*, 25, 509-511.

Hayes, J. D., Flanagan, J. U. & Jowsey, I. R. (2005). Glutathione Transferases. *Annual Review of Pharmacology and Toxicology*, 45, 51-88.

Hellou, J. (2011). Behavioural ecotoxicology, an "early warning" signal to assess environmental quality. *Environmental Science and Pollution Research*, 18, 1-11.

Hemingway, J., Hawkes, N., McCarroll, L. & Ranson, H. (2004). The molecular basis of insecticide resistance in mosquitoes. *Insect Biochemistry and Molecular Biology*, 34, 653-665.

Hodgson, E. (2010). Introduction to biotransformation (metabolism), In: *Hayes' Handbook of Pesticide Toxicology*, Krieger, R. (Ed.), 865-875, Academic Press, Burlington.

Hyne, R. V. & Maher, W. A. (2003). Invertebrate biomarkers: links to toxicosis that predict population decline. *Ecotoxicology and Environmental Safety*, 54, 366-374.

ISO (2005). Soil quality: Avoidance test for testing the quality of soils and the toxicity of chemicals – Tests with earthworms. International Organization for Standardization, Geneva.

Jemec, A., Drobne, D., Tišler, T. & Sepčić, K. (2009). Biochemical biomarkers in environmental studies—lessons learnt from enzymes catalase, glutathione S-transferase and cholinesterase in two crustacean species. *Environmental Science and Pollution Research*, 17, 571-581.

Jokanovic, M. (2001). Biotransformation of organophosphorus compounds. *Toxicology*, 166, 139-160.

Karlen, D. L., Mausbach, M. J., Doran, J. W., Cline, R. G., Harris, R. F. & Schuman, G. E. (1997). Soil quality: a concept, definition, and framework for evaluation (a guest editorial). *Soil Science Society American Journal*, 61, 4-10.

Katagi, T. (2004). Photodegradation of pesticides on plant and soil surfaces. *Reviews of Environmental Contamination and Toxicology*, 182, 1-195.

Köhne, J., Köhne, S. & Simunek, J. (2009). A review of model applications for structured soils: b) Pesticide transport. *Journal of Contaminant Hydrology*, 104, 36-60.

Koivula, M. J. & Eeva, T. (2010). Metal-related oxidative stress in birds. *Environmental Pollution*, 158, 2359-2370.

Krieg, P. & Marek, M. (1983). Protein and esterase changes haemolymph of the queens of bee, *Apis mellifera* L. in the honey. *Comparative Biochemistry and Physiology*, 75B, 513-517.

Kuster, E. (2005). Cholin- and carboxylesterase activities in developing zebrafish embryos (*Danio rerio*) and their potential use for insecticide hazard assessment. *Aquatic Toxicology*, 75, 76-85.

LaCourse, E. J., Hernandez-Viadel, M., Jefferies, J. R., Svendsen, C., Spurgeon, D. J., Barrett, J., John Morgan, A., Kille, P. & Brophy, P. M. (2009). Glutathione transferase (GST) as a candidate molecular-based biomarker for soil toxin exposure in the earthworm Lumbricus rubellus. *Environmental Pollution*, 157, 2459-2469.

Laguerre, C., Sanchez-Hernandez, J. C., Köhler, H.-R., Triebskorn, R., Capowiez, Y., Rault, M. & Mazzia, C. (2009). B-type esterases in the snail *Xeropicta derbentina*: An enzymological analysis to evaluate their use as biomarkers of pesticide exposure. *Environmental Pollution*, 157, 199-207.

Landis, D. A., Gardiner, M. M., van der Werf, W. & Swinton, S. M. (2008). Increasing corn for biofuel production reduces biocontrol services in agricultural landscapes. *Proceedings of the National Academy of Sciences*, 105, 20552-20557.

Lanno, R., Wells, J., Conder, J., Bradham, K. & Basta, N. (2004). The bioavailability of chemicals in soil for earthworms. *Ecotoxicology and Environmental Safety*, 57, 39-47.

Lavelle, P., Pashanasi, B., Charpentier, F., Gilot, C., Rossi, J. P., Derouard, L., Andre, J., Ponge, J. F. & Bernier, N. (2004). Effects of earthworms on soil organic matter and nutrient dynamics at a landscape scale over decades, In: *Earthworm Ecology*, Edwards, C. A. (Ed.), 145-160, CRC Press, Boca Raton, USA.

Lukaszewicz-Hussain, A. (2010). Role of oxidative stress in organophosphate insecticide toxicity – Short review. *Pesticide Biochemistry and Physiology*, 98, 145-150.

Maity, S., Roy, S., Chaudhury, S. & Bhattacharya, S. (2008). Antioxidant responses of the earthworm *Lampito mauritii* exposed to Pb and Zn contaminated soil. *Environmental Pollution*, 151, 1-7.

Mann, R. M., Hyne, R. V., Choung, C. B. & Wilson, S. P. (2009). Amphibians and agricultural chemicals: Review of the risks in a complex environment. *Environmental Pollution*, 157, 2903-2927.

Masson, P. & Lockridge, O. (2010). Butyrylcholinesterase for protection from organophosphorus poisons: Catalytic complexities and hysteretic behavior. *Archives of Biochemistry and Biophysics*, 494, 107-120.

Maxwell, D. M. & Brecht, K. M. (2001). Carboxylesterase: specificity and spontaneous reactivation of an endogenous scavenger for organophosphorus compounds. *Journal of Applied Toxicology*, 21, S103-S107.

Mazzia, C., Capowiez, Y., Sanchez-Hernandez, J. C., Köhler, H.-R., Triebskorn, R. & Rault, M. (2011). Acetylcholinesterase activity in the terrestrial snail *Xeropicta derbentina* transplanted in apple orchards with different pesticide management strategies. *Environmental Pollution*, 159, 319-323.

McHenery, J. G., Linley-Adams, G. E., Moore, D. C., Rodger, G. K. & Davies, I. M. (1997). Experimental and field studies of effects of dichlorvos exposure on acetylcholinesterase activity in the gills of the mussel, *Mytilus edulis* L. *Aquatic Toxicology*, 38, 125-143.

Mirsal, I. A. (2008). Planning and realisation of soil remediation, In: *Soil Pollution: Origin, Monitoring and Remediation*, (Ed.), 265-299, Springer-Verlag, Berlin, Germany.

Monserrat, J. M., Martínez, P. E., Geracitano, L. A., Lund Amado, L., Martinez Gaspar Martins, C., Lopes Leães Pinho, G., Soares Chaves, I., Ferreira-Cravo, M., Ventura-Lima, J. & Bianchini, A. (2007). Pollution biomarkers in estuarine animals: Critical review and new perspectives. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 146, 221-234.

Moulton, C. A., Fleming, W. J. & Purnell, C. E. (1996). Effects of two cholinesterase-inhibiting pesticides on freshwater mussels. *Environmental Toxicology and Chemistry*, 15, 131-137.

Newman, M., Crane, M. & Holloway, G. (2006). Does pesticides risk assessment in the Europe Union assess long-term effects? *Reviews of Environmental Contamination and Toxicology*, 187, 1-65.

Oakeshott, J., Devonshire, A., Claudianos, C., Sutherland, T., Horne, I., Campbell, P., Ollis, D. & Russell, R. (2005). Comparing the organophosphorus and carbamate insecticide resistance mutations in cholin- and carboxyl-esterases. *Chemico-Biological Interactions*, 157-158, 269-275.

OECD (2009). "OECD in figures 2009", Organisation for Economic Co-operation and Development Publications, Paris, France.

Olveravelona, A., Capowiez, Y., Mascle, O., Ortizhernandez, L. & Benoit, P. (2008). Assessment of the toxicity of ethyl-parathion to earthworms (*Aporrectodea caliginosa*) using behavioural, physiological and biochemical markers. *Applied Soil Ecology*, 40, 476-483.

Peakall, D. (1992). Animal biomarkers as pollution indicators. Chapman & Hall, London.

Pereira, J., Antunes, S., Ferreira, A., Goncalves, F. & Pereira, R. (2010). Avoidance behavior of earthworms under exposure to pesticides: Is it always chemosensorial? *Journal of Environmental Science and Health, Part B*, 45, 229-232.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O. & Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution*, 25, 345-353.

Racke, K. D. (1993). Environmental fate of chlorpyrifos. *Reviews of Environmental Contamination and Toxicology*, 131, 1-154.

Rault, M., Mazzia, C. & Capowiez, Y. (2007). Tissue distribution and characterization of cholinesterase activity in six earthworm species. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 147, 340-346.

Rault, M., Collange, B., Mazzia, C. & Capowiez, Y. (2008). Dynamics of acetylcholinesterase activity recovery in two earthworm species following exposure to ethyl-parathion. *Soil Biology and Biochemistry*, 40, 3086-3091.

Reinecke, S. & Reinecke, A. (2007). The impact of organophosphate pesticides in orchards on earthworms in the Western Cape, South Africa. *Ecotoxicology and Environmental Safety*, 66, 244-251.

Ribeiro, S., Guilhermino, L., Sousa, J. P. & Soares, A. M. V. M. (1999). Novel bioassay based on acetylcholinesterase and lactate dehydrogenase activities to evaluate the toxicity of chemicals to soil isopods. *Ecotoxicology and Environmental Safety*, 44, 287-293.

Ribera, D., Narbonne, J. F., Arnaud, C. & Saint-Denis, M. (2001). Biochemical responses of the earthworm *Eisenia fetida* andrei exposed to contaminated artificial soil, effects of carbaryl. *Soil Biology and Biochemistry*, 33, 1123-1130.

Rodríguez-Castellanos, L. & Sanchez-Hernandez, J. C. (2007). Chemical reactivation and aging kinetics of organophosphorus-inhibited cholinesterases from two earthworm species. *Environmental Toxicology and Chemistry*, 26, 1992-2000.

Rodríguez-Castellanos, L. & Sanchez-Hernandez, J. C. (2007). Earthworm biomarkers of pesticide contamination: Current status and perspectives. *Journal of Pesticide Science*, 32, 360-371.

Ross, M., Borazjani, A., Edwards, C. & Potter, P. (2006). Hydrolytic metabolism of pyrethroids by human and other mammalian carboxylesterases. *Biochemical Pharmacology*, 71, 657-669.

Ross, M. K. & Crow, J. A. (2007). Human carboxylesterases and their role in xenobiotic and endobiotic metabolism. *Journal of Biochemical and Molecular Toxicology*, 21, 187-196.

Sanchez-Hernandez, J. C. (2001). Wildlife exposure to organophosphorus insecticides. *Reviews of Environmental Contamination and Toxicology*, 172, 21-63.

Sanchez-Hernandez, J. C. & Wheelock, C. (2009). Tissue distribution, isozyme abundance and sensitivity to chlorpyrifos-oxon of carboxylesterases in the earthworm *Lumbricus terrestris*. *Environmental Pollution*, 157, 264-272.

Sanchez-Hernandez, J. C. (2010). Environmental applications of earthworm esterases in the agroecosystem. *Journal of Pesticide Science*, 35, 290-301.

Satoh, T. & Hosokawa, M. (2006). Structure, function and regulation of carboxylesterases. *Chemico-Biological Interactions*, 162, 195-211.

Scheu, S. (2003). Effects of earthworms on plant growth: patterns and perspectives. *Pedobiologia*, 47, 846-856.

Scott, G. R. & Sloman, K. A. (2004). The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. *Aquatic Toxicology*, 68, 369-392.

Sogorb, M. A. & Vilanova, E. (2002). Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. *Toxicology Letters*, 128, 215-228.

Sorensen, F. F., Bayley, M. & Baatrup, E. (1995). The effects of sublethal dimethoate exposure on the locomotor behavior of the collembolan *Folsomia candida* (isotomidae). *Environmental Toxicology and Chemistry*, 14, 1587-1590.

Spivak, M., Mader, E., Vaughan, M. & Euliss, N. H. (2011). The plight of the bees. *Environmental Science and Technology*, 45, 34-38.

Stenersen, J. (1980). Esterases of earthworms. Part I: characterisation of the cholinesterases in *Eisenia foetida* (Savigny) by substrates and inhibitors. *Comparative Biochemistry and Physiology*, 66C, 37-44.

Stenersen, J. (1984). Detoxication of xenobiotics by earthworms. *Comparative Biochemistry and Physiology*, 78C, 249-252.

Thompson, C. M. & Richardson, R. J. (2004). Anticholinesterase insecticides, In: *Pesticide Toxicology and International Regulation*, Marrs, T. C. & Ballantyne, B. (Eds.), 89-127, John Wiley & Sons, West Sussex, England.

van der Oost, R., Beyer, J. & Vermeulen, N. P. E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, 13, 57-149.

van Erp, S., Booth, L., Gooneratne, R. & O'Halloran, K. (2002). Sublethal responses of wolf spiders (Lycosidae) to organophosphorous insecticides. *Environmental Toxicology*, 17, 449-456.

van Gestel, C. A. M. & van Brummelen, T. C. (1996). Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. *Ecotoxicology*, 5, 217-225.

Walker, C.H., Hopkin, S.P., Sibly, R.M., Peakall, D.B. (2001). Principles of Ecotoxicology, 2nd ed. Taylor & Francis, London, United Kingdom.

Wheelock, C., Eder, K., Werner, I., Huang, H., Jones, P., Brammell, B., Elskus, A. & Hammock, B. (2005). Individual variability in esterase activity and CYP1A levels in Chinook salmon (*Oncorhynchus tshawytscha*) exposed to esfenvalerate and chlorpyrifos. *Aquatic Toxicology*, 74, 172-192.

Wheelock, C. E., Wheelock, A. M., Zhang, R., Stok, J. E., Morisseau, C., Le Valley, S. E., Green, C. E. & Hammock, B. D. (2003). Evaluation of α-cyanoesters as fluorescent substrates for examining interindividual variation in general and pyrethroid-selective esterases in human liver microsomes. *Analytical Biochemistry*, 315, 208-222.

Wheelock, C. E., Shan, G. & Ottea, J. (2005). Overview of carboxylesterases and their role in the metabolism of insecticides. *Journal of Pesticide Science*, 30, 75-83.

Wheelock, C. E., Phillips, B. M., Anderson, B. S., Miller, J. L., Miller, M. J. & Hammock, B. D. (2008). Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs). *Reviews of Environmental Contamination and Toxicology*, 195, 117-178.

Whyte, J. J., Jung, R. E., Schmitt, C. J. & Tillitt, D. E. (2000). Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Critical Reviews in Toxicology*, 30, 347-570.

Wogram, J., Sturm, A., Segner, H. & Liess, M. (2001). Effects of parathion on acetylcholinesterase, butyrylcholinesterase, and carboxylesterase in three-spined stickleback (*Gasterosteus aculeatus*) following short-term exposure. *Environmental Toxicology and Chemistry*, 20, 1528-1531.

Yu, S. J., Robinson, F. A. & Nation, J. L. (1984). Detoxication capacity in the honey bee, *Apis mellifera* L. *Pesticide Biochemistry and Physiology*, 22, 360-368.

12

Biomarkers of Pesticide - Contaminated Environment

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1. Introduction

Pesticide is the umbrella term for chemicals or biologicals used to control pests. The Environmental Protection Agency (EPA) defines a pesticide as any substance or mixture of substances/chemicals intended to prevent, destroy, repel or mitigate any pest (US-EPA, 2006). A pesticide need not always kill a pest: it could sterilize, or repel. Pesticides can be classified in various ways such as, by their target, chemical nature, physical state and mode of action (Ware, 2000). Classification based on the target is perhaps the most widely known as the following examples indicate; Pesticides used to manage insects are called insecticides; and those used to manage rodents are called rodenticide; those used to manage fungi are called fungicides e.t.c (Ware and Whitacre, 2004). Pesticides also include plant growth regulators, defoliants, or desiccants (Hagtrum and Subramanyam, 2006).

The environmental pollution and poisoning owing to the widespread use of pesticides in agricultural and domestic pest control may be detrimental to the health of handlers, non-target organisms and consumers. Pesticides or their residues are ubiquitous contaminants in the environmental media (air, soil, water), and in humans, plants and animal tissue samples. Pesticides uptake occurs through the skin, eyes, by inhalation, or by ingestion directly or through the food chain. The fat-soluble pesticides, and to some extent, the water-soluble pesticides are absorbed through intact skin. Sores and abrasions may facilitate uptake through the skin. The vapours of pesticides or aerosol droplets smaller than 5μm in diameters are absorbed effectively through the lungs. Larger inhaled particles or droplets may be swallowed after being cleared from the airways. A common toxic effect to the lung is the result of oxidative burden which occurs as a result of active oxidants in pesticide mixtures, especially free radicals that are generated by a variety of toxic agents and the action of lung defense cells. Much of the oxidative damage to lungs is probably done by free radicals, such as hydroxyl radical, $HO^{\cdot}$, and superoxide ion, O^{-} which initiate and mediate oxidative chain reactions. Lungs of animals exposed to oxidants have shown elevated levels of enzymes that scavenge free radicals, providing evidence for their role in oxidative damage. There is evidence to suggest that lung cells damaged by toxicants release species that convert lung O_2 to reactive superoxide anion. Pesticide ingestion can occur from the consumption of contaminated food or from using contaminated utensils. Contaminated

hands may also lead to an intake of pesticides, for example, while palm-chewing, tobacco eating or smoking, spraying, mixing, or handling the pesticides. A number of pesticides have been identified as probable and possible carcinogens, disruptors of endocrine and immune functions, genotoxins and developmental and reproductive toxins. The improper use of pesticides may engender biological effects beyond those for which they were originally manufactured. Humans and other non-target organisms are diverse in their responses to exogenous exposures because of variability in the rate of metabolism (Otitoju and Onwurah 2007).

The use of man-made chemicals for obvious positive reason(s) has inadvertently resulted in creating negative effects in the environment, and hence the need for development of methods to assess, monitor and mitigate the impact. Pesticide exposure in humans can be measured in two ways, either through direct monitoring by measuring biomarkers from individuals or developing models (animals, plants or microorganisms) to assess exposure. Biomarkers, or biological markers, are biochemicals or metabolites that can be measured in body fluid, such as urine, blood, saliva, and other body fluids. Indicator organisms also serve as biomarkers. The definition has been broadened to include biological characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to any xenobiotic exposures. Biochemical biomarkers are increasingly used in ecological risk assessments of aquatic and terrestrial ecosystems to identify the incidence of exposure to and effects caused by xenobiotics. Until recently, the most common end point measured when evaluating toxicity of chemicals were mortality values. This, unfortunately can only provide a measure of short-term acute toxicity and are not always useful for predicting the ecological consequences of exposure to a particular chemical, e.g., as seen with reproduction, where effects are observed at concentrations well below the lethal concentration (LC_{50}) value. Biomarkers can provide information on the potential adverse impacts of contaminants and can act as early warning signals of impending environmental damage. Pesticides inhibit a number of enzymes in humans. They affect several physiological systems and processes in the body – the central nervous system (CNS), reproductive, immune, endocrine, cardiovascular and respiratory systems.

The repertoire to counteract the potentially hazardous reactions initiated by metabolites from pesticide oxidation include all levels of protection, prevention, interception and repairs by certain antioxidant enzymes or biomarkers, such as glutathione S-transferase (GST), and superoxide dismutase (SOD). Glutathione S-transferase is a family of detoxifying enzymes that catalyzes the conjugation of reduced glutathione with a group of compounds having electrophilic centers e.g., nitrocompounds, organophosphates and organochlorides. Since glutathione (GSH) is essential to cellular detoxification of many toxic xenobiotics, monitoring this endogenous thiol during pesticide exposure is very important. Hence, the aim of this chapter is to evaluate some antioxidant enzymes and vitamins reported as biochemical markers for pesticide toxicity.

2. Need for biomarker development

Pesticide poisoning issue in developed and developing countries has received several attentions as can be seen from some of many press statements. Several people in crop-growing areas where pesticides are used indiscriminately almost have constant nausea, diarrhea and dizziness because persistent exposure to pesticides has depleted their immune system. There

have been several reports about pesticides making people in agricultural areas extremely ill, and experts have "no doubt" that exposure to many of the pesticides commonly used damage human health. Some researchers at the University of Cape Town's Occupational and Environmental Health Research Unit, reported that exposure to pesticides is bad for human health. Farmers might put up barriers such as trees, but the poisons could accumulate in the water or dust, and children crawling on the ground would be exposed to them.
The World Health Organisation receives reports of about three million cases a year of acute pesticide poisoning, most from developing countries, and estimates that about 20,000 people die every year from pesticide exposure. Until the 1970s, farmers in South Africa targeted pests by using equipment carried on a backpack to spray plants individually with pesticides. These days, it is normal to spray from tractors, a method that produces clouds of pesticide that can travel for kilometres. In Groblersdal, Limpopo, where citrus, grapes, cotton, vegetables and maize are grown, teenagers and a five-year-old who were growing breasts were diagnosed of chronic fatigue, nausea, muscle aches and pains, skin rashes and arthritis, particularly during the spraying season. Regular testing of their blood revealed that they have been exposed to organophosphates which attack the nervous system, and carbamate pesticides. One commonly used pesticide, endosulphan, has been shown to be linked to reproductive impairments.
The above calls for caution in the use of pesticides and abide to safety precautions without endangering the lives of people. The health and safety of the farm workers and neighbours should always have priority over the profit motive of the farmers. This is the reason scientists should develop more accurate and robust biomarkers for pesticides' toxicity assay and management so that a Policy that will pave a way for a Bill on pesticides could be passed.

3. Biomarkers

One of the greatest challenges to humanity today is the endangerment of human health due to indiscriminate use of pesticides. To estimate the biological danger thereof, knowledge of their harmful effects is necessary. In revealing the risks of such substances, every living being and life function can be considered a potential biomarker or bioindicator. Microorganisms can be used as indicator organisms (or biosensors) for toxicity tests or in risk assessment. Bioassays are ideal complement to the traditional analytical techniques employed in evaluating toxicity of pollutants or chemicals in the environment. Those tests performed with bacteria are considered to be the most reproducible, sensitive, simple, economical and rapid (Matthews, 1980). Risk assessment has relied on models that use toxicity data and physical properties of chemicals, and this approach has been effective at the ecosystem level. The use of microorganisms present in a polluted environment is an approach that provides a link between exposure and effect because chemicals are known to elicit measurable and characteristic biological responses in exposed (microbial) cells. The term "biological markers" (or biomarkers) can be taken to mean cellular, biochemical or molecular alterations) which are measurable in biological media such as the human tissue, cells or fluids as a result of exposure to environmental chemicals (Hulka, 1988). Three types of events involved are exposure, effect, and susceptibility (Schultz and Mazzuckelli, 1991). In a broad sense, biological markers are measurements in any biological specimens (such as the blood plasma, bacterial cells) that will elucidate the relationship between exposure and effect such that adverse effects could be prevented (NRC, 1992).

A crucial aspect of Ecotoxicology is the measurement of the effects of toxic substances on organisms in ecosystems and on ecosystems as a whole. This has traditionally been done by determining levels (or bioaccumulation) of toxic substances in organisms and relating these levels to detrimental effects on the organisms (biomarkers). Biomarkers can be used to identify causal associations and to make better quantitative estimates of those associations at relevant levels of exposure. They may also make it possible to identify susceptible groups or individuals who are at risk of exposure to certain types of environmental and occupational agents (Anwar, 1997). A better approach is the use of biomarkers consisting of observations and measurements of alterations in biological components, structures, processes, or behaviours attributable to exposure to xenobiotic substances as shown in Figure 1. Animals, microorganisms or plants can be used as biomarkers to evaluate the effect of chemical hazards to humans. Biomarkers, or biological markers, can also be chemicals or metabolites that can be measured in body fluid, such as urine, blood, saliva, and other body fluids. Metabolites are chemicals that were transformed by the body from an original chemical, or chemical constituents of the pesticide. The biological events detected can represent variation in the number, structure, or function of cellular or biochemical components. Recent advances in molecular and cellular biology allow for measurement of biologic events or substances that may provide markers of exposure, effect, or susceptibility in humans. Certain tests, such as DNA adduct formation, are used for measuring biologically effective dose, whereas others are considered to measure early effects, such as chromosomal aberrations. Biomarkers are predictive assays rather than diagnostic. A positive effect will be

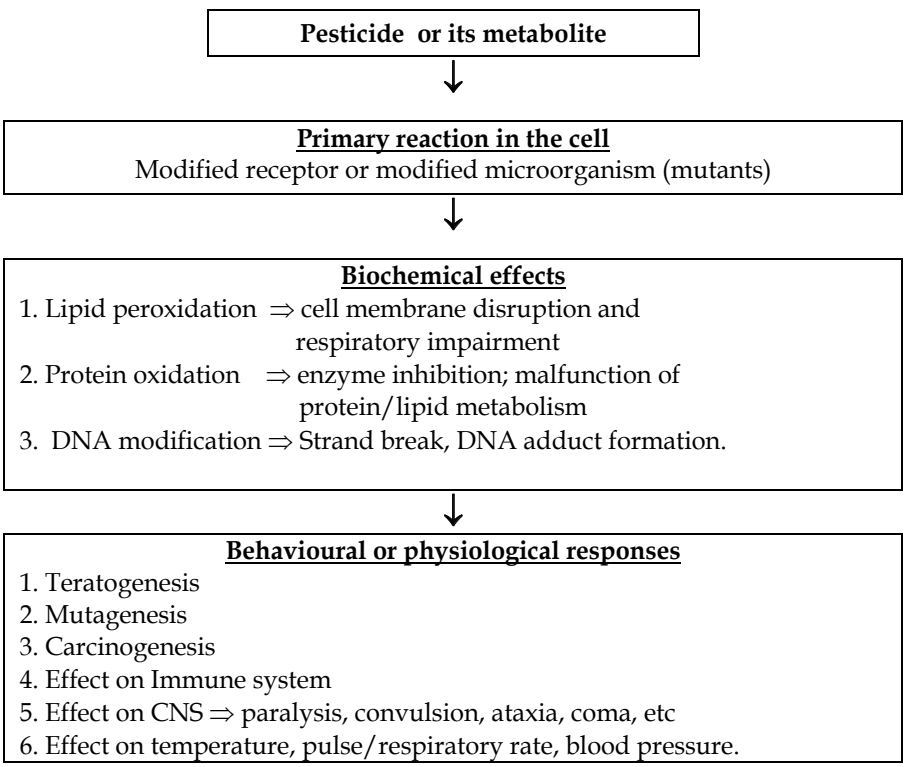

Fig. 1. Pathway of Biomarker responses after toxic interaction

indicative of exposure, but cannot be considered predictive of the future occurrence of any particular change in phenotype such as cancer. Biomarkers are used for a variety of reasons. However when assessing the toxicity of any pesticide, three types of biomarkers, each measuring different types of effects, can be distinguished and they include biomarkers of exposure, response, and susceptibility.

4. Biomarkers of exposure

A biomarker of exposure consists of the measurement of a xenobiotic substance, a metabolite of a xenobiotic or pesticide substance, or an effect directly attributable to such a substance in an organism. For example, a biomarker of human exposure to aniline might consist of measurement of it or its *p*-aminophenol metabolite in blood or urine. Pesticides or their metabolites can be measured directly in tissues obtained by biopsy, in live organisms or necropsy of deceased organisms. Urine, blood, exhaled air, faeces, and breast milk can also serve as samples and are advantageous in measurements to be made at intervals over a period of time. A particularly useful kind of biomarker used with increasing frequency during recent years consists of adducts of xenobiotics or their metabolites to biomolecules. A particularly direct example of such adducts measured for many years as evidence of exposure is carboxy-haemoglobin (COHb), produced when inhaled carbon monoxide adds to blood haemoglobin (Hb):

$$O_2Hb + CO \rightarrow COHb + O_2$$

The COHb has a distinctly different colour from the oxygenated form, O_2Hb, and can be measured spectrophotometrically. Cancer-causing compounds and carcinogenic metabolites are generally electrophilic (electron-seeking) species that cause the biochemical changes leading to cancer by adding to nucleophilic groups (electron-rich bound oxygen and nitrogen atoms) on biomolecules, particularly those in DNA. These adducts and adducts to haemoglobin can be measured as biomarkers of exposure.

In toxicity testing, doses of pesticides are assumed to be the actual level of exposure. This is fairly accurate, but it should not be presumed that 100% of the chemical has been absorbed by the subject. For this reason blood tests are conducted to achieve a more exact estimate. This level is usually similar to amounts dispersed to organs. The absorption rate of pesticides depends upon the route of exposure. However, absorption varies more through the dermal route. When alachlor was applied to the skin of rats at a low dose (14 mg/kg) nearly 75% was absorbed, while monkeys only absorbed 8 to 10% through the skin.

5. Biomarkers of response or effect

Biomarkers of effect are alterations of physiology, biochemistry, or behaviour directly attributable to exposure to a xenobiotic substance. For example, exposure to aniline can be determined by measuring it or its *p*-aminophenol in blood, but it can also be measured by its production of blood methaemoglobin (a product of haemoglobin) useless for carrying oxygen in blood in which the Fe^{2+} in haemoglobin has been oxidized to Fe^{3+}. Exposure to substances such as nerve gas organophosphates, organophosphate insecticides, and carbamate insecticides that inhibit cholinesterase enzymes required for nerve function can be determined by measurement of cholinesterase enzyme activity as a biomarker. Exposure to the insecticidal DDT metabolite, *p,p'*-DDE can be measured in the laboratory rat (*Rattus*

rattus) by induction of enzymatic cytochrome P-450 2B, an enzyme used by some organisms to detoxify xenobiotics.
Biomarkers of response are consequences of the exposure. An area of great concern for alachlor is its effects on genes which may be the basis for carcinogenesis. Alachlor is thought to be a probable carcinogen. The development of stomach, thyroid, and nasal tumors has occurred in rats given high doses of alachlor. Carcinogenesis causes an uncontrolled proliferation of cells in tissues and organs. Many cancers are caused from mutated somatic cells which disturb the genetic control. However, studies have shown that alachlor does not appear to be mutagenic. Reactions to alachlor are invoked by threshold sensitive mechanisms. Carcinogenic reactions only occur at high levels of exposure. Pharmacokinetic studies have shown that alachlor and its metabolites are transformed into a diethyl quinoneimine (DEIQ) metabolite. The presence of DEIQ produces protein adducts that lead to cytotoxicity, cell proliferation and tumor formation.

6. Biomarkers of susceptibility

Closely related to biomarkers of effect are biomarkers of susceptibility, which indicate increased vulnerability of organisms to disease, physical attack (such as low temperatures), or chemical attack from other toxicants. The most obvious biomarkers of susceptibility are those associated with weakened immune systems, which may make organisms more vulnerable to cancer, infectious diseases, or parasites. They can be seen in variations across species. Studies across different species have shown why alachlor may be more likely to be a carcinogen in certain animal species as against humans. For instance, rats are able to convert the secondary sulfide metabolite of alachlor to 2,6 – DEA, the precursor to DEIQ greater than 30 times that of monkeys and 751 times greater than humans. Variations in individual, human susceptibility may also be a result of differences in metabolism, expression of tumor suppressor genes (pharmacogenetics) and nutritional variations. Differences in metabolic phenotype, as detected in enzymes, may cause variances in required metabolic activation. Environmental carcinogen susceptibility may also have to do with phenotypes for detoxifying enzymes.
Biochemical responses to environmental chemicals (biochemical biomarkers) provide a measure of toxic effect. They are particularly valuable when used to measure the toxic effects of chemicals in the field, employing nondestructive sampling methods. A widely used biochemical biomarker is cholinesterase depression, which may involve destructive sampling (brain acetylcholinesterase [AChE]) or nondestructive sampling (serum butyrylcholinesterase). From enzyme inhibition data, it is apparent that field workers and pesticide users were exposed to significant levels of synthetic and natural toxicants. Therefore, the relationship between red blood cells (RBC), SOD, GST, catalase, vitamine A, C, E, and Zn depletion suggests a compromise of the antioxidant enzyme status.

7. Antioxidants as surrogate biomarkers

During the break down of xenobiotics and under normal metabolic processes, highly reactive compounds called free radicals are produced in the body. These compounds are inherently unstable since they have an odd number of electrons, but in order to make up for their shortage in electrons, free radicals will react with some components of the cell (lipids, proteins or DNA) and in so doing, they interfere with the cell's ability to function normally. Although, the body naturally produces free radicals, it also has a means to defend against its

harmful effects. Antioxidants which include some enzymes, are chemical substances found in the biological system that act on free radicals. Antioxidant enzymes work in several ways; they may reduce the energy of the free radical or give up some of their electrons for its use, thereby causing it to become stable. Antioxidant enzymes may also stop the free radical from forming in the first place. Similarly, they may also interrupt an oxidizing chain reaction to minimize the damage caused by free radicals. The main function of antioxidant enzymes is neutralizing free radicals produced in the body.

The human body produces several types of antioxidant enzymes which include superoxide dismutase (SOD), catalase, and glutathione peroxidase. These antioxidant enzymes neutralize many types of disease-causing free radicals, ridding the body of their harmful effects. Supplements of these antioxidant enzymes are also available. Usually they are for oral administration in the form of pills or capsules. However, the absorption of antioxidant enzymes in supplement form is minimal at best. A better way would be to supplement the body with the "building blocks" required in order for our body to manufacture its own SOD, catalase, glutathione peroxidase, and other such antioxidant enzymes. The building block nutrients of antioxidant enzymes include the minerals manganese, zinc, and copper for SOD and selenium for glutathione peroxidase. Hence this intervention programme has been explored by many researchers in order to ameliorate the toxic injury from pesticide exposure. In addition to antioxidant enzymes, many vitamins and minerals may also have antioxidant properties. These include vitamins A, C, E and nutrients such as lutein, lycopene, vitamin B_2, coenzyme Q_{10}, and cysteine. Herbs, such as bilberry, turmeric (curcumin), grape seed or pine bark extracts, and ginkgo can also provide powerful antioxidant protection for the body.

8. Non enzyme antioxidants

There are different types of antioxidants available in nature and their potential to fight free radical attacks has been widely exploited. Antioxidants from our diet appear to be of great importance in controlling damage by free radicals. Each nutrient is unique in terms of its structure and antioxidant function. Therefore, supplementation of these antioxidants in some cases of pesticide poisoning has proven to be of immense importance.

Vitamin E is actually a generic term that refers to all entities (eight found so far) that exhibit biological activity of the isomer tocopherol. Alpha-tocopherol, the most widely available isomer, has the highest biopotency, or strongest effect in the body. Because it is fat-soluble, alpha-tocopherol is in a unique position to safeguard cell membranes from damage by free radicals. Alpha-tocopherol also protects the fats in low-density lipoproteins from oxidation.

Vitamin C, also known as ascorbic acid, is a water-soluble vitamin. As such, it scavenges free radicals that are in an aqueous environment. Vitamin C works synergistically with vitamin E to quench free radicals. Vitamin C also regenerates the reduced (stable) form of vitamin E.

Beta-carotene, also a water-soluble vitamin, is the most widely studied of the 600 carotenoids identified till date. It is thought to be the best quencher of singlet oxygen. Beta-carotene is also especially excellent at scavenging free radicals in low oxygen concentration.

Selenium is a trace element that is required in very small quantities, without which we may not survive. It forms the active site of several antioxidant enzymes including glutathione peroxidase.

Similar to selenium, the minerals manganese and zinc are trace elements that form an essential part of various antioxidant enzymes.

9. Antioxidant enzymes

The antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) serve as primary line of defense in destroying free radicals. SOD first reduces the radical superoxide (O_2-) to form hydrogen peroxide (H_2O_2) and oxygen (O_2). Catalase and glutathione peroxidase (GPx) then work simultaneously with the protein glutathione to reduce hydrogen peroxide and ultimately produce water (H_2O). Glutathione (commonly abbreviated GSH) is a crucial conjugating agent in the body. This compound is a tripeptide, meaning that it is composed of three amino acids linked together. These amino acids and their abbreviations are glutamic acid (Glu), cysteine (Cys), and glycine (Gly). A glutathione conjugate may be excreted directly, although this is rare. More commonly, the GSH conjugate undergoes further biochemical reactions that produce mercapturic acids (compounds with N-acetylcysteine attached) or other species. Glutathione forms conjugates with a wide variety of xenobiotic species, including alkenes, alkyl epoxides (1,2- epoxyethylbenzene), arylepoxides (1,2-epoxynaphthalene), aromatic hydrocarbons, aromatic halides, alkyl halides (methyl iodide), and aromatic nitro compounds. The glutathione transferase enzymes required for the initial conjugation are widespread in the body. The importance of glutathione in reducing levels of toxic substances can be understood by considering that loss of H^+ from –SH on glutathione leaves an electron-rich –S– group (nucleophile) that is highly attractive to electrophiles. Electrophiles are important toxic substances because of their tendencies to bind to nucleophilic biomolecules, including nucleic acids and proteins. Such binding can cause mutations (potentially cancer) and result in cell damage. Included among the toxic substances bound by glutathione are reactive intermediates produced in the metabolism of pesticides, including epoxides and free radicals (species with unpaired electrons).

10. Superoxide dismutase (SOD)

The enzymes involved in antioxidant reactions are the superoxide dismutases, glutathione peroxidase and catalases. Superoxide dismutase (SOD) catalyses the destruction (dismutation) of superoxide free radicals produced during oxidation of pyrethroid (Otitoju and Onwurah, 2007). These ions are believed to be responsible for lipid peroxidation and peroxidative haemolysis of erythrocytes. The action of SOD therefore results in the protection of the biological integrity of cells and tissues against the harmful effects of superoxide free radicals. To ameliorate the damage caused by the hydroxyl radicals formed from superoxide radical and hydrogen peroxide, organisms have evolved mechanisms to regulate the concentrations of the two reactants. SOD is an important isoenzyme functioning as superoxide radicals' scavenger in the living organisms. It is an important enzyme family in living cells for maintaining normal physiological conditions and coping with stress (Otitoju 2005). The action of SOD therefore results in the protection of biological integrity of cells and tissues against the harmful effects of superoxide free radicals (Olusi, 2000). In order to ameliorate the damage caused by the hydroxyl radicals and hydrogen peroxide, organisms have evolved mechanisms to regulate the concentrations of the two reactants. SOD is an important isoenzyme functioning as superoxide radicals' scavengers in the living organisms. It is an important enzyme family in living cells for maintaining normal

physiological conditions and for coping with stress. The role of superoxide dismutase enzyme is to accelerate the dismutation of the toxic superoxide radical (O_2^-) produced during oxidative energy processes to hydrogen peroxide and molecular oxygen. It is known that pesticides may irritate macrophages in the lung thereby encouraging them to produce superoxide radical (O_2^-). Antioxidant enzymes are used by the organisms as natural endogenous protection against the generation of reactive oxygen species (Metwalli and El-megd, 2002; Otitoju and Onwurah, 2007). Superoxide dismutases are metalloenzymes scavengers, which destroy superoxide radicals by converting them into hydrogen peroxide and oxygen by dismutation reaction. SOD works in conjunction with two enzymes, glutathione peroxidase and catalase which converts hydrogen peroxide to water and oxygen.

In our previous work on the effect of permethrin on SOD in non-target organisms, our results showed a marked increase in SOD activity in the exposed groups (Otitoju, and Onwurah, 2007). This increase may be a coping strategy for the exposed groups which may be due to other factors such as age, concentration of the pesticide, sex, e.t.c. However, depletion of SOD activity during prolonged exposure was suggested to be due to the overwhelming influence of superoxide radicals or activated metabolites generated by the pesticide on the cell membrane of the exposed organisms. The overall effect of pesticide radicals is the increased production of free radicals in the system and the concomitant decrease in the antioxidant activity due to the utilization of the antioxidant enzymes to neutralize the free radicals generated. All the major biomolecules like lipids, proteins, and nucleic acids may be attacked by free radicals, but lipids are probably the most susceptible. The oxidative destruction of lipids is a destructive, self-perpetuating chain reaction, releasing malondialdehyde (MDA) as the end product.

11. Glutathione transferase (GST)

Pesticides are metabolised through oxidation and hydrolysis by esterases, including other reactions involving glutathione, demethylation and glucuronidation. The glutathione transferase reactions give products that are, in most cases, of low toxicity. Pesticides are mostly eliminated in the urine with lesser amounts in the faeces and expired air. Glutathione-S-transferases are a major family of detoxifying enzymes that catalyze the conjugation of GSH (as earlier stated) with electrophilic centers of lipophilic substrates, thereby increasing its solubility and aiding their excretion from the body. Increased level of GST in a challenged or exposed individual may indicate that the OP and carbamate pesticides are mainly metabolised in the liver and excreted as a conjugate of GSH. Glutathione is an ubiquitous tripeptide that plays a significant role in oxidation-reduction reactions, amino acid transport, detoxification of electrophiles and metals, metabolites of xenobiotics and many carcinogens. Glutathione (GSH) is an endogenous thiol antioxidant that has a multifaceted role in xenobiotic metabolism and is a first line of defense against oxidant-mediated cell injury (Palmeira, 1999). Studies in animal models suggest that many synthetic organophosphates and organochlorides such as endosulfan and chloryriphos modify the concentrations of GSH (Beebe *et al*., 2003). Glutathione together with glutathione dependent systems, glutathione peroxidase (GSH-Px), glutathione-S-transferase, catalase, and superoxide dismutase efficiently scavenge toxic free radicals.

12. Other antioxidants

In addition to enzymes, vitamins, and minerals, there appear to be many other nutrients (as mentioned in section 2), and compounds that have antioxidant properties. Among them is coenzyme Q_{10} (CoQ_{10}, or ubiquinone), which is essential to energy production and can also protect the body from destructive free radicals. Also, uric acid, a product of DNA metabolism, has become increasingly recognized as an important antioxidant. Additionally, substances in plants called phytochemicals are being investigated for their antioxidant activity and health-promoting potential.

13. Mechanisms of biomarker action

A critical aspect of toxicological chemistry is that which deals with the biochemical mechanisms and reactions by which pesticides or related xenobiotic compounds and their metabolites interact with biomolecules to cause an adverse toxicological effect. In order to be detected or cause a toxic response, pesticides if introduced into an organism directly or indirectly, would react before reaching a target or receptor. However, when "reactive pesticides" are produced metabolically, it may be in a location where they can further interact with a biomolecule, membrane, or tissue to cause a toxic or biomarker response. Metabolically reactive pesticides generally fall into the following four categories:

- **Electrophilic species** that are positively charged or have a partial positive charge and therefore a tendency to bond to electron-rich atoms and functional groups, particularly N, O, and S, that abound on nucleic acids and proteins (including proteinaceous enzymes), which are commonly affected by toxic substances.
- **Nucleophilic species** that are negatively charged or partially so and have a tendency to bind with electron-deficient targets. These are much less common toxicants than electrophilic species, but include agents such as CO, formed metabolically by loss of halogen and oxidation of dihalomethane compounds or cyanide, CN^-, produced by the metabolic breakdown of acrylonitrile, a biochemically reactive organic compound containing both a –CN group and a reactive C=C bond.
- **Free radicals** that consist of neutral or ionic species that have unpaired electrons. Free radicals include the superoxide anion radical $O^{\cdot -}$, produced by adding an electron to O_2, and the hydroxyl radical, $HO^{\cdot}$, produced by splitting (haemolytic cleavage) of the H_2O_2 molecule. These species can react with larger molecules to generate other free radical species. Electron transfer from cytochrome P-450 enzyme to a pesticide during its oxidation can produce the reactive, damaging free radical.
- **Redox-reactive** reagents that bring about harmful oxidation–reduction reactions. An example is the generation from nitrite esters of nitrite ion, NO_2^- , which causes oxidation of Fe^{2+} in haemoglobin to Fe^{3+}, producing methaemoglobin, which does not transport oxygen in blood. In understanding the kinds of processes by which some pesticides harm an organism, it is important to understand the concept of receptors. Here a **receptor** is taken to mean a biochemical entity that interacts with a pesticide to produce some sort of toxic effect. Generally receptors are macromolecules, such as proteins, nucleic acids, or phospholipids of cell membranes, inside or on the surface of cells. In the context of pesticide or its metabolite–receptor interactions, the substance that interacts with a receptor is called a **ligand**. Whereas an enzyme generally alters a substrate chemically (such as by hydrolysis), a toxicant does not usually change the

chemical nature of a receptor other than binding to it. In considering the interactions between the pesticide and the receptor, it may be assumed that the receptor normally binds to some endogenous substance, causing a normal effect, such as a nerve impulse. In some cases, the toxicant may activate the receptor, causing an effect similar to that of the endogenous ligand, but different enough in degree that some adverse effect results. Another possibility is that the toxicant binds to a receptor site, preventing an endogenous ligand from binding; this is known as an **antagonist action**. Yet another possibility is for the toxicant to bind to a site different from, but close enough to, the normal binding site to interfere with the binding of an endogenous substance. As a final possibility, the receptor may not have any endogenous ligands, but being bound by a toxicant nevertheless has some sort of effect.

Some pesticides can also exert their effect by interfering with enzyme activity. Enzymes are extremely important because they must function properly to enable essential metabolic processes to occur in cells. Substances that interfere with the proper action of enzymes obviously have the potential to be toxic. Many xenobiotics or pesticides that adversely affect enzymes are **enzyme inhibitors**, and thus they slow down or stop enzymes from performing their normal functions as biochemical catalysts. Stimulation of the body to make enzymes that serve particular purposes, a process called **enzyme induction**, is also important in toxicology. The body contains numerous endogenous enzyme inhibitors that serve to control enzyme catalyzed processes. When a toxicant acts as an enzyme inhibitor, however, an adverse effect usually results.

The covalent bonding of organic pesticides to enzymes can cause enzyme inhibition. Such bonding occurs most commonly through hydroxyl (–OH) groups on enzyme active sites. Covalent bonding of some pesticides is one of the major ways in which acetylcholinesterase (enzyme) crucial to the function of nerve impulses can be inhibited. An organophosphate compound, such as the nerve gas compound diisopropylphosphorfluoridate (a reactant), may bind to acetylcholinesterase, thereby inhibiting the enzyme.

14. Conclusion

Measurement of several biomarkers in albino rats at the same time for pesticides in the environment is of great importance. This approach has been advocated by several researchers who worked on different environmental toxicants (Romeo *et al*. 2003; Cajaraville *et al*. 2000). Since GST and SOD can be induced or inhibited in albino rats exposed to some pesticides, they can be used as biomarkers in risk assessment in a given human population. In fact increased levels/activities of glutathione S-transferase (GST) and superoxide dismutase (SOD) were observed in albino rats exposed to pyrethroid. Oxidative stress observed during pesticide metabolism in albino rats results in free radical and reactive oxygen species (ROS) formation. The consequence of the above is oxidation of cellular components such as membrane lipids, protein and DNA (biochemical markers), leading to adverse health effects and diseases. Based on the above, defense system against oxidative, (stress enhanced by the pesticide metabolism in rats), serves as the physiological endpoint parameters of exposure and they included superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase. Expanding the repertoire of these biomarkers of pesticide exposure and employing their multiple combinations in well-designed study protocols will provide critical tools in the evaluation of pesticide safety and

design of appropriate measures to minimize adverse exposures. Therefore, the combination of in vitro and animal data will give the best picture of biomarkers' performance.

15. References

Anwar, W. A. (1997). Biomarkers of Human Exposure to Pesticides. Environmental Health Perspectives 105(4): 801-806

Beeby ,A (1993). Measuring the effect of pollution. In "Applying Ecology" Chapman and Hall, London, New York.

Cajaraville, M.P., Bebianno, M.J., Biasco, j., Porte, C.,Sarasquete, C., Viarengo, O. (2000). The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsular : a ptactical approach. Sci. Total Environ. 247, 295 – 311.

Hagstrum, D. W and Subramanyam. B. H (2006). Fundamental of Stored-Product Entomology. American Association of Cereal Chemists International, St. Paul, Minnesota

Hulka, B.S. (1988). Biological markers. *Am J. Epidemiol.* 136, 200 -209.

Mathews, P.J. (1980). Toxicology for water scientists. *J. Environ. Mgmt.* 11,*(1)*, 1-14.

Metwalli, A.M. and El-Magd. (2002). Skin diseases and enzymatic antioxidant activity among workers exposed to pesticides. Eastern Mediterranean Health J., 8: 368 - 373.

National Research Council (NRC), (1992), Environmental Neurotoxicity" National Academy Press, Washington, DC

Olusi, S.O. (2002). Obesity is an independent risk factor for plasma lipid peroxidation and depletion of erythrocyte cytoprotective enzymes in humans. International J. Obesity, 26:1159-1164.

Otitoju, O. (2005). Sub-chronic effect of Rambo insect powder contaminated diet on superoxide dismutase (SOD) activity in Wistar albino rats. *Biochemistri.17 (2): 157-163*

Otitoju, O. and Onwurah, I.N.E. (2007). Glutathione S-transferase (GST) activity as a biomarker in ecological risk assessment of pesticide contaminated environment. African *J.* Biotech. 6 (12):1455-1459.

Palmeira, C. M. (1999). Herbicide-induced mitochondrial and cellular toxicity: A review of paraquat, dinoseb and 2,4-D effects. *Toxic Substances Mechanisms*, 18: 187 – 204.

Romeo, M., Hoarau, P., Garello, G., Gnassia-Barelli, M., Girard, J.P. (2003).Mussel traplantation and biomarkers as useful tools for assessing water quality in the NW Mediterranean. Environ. Pollut 122, 369 – 379.

Schultz, P., and Mazzuckelli (1991). Validization of biological markers for quantitative risk assessment. *Environmental Health Perspective* 90, 239 – 246.

US Environmental Protection Agency US EPA (2001). Diazinon Revised Risk Assessment and Agreement with Registrants, Office of Pesticide Programs.

Ware G.W & Whitacre D.M (2004). The Pesticide Book. 6th ed. Meister Media Worldwide, Willoughby, Ohio. pp 496

Ware, G.W (2000). The Pesticide Book. 5th Ed. Fresno, CA: Thompson Publications. pp 17.

13

Fish Cholinesterases as Biomarkers of Organophosphorus and Carbamate Pesticides

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1. Introduction

Due to reasons that last for decades, environmental monitoring of pesticides is an urgent need. Contamination by pesticides is an important public health problem, mainly in developing countries. It is estimated that only 0.1% of the applied pesticides in fact reach the target pests, while the rest spreads throughout the environment (Hart and Pimentel, 2002). In addition, among the 500,000 deaths a year related to pesticides in the developing world, approximately 200,000 occur due to the use of organophosphorus (OP) and carbamates (CB) pesticides (Eddleston et al., 2008). These are among the most important classes of insecticides/acaricides in usage and billing (Nauen and Bretschneider, 2002). The primary and most known target for the action of organophosphorus and carbamate compounds is a family of enzymes (Cholinesterases; ChEs) formed by: acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). The first is synthesized in hematopoiesis, occurs in the brain, endplate of skeletal muscle, erythrocyte membrane, and its main function is to regulate neuronal communication by hydrolyzing the ubiquitous neurotransmitter acetylcholine in synaptic cleft (Quinn, 1987; Silman and Sussman, 2005). The second is synthesized in liver and is present in plasma, smooth muscle, pancreas, adipocytes, skin, brain and heart (Çokugras, 2003). Although its physiological function is not well defined, BChE is pointed as one of the main detoxifying enzymes able to hydrolyze or scavenge a broad range of xenobiotic compounds like cocaine, heroine, anaesthetics, and pesticides (Soreq and Zakut, 1990; Taylor, 1991; Çokugras, 2003; Nicolet et al., 2003). Some studies hypothesize that one of the functions of BChE is to protect AChE against anticholinesterasic agents (Whitaker, 1980; Whitaker, 1986). Pezzementi and Chatonnet (2010) reported that ChEs emerged from a family of proteins with adhesion properties. Both play other roles in the neuronal tissue, particularly in neuronal differentiation and development, cell growth, adhesion and signalling. In addition, AChE participates even in hematopoietic differentiation (Chatonnet and Lockridge, 1989; Taylor, 1991; Johnson and Moore, 2000; Silman and Sussman, 2005).

Moreover, AChE and BChE are different concerning several other aspects: while AChE has an *in vivo* half-life of 120 days, BChE lasts 7-12 days. AChE is inhibited by substrate excess and BChE is activated by substrate excess (Lopez-Carillo and Lopez-Cervantes, 1993; Çokugras, 2003). AChE is selectively inhibited by propidium, DDM, caffeine, Nu1250, 62c47

and BW284c51 while BChE is selectively inhibited by percaine, isopestox, ethopropazine, Iso-OMPA, bambuterol and haloxon (Adams and Thompson, 1948; Austin and Berry, 1953; Aldridge, 1953; Bayliss and Todrick, 1956; Chatonnet and Lockridge, 1989; Harel et al., 1992; Kovarik et al., 2003). BChE has a larger space in its active site, which can hydrolize or be inhibited by a range of compounds. AChE has a more specific active site (Çokugras, 2003). Some of these features are governed by crucial differences in the structure of the enzymes such as: 1) the difference in size of active site can be explained by six aromatic residues lining the active site of AChE that are missing in BChE; 2) two of these (Phe-288 and Phe-290) are replaced by leucine and valine, respectively, in BChE. This feature prevents the entrance of butyrylcholine in the AChE active site; 3) peripheral site specific-ligands such as propidium does not inhibit BChE because the residue Trp-279, which is part of the peripheral anionic site located at the entrance of the active site gorge in AChE, is absent in BChE (Harel et al., 1992). According to Rosenberry (1975), AChE is more sensitive to the size of the acyl group than to the alcohol moiety (whether charged or neutral) of the substrate, while for BChE the opposite is observed. Both are inhibited by 50 μM of physostigmine (eserine), which is a condition that affords to discriminate cholinesterases (ChEs) from other esterases (Augustinsson, 1963).

The class of AChEs is more homogeneous in terms of their primary structure than the class of BChEs (Rosenberry, 1975). Despite of these differences, the amino acid sequence identity between AChE and BChE from vertebrates ranges from 53 to 60%, even in evolutionarily distant species (Chatonnet and Lockridge, 1989; Taylor, 1991). In addition, a study promoted the replacement of only two amino acids by site-directed mutagenesis in AChE for it to develop BChE activity (Harel et al., 1992). Both enzymes present the active site within a deep and narrow gorge, approximately in the middle of its globular structure, which apparently could disturb the substrate traffic. However, in fact this structure follows a rational organization which entraps substrate and transports it to the active site through the arrangement of amino acids lining the gorge. And all this occurs very efficiently (Quinn, 1987; Tõugo, 2001).

To characterize ChE, some studies used the kinetic parameters Km and Vmax, more specifically the Km and Vmax ratios for acetyl and butyrylcholine hydrolysis and their analogues by the enzymes. According to the expected values for these ratios, AChE has a low Vmax **ratio** and a Km **ratio** ≥ 1, because it presents excess substrate inhibition. BChE does not show this feature, its Vmax ratio is ≥ 1, and Km ratio < 1. (Pezzementi et al., 1991; Rodríguez-Fuentes and Gold-Bouchot, 2004).

Table 1 summarizes Km and Vmax of fish AChEs from brain, muscle and electric organ reported in the literature. The Km values varied from 0.085 (Rainbow trout brain) up to 3.339 mM (Brazilian flathead brain), whereas Vmax ranged from 0.116 (arapaima brain) up to 0.524 U/mg protein (female hornyhead turbot muscle).

Table 2 presents the values for optimum pH and maximum temperature of fish enzymes. pH values ranged from 7.5 to 8.5 for all reported species, while temperatures varied from 26ºC (bluegill brain) to 45ºC (tambaqui and pirarucu brains).

The Km values of fish BChEs presented in table 3 ranged from 0.033 (Nile tilapia liver) to 1.61 mM (tambaqui brain) and Vmax were from 0.04 (tambaqui brain) up to 0.231 U/mg protein (piaussu serum). Several studies have described that AChE accounts for most of the brain cholinesterasic activity (Rodríguez-Fuentes, 2004; Varò et al., 2004; Varò et al., 2007; Jung et al., 2007). However, our studies on brain ChEs from some fish reveal that certain

Scientific and common name	Km (mM)	Vmax (U/mg protein)	Source	Reference
Ictalurus punctatus – Channel catfish	0.375 ± 0.002	0.212 ± 0.002	Brain	Carr and Chambers, 1996
Oreochromis niloticus – Nile tilapia	0.101 ± 0.03	0.229 ± 0.014	Brain	Rodríguez-Fuentes and Gold-Bouchot, 2004
Pseudorasbora parva – topmouth gudgeon, Stone moroko	0.113 ± 0.11	0.490 ± 0.024	Brain	Shaonan et al., 2004
Carassius auratus – goldfish	0.112 ± 0.09	0.504 ± 0.027	Brain	Shaonan et al., 2004
Oncorhynchus mykiss – rainbow trout	0.085 ± 0.06	0.266 ± 0.023	Brain	Shaonan et al., 2004
Genidens genidens – guri sea catfish	0.236	nd	Brain	Oliveira et al., 2007
Paralonchurus brasiliensis – banded croaker	0.228	nd	Brain	Oliveira et al., 2007
Haemulon steindachneri – chere-chere grunt	1.035	nd	Brain	Oliveira et al., 2007
Pagrus pagrus – red porgy, common seabream	1.087	nd	Brain	Oliveira et al., 2007
Menticirrhus americanus – Southern kingcroaker	1.579	nd	Brain	Oliveira et al., 2007
Cynoscion striatus – striped weakfish	1.595	nd	Brain	Oliveira et al., 2007
Dules auriga (*Serranus auriga*)	1.624	nd	Brain	Oliveira et al., 2007
Merluccius hubbsi – Argentinean hake	3.259	nd	Brain	Oliveira et al., 2007
Percophis brasiliensis - Brazilian flathead	3.339	nd	Brain	Oliveira et al., 2007
Limanda yokohomae – Marbled sole	0.365 ± 0.16	nd	Brain	Jung et al., 2007
Limanda yokohamae – Marbled sole	0.18 ± 0.11	nd	Muscle	Jung et al., 2007
Pleuronectes vetulus - English sole	1.689 ± 0.26	0.482 ± 0.034	Muscle	Rodríguez-Fuentes et al., 2008
Pleuronichthys verticalis – hornyhead turbot	0.303 ± 0.07 (female); 0.226 ± 0.06 (male)	0.524 ± 0.032 (female); 0.120 ± 0.008 (male).	Muscle	Rodríguez-Fuentes et al., 2008
Colossoma macropomum – tambaqui	0.43 ± 0.02	0.129 ± 0.005	Brain	Assis et al., 2010
Arapaima gigas - pirarucu	0.42 ± 0.09	0.116 ± 0.002	Brain	not published results
Rachycentron canadum - cobia	0.43 ± 0.14	0.243 ± 0.02	Brain	not published results
Oreochromis niloticus – Nile tilapia	0.39 ± 0.2	0.218 ± 0.007	Brain	not published results

U = μmol of substrate hydrolyzed per minute; and nd = not determined

Table 1. Kinetic parameters of AChE from several freshwater and marine species

Scientific and common name	Optimum Temp	Optimum pH	Source	Reference
Solea solea – common sole	nd	7.5	Brain	Bocquené et al.,1990
Pleuronectes platessa – plaice	32 – 34ºC	8.5	Brain	Bocquené et al.,1990
Scomber scomber – mackerel	nd	7.5 – 8.5	Brain	Bocquené et al.,1990
Lepomis macrochirus – bluegill	26 – 27ºC	nd	Brain	Beauvais et al., 2002
Clarias gariepinus – African sharptooth catfish	nd	8.0	plasma	Mdegela et al., 2010
Colossoma macropomum – tambaqui	40 - 45ºC	7.0 – 8.0	Brain	Assis et al., 2010
Oreochromis niloticus – Nile tilapia	35ºC	8.0	Brain	not published results
Arapaima gigas - pirarucu	45ºC	8.0	Brain	not published results
Rachycentron canadum - cobia	35ºC	8.0	Brain	not published results

nd = not determined

Table 2. Values of optimal pH and temperature for AChE from several species of fish

Scientific and common name	Km (mM)	Vmax (U/mg protein)	Source	Reference
Oreochromis niloticus – Nile tilapia	0.033± 0.004	0.063 ± 0.001	Liver	Rodríguez-Fuentes and Gold-Bouchot, 2004
Oreochromis niloticus – Nile tilapia	0.123± 0.051	0.224 ± 0.016	Muscle	Rodríguez-Fuentes and Gold-Bouchot, 2004
Leporinus macrocephalus – piaussu	0.047	0.231 ± 0.008	Serum	Salles et al., 2006
Limanda yokohamae – Marbled sole	0.068 ± 0.35	nd	Muscle	Jung et al., 2007
Colossoma macropomum – tambaqui	1.61 ± 0.01	0.04 ± 0.001	Brain	not published results

U = μmol of substrate hydrolyzed per minute; nd + not determined.

Table 3. Kinetic parameters of BChE from several freshwater and marine species

species can present brain BChE or AChE with wider active sites. This is in accordance with Pezzementi and Chatonnet (2010), who reported atypical ChE activity in some fish species. Data about optimal pH and temperature of fish BChE are not presented here due to scarcity.

2. Organophosphorus and carbamates action on fish cholinesterases

OPs and CBs act by phosphorylating or carbamoylating the serine residue at the active site of the ChEs. Their structures present either similarities to the substrates or their hydrolytic intermediates and interact very slowly with the enzyme by forming stable conjugates (Quinn, 1987; Tõugu, 2001). This mechanism hinders the normal functioning of the enzyme, which cannot prevent the accumulation of the neurotransmitter in the synaptic cleft. The overstimulation caused by acetylcholine continuously firing its receptors generates a range of signs and symptoms. Because of their low environmental persistence and high toxicity, particularly to aquatic organisms, water must be continuously monitored (Beauvais et al., 2002).

Environmental monitoring may be chemical and/or biological. Chemical monitoring is the set of chemical analysis that quantify waste contaminants in a compartment or environmental matrix in a temporal or spatial scale. On the other hand, when the focus is to determine the magnitude of the effects of this contamination on organisms at individual or population level, biological monitoring is adopted (Henriquez Pérez and Sánchez-Hernández, 2003). The combined use of chemical and biological approaches in environmental monitoring is an important task for the assessment of contamination and its effects on an ecosystem. This is the basis of the concept of bioindicators.

In this scenario, when determining chemical characteristics of pollutants and their concentrations, organisms and their biomolecules represent a useful choice as bioindicators, since they afford to employ both the chemical and the biological approaches in environmental biomonitoring. Moreover, they also allow estimating the impact of these pollutants to such species that provide the target molecules (Wijesuriya and Rechnitz, 1993; Watson and Mutti, 2003). Among these compounds, enzymes play an important role due to their degree of specificity and fast response to relevant changes in the surrounding medium. The use of enzymes as bioindicators is based on the inhibition or negative interference in catalytic activity triggered by analytes (Marco and Barceló, 1996). Cholinesterase inhibition has been used as biomarker of organophosphorus and carbamate exposure. AChE is one of the oldest environmental biomarkers (Payne et al., 1996).

In general, the higher the concentration of pesticides and longer exposure time, the greater are the negative impacts, since these are the conditions when higher levels of biological organization, such as communities and ecosystems, are affected by pesticides. The effects of contaminants on low levels of biological organization (e. g., molecular and biochemical responses) occur more quickly, and the specificity of responses is generally higher. The effects on such levels can be directly related to exposure to pollutants. The presence of chemical residues and metabolites is a direct indicator of the availability of contaminants to organisms (Arias et al., 2003). In the monitoring of pesticides and other contaminants in water resources, several techniques that use organisms as bioindicators have been developed, either by estimation of population density and behavioral changes or by assessment of physiological characteristics of these organisms that make them sensitive to certain pollutants. These organisms are chosen based on features like habitat, ecology, food habits, species abundance and ease of capture (Henríquez Pérez and Sánchez-Hernández, 2003). There are two main approaches: 1) The *in vivo* approach, which exposes live specimens to the analyzed substance and collect tissues for analysis after the exposure period and 2) the *in vitro* approach, which exposes tissues or purified biomolecules directly to the analytes.

Each technique has its own advantages. In the first approach, the slow interaction between enzyme and pesticides is behind the ability ChEs has to signal inhibition several days or weeks after exposure, even when the concentrations in the water are negligible. On the other hand, the *in vitro* approach makes it possible to gain more precision in the correlation between pesticide concentrations and the resulting inhibition. In addition, the *in vitro* conditions avoid the contact between pesticides and the detoxificant complex of other tissues, allowing the use of target cholinesterases enzymes as biocomponents in electrochemical and optical devices and increasing the accuracy of data acquisition in biosensors.

In the aquatic environment pesticides and other xenobiotics can attach to suspended matter, sediments in bed of water body or be absorbed by the aquatic organisms where they undergo detoxification or bioaccumulation (Nimmo, 1985). Thus, AChE from aquatic organisms has been used due to its ability to assess the environmental impact when these compounds are not present in the water (Morgan et al., 1990; Sturm et al., 1999; Ferrari et al., 2004; Wijeyaratne and Pathiratne, 2006). Among these organisms are fish (Rodríguez-Fuentes and Gold-Bouchot, 2000; Fulton and Key, 2001; Oliveira et al, 2007; Rodríguez-Fuentes, Armstrong and Schlenk, 2008). Fish are part of ecosystems that are constantly affected by pollution from various sources, including crop fields and their pesticides and fertilizers. They occupy intermediate or higher positions in their food chains, thus undergoing accumulation of xenobiotics in their tissues and becoming a feasible alternative for environmental biomonitoring. Though it is unlikely that significant amounts of organophosphorus compounds could persist after the digestion and therefore be stored successively by higher members of the food chain, the position in the chain can influence strongly the pesticide bioaccumulation (Flint and Van der Bosch, 1981). And though the persistence of OPs in the environment is relatively short, residual life of some OP pesticides such as leptophos and fenamiphos is longer. Moreover, in general OPs may have their half-lives extended multiple times in acidic pH (WHO/IPCS/INCHEM, 1986a).

There is a lack of specificity in cholinesterase inhibition by pesticides. Several compounds are capable of inhibit them in a manner almost indistinguishable at first sight. However, such substances show different patterns of enzyme inhibition represented by time for covalent binding and type or duration of recovery. Some anticholinesterasic pesticides can interact with both active and allosteric sites of the enzyme expressing mixed inhibition mechanisms.

ChE inhibition by OP compounds follows different behaviors depending on pesticide chemical structure. OP compounds include esters, amides or thiol derivatives of phosphoric, phosphonic, phosphorotioic or phosphonotioic acids (WHO/IPCS/INCHEM, 1986a). As for the phosphoester moiety, two main groups of organophosphorus pesticides are present, the phosphate group (oxon form; P=O) and the phosphorothioate group (thion form; P=S). The first exerts direct inhibition, due to the greater electronegativity of oxygen in relation to sulphur when interacting in the active domain of the enzyme. The second group is less toxic and requires biotransformation to their oxo-analogues to become biologically active. This biotransformation occurs by oxidative desulfuration mediated by cytochrome P450 (CYP450) isoforms and flavin-containing mono-oxigenase enzymes, by N-oxidation and S-oxidation (WHO/IPCS/INCHEM, 1986a; Vale, 1998). The second group is synthesized in this form in order to resist the environmental factors and to increase the residual power of the compound, since OPs, in general, present a short half-life in the environment after biotransformation.

OPs effects can also be divided in terms of the kind of phosphorylation that takes place in the active site. Most of these pesticides contain two methyl or two ethyl (less often isopropyl) ester groups bonded to the phosphorus atom (Table 4). Depending on their structure, they can dimethyl- or diethyl-phosphorylate the serine hydroxyl group in the active center. After the release of the leaving group, dimethyl-phospho-ChE can be spontaneously reactivated slowly (starting from 0.7 hours) while diethyl-phosphoenzymes can recover their activity spontaneously in 31 hours. However, in diethyl OP compounds this recovery occurs in a minor fraction of the enzyme and this fraction can be reinhibited so that it is necessary to use oximes or other reactivation agents. On the other hand, diisopropyl-phospho-ChE has no measurable recovery (WHO/IPCS/INCHEM, 1986a; Vale, 1998; Eddlestone, 2002; Paudyal, 2008). It means that diethyl and diisopropyl-organophosphorus are able to inhibit the enzyme in long term.

Dimethyl OP	Diethyl OP	Diisopropyl OP
Dichlorvos	Diazinon	Diisopropyl fluorophosphates (DFP)
Temephos	Chlorpyrifos	Diisopropyl methylphosphonate (DIMP)
Methyl parathion	Tetraethyl pyrophosphate (TEPP)	
Malathion	Parathion	
Fenthion	Coumaphos	
Dimethoate	Sulfotepp	
Methamidophos	Ethion	

Table 4. Examples of organophosphorus pesticides according to ester groups bonded to phosphorus atom

Another feature of the interaction of OP compounds with the tissues is that most of them are lipophilic. According to Vale (1998), they are rapidly absorbed and accumulated in fat, liver, kidneys and salivary glands. Phosphorothioate compounds are more lipophilic than phosphates (Table 5).

More lipophilic	Less lipophilic
Chlorpyrifos, Diazinon, Temephos, Malathion, Parathion, Methyl-Parathion, Fenthion, Coumaphos, Dimethoate, Ethion, Sulfotepp	Tetraethyl pyrophosphate (TEPP), Trichlorfon, Dichlorvos, Methamidophos, Fenamiphos, Phosphamidon, Monocrotophos

Table 5. Examples of organophosphorus pesticides according to the lipophilicity

The loss of an alkyl group from the phosphoester bond in the enzyme-OP complex leads to the so-called aging process, which is time dependent. This process is mainly influenced by type of OP compound, pH and temperature. Since dimethyl OPs present less time for recovery, its aging half life is also short (3.7 hours). On the other hand, for diethyl OPs long time for recovery implies a longer aging half life, which may be up to 33 hours (Worek et al., 1997; Worek et al., 1999).

Oximes are nucleophilic agents which present more affinity for the OP molecules than the active center of cholinesterases. They catalyze the reactivation of enzyme and decrease the availability of enzymes subjected to the process of aging (Eddlestone, 2002). After aging, the

enzyme is not responsive to oximes treatment. Wilson (1951) reported reactivation of tetraethyl pyrophosphate-inhibited AChE by choline and hydroxylamine.

Some organophosphorus coumarinic compounds such as haloxon and coroxon present a type of inhibition which acts by phosphorylating the active site of AChE, concomitantly interacting with the peripheral site responsible for the inhibition by substrate excess. Despite being a more efficient inhibitor for BChE, haloxon and its analogues display unusual inhibition kinetics for AChE (Aldridge and Reiner, 1969).

CB pesticides are *N*-substituted esters of carbamic acid capable of readily inhibiting cholinesterases without metabolic activation, so they can induce acute toxicity effects faster than most of OP compounds. Although most CBs are not very stable in aquatic environments, some are soluble in water and can bioaccumulate in trophic levels, being particularly toxic to fish because they are metabolized slowly in such animals (Vassilieff and Ecobichon, 1982). Compared to OP compounds, CBs require larger doses to produce mortality or poisoning symptoms, because they do not bind to cholinesterases as stable as OP and do not promote aging. The half life of carbamoylated cholinesterases ranges from 0.03 to 4 h, depending on the compound (WHO/IPCS/INCHEM, 1986b).

There are two main reasons to use fish cholinesterase as biomarker. The first concerns the availability of this source: in 2009, the world fisheries and aquaculture production was 145.1 million tones, and most of the fish waste reused comes from tissues other than those that provide ChEs (FAO, 2010). Moreover, studies found very high AChE concentrations in the electric organs of the ray *Torpedo marmorata* and the eel *Electrophorus electricus* (Nachmansohn and Lederer, 1939; Leuzinger and Baker, 1967). Up to now the electric organs of *Torpedo* rays and *Electrophorus* eels (actually, they are Gymnotiformes, closer to knifefish than true eels) are still considered the most abundant source of this enzyme. These tissues are composed of structural units called electrocytes, electroplaques or electroplax, which consist in thin, flat plates of modified muscle that assemble as two large, wafer-like, roughly circular or rectangular surfaces. Each single *E. electricus* electroplaque generates a small charge because they present a potential difference of 100 mV. However, when they are piled in rows as a Voltaic pile (the arrangement in its body) they can generate a potential of approximately 600 V since there are from 5,000 to 6,000 electroplaques in its electric organ, which constitutes around 4/5 of its length. The sensitivity of fish ChEs under OP and CB exposure can be seen in tables 6, 7 and 8, which shows some differences between species *in vitro* and *in vivo*.

When measuring cholinesterases activity and inhibition, numerous differences between methodologies and laboratories become apparent, and many concerns rouse about what could be a normal level of activity for each species (Fairbrother and Bennet, 1988). In order to address these differences, some studies expressed results in terms of percentage of residual activity (Cunha Bastos et al., 1999; Villatte et al., 2002; Assis et al., 2007; Assis et al., 2010) or percentage of inhibition. According to the Food and Agriculture Organization (2007), 20% inhibition of brain AChE activity is considered the endpoint to identify the no-observed-adverse-effect-level (NOAEL) in organisms, while signs and symptoms appear when AChE is inhibited by 50% or more. Death occurs above 90% inhibition.

The most used assay for ChE activity is the Ellman method (1961). It consists in a dye-binding reaction occurring when the chromogenic reagent DTNB joins the choline or thiocholine moieties released after cholinesterases substrates breakdown. Over the years, the assay has been improved by the contribution of several works and some will be listed here.

Species	IC_{50} (µmol/L)	Ki (µmol/L)	Source	Reference
ORGANOPHOSPHATE				
Azinphos ethyl				
Cyprinus carpio	34.6	-	Muscle	Sato et al., 2007
Azinphos methyl				
Cyprinus carpio	53.7	-	Muscle	Sato et al., 2007
Chlorpyrifos				
Cyprinus carpio	810	-	Brain	Dembélé et al., 2000
Colossoma macropomum	7.6	2.61×10^{-2}	Brain	Assis et al., 2010
Arapaima gigas	7.87	2.69×10^{-2}	Brain	not published results
Rachycentron canadum	30.24	5.94×10^{-2}	Brain	not published results
Oreochromis niloticus	26.78	0.161	Brain	not published results
*Electrophorus electricus***	0.03	2.18×10^{-4}	Electric organ	not published results
Chlorpyrifos-oxon				
Gambusia affinis	0.05	-	Brain	Boone and Chambers, 1997
Gambusia affinis	0.006	-	Muscle	Boone and Chambers, 1997
Chlorpyrifos ethyl				
Cyprinus carpio	9.12	-	Muscle	Sato et al., 2007
Chlorpyrifos methyl				
Cyprinus carpio	35.48	-	Muscle	Sato et al., 2007
Chlorfenvinfos				
Cyprinus carpio	19	-	Brain	Dembélé et al., 2000
Clarias gariepinus	0.03	-	Brain	Mdegela et al., 2010
DEP				
Cyprinus carpio	12.02	-	Muscle	Sato et al., 2007
Diazinon				
Pimephales promelas	5000	-	Muscle	Olson and Christensen, 1980
Oncorhynchus mykiss	2.5	-	Brain	Keizer et al., 1995
Danio rerio	20.0	-	Brain	Keizer et al., 1995
Poecilia reticulate	7.5	-	Brain	Keizer et al., 1995
Cyprinus carpio	0.2	-	Brain	Keizer et al., 1995
Cyprinus carpio	19	-	Brain	Dembélé et al., 2000
Cyprinus carpio	2.95	-	Muscle	Sato et al., 2007
Clarias gariepinus	0.15	-	Brain	Mdegela et al., 2010
Colossoma macropomum	-	-	Brain	Assis et al., 2010
Arapaima gigas	1500	5.13	Brain	not published results
Rachycentron canadum	-	-	Brain	not published results
Oreochromis niloticus	-	-	Brain	not published results
*Electrophorus electricus***	0.3	2.18×10^{-3}	Electric organ	not published results
Diazoxon				
Cyprinus carpio	0.019	-	Muscle	Sato et al., 2007

Species	IC_{50} (μmol/L)	Ki (μmol/L)	Source	Reference
Dichlorvos				
Alburnus alburnus	0.63	-	Brain	Chuiko, 2000
Leuciscus idus	0.31	-	Brain	Chuiko, 2000
Esox lucius	0.31	-	Brain	Chuiko, 2000
Dicentrarchus labrax	33.4	-	Brain	Varò et al., 2003
Dicentrarchus labrax	44.8	-	Muscle	Varò et al., 2003
Cyprinus carpio	1.78	-	Muscle	Sato et al., 2007
Colossoma macropomum	0.04	1.37×10^{-4}	Brain	Assis et al., 2010
Arapaima gigas	2.32	7.92×10^{-3}	Brain	not published results
Rachycentron canadum	6.9	1.36×10^{-2}	Brain	not published results
Oreochromis niloticus	5.4	3.26×10^{-2}	Brain	not published results
*Electrophorus electricus***	0.16	1.16×10^{-3}	Electric organ	not published results
Dimethoate				
Clarias gariepinus	190	-	Brain	Mdegela et al., 2010
EPN oxon				
Cyprinus carpio	0.055	-	Muscle	Sato et al., 2007
Ethoprofos				
Cyprinus carpio	37.15	-	Muscle	Sato et al., 2007
Fenitrothion				
Clarias gariepinus	0.2	-	Brain	Mdegela et al., 2010
Iprobenfos				
Limanda yokohamae	1.11	-	Muscle	Jung et al., 2007
Isoxathion oxon				
Cyprinus carpio	0.00068	-	Muscle	Sato et al., 2007
Leptophos				
Cyprinus carpio	26.02	-	Muscle	Sato et al., 2007
Malaoxon				
Pimephales promelas	18	-	Muscle	Olson and Christensen, 1980
Oreochromis niloticus	0.02	-	Brain	Pathiratne and George, 1998
Pseudorasbora parva	0.81	-	Brain	Shaonan et al., 2004
Carassius auratus	0.76	-	Brain	Shaonan et al., 2004
Oncorhynchus mykiss	0.34	-	Brain	Shaonan et al., 2004
Cyprinus carpio	0.049	-	Muscle	Sato et al., 2007
Malathion				
Pimephales promelas	5700	-	Muscle	Olson and Christensen, 1980
Oreochromis niloticus	1000	-	Brain	Pathiratne and George, 1998
Cyprinus carpio	169.8	-	Muscle	Sato et al., 2007
MEP oxon				
Cyprinus carpio	2.14	-	Muscle	Sato et al., 2007

Species	IC_{50} (μmol/L)	Ki (μmol/L)	Source	Reference
Monocrotophos				
Sciaenops ocellatus	0.72	-	Brain	Ru et al., 2003
Paraoxon				
Gambusia affinis	0.27	-	Brain	Boone and Chambers, 1997
Gambusia affinis	0.06	-	Muscle	Boone and Chambers, 1997
Paraoxon ethyl				
Cyprinus carpio	0.14	-	Muscle	Sato et al., 2007
Paraoxon methyl				
Gambusia affinis	8.4	-	Brain	Boone and Chambers, 1997
Gambusia affinis	0.54	-	Muscle	Boone and Chambers, 1997
Cyprinus carpio	0.60	-	Muscle	Sato et al., 2007
Genidens genidens	0.45	-	Brain	Oliveira et al., 2007
Paralomchurus brasiliensis	0.47	-	Brain	Oliveira et al., 2007
Haemulon steindachneri	0.27	-	Brain	Oliveira et al., 2007
Pagrus pagrus	0.12	-	Brain	Oliveira et al., 2007
Menticirrhus americanus	0.29	-	Brain	Oliveira et al., 2007
Cynoscion striatu	0.21	-	Brain	Oliveira et al., 2007
Dules auriga	0.16	-	Brain	Oliveira et al., 2007
Merluccius hubbsi	0.11	-	Brain	Oliveira et al., 2007
Percophis brasiliensis	0.10	-	Brain	Oliveira et al., 2007
Parathion ethyl				
Cyprinus carpio	380	-	Muscle	Sato et al., 2007
Parathion methyl				
Cyprinus carpio	602.5	-	Muscle	Sato et al., 2007
Phoxim				
Cyprinus carpio	3.80	-	Muscle	Sato et al., 2007
Pirimiphos methyl				
Clarias gariepinus	0.003	-	Brain	Mdegela et al., 2010
Profenofos				
Clarias gariepinus	0.002	-	Brain	Mdegela et al., 2010
Temephos				
Colossoma macropomum	ne	-	Brain	Assis et al., 2010
Arapaima gigas	ne	-	Brain	not published results
Rachycentron canadum	ne	-	Brain	not published results
Oreochromis niloticus	ne	-	Brain	not published results
*Electrophorus electricus***	7.6	5.51×10^{-2}	Electric organ	not published results
TEPP				
Colossoma macropomum	3.7	1.27×10^{-2}	Brain	Assis et al., 2010
Arapaima gigas	0.009	3.07×10^{-5}	Brain	not published results

Species	IC_{50} (µmol/L)	Ki (µmol/L)	Source	Reference
Rachycentron canadum	8.1	1.59×10^{-2}	Brain	not published results
Oreochromis niloticus	20.75	0.125	Brain	not published results
*Electrophorus electricus***	0.06	4.35×10^{-4}	Electric organ	not published results
Triazophos oxon				
Pseudorasbora parva	0.13	-	Brain	Shaonan et al., 2004
Carassius auratus	0.16	-	Brain	Shaonan et al., 2004
Oncorhynchus mykiss	0.042	-	Brain	Shaonan et al., 2004
CARBAMATES				
BPMC				
Cyprinus carpio	0.76	-	Muscle	Sato et al., 2007
Carbaryl				
Pimephales promelas	10.0	-	Muscle	Olson and Christensen, 1980
Colossoma macropomum	33.8	0.116	Brain	Assis et al., 2010
Arapaima gigas	12.25	4.18×10^{-2}	Brain	not published results
Rachycentron canadum	8.31	1.63×10^{-2}	Brain	not published results
Oreochromis niloticus	9.2	5.55×10^{-2}	Brain	not published results
Electrophorus electricus	0.6	-	Electric organ	Tham et al., 2009
Clarias batrachus	0.59	-	Muscle	Tham et al., 2009
Clarias gariepinus	0.003	-	Brain	Mdegela et al., 2010
Carbofuran				
Cyprinus carpio	0.45	-	Brain	Dembélé et al., 2000
Colossoma macropomum	0.92	3.15×10^{-3}	Brain	Assis et al., 2010
Arapaima gigas	0.75	2.56×10^{-3}	Brain	not published results
Rachycentron canadum	0.082	1.61×10^{-4}	Brain	not published results
Oreochromis niloticus	0.19	1.15×10^{-3}	Brain	not published results
*Electrophorus electricus***	0.005	3.63×10^{-5}	Electric organ	not published results
Electrophorus electricus	0.02	-	Electric organ	Tham et al., 2009
Clarias batrachus	0.03	-	Muscle	Tham et al., 2009
MPMC				
Cyprinus carpio	0.98	-	Muscle	Sato et al., 2007
MTMC				
Cyprinus carpio	3.89	-	Muscle	Sato et al., 2007
NAC				
Cyprinus carpio	0.93	-	Muscle	Sato et al., 2007
PHC				
Cyprinus carpio	0.95	-	Muscle	Sato et al., 2007
XMC				
Cyprinus carpio	2.24	-	Muscle	Sato et al., 2007

ne – negligible effect.

Table 6. Pesticide IC_{50} and Ki* values for *in vitro* AChE from freshwater and marine fish.

Species	IC_{50} (μmol/L)	Source	Reference
Dichlorvos			
Alburnus alburnus	0.0063	Serum	Chuiko, 2000
Leuciscus idus	0.0016	Serum	Chuiko, 2000
Abramis ballerus	0.0008	Serum	Chuiko, 2000
Abramis brama	0.001	Serum	Chuiko, 2000
Rutilus rutilus	0.0016	Serum	Chuiko, 2000
Blicca bjoerkna	0.0008	Serum	Chuiko, 2000
Iprobenfos			
Limanda yokohamae	0.306	Muscle	Jung et al., 2007
Malathion			
Ictalurus furcatus	31	Liver	Aker et al., 2008
Ictalurus furcatus	50.2	Muscle	Aker et al., 2008
Parathion			
Gasterosteus aculeatus	0.00343a	Liver	Wogram et al., 2001
Gasterosteus aculeatus	0.00343b	Muscle	Wogram et al., 2001
Gasterosteus aculeatus	0.00343c	Gills	Wogram et al., 2001

a – 60% inhibition; b – 30% inhibition; c – 30% inhibition.

Table 7. Pesticide IC_{50} and Ki* values for *in vitro* BChE from freshwater and marine fish.

Species	Inhibition report	Source	Reference
ORGANOPHOSPHATES			
Azinphos methyl			
Sparus aurata	IC_{50} 72h - 0.0096 μM	Larvae	Arufe et al., 2007
Chlorpyrifos			
Oreochromis mossambicus	LC_{50} 96h – 0.07 μM Caused 88% inhibition in brain and gill	Brain and gill	Rao et al., 2003
Gambusia yucatana	0.43 μM 96h inhibited 80 and 50% (muscle and head, respectively)	Muscle and head	Rendón-von Osten et al., 2005
Oreochromis niloticus	IC_{50} 48 h - 0.011 μM	Brain	Chandrasekara and Pathiratne, 2007
Chlorpyrifos methyl			
Poecilia reticulate	LC50 96 h – 4.89 μM	-	Selvi et al., 2005
Diazinon			
Micropterus salmoides	295 μM 24h - 48.2%	Brain	Pan and Dutta, 1998
Cyprinus carpio	LC_{50} 96h for larvae –	Embryos and	Aydin and

Species	Inhibition report	Source	Reference
	5.03 μM and for embryos – 3.25 μM	larvae	Köprücü, 2005
Oreochromis niloticus	67% inhibition at 0.33 μM on the first day	Muscle	Durmaz et al., 2006
Oreochromis niloticus	3.3 μM - 62,5% inhibition after 24h	Brain	Üner etal., 2006
Cyprinus carpio	55.51% inhibition at 0.00012 μM after 5 days	Muscle, gill and kidney	Oruç and Usta, 2007
Dichlorvos			
Dicentrarchus labrax	LC_{50} 96h – 15.83 μM	Fingerling	Varò et al., 2003
Sparus aurata	0.23 μM 24h - 40.95% inhibition	Fingerling brain + dorsal muscle	Varò et al., 2007
Malathion			
Oreochromis niloticus	LC_{50} 96h – 6.66 μM	Brain	Pathiratne and George, 1998
Monocrotophos			
Oreochromis mossambicus	LC_{50} 96h – 51.5 μM This concentration caused 79 (brain), 89 (gill) and 43.8% (muscle) inhibition, in 24h exposure	Brain, gill and muscle	Rao, 2004
Oreochromis mossambicus	1/10 LC_{50} 96h caused 21 (liver), 40 (brain) and 28.6% (gill) inhibition in 24h exposure	Brain, liver and gill	Rao., 2006a
Parathion			
Danio rerio	0.0007 μM after 142 days inhibited 27.4%	Whole fish	Roex et al., 2003
RPR-II			
Oreochromis mossambicus	LC_{50} 96h – 0.75 μM This concentration caused 58 (brain), 90.2 (gill) and 68.5% (muscle) inhibition, in 24h exposure	Brain, gill and muscle	Rao., 2004
Oreochromis mossambicus	1/10 LC_{50} 96h caused approx. 33 (brain), 57 (gill) and 43% (muscle) inhibition, in 72h exposure	Brain, gill and muscle	Rao., 2006c
RPR-V			
Oreochromis mossambicus	LC_{50} 96h – 0.78 μM	Brain, gill and	Rao., 2004

Species	Inhibition report	Source	Reference
	This concentration caused 70.6 (brain), 86.3 (gill) and 54.8% (muscle) inhibition, in 24h exposure	muscle	
Oreochromis mossambicus	1/10 LC_{50} 96h caused approx. 30 (brain), 50 (gill) and 36% (muscle) inhibition, in 72h exposure	Brain, gill and muscle	Rao., 2006c
Temephos			
Oreochromis niloticus	ne	Head	Antwi, 1987
Sarotherodon galilaea	ne	Head	Antwi, 1987
Alestes nurse (*Brycinus nurse*)	ne	Head	Antwi, 1987
Schilbe mystus	ne	Head	Antwi, 1987
Trichlofon			
Cyprinus carpio	0.97 µM 24h - 52% inhibition	Brain	Chandrasekara and Pathiratne, 2005
Oreochromis niloticus	0.97 µM 8h - 73,6% inhibition	Axial muscle	Guimarães et al., 2007
CARBAMATES			
Aldicarb			
Danio rerio	LC_{50} 96h – 52.9 µM	-	Gallo et al., 1995
Poecilia reticulata	LC_{50} 96h – 3.5 µM	-	Gallo et al., 1995
Carbaryl			
Oncorhynchus mykiss	1.24 µM 96h inhibited 60.8%	Brain	Zinckl et al., 1987
Danio rerio	LC_{50} 96h – 46 µM	-	Gallo et al., 1995
Poecilia reticulata	LC_{50} 96h – 12.5 µM	-	Gallo et al., 1995
Oncorhynchus mykiss	3.72 µM 96h inhibited 50%	Larvae	Beauvais et al., 2001
Oncorhynchus mykiss	EC_{50} 96h – 0.095 µM	Brain and muscle	Ferrari et al., 2007
Carbofuran			
Oreochromis niloticus	LC_{50} 24h – 1.13 µM 96h – 2.17 µM	-	Stephenson et al., 1984
Carassius auratus	0.22 µM 48h inhibited 28% (brain) and 2.26 µM 48h inhibited 92% (muscle)	Brain and muscle	Bretaud et al., 1999
Gambusia yucatana	1.13 µM 24h	Muscle and	Rendón-von Osten

Species	Inhibition report	Source	Reference
	inhibited 50 and 30% (muscle and head, respectively)	head	et al., 2005
Tinca tinca	60% inhibition after 20 days of exposure of *Tinca tinca* to carbofuran at 0.1 µg/mL	Brain	Hernández-Moreno et al., 2010

ne – negligible effect.

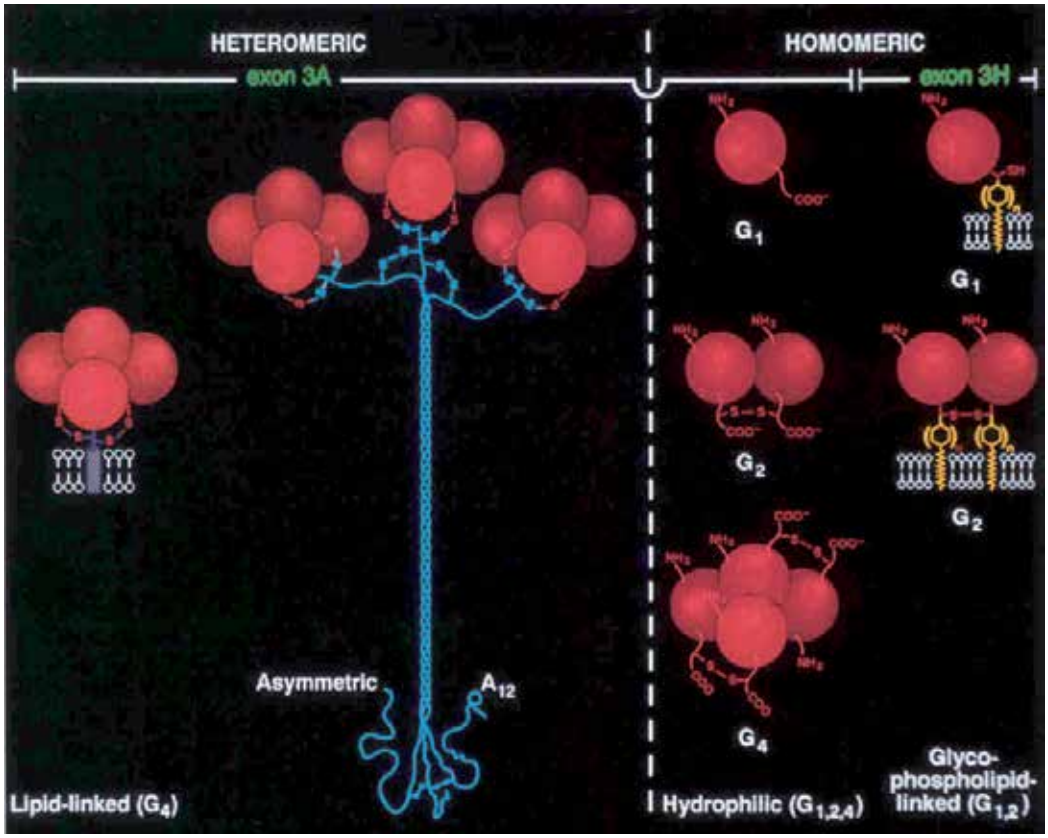
Table 8. Pesticide inhibition for *in vivo* AChE from freshwater and marine fish.

In 1960, Blaber and Creasey used ethopropazine in crude extract to prevent BChE activity when measuring AChE recovery *in vivo* (control with ethopropazine inhibited AChE by 13.7%, while BChE was inhibited by 91.5%).

Ions can alter cholinesterase activity inhibiting or activating so that some authors even propose the enzymes as biomarkers of heavy metals and other pollutants (Abou-Donia and Menzel, 1967; Mukherjee and Bhattacharya, 1974; Olson and Christensen, 1980; Tomlinson et al., 1981; Hughes and Bennett, 1985; Gill et al., 1990; 1991; Payne et al., 1996; Devi et al., 1996; Najimi, 1997; Reddy et al., 2003). This fact is not always taken into account during the use of cholinesterases as biomarkers of pesticides and can lead to false positives or negatives and misinterpretation of results. Tomlinson et al. (1981) described that activation by ions is only observed in conditions of low ionic strength, while inhibition can be noted in both low and high ionic strength.

Thus, heavy metals and ions can be present in samples of environmental matrices, as well as in food samples. Also, they are important interfering components in pesticide analysis using cholinesterases, since some of them are inhibitors or positive effectors. Nevertheless, the use of non-inhibitor chelating agents and ions with protecting enzyme activity effect could overcome these interferences.

Bocquené, Galgani and Truquet (1990) found that Tris buffer was the best extractor for fish AChE. Najimi and coworkers (1997) reported that using Tris the increasing doses of heavy metals resulted in different AChE activities though such result was not observed with phosphate buffer. It could be concluded that phosphate is the best buffer for pesticide assays and that Tris is the best alternative for heavy metals assays. However, Tomlinson et al. (1981) reported that EDTA has a protective action against divalent metallic cations which can cause some interference. Chatonnet and Lockridge (1989) reviewed cholinesterases and reported the different extracting strategies caused by ChEs molecular polymorphism: the globular forms G1, G2 and G4 are extractable in low ionic strength buffers (G2 glycophospholipid-linked is the form found in erythrocytes and in *Torpedo* electric organ, while G4 lipid-linked is present in vertebrates brain). The globular forms tightly bound to membranes require detergent for solubilization. Asymmetric forms (found mainly in vertebrate muscle and in some electric organs) are solubilized with buffers with high salt concentration. These forms contain tetrameric subunits (A4, A8 and A12) attached by disulphide bonds to a collagen-like tail (Figure 1).



Source: Taylor (1991)

Fig. 1. Molecular polymorphism of cholinesterases

Working with brain AChE, Ho and Ellman (1969) were able to solubilize the enzyme using triton X-100 and treatment with proteases. Nevertheless, in cholinesterase assays with pesticides, triton X-100 interacts with OP (oxon-form) and CB compounds or influences its rate of AChE inhibition (Marcel et al., 2000; Rosenfeld, Kousba and Sultatos, 2001).

For pesticides with larger acyl chains or higher lipophilic characteristic (for which only a small fraction reaches the target tissues), BChE can be more sensitive than AChE. The use of BChE offers some advantages, such as the facilitated plasma (its main source) separation from the other blood components and the possibility to collect samples without killing specimens. Furthermore, several studies have tried, with some success, to establish sharp correlations between inhibition in blood cholinesterases and in peripheral and central nerve tissues cholinesterases (Pope et al., 1991; Pope and Chakraborti, 1992; Chauldhuri et al., 1993; Padilla et al., 1994). Padilla (1995) working with paraoxon and chlorpyrifos, described that the strongest correlations occurred when measuring cholinesterase activity in steady-state inhibition, which is the peak inhibition time. This time depends on the inhibitor under analysis (4 hours post-dosing for paraoxon and 1-3 weeks post-dosing for chlorpyrifos).

Another concern about using fish cholinesterase as biomarker of organophosphorus and carbamate pesticides is that cyanobacterial blooms are very common in rivers, lakes and reservoirs when eutrophication raises nutrient contents in water. Some species of

cyanobacteria (*Anabaena flos-aquae* and *Anabaena lemmermannii*) produce anticholinesterasic metabolites such as anatoxin-a(s), which can be considered natural OP compounds and whose toxicity can be approximately 1000-fold higher than that of the insecticide paraoxon (Mahmood and Carmichael, 1986; Villatte et al., 2002). Moreover, cholinesterases inhibited by anatoxin-a(s) cannot be reactivated by oximes, because they are true irreversible inhibitors of these enzymes. The structure of anatoxin-a(s) resembles the shape of the organophosphorus dealkylated within the active site of the enzyme forming almost instantly an aged complex. A study obtained aged cholinesterase after 20-min incubation with this toxin (Villatte et al., 2002). However, by washing the brains before (with a solvent that does not transport it into the cells and does not affect enzymatic activity), such toxins do not interfere on *in vivo* assays using cholinesterase from this tissue, since it was observed that anatoxin-a(s) does not cross the blood-brain barrier (Cook et al., 1988; Rodríguez et al., 2006).

When comparing the use of crude extract to the use of purified enzyme, advantages and disadvantages can be observed in both, depending on the purpose. First of all, purified enzymes allow determining activity and inhibition more acutely without endogenous interfering agents. Moreover, they can be immobilized on a range of materials in particles or electrodes in order to produce electrochemical devices. Nevertheless purified enzymes require a medium to mimetize *in vivo* conditions and stabilize its activity. Besides, they are more susceptible to exogenous ions and non target compounds. The crude extract has the disadvantage of exposing the enzyme not only to the analyte. However, as mentioned before, much of OP pesticides are produced in the thion form (P=S), requiring bioactivation to reach their full toxic potential. Before biotransformation, the thion group exhibits little power of inhibition (WHO/IPCS/INCHEM, 1986a) which could hinder the correlation between pesticide concentration and ChE inhibition. Considering this, many studies use brain homogenates, since they also provide enzymatic complexes such as CYP P450 capable to transform the pesticide to its oxo-form (Mesnil, Testa and Jenner, 1984; Iscan et al., 1990; Ghersi-Egea et al., 1993).

According to Zahavi et al. (1971) and Carr and Chambers (1996), the reasons behind the species' differences in inhibitory potency has been reported to be the result of steric exclusion of the inhibitor from the active site of the enzyme. However, the difference in sensitivity between species occurs not only due to the structural diversity of inhibitors and between species cholinesterases, but also due to the balance between the activities of the detoxication complex and enzymes that promote the biotransformation of OPs. This balance can be part of enantiostatic responses to external agents which act as a device protecting against intoxication (Cunha Bastos et al., 1999; Monserrat et al., 2007).

Several attempts have been reported worldwide, in search for the best enzyme and fish source to establish methods to detect diverse organophosphorus and carbamate pesticides. In this sense, it is possible to improve monitoring protocols, obtaining data about the activation/detoxification complex of each species in use.

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4. References

Abou-Donia, M.S.; Menzel, D.B. 1967. Fish brain cholinesterase: its inhibition by carbamates and automatic assay. Comp. Biochem. Physiol. 21: 99-108.

Adams, D.H.; Thompson, R.H.S. 1948. The Selective Inhibition of Cholinesterases. Biochem. J. 42, 170.

Aker, W.G.; Hu, X.; Wang, P.; Hwang, H.-M. 2008. Comparing the Relative Toxicity of Malathion and Malaoxon in Blue Catfish *Ictalurus furcatus*. Environ Toxicol 23: 548–554.

Aldridge, W.N. 1953. The Differentiation of True and Pseudo Cholinesterase by Organophosphorus Compounds. Biochem. J. 53: 62-67.

Aldridge, W.N.; Reiner, E. 1969. Acetylcholinesterase – Two types of inhibition by an organophosphorus compound: one the formation of phosphorylated enzyme and the other analogous to inhibition by substrate. Biochem. J. 115: 147-162.

Antwi, L. A. K. 1987. Fish head acetylcholinesterase activity after aerial application of temephos in two rivers in Burkina Faso, west Africa. Bulletin of Environmental Contamination and Toxicology. 38:461-466.

Arias, A.R.L.; Buss, D.F.; Albuquerque, C.; Inácio, A.F.; Freire, M.M.; Egler, M.; Mugnai, R.; Baptista, D.F. Utilização de bioindicadores na avaliação de impacto e no monitoramento da contaminação de rios e córregos por agrotóxicos. Ciência e Saúde Coletiva, 12, n. 1, p. 61-72, 2007.

Arufe, M.I.; Arellano, J.M.; García, L.; Albendín, G.; Sarasquete, C. 2007. Cholinesterase activity in gilthead seabream (*Sparus aurata*) larvae: Characterization and sensitivity to the organophosphate azinphosmethyl. Aquat Toxicol 84: 328–336.

Assis, C.R.D.; Castro, P.F.; Amaral, I.P.G.; Maciel Carvalho, E.V.M.; Carvalho Jr, L.B., Bezerra, R.S. 2010. Characterization of acetylcholinesterase from the brain of the Amazonian tambaqui (*Colossoma macropomum*) and in vitro effect of organophosphorus and carbamate pesticides. Environ Toxicol Chem 29: 2243–2248.

Augustinsson, K.B. Cholinesterase and anticholinesterase agents. In: Handbook of experimental pharmacology. Koelle, G.B. (org). Springer-Verlag, Berlin and New York, p. 89-128, 1963.

Austin, L.; Berry, W.K. Two selective inhibitors of cholinesterase. Biochem J. 1953 July; 54(4): 695–700.

Aydın, R.; Köprücü, K. 2005. Acute toxicity of diazinon on the common carp (*Cyprinus carpio* L.) embryos and larvae. Pesticide Biochemistry and Physiology 82: 220–225.

Bayliss, B. J. & Todrick, A. (1956). The Use of a Selective Acetylcholinesterase Inhibitor in the Estimation of Pseudocholinesterase Activity in Rat Brain. Biochem. J. 62, 62-67.

Beauvais, S. L.; Cole, K. J.; Atchison, G. J.; Coffey, M. Factors affecting brain cholinesterase activity in Bluegill (*Lepomis macrochirus*). Water, Air, and Soil Pollution, v. 135, p. 249–264, 2002.

Beauvais, S. L.; Jones, S. B.; Parris, J. T.; Brewer, S. K.; Little, E. E. 2001. Cholinergic and Behavioral Neurotoxicity of Carbaryl and Cadmium to Larval Rainbow Trout (*Oncorhynchus mykiss*) Ecotoxicology and Environmental Safety 49: 84-90.

Blaber, L.C.; Creasey, N.H. 1960. The Mode of Recovery of Cholinesterase Activity in vivo after Organophosphorus Poisoning - 2. Brain cholinesterase. Biochem. J. 77: 597-604.

Bocquené G, Galgani F, Truquet P. 1990. Characterization and assay conditions for use of AChE activity from several marine species in pollution monitoring. Mar Environ Res 30: 75-89.

Boone, J.S.; Chambers, J.E. 1997. Biochemical factors contributing to toxicity differences among chlorpyrifos, parathion, and methyl parathion in mosquitofish (*Gambusia afinis*). Aquatic Toxicology, 39: 333-343.

Bretaud, S.; Toutant, J.-P.; Saglio, P. 1999. Effects of carbofuran, diuron, and nicosulfuron on acetylcholinesterase activity in goldfish (Carassius auratus). Ecotoxicology and Environmental Safety 47: 117-124

Cairns, J. Jr., Pratt, J.R. A history of biological monitoring using benthic macroinvertebrates. In: Rosenberg, D.M, Resh, V.H. Freshwater biomonitoring and benthic macroinvertebrates. New York: Chapman & Hall; 1993. p. 10-27.

Carr, R.L.; Chambers, J.E. 1996. Kinetic Analysis of the in Vitro Inhibition, Aging, and Reactivation of Brain Acetylcholinesterase from Rat and Channel Catfish by Paraoxon and Chlorpyrifos-oxon. Toxicol appl Pharmacol, 139: 365–373.

Chandrasekara, H. U.; Pathiratne, A. 2005. Influence of low concentrations of Trichlorfon on haematological parameters and brain acetylcholinesterase activity in common carp, *Cyprinus carpio* L. Aquaculture Research, 36, 144-149.

Chandrasekara, L.W.H.U.; Pathiratne, A. 2007. Body size-related differences in the inhibition of brain acetylcholinesterase activity in juvenile Nile tilapia (*Oreochromis niloticus*) by chlorpyrifos and carbosulfan. Ecotoxicology and Environmental Safety 67: 109–119.

Chatonnet, A.; Lockridge, O. 1989. Comparison of butyrylcholinesterase and acetylcholinesterase. Biochem. J. 260: 625-634.

Chauldhuri, J., Harp, P., Chakraborti, T. and Pope, C. (1993) Comparative dose-related effects of parathion and chlorpyrifos on cholinergic markers and behavior. Presented at the 1993 HERL Symposium on Biological Mechanisms and Quantitative Risk Assessment.

Cheng, Y.; Prusoff, W.H. 1973. Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol*. 22: 3099–3108.

Chuiko, G.M. 2000. Comparative study of acetylcholinesterase and butyrylcholinesterase in brain and serum of several freshwater fish: specific activities and in vitro inhibition by DDVP, an organophosphorus pesticide. Comparative Biochemistry and Physiology Part C, 127: 233–242.

Çokugras, A. N. Butyrylcholinesterase: Structure and Physiological Importance. Turkish Journal of Biochemistry. 2003; 28 (2); 54-61.

Cook, W.O.; Beasley, V.R.; Dahlem, A.M.; Dellinger, J.A.; Harlin, K.S.; Carmichael, W.W. 1988. Comparison of effects of anatoxin-a(s) and paraoxon, physostigmine and pyridostigmine on mouse brain cholinesterase activity. Toxicon. 26 (8): 750-753.

Cunha Bastos, V. L. F.; Silva Filho, M. V.; Rossini, A.; Cunha Bastos, J. The Activation of Parathion by Brain and Liver of a Brazilian Suckermouth Benthic Fish Shows Comparable in Vitro Kinetics. Pesticide Biochemistry and Physiology 64, 149–156 (1999).

Dembélé, K.; Haubruge, E.; Gaspar, C. 2000. Concentration Effects of Selected Insecticides on Brain Acetylcholinesterase in the Common Carp (*Cyprinus carpio* L.). Ecotoxicology and Environmental Safety 45: 49-54.

Devi, M., Thomas, D.A., Barber, J.T., Fingerman, M., 1996. Accumulation and Physiological and Biochemical Effects of Cadmium in a Simple Aquatic Food Chain. Ecotox. Environ. Safe. 33, 38–43.

Durmaz, H.; Sevgiler, Y.; Üner, N. 2006. Tissue-specific antioxidative and neurotoxic responses to diazinon in *Oreochromis niloticus*. Pesticide Biochemistry and Physiology, 84: 215–226

Eddleston, M.; Buckley, N. A.; Eyer, P.; Dawson, A. H. Management of acute organophosphorus pesticide poisoning. Lancet. 2008 February 16; 371(9612): 597–607.

Eddleston, M; Szinicz, L.; Eyer, P.; Buckley, N. Oximes in acute organophosphorus pesticide poisoning: a systematic review of clinical trials. Q J Med 2002;95:275-83.

Ellman, G.L.; Courtney, K.D.; Andres, V.; Featherstone, R.M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, 7: 88-95.

Fairbrother, A.; Bennett, J.K. 1988. The usefulness of cholinesterase measurement, J. Wildl. Dis. 24: 587-590.

FAO. 2007. Pesticides in food report 2007. FAO plant production and protection paper 191. Food and Agriculture Organization, Rome, IT.

FAO. 2010. The State of World Fisheries and Aquaculture 2010. Rome, IT. 197p.

Ferrari, A., Venturino, A., Pechen de D'Angelo, A.M., 2004. Time course of brain cholinesterase inhibition and recovery following acute and subacute azinphosmethyl, parathion and carbaryl exposure in the goldfish (*Carassius auratus*). Ecotoxicol. Environ. Saf. 57, 420–425.

Ferrari, A.; Venturino, A.; D'Angelo, A. M. P. 2007. Muscular and brain cholinesterase sensitivities to azinphos methyl and carbaryl in the juvenile rainbow trout Oncorhynchus mykiss. Comparative Biochemistry and Physiology, Part C 146: 308–313.

Flint, M.L. and R. van Der Bosch, 1981. Introduction to Integrated Pest Management. Plenum Press, New York, pp: 237.

Fulton, M. H.; Key, P. B. 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environ Toxicol Chem 20: 37-45.

Gallo, D.; Merendino, A.; Keizer, J.; Vittozzi, L. 1995. Acute toxicity of two carbamates to the Guppy (*Poecilia reticulata*) and the Zebrafish (*Brachydanio rerio*). The Science of the Total Environment, 171: 131-136.

Ghersi-Egea, J. F.; Perrin, R.; Leininger-Muller, B.; Grassiot, M. C.; Jeandel, C.; Floquet, J.; Cuny, G.; Siest, G.; Minn, A. Subcellular localization of cytochrome P450, and activities of several enzymes responsible for drug metabolism in the human brain, Biochem. Pharmacol. 45, 647 (1993).

Gill, T.S., Tewari, H., Pande, J., 1990. Use of the fish enzyme system in monitoring water quality: effects of mercury on tissue enzymes. Comp. Biochem. Physiol. 97C, 287-292.

Gill, T.S., Tewari, H., Pande, J., 1991. In vivo and in vitro effects of cadmium on selected enzymes in different organs of the fish *Barbus conchonius* Ham. (Rosy barb). Comp. Biochem. Physiol. 100C, 501-505.

Guimarães, A. T. B.; Silva de Assis, H. C.; Boeger, W. 2007. The effect of trichlorfon on acetylcholinesterase activity and histopathology of cultivated fish *Oreochromis niloticus*. Ecotoxicology and Environmental Safety 68: 57–62.

Harel, M.; Sussman, J.L.; Krejci, E.; Bon, S.; Chanal, P.; Massoulié, J.; Silman, I. 1992. Conversion of acetylcholinesterase to butyrylcholinesterase: Modeling and mutagenesis. Proc. Natl. Acad. Sci. USA. 89: 10827-10831.

Hart KA, Pimentel D. Environmental and economic costs of pesticide use. In: Encyclopedia of Pest Management. Pimentel D. ed.; Marcel Dekker: New York, NY, 2002: 237-239.

Henríquez Pérez, A.; Sánchez-Hernández, J. C. 2003. La amenaza de los plaguicidas sobre la fauna silvestre de las islas canarias. El indiferente, 14: 42-47.

Hernández-Moreno,D.; Soler, F.; Míguez, M.P.; Pérez-López, M. 2010. Brain acetylcholinesterase, malondialdehyde and reduced glutathione as biomarkers of continuous exposure of tench, *Tinca tinca*, to carbofuran or deltamethrin. Science of the Total Environment 408: 4976–4983

Ho, I.K.; Ellman, G.L. Triton solubilized acetylcholinesterase of brain. 1969. Journal of Neurochemistry. 16: 1505-1513.

Hughes, R.J., Bennett, J., 1985. Effect of metal ions on the activity of acetylcholinesterase. Biochem. Soc. Trans. 13, 219-220.

Iscan, M.; Reuhl, K.; Weiss, B.; Maines, M. D. Regional and subcellular distribution of cytochrome P450-dependent drug metabolism in monkey brain: The olfactory bulb and mitochondrial fraction have high levels of activity, Biochem. Biophys. Res. Commun. 169, 858 (1990).

Johnson, G.; Moore, S.W. Cholinesterases modulate cell adhesion in human neuroblastoma cells in vitro. Int. J. Dev. Neurosci., v. 18, p. 781–790, 2000.

Jung, J-H; Addison, RF; Shim, WJ. 2007. Characterization of cholinesterases in marbled sole, *Limanda yokohamae*, and their inhibition in vitro by the fungicide iprobenfos. Marine Environmental Research 63: 471–478.

Keizer, J.; D'Agostino, G.; Nagel, R.; Volpe, T.; Gnemid, P.; Vittozzi, L. 1995. Enzymological differences of AChE and diazinon hepatic metabolism: correlation of in vitro data with the selective toxicity of diazinon to fish species. The Science of the Total Environment 171: 213-220.

Kovarik, Z.; Bosak, A.; Sinko, G.; Latas, T. 2003. Exploring the Active Sites of Cholinesterases by Inhibition with Bambuterol and Haloxon. Croatica Chemica Acta. 76: 63-67.

Leuzinger, W.; Baker, A. L. 1967. Acetylcholinesterase, I. Large-scale purification, homogeneity and amino acid analysis. Proc. Nac. Acad. Sci. 57: 446-451.

Lopez-Carillo, L.; Lopez-Cervantes, M. 1993. Effect of exposure to organophosphate pesticides on serum cholinesterase levels. Archives of environmental health. 48: 359-363.

Mahmood, N.A.; Carmichael, W.W. 1986. The pharmacology of anatoxin-a(s), a neurotoxin produced by the freshwater cyanobacterium Anabaena flos-aquae NRC 525-17. Toxicon, 24: 425-434.

Marcel, V.; Estrada-Mondaca, S.; Magné, F.; Stojan, J.; Klaébé, A.; Fournier, D. 2000. Exploration of the Drosophila Acetylcholinesterase Substrate Activation Site Using

a Reversible Inhibitor (Triton X-100) and Mutated Enzymes. J Biol Chem. 275: 11603–11609.

Marco, M.-P.; Barceló, D. Environmental applications of analytical biosensors. Measuring Science Technology, v. 7, p. 1547–1562, 1996.

Mdegela, R.H.; Mosha, R.D.; Sandvik, M.; Skaare, J.U. 2010. Assessment of acetylcholinesterase activity in *Clarias gariepinus* as a biomarker of organophosphate and carbamate exposure. Ecotoxicology 19: 855–863

Mesnil, M.; Testa, B.; Jenner, P. Xenobiotic metabolism by brain monooxigenases and other cerebral enzymes, Adv. Drug Res. 13, 95 (1984).

Monserrat, J. M.; Martínez, P. E.; Geracitano, L. A.; Amado, L L.; Martins, C. M. G.; Pinho, G. L. L.; Chaves, I. S.; Ferreira-Cravo, M.; Ventura-Lima, J.; Bianchini, A. Pollution biomarkers in estuarine animals: Critical review and new perspectives. Comparative Biochemistry and Physiology, Part C 146 (2007) 221–234.

Morgan, M.J., Fancey, L.L., Kiceniuk, J.W., 1990. Response and recovery of brain AChE activity in Atlantic salmon exposed to fenitrothion. Can. J. Fish Aquat. Sci. 47, 1652–1654.

Mukherjee, S., Bhattacharya, S., 1974. Effect of some industrial pollutants on fish brain cholinesterase activity. Environ. Physiol. Biochem. 4, 226-231.

Nachmansohn, D.; Lederer, E. 1939. Sur la biochimie de la cholinesterase. I. Preparation de l'enzyme. Groupements-SH. Bull. Soc. Chim. Biol. 21: 797-808

Najimi, S., Bouhaimi, A., Daubèze, M., Zekhnini, A., Pellerin, J., Narbonne, J.F., Moukrim, A., 1997. Use of acetylcholinesterase in *Perna perna* and *Mytilus galloprovincialis* as a biomarker of pollution in Agadir marine bay (south of Morocco). Bull. Environ. Contam. Toxicol. 58, 901-908.

Nauen R, Bretschneider T. 2002. New modes of action of insecticides. *Pesticide Outlook* 13: 241-245.

Nicolet, Y.; Lockridge, O.; Masson, P.; Fontecilla-Camps, J. C.; Nachon, F. 2003. Crystal Structure of Human Butyrylcholinesterase and of Its Complexes with Substrate and Products. Journal of Biological Chemistry. 278: 41141–41147.

Nimmo, D.R. Pesticides. In: Rand, G.M.; Petrocelli, S.R., eds. Fundamentals of aquatic toxicology: methods and applications, New York: Hemisphere, p. 335-373, 1985.

Oliveira, M. M.; Silva Filho, M. V.; Cunha Bastos, V. L. F.; Fernandes, F. C.; Cunha Bastos, J. Brain acetylcholinesterase as a marine pesticide biomarker using Brazilian fishes. Marine Environmental Research 63 (2007) 303–312.

Olson, D.L., Christensen, G.M., 1980. Effects of water pollutants and other chemicals on fish acetylcholinesterase (in vitro). Environ. Res. 21, 327-335.

Oruç, E. O.; Usta, D. 2007. Evaluation of oxidative stress responses and neurotoxicity potential of diazinon in different tissues of *Cyprinus carpio*. Environmental Toxicology and Pharmacology 23: 48–55.

Padilla, S. Regulatory and research issues related to cholinesterase inhibition. Toxicology 102 (1995) 215 -220.

Padilla, S.; Moser, V.C.; Pope, C.N.; Brimijoin. W.S. (1992) Paraoxon toxicity is not potentiated by prior reduction in blood acetylcholinesterase. Toxicol. Appl. Pharmacol. 117, 110-115.

Pan, G.; Dutta, H. M. 1998. The Inhibition of Brain Acetylcholinesterase Activity of Juvenile Largemouth Bass *Micropterus salmoides* by Sublethal Concentrations of Diazinon. Environmental Research, Section A. 79: 133-137.

Pathiratne, A.; George, S.G. 1998. Toxicity of malathion to nile tilapia, *Oreochromis niloticus* and modulation by other environmental contaminants. Aquatic Toxicology 43: 261–271.

Paudyal, B.P. 2008. Organophosphorus poisoning. J Nepal Med Assoc. 47: 251-258.

Payne J F, Mathieu A, Melvin W, Fancey L L. Acetylcholinesterase, an old biomarker with a new future? field trials in association with two urban rivers and a paper mill in Newfoundland. Mar Pollut Bull, Vol. 32, No. 2, pp. 225-231, 1996.

Pezzementi, L., Chatonnet, A. 2010. Evolution of cholinesterases in the animal kingdom. Chemico-Biological Interactions 187: 27–33.

Pezzementi, L., Sanders, M., Jenkins, T., & Holliman, D. (1991). In J. Massouli e, F. Bacou, E. Barnard, A. Chatonnet, B. Doctor, & D. Quinn (Eds.), Cholinesterases: structure, function, mecanism, genetics and cell biology (pp. 24–31). USA: American Chemical Society.

Pope, C.N., Chakraborti, T.K., Chapman, M.L.. Farrar J.D. and Arthun, D. 1991. Comparison of in vivo cholinesterase inhibition in neonatal and adult rats by three organophosphorothioate insecticides. Toxicology 68: 51-61.

Pope, C.N.; Chakraborti, T.K. 1992. Dose-related inhibition of brain and plasma cholinesterase in neonatal and adult rats following sublethal organophosphate exposures. Toxicology 73: 35-43.

Quinn, D.M. Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states. Chemical Reviews, 87, p. 955-979, 1987.

Rao, J.V. 2004. Effects of monocrotophos and its analogs in acetylcholinesterase activity's inhibition and its pattern of recovery on euryhaline fish, *Oreochromis mossambicus*. Ecotoxicology and Environmental Safety 59: 217–222.

Rao, J.V. 2006a. Biochemical alterations in euryhaline fish, *Oreochromis mossambicus* exposed to sub-lethal concentrations of an organophosphorus insecticide, monocrotophos. Chemosphere 65: 1814–1820.

Rao, J.V. 2006b. Sublethal effects of an organophosphorus insecticide (RPR-II) on biochemical parameters of tilapia, *Oreochromis mossambicus*. Comparative Biochemistry and Physiology, Part C 143: 492–498.

Rao, J.V. 2006c. Toxic effects of novel organophosphorus insecticide (RPR-V) on certain biochemical parameters of euryhaline fish, *Oreochromis mossambicus*. Pesticide Biochemistry and Physiology 86: 78–84.

Rao, J.V.; Rani, C. H. S.; Kavitha, P.; Rao, R. N.; Madhavendra, S. S. 2003. Toxic effects of Chlorpyrifos to the fish *Oreochromis mossambicus*. Bulletin of Environmental Contamination and Toxicology, 70:985–992.

Rao, JV; Kavitha, P. 2010. In vitro effects of chlorpyrifos on the acetylcholinesterase activity of euryhaline fish, *Oreochromis mossambicus*. Z Naturforsch C. 65: 303-306

Reddy, G.R., Basha, M.R., Devi, C.B., Suresh, A., Baker, J.L., Shafeek, A., Heinz, J., Chetty, C.S., 2003. Lead induced effects on acetylcholinesterase activity in cerebellum and hippocampus of developing rat. Int. J. Devl. Neuroscience. 21, 347–352.

Rendón-von Osten, J.; Ortíz-Arana, A.; Guilhermino, L.; Soares, A. M. V. M. 2005. In vivo evaluation of three biomarkers in the mosquitofish (*Gambusia yucatana*) exposed to pesticides. Chemosphere, 58: 627–636.

Rodríguez, V.; Moura, S.; Pinto, E.; Pereira, C.M.P.; Braga, R.C. 2006. Aspectos toxicológicos e químicos da anatoxina-a e seus análogos. Quim. Nova, Vol. 29, No. 6, 1365-1371.

Rodríguez-Fuentes G, Armstrong J, Schlenk D. 2008. Characterization of muscle cholinesterases from two demersal flatfish collected near a municipal wastewater outfall in Southern California. Ecotoxicol Environ Saf 69: 466–471.

Rodríguez-Fuentes G, Gold-Bouchot G. 2004. Characterization of cholinesterase activity from different tissues of Nile tilapia (Oreochromis niloticus). Mar Environ Res 58: 505-509.

Rodríguez-Fuentes, G.; Gold-Bouchot, G. 2000. Environmental monitoring using acetylcholinesterase inhibition in vitro. A case study in two Mexican lagoons. Mar Environ Res 50: 357-360.

Roex, E.W.M.; Keijzers, R.; van Gestel, C.A.M. 2003. Acetylcholinesterase inhibition and increased food consumption rate in the zebrafish, *Danio rerio*, after chronic exposure to parathion. Aquatic Toxicology 64: 451-/460

Rosenberry, T.L. 1975. Acetylcholinesterase. Adv Enzymol Relat Areas Mol Biol. 43: 103-218.

Rosenfeld, C.; Kousba, A.; Sultatos, L.G. 2001. Interactions of rat brain acetylcholinesterase with the detergent Triton X-100 and the organophosphate paraoxon. Toxicol Sci 63: 208–213.

Ru, S.; Wei, X.; Jiang, M.; Li, Y. 2003. In vivo and in vitro inhibitions of red drum (Sciaenops ocellatus) brain acetylcholinesterase and liver carboxylesterase by monocrotophos at sublethal concentrations. Water, Air, and Soil Pollution 149: 17–25.

Salles, J.B.; Cunha Bastos, V.L.F.; Silva Filho, M.V.; Machado, O.L.T.; Salles, C.M.C.; Giovanni de Simone, S.; Cunha Bastos, J. 2006. A novel butyrylcholinesterase from serum of Leporinus macrocephalus, a Neotropical fish. Biochimie. 88: 59–68.

Sato, R.; Mitani, K.; Matsumoto, T.; Takahashi, S.; Yamada, R-h; Kera, Y. 2007. Effects of Insecticides In Vitro on Acetylcholinesterase Purified from Body Muscle of Koi Carp (*Cyprinus carpio*). Jpn. J. Environ. Toxicol. 10: 31-38.

Selvi, M.; Sarıkaya, R.; Erkoç, F.; Koçak, O. 2005. Investigation of acute toxicity of chlorpyrifos-methyl on guppy *Poecilia reticulata*. Chemosphere 60: 93–96.

Shaonan, L.; Xianchuan, X.; Guonian, Z.; Yajun, T. 2004. Kinetic characters and resistance to inhibition of crude and purified brain acetylcholinesterase of three freshwater fishes by organophosphates. Aquatic Toxicology 68: 293–299.

Silman, I.; Sussman, J. L. Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. Current Opinion in Pharmacology, 5, p. 293–302, 2005.

Soreq H, Zakut H. 1990. Cholinesterase genes: multileveled regulation. In Monographs in Human Genetics, ed. RS Sparkes, 13:1-102. Basel: Karger.

Sturm, A.; Wogram, J.; Hansen, P.D.; Liess, M. 1999. Potential use of cholinesterase in monitoring low levels of organophosphates in small streams: natural variability in threespined stickleback (Gasterosteus aculeatus) and relation to pollution. Environ. Toxicol. Chem. 18: 194–200.

Taylor, P. 1991. The cholinesterases. J. Biol. Chem. 266: 4025-4028.

Tham, L.G.; Perumal, N.; Syed, M.A.; Shamaan, N.A.; Shukor, M.Y. 2009. Assessment of Clarias batrachus as a source of acetylcholinesterase (AChE) for the detection of insecticides. J Environ Biol. 30: 135-138.

Tomlinson, G., Mutus, B., McLennan, I., 1981. Activation and inactivation of acetylcholinesterase by metal ions. Can. J. Biochem. 59, 728-735.

Tõugu V. 2001. Acetylcholinesterase: mechanism of catalysis and inhibition. Curr Med Chem - Cent Nerv Syst Agents 1: 155-170.

Üner, N.; Oruc, E. O.; Sevgiler, Y.; Şahin, N.; Durmaz, H.; Usta, D. 2006. Effects of diazinon on acetylcholinesterase activity and lipid peroxidation in the brain of *Oreochromis niloticus*). Environmental Toxicology and Pharmacology 21: 241–245.

Vale, J. A. 1998. Toxicokinetic and toxicodynamic aspects of organophosphorus (OP) insecticide poisoning. Toxicol. Let. 102-103: 649-652.

Varò, I.; Navarro, J.C.; Amat, F.; Guilhermino, L. 2003. Effect of dichlorvos on cholinesterase activity of the European sea bass (*Dicentrarchus labrax*). Pestic Biochem Phys 75: 61–72.

Varò, I.; Navarro, J.C.; Nunes, B.; Guilhermino, L. 2007. Effects of dichlorvos aquaculture treatments on selected biomarkers of gilthead sea bream (*Sparus aurata* L.) fingerlings. *Aquaculture* 266: 87–96.

Vassilieff, I.; Ecobichon, D.J. 1982. Stability of Matacil® in aqueous media as measured by changes in anticholinesterase potency. Bull. environ. Contam. Toxicol., 29: 366-370.

Villatte, F.; Schulze, H.; Schmid, R.D.; Bachmann, T.T. 2002. A disposable acetylcholinesterase-based electrode biosensor to detect anatoxin-a(s) in water. Anal Bioanal Chem., 372 :322–326.

Watson, W. P.; Mutti, A. Role of biomarkers in monitoring exposures to chemicals: present position, future prospects. Biomarkers, v. 9, n. 3, p. 211-242, 2004.

Whittaker, M. Cholinesterase. Karger, Basel, New York., p. 1-126, 1986.

Whittaker, M. Plasma cholinesterase variants and the anaesthetist. Anaesthesia, v. 35, p. 174-97, 1980.

WHO/IPCS/INCHEM. 1986a. Organophosphorus insecticides: a general introduction. Environmental Health Criteria 63. Geneva, CH.

WHO/IPCS/INCHEM. 1986b. Carbamate pesticides: a general introduction. Environmental Health Criteria 64. Geneva, CH.

Wijesuriya, D.C.; Rechnitz, G.A. Biosensors based on plant and animal tissues. Biosensors and Bioelectronics. V. 8, n. 3-4, p. 155-160, 1993.

Wijeyaratne, W.M.D.N., Pathiratne, A., 2006. Acetylcholinesterase inhibition and gill lesions in Rasbora caverii, an indigenous fish inhabiting rice field associated waterbodies in Sri Lanka. Ecotoxicology 15, 609–619.

Wilson, I.B. Acetylcholinesterase. XI. Reversibility of tetraethyl pyrophosphate inhibition. J Biol Chem. 1951, 190(1):111–117.

Wogram, J.; Sturm, A.; Segner, H.; Liess, M. 2001. Effects of parathion on acetylcholinesterase, butyrylcholinesterase, and carboxylesterase in three-spined stickleback (*Gasterosteus aculeatus*) following short-term exposure. Environ Toxicol Chem. 20: 1528-1531.

Worek, F.; Backer, M.; Thierman, H. 1997. Reappraisal of indications and limitations of oxime therapy in organophosphate poisoning. Hum Exp Toxicol. 16: 466-472.

Worek, F.; Diepold, C.; Eyer, P. 1999. Dimethylphosphoryl-inhibited human cholinesterases: inhibition, reactivation and ageing kinetics. Arch Toxicol. 73: 7-14.

Zahavi, M., Tahori, A. S., and Klimer, F. (1971). Insensitivity of acetylcho linesterases to organophosphorus compounds as related to size of esteratic site. Mol. Pharmacol, 7: 611–619.

Zinkl, J.G.; Shea, P.J.; Nakamoto, R.J.; Callman, J. 1987. Brain cholinesterase activity of rainbow trout poisoned by carbaryl. Bulletim of Environmental Contamination and Toxicology. 38: 29-35.

14

The Effects of Pesticides on *Dictyostelium* Cholinesterase, from Basic to Applied Research

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1. Introduction

Numerous organisms have been proposed as biotests in standardised laboratory procedures to evaluate contamination caused by pesticides. In this context, protozoa are regarded as a valuable assay to be exploited in laboratory investigations because of their fundamental features. Due to their nature as eukaryotic cells/organisms, protozoa exhibit a relatively simple organisation and a high degree of specialisation. As eukaryotic organisms, protozoa behave like animals, responding directly to environmental stimuli, but, as single-cells, they are more sensitive to environmental changes than the cells of higher organisms. Like micro-organisms generally, protozoa multiply through short cell-cycles and this makes it possible to detect the effects of pesticides on large and genetically homogeneous cell populations and on their progeny as well. These peculiarities of protozoa are more interesting if we consider how the test on protozoa can assuage public opinion, more and more sensible to bioethical matters and meet the requests of both the ICCVAM and the ECVAM for compliance with the 3Rs strategy. Furthermore, it is important to highlight that the identification of molecules functionally related to neurotrasmission in protozoa (such as the GABAergic system in *Paramecium* and *Dictyostelium*, the nitrergic system in *Paramecium* and the cholinergic system in *Paramecium*, *Dictyostelium* and *Euplotes*, through which they can react to environmental stress, like the molecules of macroinvertebrate and vertebrate models) provides a new method for using protozoa in neurotoxicity tests and as ecological indicators in biomonitoring. This paper will focus on the inhibition of *Dictyostelium* ChE activity as a biomarker of exposure to neurotoxic pesticides and how this biomarker can be used in the field for the pre-chemical screening of estuarial zones. The area investigated was the western coast of Liguria, an area stretching from Genoa to the French border, whose economy is based on greenhouse market gardening, olive oil production and summer tourism. In this area, it is important to convince people of the need for continuous biomonitoring to maintain the balance between intensive farming and tourism on the one hand and environmental and public health on the other for sustainable development.

2. Protozoa, a micro-organism model for modern environmental biomonitoring

To assuage ever-increasing public concern over bioethical matters such as laboratory-animal suffering, the scientific community has tried to find alternative research models

(Schechtman, 2002). This has been a centuries-long process, beginning in Great Britain in the early nineteenth century with the first bioethical law, "The Cruelty to Animals Act", as reported by Orlans (1993), which had the objective of controlling animal experimentation (Flecknell, 2002). However, it was only with the publication of the article of Russel & Burch (1959), entitled "The Principles of Humane Experimental Technique", that it was clearly stated that in experimentation every effort must be made to ensure that animals do not suffer.

In this article the authors proposed the use of the 3Rs strategy:

Replace = the use of animals must be replaced with alternative techniques or avoided altogether;

Reduce = the number of animals involved must be reduced to an absolute minimum;

Refine = the experimental parameters must be refined to make sure animals suffer as little as possible (Russell & Burch, 1959).

Unfortunately, this ethical method has received scarce attention from the scientific community in the past and only recently has it obtained the interest of the Interagency Coordinating Commitee on the Validation of Alternative Methods (ICCVAM) and the European Centre for the Validation of Alternative Methods (ECVAM), due to proposals for new laws to regulate animal experimentation (Balls et al., 1995; Stokes & Hill, 2000; Schechtman, 2002). The need to regulate animal experimentation is made more urgent if you consider the data reported by the EU system for the Registration, Evaluation and Authorisation of Chemicals (REACH), which lists about 3,000 new chemical substances, including many pesticides, that will have to be analysed over the next twelve years before being released on the market (Louekari et al., 2006), and which, according to the European Commission Joint Research Centre, will require between 2.1 and 3.9 million animals to complete all the necessary tests. Given the numbers involved, it is not surprising that interest in alternative experimental methods and the 3Rs has increased, even in the industrial sector, which must reduce the costs of experimentation by between 1,143 and 2,274 million euro to satisfy the criteria dictated by REACH (Grindon & Combes, 2006; Grindon et al., 2006).

In recent years numerous organisms have been proposed as alternative vertebrate experimental models for the evaluation of toxicological risks from man-made compounds. In this context, the protozoa, unicellular eukayotic organisms that have long inhabited aquatic and terrestrial habitats and so developed adaptment strategies to survive environmental perturbations (Fenchel, 1987), can be considered excellent laboratory models (Apostol, 1973).

In fact, due to their nature as eukaryotic single cells/organisms, the protozoa exhibit a relatively simple organisation and a high degree of specialisation. As organisms, the protozoa respond directly to environmental stimuli like the metazoan, while, as single cells, which expose their receptors directly to the surrounding environment, they are more sensible to environmental modifications than the metazoa cells, which have developed complex apparatuses and structures that respond to environmental stimuli according to their diverse functions. Furthermore, as the ancestors of the metazoan, the experimental response of the protozoa can be correlated with those of the more developed organisms.

An advantage of using protozoa in toxicological studies is that their short cell cycles allow the analysis of the toxic effects of a contaminant on a conspicuous number of cells, genetically homogeneous populations and successive generations in a short time. In addition, the absence of a cellular sheath in the vegetative state allows the protozoa to

respond to the stimulus more rapidly than bacteria and yeasts. Furthermore, they can be cultured in the laboratory under conditions very similar to those in nature, making their response more reliable than that of animal-cell cultures cultured in artificial conditions (Delmonte Corrado et al., 2006).

Finally, it is important to emphasise how in protozoa, the identification of molecules responsible for neurotransmission in metazoan, such as those belonging to the GABAergic system in *Paramecium primaurelia* (Ramoino et al., 2010) and *Dictyostelium discoideum* (Anjard & Loomis 2006), to the nitergic system in *Paramecium primaurelia* (Amaroli et al., 2010), and to the cholinergic system in *Paramecium primaurelia*, *Dictyostelium discoideum*, *Euplotes crassus* (Delmonte Corrado et al.,1999; Amaroli et al. 2003; Trielli et al., 2007), with characteristics similar to those of the vertebrates, has opened the way to their use in neurotoxicological studies. The genomal sequencing of several protozoa (Dessen et al. 2001; Turkewitz et al., 2002; Eichinger et al., 2005), has demonstrated that they have conserved gene sequences compared to human genome and this has stimulated the interest of the scientific community in their use in field studies on human health, as in the case of *Dictyostelium discoideum* included in the eight alternative models to be used instead of vertebrates in human health studies in the USA (Williams et al., 2006).

The characteristics listed above make the protozoa excellent biological assay tools, combining the reliability of *in vivo* results with the practicality of *in vitro*, to be used in toxicological studies and wider studies of the effects of environmental perturbations (Persoone & Dive, 1978; Denis et al., 1992; Ricci, 1995; Sauvant et al., 1999; Miyoshi et al., 2003; Delmonte Corrado et al., 2006;).

3. Basic research into the effects of pesticides on *Dictyostelium* cholinesterase

The cholinergic system has been extensively studied as the cholinergic molecules play the role of specialised transmitters of nervous signals at myoneural junctions, regulating the intercellular messages by varying the ionic content of the cytoplasm.

The cholinergic transmitter system includes the signal molecule acetylcholine (ACh), synthesised by the enzyme choline acetyltransferase (ChAT, E.C. 2.3.1.6), which catalyses the acetylation of choline with acetyl coenzyme-A (Nachmansohn & Machado, 1943), and both classes of ACh receptors, the nicotine-sensitive ones (Stroud et al., 1990) and the muscarine-sensitive ones (Birdsall et al., 1978), which play different roles in excitable cells, even though activated by the same signal molecule. The activity of the ACh lytic enzyme acetyl-cholinesterase (AChE) modulates the reception function by hydrolysing the receptor-bound ACh on the surface of target cells. More generally, the ChEs refer to enzymes able to hydrolyse ACh and other choline esters into their respective components, choline and acetate, or butyrate or propionate (Stedman & Easson, 1932).

Depending on the species, different ChEs have been detected and characterised on the basis of their catalytic properties, depending both on the substrate hydrolysed by their activity and on their sensitivity to specific inhibitors (Mendel & Rudney, 1943; Talesa et al., 1990).

AChE activity, also referred to as "true" AChE (E.C. 3.1.1.7) and present in higher organisms with specialised synapses (Massoulié et al., 1993), hydrolyses either ACh with high affinity and the substrate acetyl-β-methyl thiocholine iodide (AcTChI). This enzyme activity is inhibited by eserine, a carbamate compound, and more specifically, by BW 284c51, a phenol

ester. Among the other choline esters are butyrylcholinesterase (BChE, E.C. 3.1.1.8) and propionyl-cholinesterase (PrChE), also referred to as "pseudocholinesterases", that preferentially hydrolyse the substrates butyryl thiocholine iodide (BTChI) and propionyl thiocholine iodide (PrChI) respectively (Talesa et al., 1990). Both BChE and PrChE enzyme activities are inhibited by eserine and, more specifically, by iso-OMPA, a phosphoramide compound.

As reported in Section 2, there is some information on the presence and functional role of the molecules related to the cholinergic system in protozoa. In particular, in this review, attention is focused on the ChE of *Dictyostelium discoideum* single-cell amoebae. This protozoa, often referred to as "slime mould", exhibits a very simple organisation with a very complex developmental cycle. The life cycle of *D. discoideum* (Fig. 2) starts with the multiplication phase by binary fission of single-cell amoebae feeding on bacteria. Depletion of the food source triggers the aggregation and differentiation phase. The amoebae aggregate and migrate towards an extracellular cyclic AMP signalling source by chemotaxis, giving rise to the first multicellular stage, which undergoes the process of culmination leading to the formation of the fruiting body (Loomis, 1975; Escalante & Vicente 2000). In *D. discoideum,* we citochemically and spectrophotochemically detected ChE activities able to cleave the substrate PrChI with high affinity and the substrate AcTChI, with less affinity (Falugi et al., 2002). The non-denaturing electrophoresis pattern showed that the PrChI substrate was hydrolysed by a single enzyme activity, while the AcTChI susbstrate was hydrolysed by two enzyme activities, one migrating like the 260-kDa molecular form of *Electrophorus electricus* AChE and the other corresponding to the molecular form revealed by the PrTChI substrate (Falugi et al., 2002). To characterise the PrChE, we spectrophotometrically evaluated the effects of pH, temperature variations and specific inhibitors. It is important to underline that a 10^{-5} M iso-OMPA concentration completely blocked the PrChE activity (Delmonte Corrado et al., 2005). Moreover, the full genome sequencing of *Dictyostelium discoideum* enabled us to detect a putative sequence of a protein, that showed a similarity to a pseudocholinesterase of the vertebrates.

The inhibition of ChE activity was one of the first validated environmental biomarkers and it has been employed widely for over thirty years as a specific biomarker of exposure to pesticides, such as the organophosphate (OP) and carbamate (CA) compounds used world wide for agriculture pest control (Kennedy, 1991; Hassal, 1990). These compounds, less persistent in the environment but more toxic than the organochlorine pesticides, are anticholinesterase agents that have been designed to be effective inhibitors of ChE activity. The organophosphate pesticides are esters or thiols derived from phosphoric, phosphonic, phosphinic or phosphoramidic acid. Their basic chemical structure is shown in Fig. 1A. generally, R1 and R2 are aryl or alkyl groups, while X refers to a wide range of groups: halogen, aliphatic, aromatic and heterocyclic (Kuhr & Dorough, 1976). The OP pesticides destroy ChE activity by phosphorylating a specific serine residue within the ChE catalytic centre and under this condition the enzyme is unable to hydrolyse the choline esters (Kennedy, 1991; Gallo & Lawryk, 1991). The CA pesticides are derivates of carbamic acid. Their basic chemical structure is shown in Fig. 1B. Generally R1 and R2 are organic radicals or a metal. Carbamates have insecticide properties when R2 is an H and R1 a methyl; herbicidal properties when R1 is an aromatic group; fungicidal properties when R1 is a component of benzimidazole. The CAs, like the OPs, act on the serine within the ChE catalytic centre through a carbamylation reaction. However, unlike the OPs, the inhibition of ChE activity by CAs is a reversible process (Kuhr & Dorough, 1976; Baron, 1991).

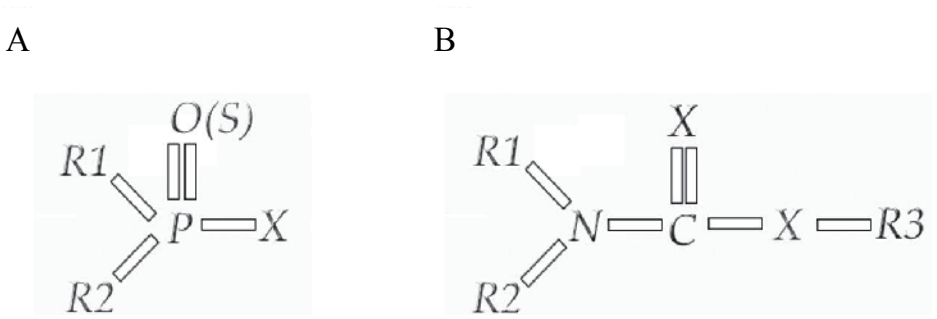

Fig. 1. Chemical structure of organophosphate (A) and carbamate (B) compounds.

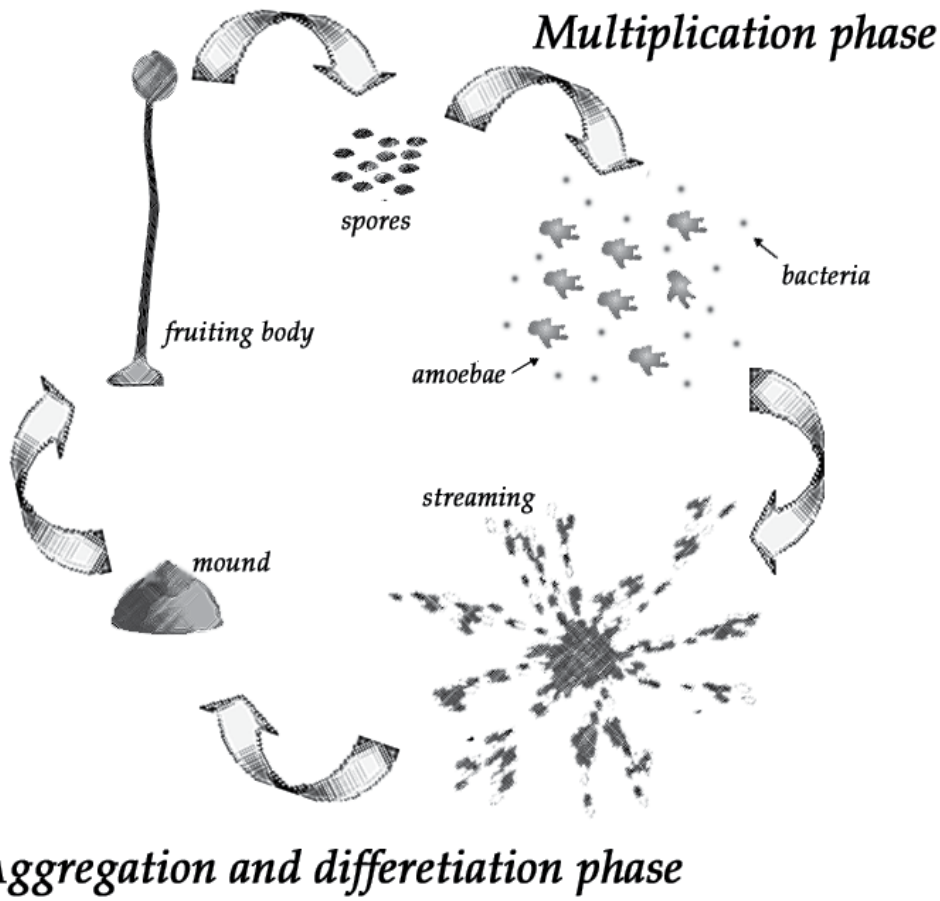

Fig. 2. A simplified scheme of the developmental cycle of *Dictyostelium discoideum*. The life cycle start from the multiplication phase, during which single-cell amoebae feed bacteria, grow, and reproduce by binary fission. The next phase starts from the depletion of food supply that triggers aggregation of the amoebae in stream to form multicellular aggregate, the mound. The final phase culminates in the development of the fruiting body, containing the apical spore able to reproduce under suitable environmental conditions, and the stalk cells unable to multiply.

To evaluate the possibility of exploiting the PrChE activity detected in *D. discoideum* as a specific biomarker of exposure to neurotoxic pesticides, we spectrophotometrically (Ellman et al., 1961) tested the sensitivity of this enzyme to OPs and CAs. The OPs employed were diazinon and cidial, which caused an inhibition of 67% and 52% respectively at a concentration of 10^{-6} M, whereas the CAs were carbaryl and pirimicarb, which caused an inhibition of 63% and 61% at the same concentration as the OPs (Falugi et al., 2002).
In addition, a dose-dependent inhibition of PrChE activity was found for diazinon concentrations ranging from 10^{-7} M to 10^{-3} M. The identification of ChE activity, sensitive to exposure to OP and CA pesticides, in *D. discoideum* can avoid the interference of several stress factors in testing. In fact, several non-contaminant factors, such as type of tissue, species, genetic variation, age, sex, circadian and seasonal rhythms, reproductive state, and endocrine regulation can affect ChE activity, when invertebrates and vertebrates are exploited as test organisms for detecting the presence of pesticides (Rettner & Fairbrother, 1991). On the contrary, these interference factors are excluded by the use of *D. discoideum* as the asexual reproduction of this unicellular organism gives rise to phenotypically and genotypically homogeneous cell lines. Furthermore, the stress factors that can interfere with *D. discoideum* PrChE activity are linked to the nutritional and physiological state of the culture and the culturing temperature and are eliminated by the standard conditions under which they are cultured.

4. Applied research into the effects of pesticides on *Dictyostelium* cholinesterase

Considering the results presented in the preceding section, we have examined the possibility of using the PrChE of *D. discoideum* as a biomarker for the pre-chemical screening of estuarial environments subject to pesticide pollution.
New Italian (D. L. n. 152/1999 and D. L. n. 258/2000) and European (Direttive CE 2000/60), regulations give particular attention to the quality of water and sediment, not only for their chemico-physical characteristics but also for their biocenotic content. In fact, the European Union is particularly interested in promoting chemico-physical and biological monitoring programmes for evaluating the health of the aquatic environment (Sandulli, 2004).
It has been noted how pollutants dispersed in the environment often finish, more or less rapidly, in the aquatic environment, where they can be absorbed by suspended particulate matter and then accumulated in the sediments. Therefore, the sedimentary deposits in contiguous marine and continental zones represent filters and storage for pollutants, constituting important sources of contamination for organisms (Geyer et al., 1984; Fulton & Key, 2001; Bolton-Warberg et al., 2007; Moserrat et al., 2007). These coastal, estuarial and wet zones are particularly noted for their great biodiversity and are very important biotopes for the reproduction of numerous animal and vegetal species. Furthermore, pollution in these zones can have repercussions for the health of the human populations using their resources (McCauley et al., 2000; Pal et al., 2010; Azizullah et al., 2011). In fact, it has been noted that the residues of these substances act not only on the cholinergic systems of the pest invertebrates inhabiting these zones but also on the human cholinergic system and other enzymes such as the aliesterases, lipases, trypsin, chymotrypsin, succinoxidase, ascorbic acid oxidase, dehydrogenase, sulfhydryl enzymes (Gallo & Lawryk, 1991). In a review, Gilden et al. (2010) noted that many articles described the toxic and epidemiological effects of exposure to pesticides, and particularly their effect on infant development. Furthermore, it has been reported that exposure to pesticides can be an important risk factor in the

development of infant leukemia and it is necessary to protect the mother during pregnancy (Van Maele-Fabry et al., 2011). Numerous observations have emphasised the higher risk of people exposed to pesticides developing neurodegenerative diseases such as Parkinson's Disease (Weisskopf et al., 2010), and the relationship between exposure to pesticides and the development of tumors (Gold et al., 2001; Weichenthal el al., 2010).

Our study encompassed a stretch of the Ligurian coast between Genoa and the French border, whose economy is based largely on summer tourism and intensive market gardening in the areas between urban and tourist settlements. In fact, specialist fruit vegetable and flower producers, in close competition with northern European producers and, for some years, with those of South East Asia, have tended to rely increasingly on pesticides. The data is in line with that of the rest of the world and emphasised by The Worldwatch Institute (2006), according to which the consumption of pesticides has increased exponentially in recent decades, from 0.49 kg/ha in 1961 to 2kg/ha in 2004, with the consequences that these substances are now found in many foods (Akland et al., 2000).

It is, therefore, obvious that continuous environmental biomonitoring is necessary in this area to reach sustainable development, to balance agriculture and tourism and to protect the environment and the health of the inhabitants and visitors.

Our samples were taken from the Argentina Torrent in the Province of Imperia and the Centa Torrent in the Province of Savona, both critical areas subject to particular anthropic pressure, and the Arresta Torrent in the Province of Genoa as control area, as it has hardly been touched by anthropic development.

The Centa and Argentina torrents lie in areas of intensive agriculture, mainly specialising in olive oil production and growing flowers and plants, and there is a notable increase in the population at nearby seaside resorts in summer. Differently, the Arresta Torrent, lying on the border between the provinces of Genoa and Savona, flows through the Parco del Beigua natural area and geopark, and only near its mouth flows through scarcely inhabited and cultivated land.

The monitoring programme for the three torrents was based on the location of their cultivation and the characteristics of each watercourse (accessibility of the stream bed, granulometry of the sediment). In the case of the Argentina Torrent, two sites were studied, 100 m and 400 m from the swash zone, for the Centa, two sites 50 m and 400 m from the swash zone, and for the Arresta two sites 50 m and 200 m from the swash zone.

Samples of sediments and surface water were collected from each site. The sampling was performed monthly over the period 2007-10, taking meteorological and marine conditions into consideration. The samples were buffered at pH 8.0 and the salinity determined before spectrophotometric analysis following Ellman et al. (1961).

The amoebae of *D. discoideum* exposed to water (data not shown) and elutriated sediment (Fig. 3) samples from the Arresta Torrent over the period 2007-10 did not show PrChE activity significantly different ($p>0.05$) from the control (commercial mineral water).

The amoebae of *D. discoideum* exposed to surface water from the Argentina Torrent did not demonstrate alterations in PrChE activity ($p>0.05$) (data not shown). The situation was different for the amoebae exposed to samples of elutriated sediment from the Argentina Torrent, where the amoebae exposed to the sediment from the site 400 m from the swash zone had significantly increased PrChE activity ($p<0.05$) in November 2007 (Fig. 4A), November and December 2008 (Fig. 4B) and November and December 2009 (Fig. 4C). An analogous situation ($p<0.05$) was observed for the amoebae exposed to the elutriated sediment from the site 100 m from the swash zone in December 2007 (Fig. 4E) and December 2008 (Fig. 4F)

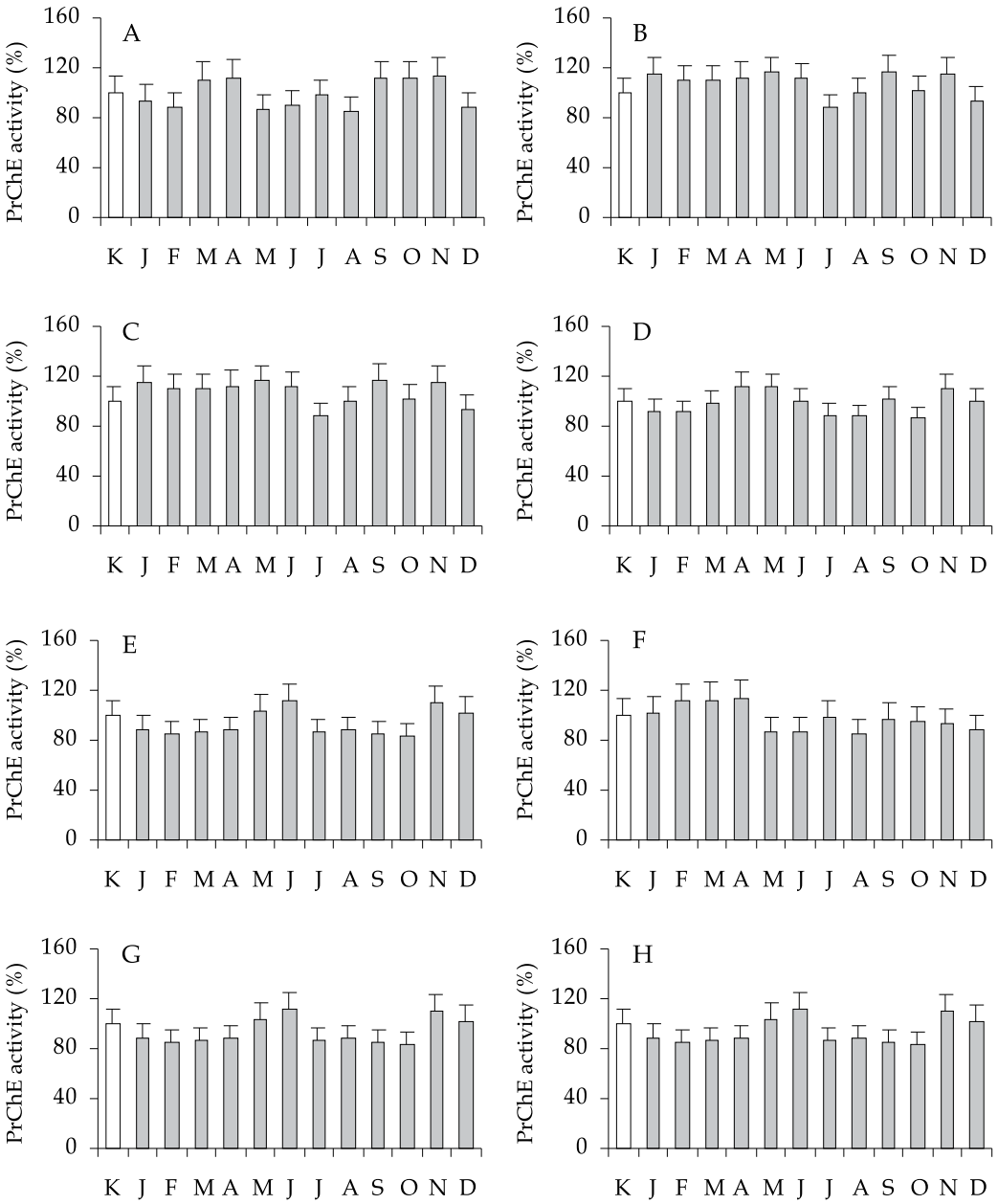

Fig. 3. PrChE activity in *D. discoideum,* measured after exposure to elutriated sediments from the Arresta Torrent. Samples collected 200 m from the swash zone in 2007 (A), 2008 (B), 2009 (C), and 2010 (D). Samples collected 50 m from the swash zone in 2007 (E), 2008 (F), 2009 (G), and 2010 (H). Sampling months shown along the x-axis. The black columns indicate significant differences ($p<0.05$) from the control (commercial mineral water) (K).

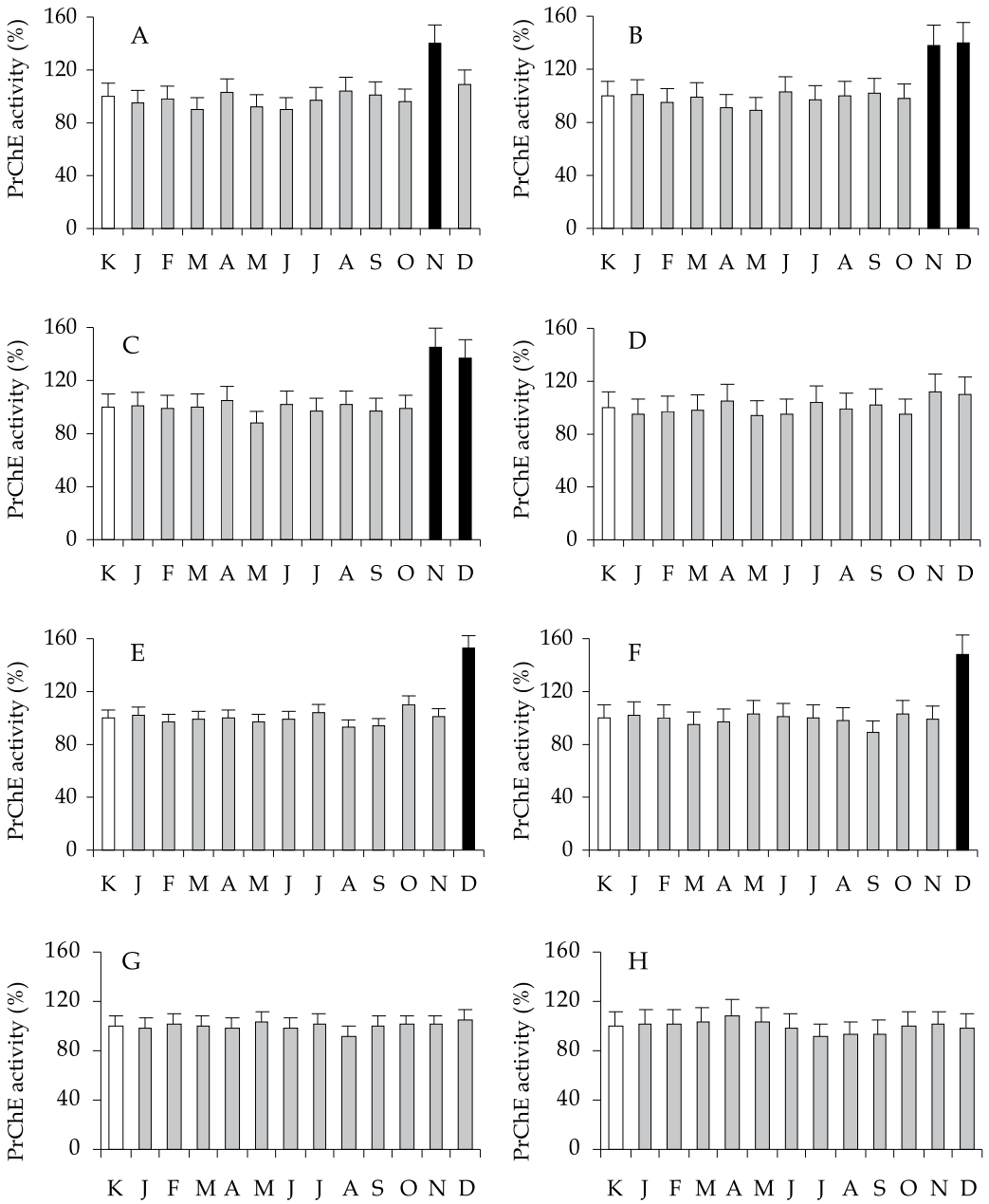

Fig. 4. PrChE activity in *D. discoideum,* measured after exposure to elutriated sediments from the Argentina Torrent. Samples collected 400 m from the swash zone in 2007 (A), 2008 (B), 2009 (C), and 2010 (D). Samples taken 100 m from the swash zone in 2007 (E), 2008 (F), 2009 (G), and 2010 (H). Sampling months shown along the x-axis. The black columns indicate significant differences ($p<0.05$) from the control (commercial mineral water) (K).

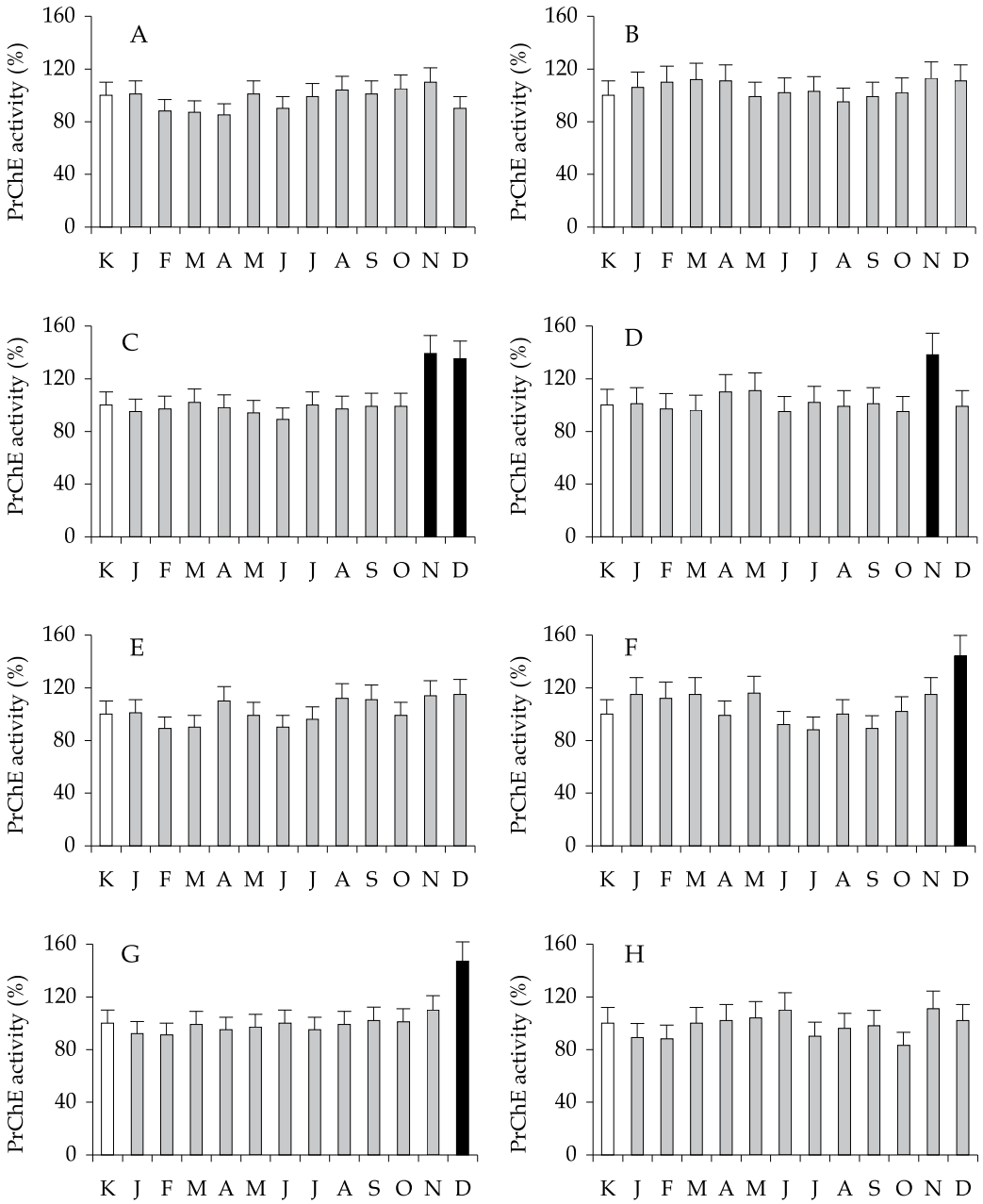

Fig. 5. PrChE activity in *D. discoideum,* measured after exposure to elutriated sediments from the Centa Torrent. Samples collected 400 m from the swash zone in 2007 (A), 2008 (B), 2009 (C), and 2010 (D). Samples taken 50 m from the swash zone in 2007 (E), 2008 (F), 2009 (G), and 2010 (H). Sampling months shown along the x-axis. The black columns indicate significant differences ($p<0.05$) from the control (commercial mineral water) (K).

The amoebae of *D. discoideum* exposed to surface water from the Centa torrent did not demonstrate alterations in PrChE activity ($p>0.05$) (data not shown), nor did those exposed to the elutriated sediments from the site 400 m from the swash zone in 2007 and 2008 (Fig. 5A, Fig. 5B) and the station 50 m from the swash zone in 2007 and 2010 (Fig. 5E, Fig. 5H). Instead, the amoebae exposed to the elutriated sediments from the site 400 m from the swash zone showed a significant increase ($p<0.05$) in PrChE activity in November and December 2009 (Fig. 5C) and November 2010 (Fig. 5D), as did those exposed to the sediments from the site 50 m from the swash zone in December 2008 (Fig. 5F) and December 2009 (Fig. 5G).
These increases in the PrChE activity of the amoebae of *D. discoideum* exposed to sediments from the estuarial zones of the Centa and Argentina Torrents indicates recurrent environmental stress in this area in the autumn-winter period. This result is more evident when compared to the situation of the Arresta Torrent, where no water or sediment sample induced a significant increase in PrChE activity in the exposed cells. Finally, the laboratory analyses exclude the possibility that the results are "artefacts" due to variations in the pH or salinity of the samples.

5. Conclusion

In conclusion, it is important highlight as, *in vitro* analysis, parallel studies have shown how some heavy metals and non-OP and -CA pesticides can induce a significant increase in the PrChE activity of exposed *D. discoideum* amoebae (Delmonte Corrado et al., 2006), so, it is possible to assume the presence of these substances in the sediments of Argentina Torrent and Centa Torrent. Analyses of the sediments of the zones we sampled have revealed the presence of pyrethroid pesticides in the autumn-winter period. These pesticides are able to induce a significant increase in cholinesterase as shown by studies on bees (Badiou & Belzunces, 2008). The pyrethroid pesticides can enter the watercourses and then the estuarial sediments as a result of the abundant rain that falls in this period and floods the land dedicated to olive production treated with such pesticides in the fight against the olive fruit fly and other insects (Ruano et al., 2010), or the runoff from olive groves, which various studies have revealed contains high levels of pesticides (Karaouzas et al., 2010).
In conclusion, *D. discoideum* cholinesterase activity is a sensitive and reliable biomarker of the presence of pesticides in the sediments of watercourses subject to heavy anthropic pressure. A characteristic of great importance if we consider that this enzymatic activity occurs in a protozoa that responds to the conditions proposed by the 3Rs strategy and can assuage increasing international public concern over bioethical matters.

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7. References

Akland, GG.; Pellizzari, ED.; Hu, Y.; Roberds, M.; Rohrer, CA.; Leckie, JO. & Berry, MR. (2000). Factors influencing total dietary exposures of young children. *Journal of*

Exposure Analysis and Environmental Epidemiology, Vol.10, pp. 710-722, ISSN 1053-4245.

Amaroli, A.; Gallus, L.; Passalacqua, M.; Falugi, C.; Viarengo, A. & Delmonte Corrado, MU. (2003). Detection of cholinesterase activities and acetylcholine receptors during the developmental cycle of *Dictyostelium discoideum*. *European Journal of Protistology*, Vol.39, pp. 213-222, ISSN 0932-4739.

Amaroli, A.; Trielli, F.; Sifredi F.; Chessa, MG. & Delmonte Corrado, MU. (2010). Nitric oxide production inhibited by xenobiotic compounds in the protozoan *Paramecium primaurelia*. *Ecological Indicators*, Vol.10, No.2, pp. 212-216, ISSN 1470160x.

Anjard, C. & Loomis, WF. (2006). GABA induces terminal differentiation of *Dictyostelium* through a GABAB receptor. *Development*, Vol.133, No.11, pp. 2253-2261, ISSN 1011-6370.

Apostol, S. (1973). A bioassay of toxicity using protozoa in the study of aquatic environment pollution and its prevention. *Environmental Research*, Vol.6, No.4, pp. 365-72, ISSN 0013-9351.

Azizullah, A.; Khattak, MN.; Richter, P. & Häder, DP. (2011). Water pollution in Pakistan and its impact on public health--a review. *Environment International*, Vol.37, pp. 479-497, ISSN 0160-4120.

Badiou, A. & Belzunces, LP. (2008). Is acetylcholinesterase a pertinent biomarker to detect exposure of pyrethroids? A study case with deltamethrin. *Chemico-Biological Interactions*, Vol.175, pp. 406-409, ISSN 0009-2797.

Balls, M.; Goldberg, AM.; Fentem, JH.; Broadhead, CL.; Burch, RL.; Festing, MF.; Frazier, JM.; Hendriksen, CF.; Jennings, M.; van der Kamp, MD.; Morton, DB.; Rowan, AN.; Russell, C.; Russell, WM.; Spielmann, H.; Stephens, ML.; Stokes, WS.; Straughan, DW.; Yager, JD.; Zurlo, J. & van Zutphen, BF. (1995). The three Rs: the way forward: the report and recommendations of ECVAM Workshop 11. *ATLA Alternatives to Laboratory Animals*, Vol.23, pp. 838-866, ISSN 02611929.

Baron, RL. (1991). Carbamate insecticides, In: *Handbook of Pesticide Toxicology*, JH. Wayland, RL. Edward, (Eds), 1125-1171, ISBN 978-0123341600, San Diego, USA.

Birdsall, NJ.; Burgen, AS. & Hulme, EC. (1978). The binding of agonists to brain muscarinic receptors. *Molecular Pharmacology*, Vol.14, No.5, pp. 723-736, ISSN 0026-895X.

Bolton-Warberg, M.; Coen, LD. & Weinstein, JE. (2007). Acute toxicity and acetylcholinesterase inhibition in grass shrimp (*Palaemonetes pugio*) and oysters (*Crassostrea virginica*) exposed to the organophosphate dichlorvos: laboratory and field studies. *Archives of Environmental Contamination and Toxicology*, Vol.52, pp. 207-216, ISSN 0090-4341.

Delmonte Corrado, MU.; Amaroli, A.; Trielli, F. & Falugi, C. (2006). Cholinesterase enzyme activity in protists and environmental biomonitoring. *Current Trends in Microbiology*, Vol.2, pp. 123-136, ISSN 0972-7736.

Delmonte Corrado, MU.; Politi, H.; Trielli, F.; Angelini, C. & Falugi C. (1999). Evidence for the presence of a mammalian-like cholinesterase in *Paramecium primaurelia* (Protista, Ciliophora) developmental cycle. *Journal of Experimental Zoology*, Vol.283, No.1, pp. 102-105.

Delmonte Corrado, MU.; Trielli, F.; Amaroli, A.; Ognibene, M. & Falugi, C. (2005). Protists ad Tools for Environmental Biomonitoring: Importance of Cholinesterase Enzyme

Activities, In: *Water Pollution – New Research*. AR. Burk (Ed), 181-200, ISBN 1-59454-393-3, New York, USA.

Denis, H.; Gilron, L. & Gilron, GL. (1992). A brief review of approaches using ciliated protists to assess aquatic ecosystem health. *Journal of Aquatic Ecosystem Stress and Recovery*, Vol.1, No.4, pp. 263-270, ISSN 1386-1980.

Dessen, P.; Zagulski, M.; Gromadka R.; Plattner, H.; Kissmehl, R.; Meyer E.; Bétermier, M.; Schultz, JE.; Linder, JU.; Pearlman, RE.; Kung, C.; Forney, J.; Satir, BH.; Van Houten, JL.; Keller, AM.; Froissard, M.; Sperling, L. & Cohen, J. (2001). *Paramecium* genome survey: a pilot project. *Trends in Genetics*, Vol.17, pp. 306-308, ISSN 0168-9525.

Eichinger, L., Pachebat, JA., Glöckner, G., Rajandream, MA., Sucgang, R., Berriman, M., Song, J., Olsen, R., Szafranski, K., Xu, Q., et al. (2005). The genome of the social amoeba *Dictyostelium discoideum*. *Nature*, Vol.435, pp. 43-57, ISSN 0028-0836.

Ellman, GL.; Courtney, KD.; Andreis, V. & Feather-Stone, RM. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, Vol.7, pp. 88-95, ISSN 0006-2952.

Escalante, R. & Vicente, JJ. (2000). *Dictyostelium discoideum*: a model system for differentiation and patterning. *The International Journal of Developmental Biology*, Vol.44, No.8, pp. 819-835, ISSN 0214-6282.

Falugi, C.; Amaroli, A.; Evangelisti, V.; Viarengo, A. & Corrado, MU. (2002). Cholinesterase activity and effects of its inhibition by neurotoxic drugs in *Dictyostelium discoideum*. *Chemosphere*, Vol.48, No.4, pp. 407-14, ISSN 0045-6535.

Fenchel, T. (1987). *Ecology of Protozoa: The Biology of Free-Living Phagotrophic Protists.* Madison/Springer-Verlag, ISBN 978-3540169604, Berlin, Germany.

Flecknell, P. (2002). Replacement, Reduction and Refinement. *ALTEX*, Vol.19, No.2, pp. 73-78, ISSN 0946-7785.

Fulton, MH. & Key, PB. (2001). Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environmental Toxicology & Chemistry*, Vol.20, pp. 37-45, ISSN 0730-7268.

Gallo, MA. & Lawryk, NJ. (1991). Organic Phosphorous Pesticides, In: *Handbook of Pesticide Toxicology*, JH. Wayland, RL. Edward, (Eds), 917-933, ISBN 978-0123341600, San Diego, USA.

Geyer, H.; Freitag, D. & Korte, F. (1984). Polychlorinated biphenyls (PCBs) in the marine environment, particularly in the Mediterranean. *Ecotoxicology and Environmental Safety*, Vol.8, pp. 129-151, ISSN 0147-6513.

Gilden, RC.; Huffling, K. & Sattler, B. (2010). Pesticides and health risks. *Journal of Obstetric, Gynecologic, and Neonatal Nursing (JOGNN)*, Vol.39, pp. 103-110, ISSN 0884-2175.

Gold, LS.; Slone, TH. & Ames, BN. (2001). Pesticide Residues in Food and Cancer Risk: a Critical Analysis, In: *Handbook of Pesticide Toxicology, Second Edition*, R. Krieger (Ed.), 799-843, ISBN 978-0124262607, San Diego, USA.

Grindon, C. & Combes, R. (2006). Introduction to the EU REACH legislation. *ATLA Alternatives to Laboratory Animals,* Vol.34, Suppl.1, pp. 5-10, ISSN 02611929.

Grindon, C.; Combes, R.; Cronin, MT.; Roberts, DW. & Garrod, J. (2006). A review of the status of alternative approaches to animal testing and the development of integrated testing strategies for assessing the toxicity of chemicals under REACH - a summary of a DEFRA-funded project conducted by Liverpool John Moores

University and FRAME. *ATLA Alternatives to Laboratory Animals,* Vol.34, Suppl.1, pp. 149-158, ISSN 02611929.

Hassall, KA. (1990). *The Biochemistry and Use of Pesticides. Structure, Metabolism, Mode of Action and Use in Crop Protection*. MacMillan, ISBN 978-0895739766, NewYork, USA.

Karaouzas, I.; Cotou, E.; Albanis, TA.; Kamarianos, A.; Skoulikidis, NT. & Giannakou, U. (2010). Bioassays and biochemical biomarkers for assessing olive mill and citrus processing wastewater toxicity. *Environmental Toxicology*, n/a, ISSN 1520-4081.

Kennedy, SW. (1991). The Mechanism of Organophosphate Inhibition of Cholinesterase - Proposal for a New Approach to Measuring Inhibition, In: *Cholinesterase-inhibiting Insecticides: Their Impact on Wildlife and the Environmental. Chemical in Agriculture*, P. Mineau (Ed.), Vol.2, 74-87, ISBN 9780444887078, Amsterdam, Holland.

Kuhr, RJ. & Dorough, HW. (1976). Carbamate Insecticides: Chemistry, Biochemistry, and Toxicology, In: *Handbook of Pesticide Toxicology*, JH. Wayland, RL. Edward, (Eds), 41-70, ISBN 9780878190522, Cleveland, (USA).

Loomis, WF. (1975). *Dictyostelium discoideum: A Developmental System,* Academic Press, ISBN 9780124561502, New York, USA.

Louekari, K.; Sihvonen, K.; Kuittinen, M. & Sømnes, V. (2006). In vitro tests within the REACH information strategies. *ATLA Alternatives to Laboratory Animals,* Vol.34, No.4, pp. 377-386, ISSN 02611929.

Massoulié, J.; Pezzementi, L.; Bon, S.; Krejci, E. & Vallette FM. (1993). Molecular and cellular biology of cholinesterases. *Progress in Neurobiology*, Vol.41, No.1, pp. 31-91, ISSN 0555-4047.

McCauley, DJ.; DeGraeve, GM. & Linton, TK. (2000). Sediment quality guidelines and assessment : overview and research needs. *Environmental Science & Policy*, Vol.3, pp. 133-144, ISSN 1462-9011.

Mendel, B. & Rudney, H. (1943). Studies on cholinesterase: 1. Cholinesterase and pseudo-cholinesterase. *The Biochemical Journal*, Vol.37, No.1, pp. 59-63, ISSN 0264-6021.

Miyoshi, N.; Kawano, T.; Tanaka, M.; Kadono, T.; Kosaka, T.; Kunimoto, M.; Takahashi, T. & Hosoya, H. (2003). Use of *Paramecium* species in bioassays for environmental risk management: determination of IC50 values for water pollutants. *Asian Journal of Health Science*. Vol.46, No.6, pp. 429-435, ISSN 1671-5927.

Monserrat, JM.; Martínez, PE.; Geracitano, LA.; Amado, LL.; Martins, CM.; Pinho, GL.; Chaves, IS.; Ferreira-Cravo, M.; Ventura-Lima, J. & Bianchini, A. (2007). Pollution biomarkers in estuarine animals: critical review and new perspectives. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, Vol.146, pp. 221-234, ISSN 1532-0456.

Nachmansohn, D. & Machado, AL. (1943). The formation of acetylcholine. A new enzyme choline acetylase. *Journal of Neurophysiology*, Vol.6, pp. 397-403, ISSN 0022-3077.

Orlans, BF. (1993). In the Name of Science, In: *Responsible Animal Experimentation*, Oxford University Press, 312, ISBN 978-0195108712, New York, USA.

Pal, A.; Gin, KY.; Lin, AY. & Reinhard, M. (2010). Impacts of emerging organic contaminants on freshwater resources: review of recent occurrences, sources, fate and effects. *Science of the Total Environment*, Vol.408, pp. 6062-6069, ISSN 0048-9697.

Persoone, G. & Dive, D. (1978). Toxicity tests on ciliates: a short review. *Ecotoxicology and Environmental Safety*, Vol.2, No.2, pp. 105-14, ISSN 0147-6513.

Ramoino, P.; Milanese, M.; Candiani, S.; Diaspro, A.; Fato, M.; Usai, C. & Bonanno, G. (2010). Gamma-amino butyric acid (GABA) release in the ciliated protozoon *Paramecium* occurs by neuronal-like exocytosis. *The Journal of Experimental Biology*, Vol.213, pp. 1251-1258, ISSN 0022-0949.

Rattner, B. & Fairbrother, A. (1991). The Mechanism of Organophosphate Inhibition of Cholinesterase - Proposal for a New Approach to Measuring Inhibition, In: *Cholinesterase-inhibiting Insecticides: Their Impact on Wildlife and the Environmental. Chemical in Agriculture*, P. Mineau (Ed.), Vol.2, 89-101, ISBN 9780444887078, Amsterdam, Holland.

Ricci, N. (1995). Protozoa; Bioindicators of Environmental Monitoring and Pollution Control, In: *Pollution and Biomonitoring*, Tata Mc Graw-Hill, 240-269, New Delhi, India.

Ruano, F.; Campos, M.; Sánchez-Raya, AJ. & Peña, A. (2010). Olive trees protected from the olive bark beetle, *Phloeotribus scarabaeoides* (Bernard 1788) (Coleoptera, Curculionidae, Scolytinae) with a pyrethroid insecticide: Effect on the insect community of the olive grove. *Chemosphere*, Vol.80, pp. 35-40, ISSN 0045-6535.

Russel, WMS. & Burch RL. (1959). *The Principles of Humane Experimental Tecnique*. Metheuen and Co Ltd, ISBN 0-900767-78-2, London, England.

Sandulli, R. (2004). Il ruolo degli indicatori biologici nella valutazione dello stato dell'ambiente marino. *Biologia Marina Mediterranea*, Vol.11, pp. 185-192, ISSN 1123-4245.

Sauvant, MP.; Pepin, D. & Piccinni, E. (1999). *Tetrahymena pyriformis*: a tool for toxicological studies. A review. *Chemosphere*, Vol.38, No.7, pp. 1631-1669, ISSN 0045-6535.

Schechtman, LM. (2002). Implementation of the 3Rs (refinement, reduction, and replacement): validation and regulatory acceptance considerations for alternative toxicological test methods. *ILAR Journal*, Vol.43, pp. S85-S94, ISSN 1084-2020.

Stedman, E.; Stedman, E. & Easson, LH. (1932). Choline-esterase. An enzyme present in the blood-serum of the horse. *The Biochemical journal*, Vol.26, No.6, pp. 2056-66, ISSN 0264-6021.

Stokes, WS. & Hill, RN. (2002). The Role of ICCVAM in Evaluating New and Alternative Test Methods. *Lab Animal*, Vol.31, No.7, pp. 26-32, ISSN 0093-7355.

Stroud, RM.; McCarthy, MP. & Shuster, M. (1990). Nicotinic acetylcholine receptor superfamily of ligand-gated ion channels. *Biochemistry*, Vol.29, No.50, pp. 11009-11023, ISSN 0006-2960.

Talesa, V.; Contenti, S.; Mangiabene, C.; Pascolini, R.; Rosi, G. & Principato GB. (1990). Propionylcholinesterase from *Murex brandaris*: Comparison with other invertebrate cholinesterases. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, Vol.96, No.1, pp. 39-43, ISSN 1532-0456.

The Worldwatch Institute. (2006). *Vital Signs 2006-2007: the trends that are shaping our future*. WW. Norton, (Ed), ISBN 0393328724, New York, (USA).

Trielli, F.; Amaroli, A.; Sifredi, F.; Marchi, B.; Falugi, C. & Delmonte Corrado, MU. (2007). Effects of xenobiotic compounds on the cell activities of *Euplotes crassus*, a single-cell eukaryotic test organism for the study of the pollution of marine sediments. *Aquatic Toxicology*, Vol.83, No.4, pp. 272-283, ISSN 0166-445X.

Turkewitz, AP.; Orias, E. & Kapler, G. (2002). Functional genomics: the coming of age for *Tetrahymena thermophila*. *Trends in Genetics*, Vol.18, pp. 35-40, ISSN 0168-9525.

Van Maele-Fabry, G.; Lantin, AC.; Hoet, P. & Lison, D. (2011). Residential exposure to pesticides and childhood leukaemia: a systematic review and meta-analysis. *Environment International*, Vol.37, pp. 280-291, ISSN 0160-4120.

Weichenthal, S.; Moase, C. & Chan, P. (2010). A review of pesticide exposure and cancer incidence in the Agricultural Health Study cohort. *Environmental Health Perspectives*, Vol.118, pp. 1117-1125, ISSN 0091-6765.

Weisskopf, MG.; Knekt, P.; O'Reilly, EJ.; Lyytinen, J.; Reunanen, A.; Laden, F.; Altshul, L. & Ascherio, A. (2010). Persistent organochlorine pesticides in serum and risk of Parkinson disease. *Neurology*, Vol.74, pp. 1055-1061, ISSN 0028-3878.

Williams, RS.; Boecheler, K.; Grod, R.; Gräf, R.; Müller-Taubenberger, A.; Li, Z.; Isberg, RR.; Wessels, D.; Soll, DR.; Alexander, H. & Alexander, S. (2006). Towards a molecular understanding of human diseases using *Dictyostelium discoideum*, Trends in Molecular Medicine, Vol.12, pp. 415-424, ISSN 1471-4914.

Part 3

Pesticides Toxicity

15

Molecular Mechanisms of Pesticide Toxicity

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1. Introduction

The environment represents a key contributor to human health and disease. Exposure to many environmental stressors such as pesticides have detrimental effects on health and are considered to contribute substantially to most diseases of major public health significance. Pesticide toxicity has been clearly demonstrated to alter a variety of physiological functions. In addition, evidence suggests that pesticide exposure increases the risk of cancer and neurodegenerative diseases. Recent evidence also demonstrates the ability of pesticides to act as endocrine disruptors, contributing to various adverse effects associated with reproductive and developmental toxicity (Colborn, 2006; Eskenazi et al., 1999). Thus, it is now evident that research towards understanding how pesticides influence the development and progression of disease will lead to further improvements in public health. A key for Environmental Sciences is identifying and understanding the basic biological processes that are altered or regulated by environmental factors, and that stimulate disease processes to begin, or the course of the disease to be substantially altered. For this, basic biology research with potential for future translation into the clinic must be pursued to understand the fundamental changes caused by exposure to environmental agents especially pesticides that will drive the scientific basis for health decisions. Cells respond and adapt to environmental signals such as toxicants or stressors through multiple mechanisms that involve communication pathways or signal transduction processes. A number of receptors sense the presence of foreign compounds in the cell and induce a cascade of events that is intended to lead to neutralization and excretion of these compounds. However, in many cases the metabolism of xenobiotic substances can give rise to toxic metabolites or to reactive oxygen species (ROS) that can harm the cell further. Additionally, the metabolism of foreign compounds can disturb other essential processes in the body, such as production and metabolism of certain hormones. Alterations in biochemical systems are often more sensitive indicators than those at higher levels of biological organization. Indeed, changes at the molecular level will underlie the effects at higher levels of organization.

In this chapter, we focus on a number of molecular pathways implicated in responses to pesticides. In many cases, these responses are adaptive. However, the same systems are involved in reactions leading to toxic effects. They are crucial to the health effects associated with pesticide insult and can be linked to adverse toxic effects and pathologies at higher levels of organization. These systems are:

- Endocrine disruption that can take place at different physiological levels: A) Altering (inhibiting or stimulating) the secretion of hormones. This possible effect is related to

mechanisms that control both the release of hormones from endocrine cells and synthesis of these hormones. B) Interfering with hormone-receptor interaction. C) Modifying the metabolism of circulating hormones, that is, by increasing or decreasing their excretion rate and/or biotransformation in the liver and other organs.

- Oxidant-mediated responses enhance the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the cell. Increased fluxes of these species can induce a number of antioxidant enzymes, alter concentrations of other antioxidants, and/or produce biochemical lesions associated with oxidative damage.

The present effort expands upon those earlier works, benefiting from the advances made in biochemistry and molecular biology in recent years. This chapter is meant to be substantive, but not exhaustive. Each section could be a chapter in itself.

2. Endocrine disruption

An "Endocrine Disrupting Chemical" (EDC) is best defined as "an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function" (European workshop on the impact of endocrine disrupters on human health and wildlife [EEC], 1996). Endocrine disruption refers to a mechanism of toxicity that hinders the ability of cells, tissues and organs to communicate hormonally, resulting in a wide variety of adverse health outcomes including reduced fertility and fecundity, spontaneous abortion, skewed sex ratios within the offspring of exposed communities, male and female reproductive tract abnormalities, precocious puberty, polycystic ovary syndrome, neurobehavioral disorders, hypothyroidism, hyperthyroidism, impaired immune function and a wide variety of cancers. Links between exposure to pesticides and endocrine disruption were suggested as early as 1949 when low sperm counts were observed in men involved in the aerial application of dichlorodiphenyltrichloroethane (DDT) (Singer, 1949). More recently, exposure to endocrine disrupting pesticides (EDPs) has been implicated in the etiologies of various cancers (Garry, 2004; Mathur et al., 2002), miscarriage and other reproductive disorders (Garry, 2004; Nicolopoulou and Stamanti, 2001), genital deformities (Baskin et al., 2001), other birth defects (Schreinemachers, 2003), behavioral abnormalities (Zala & Penn, 2004) and skewed offspring sex ratios (Garry, 2004; Mackenzie & Constanze, 2005). EDPs can affect the endocrine systems of an organism in a wide variety of ways. These include mimicking natural hormones, antagonizing their action or modifying their synthesis, metabolism, and transport. Moreover, these substances can act via multiple pathways including membrane receptors, or the enzymatic machineries involved in hormone biosynthesis/metabolism. However, most of the reported harmful effects of EDPs are attributed to their interference with hormonal signaling mediated by nuclear hormone receptors (NRs) (Swedenborg et al., 2009; Toppari, 2008).

In this section, we first describe receptors that mediate toxicity. We then give a short overview of pesticides that induce receptor-mediated events and finally discuss mechanisms of endocrine disruption.

2.1 Nuclear receptors involved in pesticide toxicity

Human NRs are a family of 48 transcription factors, many of which have been shown to be activated by ligands. NRs regulate cognate gene networks involved in key physiological functions such as cell growth and differentiation, development, homeostasis, or metabolism (Germain et al., 2006; Gronemeyer et al., 2004).

Receptors mediating toxicity can be roughly divided into two groups: dedicated xenosensors and hormone receptors with no primary role in the defense against xenobiotic insult. Upon binding of a xenobiotic compound, the dedicated xenosensors induce a response intended to metabolize and excrete the compound. Activation of the hormone receptors by xenobiotic substances leads to interference with the hormonal system of the exposed organism. To eliminate the harmful effects of an exogenous chemical, the cell attempts to change the compounds to an inactive state, make them water soluble, and excrete them. Metabolism of xenobiotics occurs in three phases: in phase I, the chemical is oxidized; in phase II, the oxidized products are conjugated to glutathione, sulfuric acid, or glucuronic acid, resulting in hydrophilic molecules; and finally in phase III, these substances are transported out of the cell by ATP-dependent export pumps (Nakata et al., 2006). In mammals, three different transcription factor superfamilies are responsible for the induction of xenobiotic metabolizing enzymes, basic-helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) proteins, nuclear receptors (NRs) and basic leucine zipper (bZIP) proteins (Kliewer & Willson, 2002). Although bZIP proteins are important in the detoxification process, they do not bind xenobiotics but rather mediate the cellular response to oxidative stress.
Other receptors involved in the xenobiotic recognition are the NRs constitutive androstane receptor (CAR), rodent pregnane X receptor/steroid and its human orthologue human steroid X receptor (PXR/SXR) (Tolson & wang, 2010). Together with PXR, the constitutive androstane receptor (CAR) acts as an intracellular sensor for foreign chemicals and endogenous lipophilic substances (Willson & Kliewer, 2002). However, CAR differs from PXR in having high constitutive activity in the absence of ligand (Xu et al., 2004) and has been only identified in mammals (Reschly & Krasowski, 2006 ; Tolson & wang, 2010). The NRs farnesoid X receptor (FXR), liver X receptor (LXR), and peroxisome proliferator-activated receptor (PPAR) are also able to induce enzymes involved in the metabolism of xenobiotics. However, as these receptors are not primarily activated by xenobiotics, they are not considered xenosensors.

2.2 Pesticides that induce receptor-mediated toxicity

Receptor-mediated toxicity is induced by a number of pesticides. In contrast to genotoxic substances that are directly carcinogenic by inducing DNA damage, the receptor-mediated effects are versatile and often more subtle, and thus more difficult to identify. In this section, we give examples of pesticides inducing receptor-mediated events that can ultimately lead to toxicity to the organism. 127 pesticides were identified as having endocrine disrupting properties, including the 91 listed by the Pesticide Action Network (PAN) (2005). These pesticides have been used widely over the last 50 years, and the incidences of the diseases linked to them have increased markedly over the same time period and has led many scientists to suggest a connection, despite the inherent difficulty in proving any connection using epidemiological data (Mc Kinlay et al., 2008).
Many of these pesticides are structurally related to steroid hormones and may thus act on the respective hormone receptor. Actually, the first man-made chemicals identified as estrogen receptor (ER) disruptors were pesticides. Symptoms in men working with the manufacture of these compounds led to the identification of dichloro-diphenyl-trichloroethane (DDT) as an ER agonist. Other examples of pesticides that activate the ER are the DDT metabolite dichloro-diphenyl-dichloroethylene (DDE), methoxychlor, and dieldrin (Lemaire et al., 2006). In addition DDE and the fungicide vinclozolin can also affect the function of other hormone receptors: they both act as androgen receptor (AR) antagonists (Kavlock & Cummings, 2005), while vinclozolin can further antagonize the

activity of progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) *in vitro (*Molina-Molina et al., 2006). Androgen receptors are also affected by carbendazim. Premating exposures of male and female rats to carbendazim produced androgen-mediated birth defects in the resultant offspring which could be prevented by cotreatment with the androgen-receptor antagonist flutamide (Morinaga et al., 2004). Tetramethrin and Bioallethrin have also been shown to be estrogen antagonists (Garey and Wolff, 1998). The endocrine disrupting properties of pyrethroid metabolites are poorly understood. Cypermethrin and permethrin both produce a variety of metabolites with structures similar to 17-β-estradiol, some of which have been shown to be weakly estrogenic in vitro (McCarthy et al., 2006). The effects of these metabolites *in vivo* and in combination with other chemicals are unknown, as are the effects of other pyrethroid metabolites.
It has been demonstrated that some pesticides can act as endocrine disrupters on the basis of their basic chemistry, including Quantitative Structure Activity Relationships (QSAR), experimental studies on laboratory animals, wildlife studies and some human epidemiological studies like the International Program on Chemical Safety (IPCS, 2002). The effects of different groups of pesticides on hormone systems and their modes of action are listed in Table 1.

Pesticide	Uses	Hormones affected	Mechanism	References
Organochlorines	Insecticides	Androgens, oestrogens, prolactin	Competitive inhibitor of androgen receptors, inhibits oestrogen-sensitive reporter binding to androgen receptors. Some induce the production of aromatase, an enzyme that converts androgen to oestrogen	Daxenberger, 2002, Lemaire et al., 2004 ; Scippo, 2004 ; Sonnenschein & Soto,1998;
Organophosphates	Mostly Insecticides	Oestrogens, thyroid hormones binding.	Prevents thyroid hormone-receptor Increases the expression of oestrogen responsive genes	Gwinn et al., 2005 ; Jeong et al., 2006, Kang et al., 2004
Carbamates	Herbicides Fungicides	Androgens, oestrogens, Steroids	Still largely unknown. Thought to affect androgen- and androgen-receptor-dependant mechanisms. Shown to interfere with cellular microtubule formation in oestrogen-sensitive cells.	Daxenberger, 2002 ; Goad et al., 2004 ; Lu et al., 2004, Morinaga et al., 2004
Triazines	Herbicides	Androgens	Inhibition of natural ligands that bind to androgen receptors and androgen-binding proteins. Some induce or inhibit the production of aromatase, an enzyme that converts androgen to oestrogen	Hayes et al., 2002 ; Ishihara et al., 2003 ; Meulenberg, 2002
Pyrethroids	Insecticides	Oestrogens, Progesterone	Different compounds antagonise or potentiate the action of oestrogen by acting on the oestrogen receptor or possibly an alternative signalling pathway. Some inhibit the action of progesterone by affecting the hormone itself.	Garey & Wolff, 1998 ; Kim et al., 2004

Table 1. Common endocrine disrupting pesticides: their effects and modes of action

2.3 Pesticide-induced endocrine disruption mechanisms

The human endocrine system is a body-wide network of signaling pathways in which hormones deliver messages, directly or indirectly affecting all aspects of physiology. The phenomenon of exogenous compounds causing dysregulation of the endocrine system has been termed endocrine disruption, and the compounds are collectively referred to as endocrine disrupting chemicals (EDCs). Although pesticides could theoretically disturb the function of all hormone receptors, research to date has mainly focused on their effects on ER, AR and TR. In particular, the ER is recognized as a target for many pesticides. There are two main pathways by which a pesticide can disrupt hormone signaling through receptor-mediated mechanisms: (1) by directly binding to the hormone receptors (Fig. 1), or (2) by binding to xenosensors like PXR and CAR and indirectly affecting hormone signaling by various mechanisms (Fig. 2). Direct binding to hormone receptors is by far the most studied mechanism of endocrine disruption. However, there are several other mechanisms by which pesticides may indirectly affect hormonal pathways, such as inhibition of steroidogenic enzymes and binding to steroid transport proteins, reflecting how complex and interwoven the endocrine and pesticide detoxification systems are.

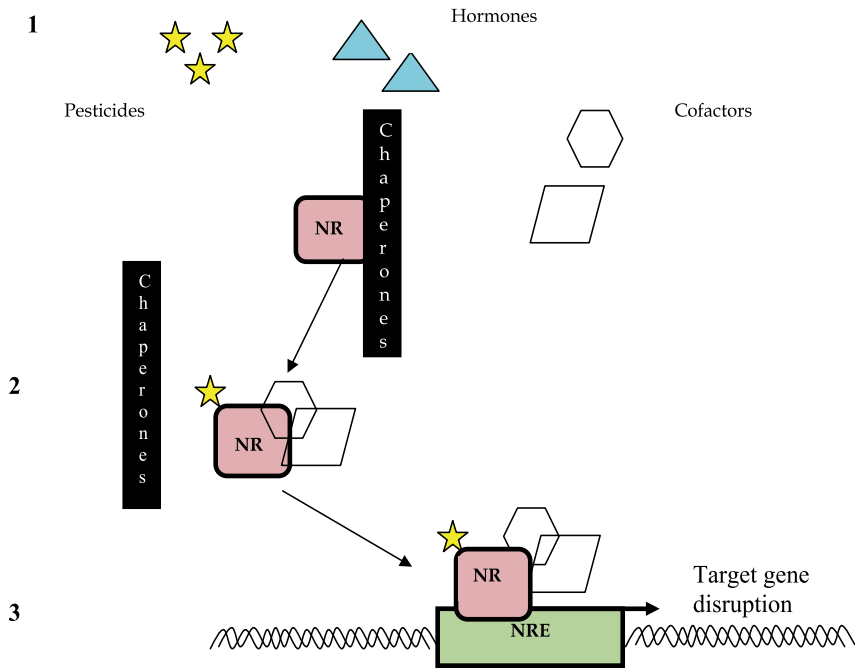

Fig. 1. Mechanistic representation of receptor-mediated endocrine disruption. (1) Endocrine disruptive Pesticides of suitable structure and affinity (illustrated with stars) bind to a nuclear receptor (NR) instead of the cognate hormone (illustrated with triangles). (2) Depending on the receptor conformation induced by the ligand, different cofactors are recruited to the ligand-NR complex. (3) The transcriptional activity of the receptor is governed through the ligand and cofactors, and may lead to inappropriate activation or repression of genes containing NR response elements (NRE) in their regulatory regions (Adapted from Rüegg et al., 2009, with modification).

2.3.1 Direct binding to the nuclear hormone receptors

The nuclear hormone receptors regulate gene transcription in response to their cognate ligands. Nuclear receptor (NR) is bound to chaperone proteins in the absence of ligand. Upon ligand binding, the chaperone complex dissociates from the receptor, leading to increased affinity of the receptor for cofactors and nuclear receptor response elements (NRE) on DNA. The activated receptor complex binds to the response elements and regulates the expression of the target gene (activation or inhibition). As many pesticides structurally resemble these cognate ligands, they too can bind to the receptors causing inappropriate signals in the cell. A pesticide with suitable structural features binds to the hormone-binding cavity or in some instances to some other part of the receptor, causing a conformational change of the receptor. This change determines the activity of the receptor: an agonistic conformation leads to recruitment of coactivators and thus increased transcriptional activity of the receptor, whereas an antagonistic conformation prevents coactivator recruitment and/or attracts corepressors, leading to decreased transcriptional activity of the receptor (Rüegg et al., 2009) (Fig. 1).

2.3.2 Xenosensor-mediated endocrine disruption competition for common coactivators

The biological function of transcription factors, including the NRs, is dependent on the availability of transcriptional cofactors. Many of these cofactors are not receptor specific but are used in several different signaling pathways in the cell. Hence, the cofactors are a potential target for endocrine disruption. If one receptor is persistently activated, the continuous recruitment of cofactors to this receptor may reduce the availability of the cofactors to other receptors, thereby impairing their activity (Fig. 2A).

2.3.3 Dysregulation of hormone metabolism

Steroid hormones are small lipophilic molecules that are synthesized from cholesterol. They have a cyclopentano-perhydrophenanthrene four-ring hydrocarbon nucleus, the so-called steroid nucleus, as a core structure. The synthesis of steroid hormones occurs primarily in the adrenal cortex, gonads (testes and ovaries) and placenta. The catabolism of sex steroids occurs in the liver in a process that closely resembles the metabolism of pesticides. To render the steroid hormones hydrophilic, they are hydroxylated and conjugated. Hydroxylation reactions are carried out by steroid hydroxylases of the cytochrome P450 family (CYP), whereas sulfotransferases and the UDP-glucuronosyltransferases are responsible for steroid conjugation. As a consequence of the similarities between pesticide and steroid metabolism, activation of the pesticide response can affect metabolism of hormones (Fig. 2B). Enzyme activation upon exposure to pesticides can lead to alterations in the endogenous levels of hormones in the organism, and subsequently compromise hormone signaling. The most important regulators of the activity of these enzymes are PXR and CAR. For example, DDT and its metabolite DDE activate PXR and CAR in rodents. In turn, they induce CYP2B, CYP3A and other steroid hydroxylases, thereby potentially perturbing normal steroid metabolism (You, 2004). We have demonstrated previously that treatment of rats with 50 and 100 mg/kg of p,p'-DDT or with 3 and 6 mg/kg of dieldrin for 10 consecutive days induced a dose-dependent decrease of the number and the motility of epididymal spermatozoa and we have hypothesized that DDT/dieldrin induction of testosterone metabolism may play a role by reducing testosterone concentrations (Ben Rhouma et al., 2001; Hallègue et al., 2003). Thyroid metabolism is another target for endocrine disruption by pesticides.

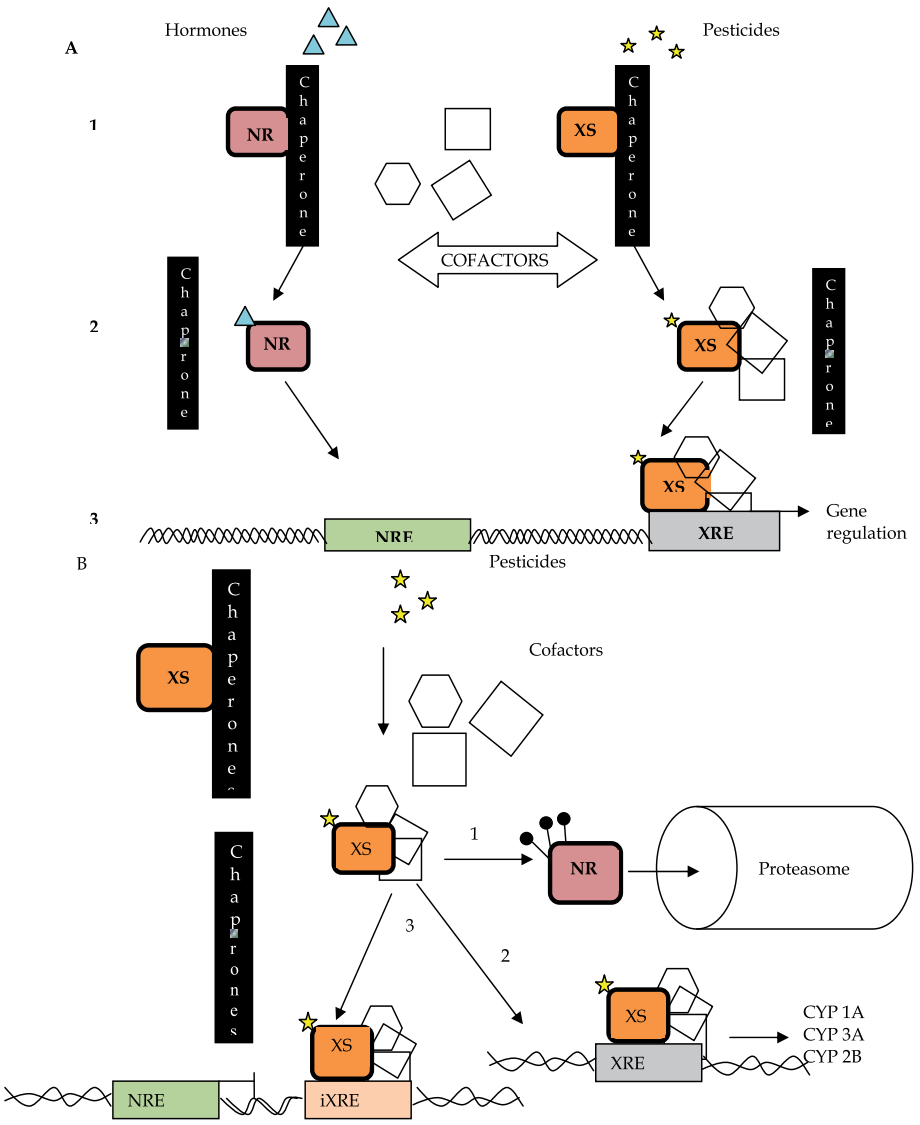

Fig. 2. Indirect mechanisms of receptor-mediated endocrine disruption. (A) Pesticides can disrupt nuclear receptor (NR) activity indirectly through competition for common cofactors: (1) When both NR and xenosensor (XS)-mediated signaling occurs in the same cell, common cofactors may become limiting factors in the signaling event. (2) When the cofactors are recruited to XS pathway, they are not sufficiently available for NR signaling. (3) NR signaling is hampered, while XS-mediated activity occurs normally. (B) More indirect mechanisms of endocrine disruption include: (1) Targeted degradation of NR in proteasome as a consequence of XS-induced ubiquitination (depicted with pins) of the receptor; (2) XS-induced transcription of enzymes involved in hormone metabolism; and (3) Binding of XS to inhibitory xenobiotic response elements (iXRE) in close proximity of NREs on DNA, blocking gene regulation through the NRE (Adapted from Rüegg et al., 2009, with modification).

We have recently shown that the same treatment with p,p'-DDT increased the metabolism of thyroid hormones and led to a decrease in serumT4 levels and hypothyroidism in rats (Tebourbi et al., 2010). Alteration of the enzymes involved in the thyroid hormone metabolism has been described as a mechanism for reducing the amount of available hormone (Curran and De Groot, 1991). Yet, we and others have shown that hepatic microsomal enzyme inducers, like DDT enhanced the glucuronidation and biliary excretion of thyroid hormones *via* induction of the hepatic T4 uridinediphospho-glucuronyltransferase (UDP-GT) (Bastomsky, 1974; Tebourbi et al., 2010).

2.3.4 Dysregulation of receptor stability

The stability of hormone receptors is an integral feature of receptor biology. Since cells need to rapidly respond to fluctuating hormone levels, the amount of the receptors has to be tightly and rapidly regulated. For example, in the absence of estrogens the ER levels are up-regulated and upon estrogen treatment, the levels quickly decrease. The degradation of several NRs occurs in the ubiquitin-proteasome pathway. Receptors are targeted for degradation with ubiquitin, a 76-amino acid protein. Polyubiquitinated proteins are transported to the proteasome, a multi-protein complex, where they are degraded. Inhibition of this pathway affects different hormone receptors differently. For instance, upon ubiquitination the transcriptional activity of AR (Lin et al., 2002) or GR (Deroo et al., 2002) is increased, whereas the activity of ER is decreased (Wijayaratne & McDonnell, 2002). Therefore, activation of CAR/PXR by pesticides may alter the amount of steroid hormone receptors in the cell, leading to disrupted estrogen and androgen signaling (Fig. 2B).

2.3.5 Inhibitory xenobiotic response elements

Ligand-activated xenosensors bind to xenobiotic response elements (XREs) in the DNA and induce the expression of target genes. Interestingly, many NR-regulated genes have also XRE-like elements in their promoters. These inhibitory XREs (iXREs) differ slightly in base composition from the response elements on xenobiotic-responsive genes, rendering the sequence capable of binding xenosensors but not of activating the downstream gene. Binding of the xenosensor (XS) to inhibitory xenobiotic response elements (iXRE) in close proximity of NREs on DNA, blocks gene regulation through the NR response elements (NRE) (Fig. 2B) (Nagel et al., 2001; Rüegg et al., 2009).

2.4 Endocrine disruption as a common causal mechanism of pesticide-induced carcinogenesis

The increased incidence of cancer over the last 50–60 years may be largely attributed to two factors: the ageing of the population and the diffusion of carcinogenic agents, present not only in the occupational, but also in the general environment. There are studies supporting evidence that lifespan exposure to carcinogenic agents, beginning during developmental life, produces an overall increase in carcinogenic processes (Soffritti et al., 2008). There are scientific evidences linking environmental changes over the time period preceding the growing incidence of some types of cancer. These changes have not stopped and the accumulation of carcinogens keeps growing (Irigaray et al., 2007). Genetic alterations, immune suppression, and malignant transformation are phenomena linked to the origin of cancer. Cancer is generally believed to arise from a single cell which has become "initiated" by mutation of a few crucial genes, caused by random errors in DNA replication or a

reaction of the DNA with free radicals or other chemical species of exogenous or endogenous origin (Hanahan & Weinberg, 2000). Carcinogenesis is indeed an extremely complex and long lasting biological process involving initiation, promotion and progression, which are three individualized steps that chronologically and sequentially contribute to cancer genesis and development through the interplay of a myriad of endogenous and exogenous causal factors (Belpomme et al., 2007). Among the numerous man made environmental chemicals used, pesticides, because of their estrogenic properties and carcinogenic potential, may in fact be common causal agents of cancers.

2.4.1 Pesticides as tumor promoters

Some xenoestrogens may possess a 1000 times lower affinity for nuclear estrogen receptors (ERs) than estradiol (Lemaire et al., 2006), meaning that they could not efficiently combine with and activate or inhibit ERs. However activation or inhibition of ERs is an extremely complex ligand-structure-dependent phenomenon, which also depends on several other factors including cell tumor-specific expression of coactivator/coregulatory proteins, gene promoters and cell environment (Katzenellenbogen et al., 1996). More precisely, mechanisms of estrogen activation involve ligand-induced dimerization of ERs, interactions with estrogen responsive elements in target gene promoters and transcriptional activation (Hall et al., 2001). ERα and ERβ are two major ER subtypes that have been evidenced in estrogen-dependent tissues. Activation of ERα stimulates cell proliferation and is associated with cancer-causing effects through tumor promotion, whereas activation of ERβ stimulates terminal cell differentiation and may contribute to anticancer effects (Foster et al., 2004). Many xenoestrogens, especially organochlorine pesticides have been shown to disrupt endocrine processes by acting as agonists on ERα and/or antagonists on ERβ (Lemaire et al., 2006) and also possibly as antagonists on androgenic receptors (ARs) (Escriva et al., 2004; Sonnenschein & Soto, 1998). Indeed, in addition to the induction of a more or less agonistic effects by interacting with ERα, many pesticides used such as chlordecone, endosulfan, aldrin, dieldrin and endrin have been shown to be associated with antagonistic effects by activating ERβ, meaning that agonistic effects involving ERα in addition to antagonistic effects involving ERβ may strongly contribute to the tumor promoting effects of these pesticides (Lemaire et al., 2006). In addition, several of the aforementioned pesticides or their metabolites have been shown to exhibit antiandrogenic effects by binding to ARs and competing with endogenous androgens, a property that reinforce their estrogenic effect. This is particularly true for p,p'-DDE (Kelce et al., 1995), HCH (Schrader & Cooke, 2000), dieldrin (Andersen et al., 2002) and chlordecone (Schrader & Cooke, 2000). Pesticides which stimulate α-aromatase that converts testosterone to 17β-estradiol and androstenedione to estrone, more precisely p,p'-DDE (You et al., 2001), chlordanes, aldrin and dieldrin (Laville et al., 2006), toxaphene (Yang & Chen, 1999) and atrazin (Laville et al., 2006; Sanderson & van den Berg, 2003) may also indirectly contribute to prostate and breast cancer promotion by increasing concentration of endogenous natural estrogens in peripheral tissues as well as in the intratumoral milieu. Also, it has been shown *in vitro* that p,p'-DDE at high concentrations could function as an inhibitor of 5α-reductase, an intraprostatic enzyme that converts testosterone to dihydrotestosterone (DHT) (Lo et al., 2007). However because it cannot be aromatized to estrogen, DHT hardly induces prostate cancer, suggesting that in addition to androgens, estrogens may play locally a major critical role in prostate carcinogenesis (Bosland, 2006).

2.4.2 Pesticides as tumor initiators

In addition to tumor promotion-induced endocrine disruption mechanisms, pesticides may be directly or indirectly mutagenic through free radical production (Cassidy et al., 2005) and may cause both tumor initiation and subsequent tumor promotion by inhibiting Gap junctional intercellular communication (GJIC) (Kang et al., 1996). Inhibition of GJIC has clearly been shown in normal epithelial breast tissue exposed to relatively high concentrations of organochlorine pesticides. Indeed DDT, dieldrin, toxaphene or mixtures of one of these pesticides with HCB (Kang et al., 1996) have been shown to inhibit GJIC and therefore may contribute to carcinogenesis through this mechanism. Indeed, during tumor initiation, blockage of GJIC between normal and preneoplasic cells consequently create an appropriate intratissue microenvironment leading initiated cells to escape growth control from normal surrounding cells and therefore indirectly contribute to tumor promotion (Klaunig et al., 1990; Klaunig & Ruch, 1990). This may also be the case for several non organochlorine pesticides more recently used, such as the quinonoid herbicide Paraquat (Ruch & Klaunig, 1988), which has been proved to block GJIC in mouse hepatocytes (Klaunig et al., 1990; IARC, 1997) and thus possibly contribute to carcinogenesis through this mechanism.

3. Oxidant-mediated responses to pesticide exposure

The areas of oxidative stress have received intensive investigation by the biomedical community in recent years. These studies have elucidated endogenous and xenobiotic-mediated mechanisms of reactive oxygen specie (ROS) and reactive nitrogen specie (RNS) production, antioxidant defense mechanisms and deleterious consequences of oxyradical fluxes that outstrip detoxification pathways. Oxidant-mediated effects include adaptive responses (such as increased activities of antioxidant enzymes) and oxidant-mediated toxicities (such as oxidations of proteins, lipids, nucleic acids and disrupted tissue redox status). Oxidative stress in the context of pesticide metabolism is briefly reviewed here and specific biochemical responses to examples of pesticides with relevance to some common diseases are discussed.

3.1 Overview of oxygen toxicity and antioxidant defenses

Oxidative stress is caused by an imbalance between the production of reactive oxygen and the ability to: (1) detoxify the reactive intermediates produced or (2) repair the resulting damage. Ultimately, oxidative stress conveys the biomolecular alteration in cellular function caused by the reaction of reactive species with cellular constituents. Reactive oxygen species (ROS) include oxygen (O_2)-derived free radicals (defined as molecules with one or more unpaired electrons in an outermost valence shell) such as superoxide anion ($\bullet O_2^-$) and the hydroxyl radical ($\bullet OH$), as well as nonradical derivatives of O_2 such as hydrogen peroxide (H_2O_2). ROS production is the result of an aerobic environment. A significant amount of O_2 consumed by mitochondria is converted to $\bullet O_2^-$, although it can be produced through various enzymatic oxidation reactions catalyzed by cytochrome P450 (phase I detoxifying enzymes), other oxidoreductases and also by nicotinamide adenine dinucleotide phosphate)-oxidase (NADPH oxidase). $\bullet O_2^-$ reacts at diffusion-controlled rates with nitric oxide ($\bullet NO$) produced by $\bullet NO$ synthases (NOS) leading to the formation of a wide diversity of oxidizing and nitrosating/nitrating species such as peroxynitrite ($ONOO^-$) (Fig. 3). $\bullet O_2^-$ is also dismutated non enzymatically or

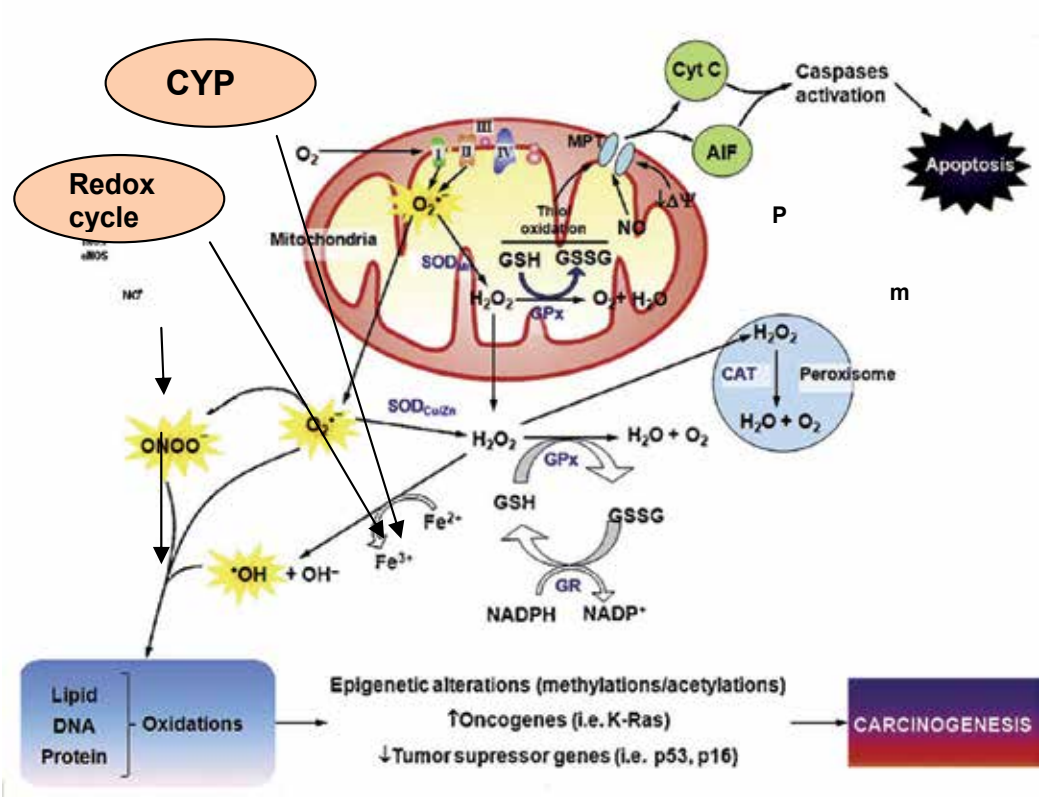

Fig. 3. Pesticides promote oxidative stress leading to cell death or procarcinogenic mutations. This figure schematically illustrates the complex molecular network activated different pesticides. Abbreviations used are: AIF, apoptosis-inducing factor; CAT, catalase; CYP, Cytochrome P450; CytC, cytochrome C; eNOS, endothelial nitric oxide synthase; GSH, glutathione; GSSG, glutathione disulphide; iNOS, inducible nitric oxide synthase; MPTP, mitochondrial permeability transition pore; SODCu/Zn, copper/zinc-type superoxide dismutase; SODMn, mangano-type superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; ΔΨm, mitochondrial transmembrane potential (Adapted from Mena et al., 2009, with modification).

enzymatically with the aid of superoxide dismutases (SODs) to hydrogen peroxide (H_2O_2). H_2O_2 can be also utilized by myeloperoxidases (MPO) to produce hypochlorous acid and other noxious chlorine-derived oxidants. Furthermore, H_2O_2 can be reduced to $\bullet OH^-$ through Fenton type reactions (Fig. 3). Thus it is clear that the formation of a reactive species can ultimately lead to an amplification chain generating other more toxic reactive species. Cells have intrinsic antioxidant mechanisms to detoxify ROS generated under both physiological and pathological conditions. Reduced glutathione (GSH) is the most important antioxidant molecule in the cell, and due to its high cytosolic concentration, it can directly scavenge ROS such as $\bullet O_2^-$, •OH and •NO. H_2O_2 is reduced to H_2O by GSH peroxidases (GPX) and catalase (CAT). The GSH reductase (GR) and thioredoxin (Trx)/thioredoxin reductase systems regenerate cellular GSH or reduced Trx, respectively, at the expense of NADPH (Fig. 3). Other antioxidant molecules (such as ascorbate or

vitamin E) and enzymes (such as peroxiredoxins) are important defenses against oxidative stress. Oxidative stress arises if detoxification systems and antioxidants are compromised or if ROS production is excessive, resulting in DNA, protein, and lipid oxidation (Fig. 3) (Franco et al., 2009, 2010; Ryter et al., 2007). Oxidative damage to DNA leads to the formation of lesions such as 8-oxo-deoxyguanosine, 8-oxo-deoxyadenosine, and deoxythymidine glycol which are selectively excised from DNA by DNA glycosylases. Lipid peroxidation refers to the oxidative degradation of lipids. Lipid peroxidation is initiated through a radical-mediated abstraction of a hydrogen atom from polyunsaturated fatty acids to make water and a fatty acid radical. Lipid peroxyl radicals (LOO•) are formed by the reaction of free fatty acid radicals with oxygen that subsequently reacts with another free fatty acid producing a different fatty acid radical and a lipid peroxide propagating the damage. Lipid peroxidation generates a number of lipid hydroperoxide products such as malondialdehyde, 4-hydroperoxy-2-nonenal, 4-oxo-2-nonenal and 4-hydroxy-2-nonenal (4HNE). These aldehyde products react with individual nucleotides and nucleophilic amino acids, thus inducing several signaling effects (West & Marnett, 2006). Oxidative protein modifications have been shown to regulate the activity of a wide variety of proteins such as kinases, phosphatases, proteases (caspases), molecular adaptors and chaperones, and transcription factors. Amino acids such as cysteine, methionine, tryptophan, and tyrosine residues are prone to specific oxidative modification. Oxidative protein modifications in general can be classified as reversible and irreversible. Highly reactive oxidant species such as hypochlorous acid, $ONOO^-$, and •OH are thought to oxidize biomolecules leading to the irreversible formation of, for example, 3-nitrotyrosine and protein carbonyls. Physiological oxidants such as $•NO$, $•O_2^-$ and H_2O_2, have been implicated in reversible protein modifications at the cysteine level (nitrosylation, hydroxylation, glutathionylation, disulfide bond formation) that underlie homeostatic control and diverse biological responses. A wide variety of enzymes regulate these post-translational modifications including sulfiredoxins, thioredoxins, glutaredoxins and methionine sulfoxide reductases (Dalle-Donne et al., 2008; Janssen-Heininger et al., 2008).

Pesticides are known to induce oxidative stress by: (1) the induction of reactive oxygen species (ROS) as byproducts of detoxifying metabolism, (2) alterations in the mitochondrial respiration or (3) by their own redox (reduction/oxidation) cycling properties per se. In the redox cycle, the parent compound typically is the first enzymatically reduced by a NADPH-dependent reductase (such as NADPH-cytochrome P450 reductase) to yield the pesticide radical. This radical donates its unshared electron of O2, yielding (• $O2^-$) and the parent compound. The latter can undergo another cycle. At each turn of the cycle, therefore, two potentially deleterious events have occurred: a reductant has been oxidized and an oxyradical has been produced.

3.2 Oxidative stress and cell death

3.2.1 Types of cell death

It is well known that environmental toxicants exert their toxicity, at least in part, by triggering cell death. Cell death is classified by biochemical and morphological criteria. According to the recommended classification of cell death (Kroemer et al., 2009), three distinct types of cell death pathways can be defined according to morphological criteria which are necrosis, apoptosis and autophagy, although there are numerous examples in which cell death displays mixed features.

Necrotic cell death is characterized by a gain in cell volume, swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents. It is now recognized that execution of necrotic cell death may be regulated by a series of signal transduction pathways and catabolic processes (Kroemer et al., 2009).

Autophagy is a major catabolic pathway by which eukaryotic cells degrade and recycle macromolecules and organelles, leading to cell survival. It plays an essential role in differentiation and development, as well as in cellular response to stress. Autophagy can be activated during amino acid deprivation and has been associated with neurodegenerative diseases. Autophagy is initiated by the surrounding of cytoplasmic constituents by the crescent-shaped isolation membrane/phagophore, which forms a closed double membrane structure, called autophagosome. The autophagosome subsequently fuses with a lysosome to become an autolysosome, and its content is degraded by acidic lysosomal hydrolases. When autophagy is prolonged, this could lead to cell death. Autophagic cell death is morphologically defined by massive autophagic vacuolization of the cytoplasm in the absence of chromatin condensation (Kroemer et al., 2009).

Apoptosis, or programmed cell death, is an evolutionary conserved homeostatic mechanism involved in a variety of biological processes. Under physiological conditions, apoptosis is important not only for the constant turnover of cells in all tissues but also during the normal development and senescence of the organism. Apoptosis is a highly organized process characterized by the progressive activation of precise signaling pathways leading to specific biochemical and morphological alterations. Initial stages of apoptosis are characterized by cell shrinkage, loss of membrane lipid asymmetry and chromatin condensation, while later stages are associated with the activation of execution caspases (cysteine-dependent aspartate-directed proteases) and endonuclease, apoptotic body formation and cell fragmentation Galluzzi et al., 2007; Hengartner, 2000). Both extrinsic and intrinsic pathways have been described for the activation of apoptosis. Induction of apoptosis via the extrinsic pathway is triggered by the activation of death receptors leading to the formation of the death-inducing signaling complex (DISC) by the recruitment of the Fas-associated death domain (FADD) and initiator caspase 8. Death receptor-induced apoptosis is amplified by cleavage of the B-cell lymphoma 2 (Bcl-2) family protein Bid by caspase 8, which triggers the mitochondrial pathway of apoptosis (Barnhart et al., 2003; Khosravi-Far & Esposti, 2004) (Fig. 4). The intrinsic pathway of apoptosis, also referred to as the mitochondrial pathway, is activated by a wide variety of cytotoxic stimuli or environmental stressors. Although the mechanisms by which these stimuli trigger apoptosis differ between them, they convey the opening of mitochondrial permeability transition pores (MPTP) that mediates the disruption of the mitochondrial transmembrane potential ($\chi\Psi m$) and the release of pro-apoptotic proteins from the mitochondria including cytochrome C (Fig. 3 & Fig. 4).

However, the exact mechanisms mediating cytochrome C release are still controversial (Franco et al., 2010). Distinct mitochondrial components and mitochondrial released proteins such as apoptosis inducing factor (AIF), endonuclease G (EndoG), adenine nucleotide translocase (ANT), cyclophilin D, Bit1, p53-regulated Apoptosis Inducing Protein 1 (p53AIP), gene associated with retinoic-interferon-induced mortality 19 (GRIM-19), death associated protein 3 (DAP3), Nerve Growthfactor IB (Nur77/TR3/NGFB-1), HtrA serine peptidase 2 (HtrA2)/ second mitochondria-derived activator of caspases (Omi and Smac)/Diablo have been proposed to participate in the mitochondrial pathway to apoptosis (Ekert & Vaux, 2005). The intrinsic pathway is also regulated by the Bcl-2 family of proteins.

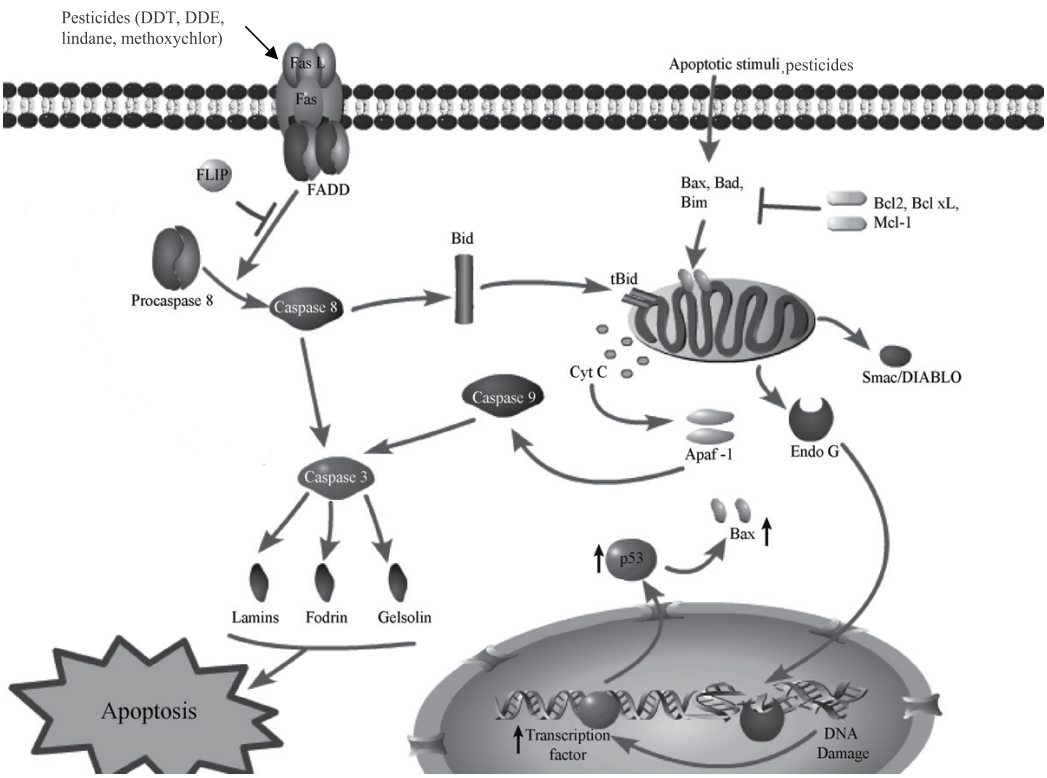

Fig. 4. A schematic diagram shows the various molecular components involved in the extrinsic and the intrinsic pathways of apoptosis. Two pathways are represented. The intrinsic pathway involving the mitochondria and the extrinsic pathway with the Fas/FasL with their corresponding downstream regulators are shown. The substrates of caspase 3, including lamins, fodrin and gelsolin are indicated as these are the primary components that are acted upon by caspase 3 leading to fragmentation of the cell. The activation of p53 after DNA damage is shown (Adapted from Tripathi et al., 2009, with modification)

The Bcl-2-associated death promoter (Bad), Bcl-2-interacting domain (Bid), Bcl-2-interacting killer (Bik), NOXA, and p53 upregulated modulator of apoptosis (PUMA) regulate the anti-apoptotic Bcl-2 proteins : Bcl-2 and B-cell lymphoma-extra large (Bcl-xl) to promote apoptosis. Bcl-2 and Bcl-xl inhibit Bcl-2–associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak) by direct binding and inhibition of Bcl-2 and other anti-apoptotic family members. Bax and Bak are known to mediate the release of cytochrome C. Released cytochrome C leads to the recruitment of the apoptotic protease activating factor 1 (APAF1) into the apoptosome and activation of caspase-9 (Ravagnan et al., 2002; Youle & Strasser, 2008) (Fig. 4). Once activated, initiator caspases converge in the cleavage/activation of execution caspases 3, 6 and 7 which further cleave different cellular substrate leading to the organized demise of the cell. A wide variety of enzymes such as protein kinases, phosphatases, calpains, transcription factors and several other adaptor or scaffolding proteins have been described to participate in several pathways of apoptosis in distinct ways (Saelens et al., 2004; Youle & Strasser, 2008). Other intrinsic pathways of apoptosis, such as endoplasmic reticulum (ER) stress and DNA damage, have been described, which can be dependent or independent from the mitochondrial pathway. The ER is highly

sensitive to stressors that perturb cellular energy levels, the redox state and/or Ca^{2+} concentration. Such stress results in the accumulation and aggregation of unfolded proteins which are toxic to cells. ER stress leads to the activation of the stress-activated protein kinase (SAPK), c-Jun N-terminal kinases (JNK) and induction of C/EBP homologous protein (CHOP), which by impairment of the anti-apoptotic function of Bcl-2, lead to the activation of Bim, Bax and Bak, transmission of the signal from the ER to the mitochondria and execution of death by activation of caspases (Boyce & Yuan, 2006; Szegezdi et al., 2006). DNA damage is also known to trigger apoptosis. Blockage of DNA replication associated with DNA damage leads to the activation of p53 which induces the transcriptional activation of pro-apoptotic factors. However, non-transcriptional regulation of apoptosis by p53 and p53-independent pathways have also been described (Roos & Kaina, 2006).

3.2.2 Oxidative stress and apoptosis

Although oxidative stress has been largely linked to the activation of distinct apoptotic enzymes, the direct mechanisms involved have remained largely elusive. Oxidative stress-induced apoptosis has been largely associated to the activation of the intrinsic pathways of apoptosis at the level of the mitochondria. One important target of ROS is the mitochondrial DNA (mtDNA) due to the close proximity to the electron transport chain and the lack of protective histones. Oxidative mtDNA damage induced by ROS leads to lethal cell injury due to mitochondrial genomic instability leading to respiratory dysfunction through the disruption of electron transport, mitochondrial membrane potential, and ATP generation. ROS in the mitochondria can also directly oxidize and inactivate proteins such as mitochondrial aconitase and complex I NADH oxidase leading to further ROS overload. Lipid peroxidation at the level of the mitochondria also impairs mitochondrial metabolism and induction of the mitochondrial permeability transition pores (MPTP) (Ott et al., 2007). In addition, oxidative reactions causing glutathione (GSH) exhaustion, followed by thiol oxidation would favor MPTP opening (Marchetti et al., 1997). Similarly, loss of GSH *via* non oxidative mechanisms, as in the case after Fas-cross-linking, might indirectly favor this induction of MPTP (Vandendobberlsteen et al., 1996). However, the relationship between MPTP and thiol redoxidation processes is probably not unilateral. Thus, MPTP itself affects cellular GSH levels. Due to its uncoupling effect on the respiratory chain, MPTP causes an immediate depletion of reduced $NADH_2$/$NADPH_2$, which, in turn, causes the oxidation of mitochondrial and cytosolic GSH *via* the glutathione peroxidase reaction (Hoek & Rydström, 1988). Moreover, one consequence of MPTP is uncoupling of the respiratory chain with hyperproduction of superoxide anions, which would favor GSH depletion (Zamzami et al, 1995). This depletion can be prevented by Bcl2, which functions as an endogenous inhibitor of MPTP induction and thus acted as an antioxidant (Hockenberry et al., 1993). Finally, cytochrome C which is bound to the inner mitochondrial membrane by an association with the anionic phospholipid cardiolipin has been shown to be released *via* oxidation of cardiolipin during apoptosis which precedes its release to the cytosol (Ott et al., 2007). ROS and RNS have been shown to directly trigger the activation of distinct signaling cascades induced by ER stress including activation of JNK and dissociation of the tumor necrosis factor receptor-associated factor-apoptosis signal-regulating kinase1 (TRAF2–ASK1) complex, transcriptional activation of CHOP, and caspase activation (Fig. 5). Protein disulfide isomerase (PDI) which is the most abundant chaperone in the ER facilitates the folding and disulfide bond formation of its protein substrates. PDI is regulated not only by post-translational oxidative modifications (nitrosylation and glutathionylation) but also by the ER oxidase (ERO1), which restores reduced PDI to an oxidized state through disulfide exchange with ERO1. ERO1 activity is also

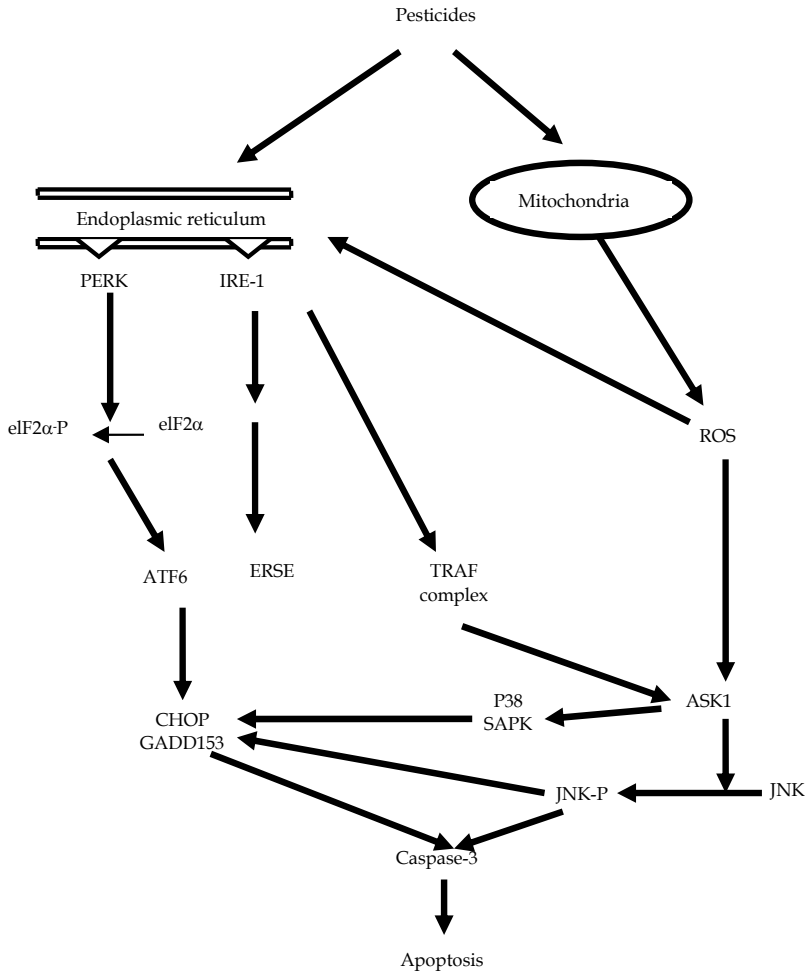

Fig. 5. Oxidant and ER stress pathways involved in apoptosis. Abbreviations used are: ASK, apoptosis signal-regulating kinase; ATF, activating transcription factor; CHOP, C/EBP homologous protein; elF2α, eukaryotic initiation factor 2α; ERSE, endoplasmic stress response element; GADD, growth arrest and DNA damage; IRE, inositol-requiring ER-to-nucleus signal kinase; JNK, c-jun N-terminal kinase; PERK, protein kinase-like ER kinase; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; TRAF, tumor necrosis factor receptor-associated factor (Adapted from Katsoulieris et al., 2010, with modification).

regulated through modulation of non catalytic cysteine residues and has been shown to be inhibited under oxidized conditions in the ER (Townsend, 2007). Redox imbalance in the ER lumen is the most frequent cause of ER stress-induced apoptosis, which involves the impairment of oxidative protein folding, the accumulation of unfolded/misfolded proteins in the lumen and the initiation of the unfolded protein response (UPR). In the ER, changes in the luminal redox state are sensed by secretory proteins to be folded (via ER chaperone Bip),

and also directly by transmembrane proteins involved in signaling such as activating transcription factor 6 (ATF6) (Fig. 5).
Several antioxidant protective systems such as glutathione, ascorbate, flavin adenine dinucleotide (FAD), tocopherol and vitamin K exist in the ER. Formation of disulfide bonds is required for the proper folding of secretory and membrane proteins in the ER and thus, redox imbalance leads to the accumulation of unfolded/misfolded proteins in the ER lumen. Redox-sensitive thiols in the ryanodine receptor calcium channel are also targets for oxido-reduction that regulates the open probability of the channel. More recently, the InsP3 receptor (InsP3R) has also been demonstrated to be regulated by ER luminal redox status through a direct interaction with ERp44, a thioredoxin family member (Banhegyi et al., 2007).
Thiram chemicals have been reported to induce GSH depletion which is paralleled by protein carbonylation, lipid peroxidation and subsequent apoptotic cell death (Grosicka et al., 2005). Carbamate derivatives such as mancozeb and pyrethroids such as cyermethrin have also been shown to induce oxidative stress, DNA damage and activation of the mitochondrial pathway of apoptosis (Calviello et al., 2006; Jin et al., 2011).

3.2.3 Oxidative stress and autophagy

Interaction mechanisms between autophagy and oxidative stress induction are still not clear. It was demonstrated that ROS can induce autophagy through several distinct mechanisms involving autophagic gene 4 (Atg4), catalase, and the mitochondrial electron transport chain (mETC). This leads to both cell survival and cell death responses. $\bullet O_2^-$ is the major ROS regulating autophagy (Chen et al., 2009). Recent studies provide strong evidences for the involvement of mitochondrially-generated ROS production in the induction of autophagy (Chen & Gibson, 2008; Chen et al., 2009). Recently, it has been demonstrated that targeting mETC complex I by rotenone, induces autophagic cell death through a ROS-mediated mechanism (Chen et al., 2007).

3.2.4 Oxidative stress and necrosis

Exposure to oxidants causes multiple intracellular alterations, including elevation of cytosolic Ca2+, depletion of ATP, oxidation of NADH, reduced glutathione (GSH) and lipids (Slater et al., 1995). Necrosis is the characteristic end point of such a dramatic disturbance in cell homeostasis. Many studies have demonstrated that raising the concentration of the oxidant was observed to shift the form of cell death away from apoptosis towards necrosis, probably via a rapid ATP and GSH depletion induced by acute oxidant doses (Orrenius, 1993; Slater et al., 1995). Inhibition of caspase activity or transient inhibition of the production of caspase can switch cells from an apoptotic to necrotic death mode (Nicotera & Melino, 2004). Cells with low levels of Bcl-xL die by apoptosis, whereas high levels of Bcl-xL can induce necrotic cell death (Abdelhaleem, 2002). Cell lysis seems to be a more prominent route of cell death when apoptosis is defective, presumably due to the limited options by which a cell has to die (Degenhardt et al., 2006). Autophagy can delay cell death by apoptosis in response to metabolic stress. Moreover, cells with defective apoptosis, and in which the autophagic pathway is disrupted, are forced to die by necrosis when stressed (White, 2008). Recently, it has been shown that lindane disrupts the autophagic pathway and inhibits spontaneous apoptosis, leading to necrosis in primary cultured rat hepatocytes (Zucchini-Pascal et al., 2009).

4. Oxidative stress-mediated cell death/survival of pesticides: relevance to common diseases

The majority of cases of diseases are not inherited and thus it has been proposed that a complex array of environmental factors and gene interactions might exert synergetic effects toward the predisposition to sporadic diseases. In this section, we will focus on the pesticide-induced oxidative stress as possible mechanism involved in the regulation of death/survival signaling pathways in cells due to their relevance to some diseases.

4.1 Cancer

It is generally accepted that ROS eventually cause DNA damage. In addition phase I pesticide detoxifying enzymes generate electrophilic compounds like carcinogenic epoxides, whereby insufficient cellular repair mechanisms may contribute to premature aging and apoptosis. Conversely, ROS-induced imbalances of the signaling pathways for metabolic protein turnover may also result in opposite effects to recruit malfunctioning aberrant proteins and compounds that trigger tumorigenic processes (Wang, 2008). Consequently, DNA damage plays a role in the development of carcinogenesis, but is also associated with an aging process in cells and organisms (Bertram & Hass, 2008). Hence, additional actions of ROS must be important, possibly their effects on p53, cell proliferation, invasiveness and metastasis. Chronic inflammation predisposes to malignancy, but the role of ROS in this is likely to be complex because ROS can sometimes act as anti-inflammatory agents (Halliwell, 2007). The mutagenic origin of cancer may be due, in some cases, to exposition to different carcinogens present in our environment (Claxton & Woodall, 2007). These mutations can affect genes of relevant xenobiotic metabolizing enzymes, produce polymorphisms leading to altered ligand affinity and activity, or influencing the expression of downstream target genes (Dong et al., 2008). Besides, although polymorphisms in oxidative stress-related genes (e.g. mangano-type superoxide dismutase, catalase, or glutathione peroxidase) may not be directly associated with cancer risk, it is possible that accumulative defects in protection from oxidative stress may result in increased risk of the disease (Ryter et al., 2007).

Piperonyl butoxide (PBO), is a pesticide widely used along with pyrethroids as grain protector and domestic insecticide (Muguruma et al., 2007). PBO is capable of increasing the gene expression of *CYP1A1*, a cytochrome P-450 isoform and the most active enzyme catalyzing procarcinogens (Canistro et al., 2002). PBO has a liver-tumor-promoting effect increasing production of ROS as a byproduct of hepatic microsomal oxidation in mice (Muguruma et al., 2007). The increase of ROS is due to PBO-induced regulation of glutathione reductase (GR), NADPH: quinone oxidoreductase 1 (NQO_1), and other antioxidant enzymes. This regulation is under the nuclear factor erythroid 2 p45-related factor 2 (Nrf2) (Ellinger-Ziegelbauer et al., 2005). NQO_1 that detoxifies quinones and thus decreases ROS formation (Yang et al., 2005), has been found within solid tumors of the breast, ovary, lung, colon, thyroid, and adrenal gland in an elevated concentration (Siegel & Ross, 2000). Organochlorine pesticides like DDT and dieldrin, are persistent organic pollutants that may accumulate in the environment and food. They are lipophilic and can be detected, after ingestion, in human breast milk and adipose tissue (Cok et al., 1997; Malarvannan et al., 2009). They are particularly harmful during pregnancy (these compounds cross the placenta and reach the fetus blood) and after birth (neonates are exposed through the breast milk) (Perera et al., 2005). These pesticides induce hepatic cell proliferation and are known as non genotoxic hepatocarcinogens (Stevenson et al., 1999).

4.2 Parkinson's disease

Parkinson's disease (PD) is characterized by abnormalities of motor control such as resting tremors, bradykinesia (slowness of voluntary movement), rigidity, and a loss of postural reflexes. A number of epidemiologic studies have found an association between PD and exposure to pesticides. Furthermore, increased levels of pesticides have been found in brains of PD cases versus controls. Contradictory studies have also been published demonstrating no association between PD and pesticide exposure (Brown et al., 2006; Hatcher et al., 2008; Landrigan et al., 2005). It is clear now that PD is a multi-factorial disease with a complex etiology including genetic risk factors, environmental exposure and aging (Benmoyal-Segal & Soreq, 2006; Palomo et al., 2004). Nevertheless, the study of neurotoxic properties of paraquat, diquat, maneb (Meco et al., 1994; Zhang et al., 2003), rotenone (Coulom & Birman, 2004; Hartley et al., 1994), glyphosate pesticides (Negga et al., 2011) and organochlorine pesticides (DDT and dieldrin) (Fleming et al., 1994), has provided valuable information regarding the potential mechanisms involved in the progression of neurodegeneration associated with environmental toxicity. Parkinson's disease (PD) is characterized by a selective degeneration of dopaminergic neurons in the *substantia nigra* (SN) *pars compacta* attributed to the toxic accumulation and aggregation of proteins, mitochondrial dysfunction and oxidative stress. The occurrence of oxidative stress has been observed in the SN of PD brains as evidenced by increased lipid, protein, and DNA oxidation, increased total iron content, and significant decreases in GSH and GSH/glutathione disulfide (GSSG) ratio (Mattson, 2006). The main pathway of cell toxicity in PD involves misfolding and aggregation of α-synuclein (Okouchi et al., 2007). Failure of α-synuclein clearance by the ubiquitin-proteasome system (UPS) leads to its accumulation over time and to the formation of fibrillar aggregates and Lewy bodies. α-Synuclein protofibrils can directly lead to oxidative stress that can further impair the UPS by reducing ATP levels, inhibiting the proteasome, and by the oxidation of parkin. Exposure to paraquat maneb rotenone and dieldrin has been shown to induce proteasome dysfunction and α-synuclein aggregation (Barlow et al., 2005; Ding & Keller, 2001; Fei et al., 2008; Sun et al., 2005; Zhou et al., 2004). Furthermore, paraquat has been shown to potentiate α-synuclein-induced toxicity (Norris et al., 2007). It has been hypothesized that mutated α-synuclein induces a reduction in vesicle number and the accumulation of cytoplasmic dopamine in association with enhanced ROS generation and initiation of the apoptotic cascade (Wood-Kaczmar et al., 2006). In the cytosol, dopamine is metabolized by monoamine oxidase which generates H_2O_2, or is auto-oxidized generating $\bullet O_2^-$, H_2O_2 and dopamine-quinone species (Abou-Sleiman et al., 2006). In the brain, specific cell types have been reported to express cytochrome P450 enzymes. The presence of cytochrome P450 enzymes in the brain should also be important in inducing bioactivation and cellular damage of pesticides. Many results support the possibility of a local metabolism of pesticides and other pollutants in the brain by cytochrome P450 enzymes into neurotoxic compounds, suggesting that brain metabolism could be a factor modulating the individual susceptibility to Parkinson's disease during pesticide exposure. Moreover, the involvement of mitochondrial superoxide in the neurodegenerative process is demonstrated. Recently, paraquat, maneb and dieldrin was reported to act at the level of complex III to generate ROS, whereas rotenone inhibit electron flow through complex I (Castello et al., 2007; Drechsel & Patel, 2009). It is clear that oxidative stress is a central player in the regulation of paraquat-induced neuronal cell death. The ability of paraquat to cause oxidative damage through a free radical mechanism may explain the selective vulnerability of dopaminergic neurons which are highly susceptible to oxidative damage

due to the pro-oxidant properties of dopamine. Interactions with glial cell types play a role in potentiating or reducing oxidative stress (Vogt et al., 1998). Paraquat has been reported to induce necrosis when injected into different areas of the rat brain. However, this effect might be observed just at high doses (Calo et al., 1990). Neurons from patients with PD display characteristics of autophagy. Recent studies have demonstrated that low concentrations of paraquat induce autophagy which is followed by apoptosis. Because inhibition of autophagy potentiated apoptosis induced by paraquat, it was proposed that autophagy might be acting as a protective mechanism against cell death progression (Gonzalez-Polo et al., 2007). Neurodegenerative diseases are most commonly associated with selective neuron loss by apoptosis. Paraquat-induced neural cell death has been demonstrated to involve primarily the activation of apoptosis (Franco et al., 2010). In PD, cell death by apoptosis has been proposed to result from mitochondrial dysfunction, leading to an increase in oxidative stress and a decline in ATP production. Paraquat and rotenone induce cytochrome C release (Ahmadi et al., 2003; Fei et al., 2008) and caspase-9 activation, which are preceded by the induction/activation of pro-apoptotic Bax and Bak (Fei et al., 2008; Yang & Tiffany-Castiglioni, 2008). Induction of pro-apoptotic Bax and apoptosis in response to paraquat have also been reported to be dependent on p53 (Yang & Tiffany-Castiglioni, 2008). Paraquat neurotoxicity has also been recently reported to require the activation of stress activated protein kinases (SAPK) (Niso-Santano et al. 2010; Yang et al., 2009). Interestingly, exposure to maneb enhances Bax-dependent cell death through an increased induction of Bax-activators Bik and Bim (Fei & Ethel, 2008). Dieldrin has been shown to induce apoptosis *via* GSH depletion and oxidative stress, triggering the intrinsic mitochondrial apoptotic pathway (Kitazawa et al., 2004). Dichlorodiphenyltrichloroethane (DDT) derivatives have been shown to induce neural cell death by apoptosis through the activation of mitogen-activated protein kinases (MAPKs) (Shinomiya & Shinomiya, 2003). Recently, paraquat and rotenone have been shown to induce DNA damage and ER stress. ER stress was associated with the activation of the inositol-requiring enzyme 1 (IRE1), apoptosis signal regulating kinase 1 (ASK1), and JNK (Niso-Santano et al. 2010; Yang et al., 2009). Rotenone has also been reported to trigger ER stress via the activation of the PKR-like ER kinase (PERK) (Ryu et al., 2002). Rotenone has been demonstrated to induce activation of the glycogen synthase kinase 3 (GSK-3), JNK and p38 kinases, whose activity seems to be required for the progression of apoptosis (Newhouse et al., 2004).The molecular mechanisms linking paraquat-induced oxidative stress and neural apoptosis are still largely elusive. Recently, paraquat-induced oxidation of Trx has been reported as a possible mechanism for the activation of the ASK1/JNK signaling pathways. Accordingly, Nrf2-dependent regulation of Trx levels determines the sensitivity of paraquat toxicity by activation of the ASK1/JNK p38 signaling (Niso-Santano et al. 2010). Paraquat-induced tyrosine nitration and lipid peroxidation (4HNE) have been recently demonstrated (McCormack et al., 2005). However, the molecular targets for these signaling events remain to be elucidated. It was recently demonstrated that oxidative stress induced by paraquat generates protein aggregation of the plasma membrane Ca2+-ATPase (PMCA) and its degradation by calpain (Zaidi et al., 2009).

4.3 Reproductive disorders

A scientific finding on human reproduction showed that infertility may affect 15–20% of couples in industrialized countries (Oehninger, 2001) compared to 7–8% during early 1960s. The concern over decreased sperm count and male reproductive capacity was triggered by a

report of Carlsen et al. (1992) on the meta-analysis of 61 sperm count studies which showed a nearly 50% decrease in sperm counts between 1940 and 1990 worldwide and this decrease amounts to about two percent per year over the last two decades (Auger et al., 1995). The full mechanism of male infertility is poorly understood. The testis is one tissue where a large incidence of apoptosis occurs to discard excessive germ cells, or whereby germ cells damaged by toxins are removed (Hikim et al., 2003). Few studies have been conducted on pesticide-induced cell death in reproductive tract. Song et al. (2008) showed that p,p'-DDE could activate apoptosis of cultured rat Sertoli cells in a pro-oxidant and mitochondria-dependent manner by activating the intrinsic programmed cell death pathway. The same authors have demonstrated also that p,p'-DDE increased the apoptotic rate of isolated Sertoli cells (Shi et al., 2009) and germinal cells *in vivo* (Shi et al., 2010), by a mechanism possibly involving FasL-dependent pathway. Exposure to p,p'-DDE can enhance ROS and oxidative stress, then induce activation of Fas-FasL. Consequently, an intrinsic program of apoptotic death is stimulated in a target cell leading to the activation of caspase 8. Finally, apoptosis of Sertoli cells and germinal cells is mediated by a terminal executioner, caspase 3, thereby disturbing the spermatogenic process (Shi et al., 2010). More recently, p,p'-DDE has also been reported to induce Sertoli cell apoptosis via p38 MAPK pathway (Song et al., 2011). Similarly, lindane and methoxychlor have been shown to induce testicular apoptosis in rats through the involvement of Fas-FasL and mitochondria-dependent pathways (Saradha et al., 2009; Vaithinathan et al., 2010). These data provided additional mechanisms explaining pesticide-induced endocrine disruption. However, there's a striking lack of data concerning pesticide-induced cell death in females.

4.4 Immune dysfunction

It is important to remember that apoptosis plays a variety of important roles under normal physiological conditions, but when it is out of regulation, apoptosis can contribute to several diseases as immunodeficiency, autoimmunity diseases and cancer. It seems evident that the uncontrolled elimination of immune cells may account for immunosupression or immune dysregulation (Gougeon et al., 1996). In a previous study, we have reported the induction of apoptosis in rat thymocytes exposed for 6 hours to p,p'-DDT at a concentration of 7 µg/mL (20 µM/mL) (Tebourbi et al., 1998). As shown in Fig. 6, DNA fragmentation is negligible in thymocytes immediately after isolation and for control cells after 6 hours of incubation in free medium, whereas thymocyte exposure to DDT resulted in a DNA ladder, typical of apoptosis, similar to that observed in dexamethasone (DEXA)-treated cells. Furthermore, as for DEXA, DDT fragmentation induced by DDT is inhibited by zinc (1mM) which is known as an antioxidant. This result suggests the involvement of oxidative stress mechanism in the apoptotic action of DDT. By this apoptotic effect, DDT could have a profound immunotoxic action similar to that caused by corticosteroid hormones (Tebourbi et al., 1998). Pérez-Maldonado et al. (2004) have demonstrated that *in vitro* o'p-DDT, p'p-DDT, p'p-DDE and p'p-DDD are able to induce apoptosis of human peripheral blood mononuclear cells (PBMC), through the production of reactive oxygen species (Pérez-Maldonado et al., 2005). Furthermore, a preliminary association between the percentage of apoptotic cells and the levels of DDT and its metabolites in blood of exposed children was reported in a pilot study (Pérez-Maldonado et al., 2006).

Organophosphorus pesticides induce apoptosis in immune cells *via* the mitochondrial pathway (Das et al., 2006; Saleh et al., 2003). Chlorpyrifos and dichlorvos have been shown

to induce caspase-dependent apoptosis associated to oxidative stress. This apoptosis was detected in human natural killer lymphocytes (Li, et al., 2007), in human T cells (Li et al., 2009) and in human monocyte cell line (Nakadai et al., 2006). Recently, phosphamidon and endosulfan have been shown to induce apoptosis of human peripheral blood mononuclear cells *via* cytochrome C release. Coadministration of the antioxidants N-acetylcysteine, which enhances GSH synthesis, attenuated phosphamidon-induced apoptosis. This work supports the hypothesis that oxidative stress, as indicated by GSH depletion, results in the induction of apoptosis by release of cytochrome C (Ahmed et al., 2008; Ahmed et al., 2010).

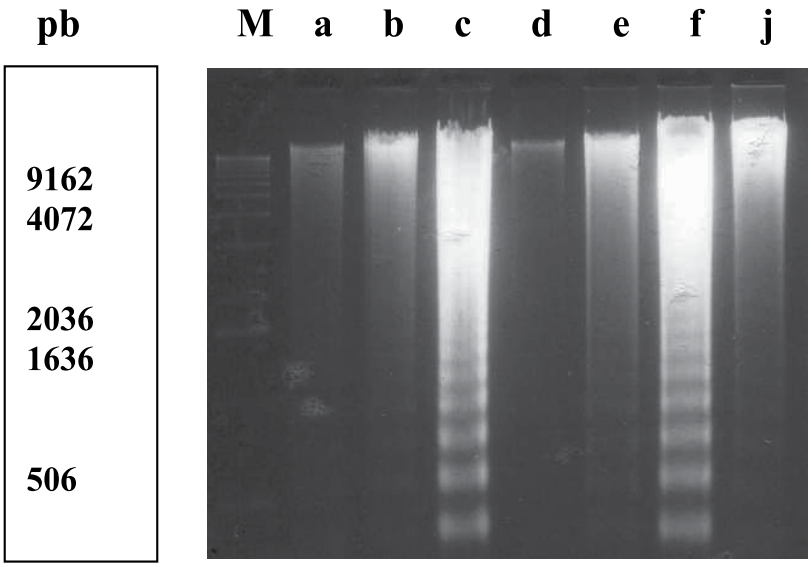

Fig. 6. Agarose gel analysis of DNA samples obtained from thymocytes not incubated (lane a) and incubated 6 hours in control medium (lane b), medium containing 10^{-5} M DEXA (lane c), 1 mM zinc (lane d), 10^{-5} M DEXA plus 1 mM zinc (lane e), 2.10^{-5} M DDT (lane f), 2.10^{-5} M DDT plus 1 mM zinc (lane j), 1 Kb molecular weight DNA marker (lane M) (Tebourbi et al.; 1998).

5. Conclusion

Through food, water and air, humans are exposed daily to a diversity of pesticides, which are new to the cellular detoxification system and may present a hazard to health. As described in this chapter, many problems may arise upon exposure to pesticides, for example endocrine disruption and oxidative stress. Indeed, it is alarming that, while the manufacture and use of pesticides has drastically increased during the last decades, the incidence of hormone/oxidative stress-related diseases, such as cancers of breast and prostate and neurodegenerative diseases, has increased markedly all over the world. Moreover, the difficulty of identifying receptor-mediated human health hazards based on *in vitro* methodology depends partly on poor understanding of the function of the receptors with respect to disease development. It is not clear which endpoints should be considered alarming, or which end-points should be used to identify all possible biological effects of pesticides. To tackle these problems, basic research on the function of xenosensors and

hormone receptors is needed. The growing field of genomics, proteomics (analysis of protein expression and interactions), and structural biology will contribute to a more detailed understanding of the complexity of the signaling of these receptors. Research should also consider the action of pesticides on other hormonal systems other than steroid and thyroid hormone signaling. To date, only little is known about how pesticides can affect the pathways of peptide hormones and fatty acid derivatives. The data summarized above demonstrate that redox signaling is one of the central mechanisms by which many of these pesticides modulate/trigger apoptosis. In certain circumstances pesticides might trigger other types of cell death pathways such as necrosis and autophagy. Redox signaling induced by pesticides involves both alterations in antioxidant defenses and accumulation of ROS leading to oxidative stress. These biochemical events mediate a number of redox-dependent processes such as oxidative protein modifications, oxidative DNA damage, ER stress and alterations in mitochondrial function which in turn trigger the activation of specific signaling cascades. Activation of SAPKs such as JNK and of transcription-dependent p53 signaling cascades act as important sensors for xenobiotic stress and the induction of apoptotic cell death. Interestingly, pesticides also induce the activation of survival responses including, DNA repair mechanisms, Mitogen-activated protein kinase/ phosphatidylinositol 3-kinase (MAPK/ PI3K) signaling cascades and up-regulation of antioxidant defenses in an attempt to counteract the deleterious effects of cell death pathways. In fact, in most cases both apoptotic and survival signaling cascades have been observed to be activated in parallel in response to pesticide toxicity. Tipping the balance towards either cell death or survival depends in most cases on the intensity, length and type of exposure. In this case, not only the extent and duration of redox signals are important to determine subsequent cell fate, but also the intracellular localization of the redox signaling and the surrounding cellular environment. Finally, deregulated activation of survival signals as a consequence of mutagenesis is well-known to promote cellular transformation aroused by the impairment of apoptotic signaling. When facing this challenge it is important to state that for many pesticides, there is a lack of research and information about complete mechanistic events involved in the induction of cell death/survival by these toxicants. Thus, future research in the understanding of both pesticide-induced cytotoxicity and pesticide-induced cellular transformation is necessary for a complete understanding of the human health consequences to pesticide exposure, in order to establish improved usage regulations and reduction of exposure risk.

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7. References

Abdelhaleem, M. (2002). Differential effect of Bcl-xl over-expression on cell death of the monocytic leukemia cell line U937. *Anticancer Research,* 22., 3911–3915.

Abou-Sleiman, P.M.; Muqit, M.M. & Wood, N.W. (2006). Expanding insights of mitochondrial dysfunction in Parkinson's disease. *Nature Reviews Neuroscience*, 7., 207–219.

Ahmadi, F.A.; Linseman, D.A.; Grammatopoulos, T.N.; Jones, S.M.; Bouchard, R.J. Freed, C.R.; Heidenreich, K.A. & Zawada, W.M. (2003). The pesticide rotenone induces caspase-3-mediated apoptosis in ventral mesencephalic dopaminergic neurons. *Journal of Neurochemistry*, 87., 914–921.

Ahmed, T.; Tripathi, A.K. ; Ahmed, R.S.; Das, S., Suke, S.G.; Pathak, R.; Chakraboti, A. & Banerjee, B.D. (2008). Endosulfan-induced apoptosis and glutathione depletion in human peripheral blood mononuclear cells: Attenuation by N-acetylcysteine. *Journal of Biochemical and Molecular Toxicology,* 22., 299-304.

Ahmed, T.; Tripathi, A.K.; Ahmed, R.S. & Banerjee, B.D. (2010). Assessment of phosphamidon-induced apoptosis in human peripheral blood mononuclear cells: protective effects of N-acetylcysteine and curcumin. *Journal of Biochemical and Molecular Toxicology,* 24., 286-292.

Andersen, H.R.; Vinggaard, A.M.; Rasmussen, T.H.; Gjermandsen, I.M. & Bonefeld-Jørgensen, E.C. (2002). Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. *Toxicology and Applied Pharmacology,* 179., 1-12.

Auger, J.; Kunstmann, J.M.; Czyglik, F. & Jouannet, P. (1995). Decline in sperm quality among fertile men in Paris during the past 20 years. *The New England Journal of Medicine,* 332., 281–285.

Banhegyi, G.; Benedetti, A.; Csala, M. & Mandl, J. (2007). Stress on redox. *The Federation of European Biochemical Societies Letters,* 581., 3634–3640.

Barlow, B.K.; Lee, D.W.; Cory-Slechta, D.A. & Opanashuk, L.A. (2005). Modulation of antioxidant defense systems by the environmental pesticide maneb in dopaminergic cells. *Neurotoxicology,* 26., 63–75.

Barnhart, B.C.; Alappat, E.C.M. & Peter, E. (2003). The CD95 type I/type II model. *Seminars in Immunology*, 15., 185-193.

Baskin, L.S.; Himes, K. & Colborn, T. (2001). Hypospadias and endocrine disruption: Is there a connection? *Environmental Health Perspectives,* 109., 1175-1183.

Bastomsky, C.H. (1974). Effects of a polychlorinated biphenyl mixture (Aroclor 1254) and DDT on biliary thyroxine excretion in rats. *Endocrinology,* 95., 1150-1155.

Belpomme, D.; Irigaray, P.; Hardell, L.; Clapp, R.; Montagnier, L. & Epstein, S. (2007). The multitude and diversity of environmental carcinogens. *Environmental Research,* 105., 414-429.

Ben Rhouma, K.; Tebourbi, O.; Krichah, R. & Sakly, M. (2001). Reproductive toxicity of DDT in adult male rats. *Human and Experimental Toxicology,* 20., 393-397.

Benmoyal-Segal, L. & Soreq, H. (2006). Gene–environment interactions in sporadic Parkinson's disease. *Journal of Neurochemistry*. 97., 1740–1755.

Bertram, C. & Hass, R. (2008). Cellular responses to reactive oxygen species-induced DNA damage and aging. *Biological Chemistry*, 389., 211–220.

Bosland, MC. (2006). Sex steroids and prostate carcinogenesis: integrated, multifactorial working hypothesis. *Annals of the New York Academy of Sciences*, 1089., 168-176.

Boyce, M.& Yuan, J. (2006). Cellular response to endoplasmic reticulum stress: a matter of life or death, *Cell Death and Differentiation*, 13., 363-373.

Brown, T.P.; Rumsby, P.C.; Capleton, A.C.; Rushton, L. & Levy, L.S. (2006). Pesticides and Parkinson's disease is there a link? *Environmental Health Perspectives*, 114., 156–164.

Calo, M.; Iannone, M.; Passafaro, M. & Nistico, G. (1990). Selective vulnerability of hippocampal CA3 neurones after microinfusion of paraquat into the rat substantia nigra or into the ventral tegmental area. *Journal of Comparative Pathology*, 103., 73–78.

Calviello, G.; Piccioni, E.; Boninsegna, A.; Tedesco, B.; Maggiano, N.; Serini, S. & Wolf, F.I.; & Palozza, P. (2006). DNA damage and apoptosis induction by the pesticide Mancozeb in rat cells: involvement of the oxidative mechanism. *Toxicology and Applied Pharmacology,* 211., 87–96.

Canistro, D.; Cantelli-Forti, G.; Biagi, G.L.& Paolini, M. (2002). Re: dioxin increases reactive oxygen production in mouse liver mitochondria. *Toxicology and Applied Pharmacology*, 178., 15–21.

Carlsen, E.; Giwercman, A.; Keiding, N. & Skakkebaek, N.E. (1992). Evidence for the decreasing quality of semen during the past 50 years. *British Medical Journal,* 305., 609–613.

Cassidy, R.A.; Natarajan, S.& Vaughan, G.M. (2005). The link between the insecticide heptachlor epoxide, estradiol, and breast cancer. *Breast Cancer Research and Treatment,* 90., 55-64.

Castello, P.R.; Drechsel, D.A. & Patel, M. (2007). Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. *Journal of Biological Chemistry*, 282., 14186–14193.

Chen, Y.; McMillan-Ward, E.; Kong, J.; Israels, S.J. & Gibson, S.B. (2007). Mitochondrial electron-transport-chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species. *Journal of Cell Science,* 120., 4155-4166.

Chen, Y. & Gibson, S.B. (2008). Is mitochondrial generation of reactive oxygen species a trigger for autophagy? *Autophagy,* 16., 246-248.

Chen, Y.; Azad, M.B & Gibson, S.B. (2009). Superoxide is the major reactive oxygen species regulating autophagy. *Cell Death and Differentiation,* 16., 1040-1052.

Claxton, L.D. & Woodall, G.M. (2007). A review of the mutagenicity and rodent carcinogenicity of ambient air, *Mutation Research,* 636., 36–94.

Cok, I.; Bilgili, A.; Ozdemir, M.; Ozbek, H.; Bilgili, N. & Burgaz, S. (1997). Organochlorine pesticide residues in human breast milk from agricultural regions of Turkey, 1995–1996. *Bulletin of Environmental Contamination and Toxicology,* 59., 577–582.

Colborn, T. (2006). A case for revisiting the safety of pesticides: a closer look at neurodevelopment, *Environmental Health Perspectives*, 114., 10-17.

Coulom, H. & Birman, S. (2004). Chronic exposure to rotenone models sporadic Parkinson's disease in Drosophila melanogaster. *Journal of Neuroscience*, 24., 10993–10998.

Curran, P.G. & De Groot, L.J. (1991). The effect of hepatic enzyme-inducing drugs on thyroid hormones and the thyroid gland. *Endocrine Reviews*, 12., 135-150.

Dalle-Donne, I.; Rossi, R.; Colombo, R.; Giustarini, D. & Milzani, A. (2006). Biomarkers of oxidative damage in human disease. *Clinical Chemistry*, 52., 601–623.

Dalle-Donne, I.; Milzani, A.; Gagliano, N.; Colombo, R.; Giustarini, D. & Rossi, R. (2008). Molecular mechanisms and potential clinical significance of S-glutathionylation, *Antioxidants and Redox Signaling*, 10., 445-473.

Das, G.P.; Shaik, A.P. & Jamil, K. (2006). Estimation of apoptosis and necrosis caused by pesticides in vitro on human lymphocytes using DNA diffusion assay. *Drug and Chemical Toxicology,* 29., 147–156.

Daxenberger, A.A. (2002). Pollutants with androgen-disrupting potency. *European Journal of Lipid Science and Technology*, 104., 124–130.

Degenhardt, K.; Mathew, R.; Beaudoin, B.; Bray, K.; Anderson, D.; Chen, G.; Mukherjee, C.; Shi, Y.; Gélinas, C.; Fan, Y.; Nelson, D.; Jin, S. & White, E. (2006). Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell,* 10., 51–64.

Deroo, B.J.; Rentsch, C.; Sampath, S.; Young, J.; DeFranco, D.B. & Archer, T.K. (2002). Proteasomal inhibition enhances glucocorticoid receptor transactivation and alters its subnuclear trafficking. *Molecular and Cellular Biology*, 22., 4113-4123.

Dhalla, N.S.; Temsah, R.M. & Netticadan, T. (2000). Role of oxidative stress in cardiovascular diseases. *Journal of Hypertension*, 18., 655–673.

Ding, Q. & Keller, J.N. (2001). Proteasome inhibition in oxidative stress neurotoxicity: implications for heat shock proteins. *Journal of Neurochemistry,* 77., 1010–1017.

Dong, L.M.; Potter, J.D.; White, E.; Ulrich, C.M.; Cardon, L.R. & Peters, U. (2008). Genetic susceptibility to cancer: the role of polymorphisms in candidate genes. *Journal of the American Medical Association,* 299., 2423–2436.

Drechsel, D.A. & Patel, M. (2009). Differential contribution of the mitochondrial respiratory chain complexes to reactive oxygen species production by redox cycling agents implicated in parkinsonism. *Toxicological Sciences*, 112., 427-434.

European workshop on the impact of endocrine disrupters on human health and wildlife (EEC). (1996). Weybridge: UK.

Ekert, P.G. & Vaux, D.L. (2005). The mitochondrial death squad: hardened killers or innocent by standers? *Current Opinion in Cell Biology*, 17., 626-630.

Ellinger-Ziegelbauer, H.; Stuart, B.; Wahle, B.; Bomann, W. & Ahr, H.J. (2005). Comparison of the expression profiles induced by genotoxic and nongenotoxic carcinogens in rat liver. *Mutation Research*, 575., 61–84.

Escriva, H.; Bertrand, S. & Laudet, V. (2004). The evolution of the nuclear receptor superfamily. *Essays in Biochemistry*, 40., 11-26.

Eskenazi, B.; Bradman, A.; Castorina, R. (1999). Exposures of children to organophosphate pesticides and their potential adverse health effects, *Environmental Health Perspectives*, 107., 409-419.

Fei, Q. & Ethel, D.W. (2008). Maneb potentiates paraquat neurotoxicity by inducing key Bcl-2 family members. *Journal of Neurochemistry*, 105., 2091-2097.

Fei, Q.; McCormack, A.L.; Di Monte, D.A. & Ethel, D.W. (2008). Paraquat neurotoxicity is mediated by a Bak-dependent mechanism. *Journal of Biological Chemistry*, 283., 3357–3364.

Fleming, L.; Mann, J.B.; Bean, J. & Briggle, T. & Sanchez-Ramos, J.R. (1994). Parkinson's disease and brain levels of organochlorine pesticides. *Annals of Neurology*, 36., 100–103.

Foster, W.G.; Younglai, E.V.; Boutross-Tadross, O.; Hughes, C.L. & Wade, M.G. (2004). Mammary gland morphology in sprague-dawley rats following treatment with an organochlorine mixture in utero and neonatal genistein. *Toxicological Sciences*, 77., 91-100.

Franco, R.; Sanchez-Olea, R.; Reyes-Reyes, E.M. & Panayiotidis, M.I. (2009). Environmental toxicity, oxidative stress and apoptosis: ménage à trois. *Mutation Research*, 674., 3-22.

Franco, R.; Li, S.; Rodriguez-Rocha, H.; Burns, M.; Panayiotidis, M.I. (2010). Molecular mechanisms of pesticide-induced neurotoxicity: Relevance to Parkinson's disease. *Chemico-Biological Interactions,* 188., 289-300.

Galluzzi, L.; Maiuri, M.C.; Vitale, I.; Zischka, H.; Castedo, M.; Zitvogel, L. & Kroemer, G. (2007). Cell Death Modalities: classification and pathophysiological implications. *Cell Death and Differentiation*, 14., 1237-1243.

Garey, J. & Wolff, M.S. (1998). Estrogenic and antiprogestagenic activities of pyrethroid insecticides. *Biochemical and Biophysical Research Communications*, 251., 855-859.

Garry, V.V. (2004). Pesticides and children. *Toxicology and Applied Pharmacology,* 198., 152-163.

Germain, P.; Staels, B.; Dacquet, C.; Spedding, M. & Laudet, V. (2006). Overview of nomenclature of nuclear receptors. *Pharmacological Reviews*, 58., 685-704.

Goad, R.; Goad, J.; Atieh, B. & Gupta, R. (2004). Carbofuran-induced endocrine disruption in adult male rats. *Toxicology Mechanisms and Methods,* 14., 233–239.

Gonzalez-Polo, R.A.; Niso-Santano, M.; Ortiz-Ortiz, M.A.; Gomez-Martin, A; Moran, J.M.; Garcia-Rubio, L.; Francisco-Morcillo, J.; Zaragoza, C.; Soler, G. & Fuentes, J.M. (2007). Relationship between autophagy and apoptotic cell death in human neuroblastoma cells treated with paraquat: could autophagy be a "brake" in paraquat-induced apoptotic death? *Autophagy,* 3., 366–367.

Gougeon, M.L.; Lecoeur, H. & Dulioust, A. (1996). Programmed cell death in peripheral lympocytes from HIV-infected persons. *Journal of Immunology,* 156., 3509–3520.

Gronemeyer, H.; Gustafsson, JA. & Laudet, V. (2004) Principles formodulation of the nuclear receptor superfamily. *Nature Reviews : Drug Discovery*, 3., 950-964.

Grosicka, E.; Sadurska, B.; Szumilo, M.; Grzela, T.; Lazarczyk, P.; Niderla-Bielinska, J. & Rahden-Staron, I. (2005). Effect of glutathione depletion on apoptosis induced by thiram in Chinese hamster fibroblasts. *International Immunopharmacology,* 5., 1945–1956.

Gwinn, M.R.; Whipkey, D.L.; Tennant, L.B. & Weston, A. (2005). Differential gene expression in normal human mammary epithelial cells treated with malathion monitored by DNA microarrays. *Environmental Health Perspectives,* 113., 1046–1051.

Hall, J.M.; Couse, J.F. & Korach, K.S. (2001). The multifaceted mechanisms of estradiol and estrogen receptor signaling. *Journal of Biological Chemistry*, 276., 36869-36872.

Hallegue, D.; Ben Rhouma, K.; Tebourbi, O. & Sakly, M. Impairment of testicular endocrine and exocrine functions after dieldrin exposure in adult rat. *Polish Journal of Environmental Studies*, 12., 557-561.

Halliwell, B. (2007). Oxidative stress and cancer: have we moved forward? *Biochemical Journal*. 401., 1–11.

Hanahan, D. & Weinberg, R.A. (2000). The hallmarks of cancer. *Cell*. 100., 57–70.

Hartley, A.; Stone, J.M.; Heron, C.; Cooper, J.M. & Schapira, A.H. (1994). Complex I inhibitors induce dose-dependent apoptosis in PC12 cells: relevance to Parkinson's disease. *Journal of Neurochemistry*, 63., 1987–1990.

Hatcher, J.M.; Pennell, K.D. & Miller, G.W. (2008). Parkinson's disease and pesticides: a toxicological perspective. *Trends in Pharmacological Sciences*, 29., 322–329.

Hayes,T.; Haston, K.; Tsui, M.; Hoang, A.; Haeffele, C. & Vonk, A. (2002). Herbicides: feminization of male frogs in the wild. *Nature,* 419., 895–896.

Hengartner, M.O. (2000). The biochemistry of apoptosis. *Nature,* 407., 770-776.

Hikim, A.P.; Lue, Y.; Yamamoto, C.M.; Vera, Y.; Rodriguez, S.; Yen, P.H.; Soeng, K.; Wang, C. & Swerdloff, R.S. (2003). Key apoptotic pathways for heat-induced programmed germ cell death in the testis. *Endocrinology,* 144., 3167–3175.

International Program on Chemical Safety (IPCS). (2002). Global assessment of the state-of-the-science of endocrine disruptors. *World Health Organization*: [http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/].

Hockenberry, D.M.; Oltvai, Z.N.; Yin, X.M.; Milliman, C.L. & Korsmeyer, S.J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell,* 75., 241-251.

Hoek, J.B. & Rydström, J. (1988). Physiological roles of nicotinamide nucleotide transhydrogenase. *Biochemical Journal,* 254., 1-10.

Irigaray, P.; Newby, J.A.; Clapp, R.; Hardell, L.; Howard, V.; Montagnier, L.; Epstein, S. & Belpomme, D. (2007). Lifestyle-related factors and environmental agents causing cancer: an overview. *Biomedicine and Pharmacotherapy*, 61., 640–658.

Ishihara, A.; Nishiyama, N.; Sugiyama, S.I. & Yamauchi, K. (2003). The effect of endocrine disrupting chemicals on thyroid hormone binding to Japanese quail transthyretin and thyroid hormone receptor. *General and Comparative Endocrinology*, 134., 36–43.

Janssen-Heininger, Y.M.; Mossman, B.T.; Heintz, N.H.; Forman, H.J.; Kalyanaraman, B.; Finkel, T.; Stamler, J.S.; Rhee, S.G. & Van Der Vliet, A. (2008). Redox-based regulation of signal transduction: principles, pitfalls, and promises. *Free Radical Biology and Medicine*, 45., 1-17.

Jeong, S.H.; Kim,B.Y.; Kang, H.G.; Ku, H.K. & Cho, J.H. (2006). Effect of chlorpyrifos-methyl on steroid and thyroid hormones in rat F0- and F1-generations. *Toxicology*, 220., 189–202.

Jin, Y.;Zheng, S.; Pu, Y.; Shu, L.; Sun, L.; Liu, W. & Fu, Z. (2011). Cypermethrin has the potential to induce hepatic oxidative stress, DNA damage and apoptosis in adult zebrafish (Danio rerio). *Chemosphere,* 82., 398-404.

Kang, K.S.; Wilson, M.R.; Hayashi, T.; Chang, C.C. & Trosko, J.E. (1996). Inhibition of gap junctional intercellular communication in normal human breast epithelial cells after treatment with pesticides, PCBs, and PBBs, alone or in mixtures. *Environmental Health Perspectives,* 104., 192-200.

Kang, H.G.; Jeong, S.H.; Cho, J.H.; Kim, D.G.; Park, J.M. & Cho, M.H. (2004). Chloropyrifos-methyl shows anti-androgenic activity without estrogenic activity in rats. *Toxicology*, 199., 219–230.

Katsoulieris , E.; Mabley, J.G.; Samai, M.; Sharpe, M.A.; Green, I.C. & Chatterjee, P.K. (2010). Lipotoxicity in renal proximal tubular cells: relationship between endoplasmic reticulum stress and oxidative stress pathways *Free Radical Biology and Medicine*, 48., 1654-1662.

Katzenellenbogen, J.A.; O'Malley, B.W. & Katzenellenbogen, B.S. (1996). Tripartite steroid hormone receptor pharmacological interaction with multiple effector sites as a basis for the cell- and promoter-specific action of these hormones. *Molecular Endocrinology,* 10., 119-131.

Kavlock, R. & Cummings, A. (2005). Mode of action: Inhibition of androgen receptor function-Vinclozolin-induced malformations in reproductive development. *Critical Reviews in Toxicology*, 35., 721-726.

Kelce, W.R.; Stone, C.R.; Laws, S.C.; Gray, L.E.; Kemppainen, J.A. & Wilson, E.M. (1995). Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. *Nature,* 375., 581-585.

Khosravi-Far, R. & Esposti, M.D. (2004). Death receptor signals to mitochondria. *Cancer Biology and Therapy*, 3.,1051-1057.

Kim, I.Y.; Shin, J.H.; Kim, H.S.; Lee, S.J.; Kang, I.H. & Kim, T.S. (2004). Assessing estrogenic activity of pyrethroid insecticides using in vitro combination assays. *The Journal of Reproduction and Development*, 50., 245–255.

Kitazawa, M.; Anantharam, V.; Kanthasamy, A. & Kanthasamy, A.G. (2004). Dieldrin promotes proteolytic cleavage of poly(ADP-ribose) polymerase and apoptosis in dopaminergic cells: protective effect of mitochondrial anti-apoptotic protein Bcl-2. *Neurotoxicology,* 25., 589–598.

Klaunig, J.E. & Ruch, R.J. (1990). Biology of disease: role of inhibition of intercellular communication in carcinogenesis. *Laboratory Investigation,* 62., 135-146.

Klaunig, J.E.; Hartnett, J.A.; Ruch, R.J.; Weghorst, C.M.; Hampton, J.A. & Schafer, L.D. (1990). Gap junctional intercellular communication in hepatic carcinogenesis. *Progress in Clinical and Biological Research,* 340., 165-174.

Kliewer, S.A. & Willson, T.M. (2002). Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor. *Journal of Lipid Research*, 43., 359-364.

Kroemer, G.; Galluzzi, L.; Vandenabeele, P.; Abrams, J.; Alnemri, E.S.; Baehrecke, E.H.; Blagosklonny, M.V.; El-Deiry, W.S.; Golstein, P.; Green, D.R.; Hengartner, M.; Knight, R.A.; Kumar, S.; Lipton, S.A.; Malorni, W.; Nunez, G.; Peter, M.E.; Tschopp, J.; Yuan, J.; Piacentini, M.; Zhivotovsky, B. & Melino, G. (2009). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009, *Cell Death and Differentiation*, 16., 3-11.

Landrigan, P.J.; Sonawane, B.; Butler, R.N.; Trasande, L.; Callan, R. & Droller, D. (2005). Early environmental origins of neurodegenerative disease in later life. *Environmental Health Perspectives*, 113., 1230–1233.

Laville, N.; Balaguer, P.; Brion, F.; Hinfray, N.; Casellas, C.& Porcher, J.M. (2006). Modulation of aromatase activity and mRNA by various selected pesticides in the human choriocarcinoma JEG-3 cell line. *Toxicology,* 228., 98-108.

Lemaire, G.; Terouanne, B.; Mauvais, P.; Michel, S. & Rahmani R. (2004). Effect of organochlorine pesticides on human androgen receptor activation in vitro. *Toxicology and Applied Pharmacology,* 196.,235–246.

Lemaire, G.; Mnif, W.; Mauvais, P.; Balaguer, P. & Rahmani, R. (2006). Activation of alpha- and beta-estrogen receptors by persistent pesticides in reporter cell lines. *Life Sciences,* 79., 1160-1169.

Li, Q.; Kobayashi, M. & Kawada, T. (2007). Organophosphorus pesticides induce apoptosis in human NK cells, *Toxicology,* 239., 89–95.

Li, Q.; Kobayashi, M. & Kawada, T. (2009). Chlorpyrifos induces apoptosis in human T cells. *Toxicology,* 255., 53–57.

Lin, H.K.; Altuwaijri, S.; Lin, W.J.; Kan, P.Y.; Collins, L.L. & Chang, C. (2002). Proteasome activity is required for androgen receptor transcriptional activity via regulation of

androgen receptor nuclear translocation and interaction with coregulators in prostate cancer cells. *Journal of Biological Chemistry*. 277., 36570-36576.

Lo, S.; King, I.; Allera, A. & Klingmuller, D. (2007). Effects of various pesticides on human 5alpha-reductase activity in prostate and LNCaP cells. *Toxicology in Vitro,* 21., 502-508.

Lu, S.Y.; Liao, J.W.; Kuo, M.L.; Wang, S.C.; Hwang, J.S. & Ueng, T.H. (2004). Endocrine disrupting activity in carbendazim-induced reproductive and developmental toxicity in rats. *Journal of Toxicology and Environmental Health Part A*, 67., 1501–1515.

Mackenzie, C.A. & Constanze, A. (2005). Declining sex ratio in a first nation community. *Environmental Health Perspectives*, 113., 1295-1298.

Malarvannan, G.; Kunisue, T.; Isobe, T.; Sudaryanto, A.; Takahashi, S.; Prudente, M.; Subramanian, A. & Tanabe, S. (2009). Organohalogen compounds in human breast milk from mothers living in Payatas and Malate, the Philippines: levels, accumulation kinetics and infant health risk. *Environmental Pollution*, 157., 1924–1932.

Manning-Bog, A.B.; McCormack, A.L.; Li, J.; Uversky, V.N.; Fink, A.L. & Di Monte, D.A. (2002). The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. *Journal of Biological Chemistry,* 277., 1641–1644.

Marchetti, P.; Decaudin, D.; Macho, A.; Zamzami, N.; Hirsch, T.; Susin, S.A. & Kroemer, G. (1997). Redox regulation of apoptosis: impact of thiol redoxidation on mitochondrial function. *European Journal of Immunology*, 27., 2889-2896.

Mathur, V.; Bhatnagar, P.; Sharma, R.G.; Acharya, V. & Sexana R. (2002). Breast cancer incidence and exposure to pesticides among women originating from Jaipur. *Environment International*, 228., 331-336.

Mattson, M.P. (2006). Neuronal life-and-death signaling, apoptosis, and neurodegenerative disorders, *Antioxidants and Redox Signaling,* 8., 1997–2006.

McCarthy, A.R.; Thomson, B.M.; Shaw, I.C. & Abella, A.D. (2006). Estrogenicity of pyrethroid insecticide metabolites. *Journal of Environmental Monitoring*, 8., 197-202.

McCormack, A.L.; Atienza, J.G.; Johnston, L.C.; Andersen, J.K.; Vu, S. & Di Monte, D.A. (2005). Role of oxidative stress in paraquat-induced dopaminergic cell degeneration. *Journal of Neurochemistry*, 93., 1030–1037.

McKinlay, R.; Plant, J.A. & Bell, J.N.B.; Voulvoulis, N. (2008). Endocrine disrupting pesticides: Implications for risk assessment. *Environment International,* 34., 168-183.

Meco, G.; Bonifati, V.; Vanacore, N. & Fabrizio, E. (1994). Parkinsonism after chronic exposure to the fungicidemaneb(manganese ethylene-bis-dithiocarbamate). *Scandinavian Journal of Work, Environment and Health,* 20., 301–305.

Mena, S.; Ortega, A. & Estrela, J.M. (2009). Oxidative stress in environmental-induced carcinogenesis. *Mutation Research,* 674., 36–44.

Meulenberg, E.E. (2002). A new test to identify endocrine disruptors using sex hormone binding globulins from human serum. *European Journal of Lipid Science and Technology*, 104., 131–136.

Molina-Molina, J.M.; Hillenweck, A.; Jouanin, I.; Zalko, D.; Cravedi, J.P.; Fernandez, M.F.; Pillon, A.; Nicolas, J.C.; Olea, N. & Balaguer, P. (2006). Steroid receptor profiling of vinclozolin and its primary metabolites. *Toxicology and Applied Pharmacology*, 216., 44-54.

Morinaga, H.; Yanase, T.; Nomura, M.; Okabe, T.; Goto, K. & Harada, N. (2004). A benzimidazole fungicide, benomyl, and its metabolite, carbendazim, induce aromatase activity in a human ovarian granulose-like tumor cell line (KGN). *Endocrinology,* 145., 1860-1869.

Muguruma, M.; Unami, A.; Kanki, M.; Kuroiwa, Y.; Nishimura, J.; Dewa, Y.; Umemura, T.; Oishi, Y.& Mitsumori, K. (2007). Possible involvement of oxidative stress in piperonyl butoxide induced hepatocarcinogenesis in rats. *Toxicology,* 236., 61–75.

Nagel, S.C.; Hagelbarger, J.L. & McDonnell, D.P. (2001). Development of an ER action indicator mouse for the study of estrogens, selective ER modulators (SERMs), and xenobiotics. *Endocrinology,* 142., 4721-4728.

Nakadai, A.; Li, Q. & Kawada, T. (2006). Chlorpyrifos induces apoptosis in human monocyte cell line U937. *Toxicology,* 224., 202–209.

Nakata, K., Tanaka, Y., Nakano, T., Adachi, T., Tanaka, H., Kaminuma, T. & Ishikawa, T. (2006). Nuclear receptor-mediated transcriptional regulation in phase I, II, and III xenobiotic metabolizing systems. *Drug Metabolism and Pharmacokinetics,* 21., 437-457.

Negga, R.;Rudd, D.A.; Davis, N.S.; Justice, A.N; Hatfield, H.E; Valente, A.L.; Fields, A.S. & Fitsanakis, V.A. (2011). Exposure to Mn/Zn ethylene-bis-dithiocarbamate and glyphosate pesticides leads to neurodegeneration in Caenorhabditis elegans. *Neurotoxicology,* doi:10.1016/j.neuro.2011.02.002.

Newhouse, K.; Hsuan, S.L.; Chang, S.H.; Cai, B.; Wang, Y. & Xia, Z. (2004). Rotenone-induced apoptosis is mediated by p38 and JNK MAP kinases in human dopaminergic SH-SY5Y cells. *Toxicological Sciences*. 79., 137–146.

Nicolopoulou, P. & Stamanti, P. (2001). The impact of endocrine disrupters on the female reproductive system. *Human Reproduction Update,* 7., 323-330.

Nicotera, P. & Melino, G. (2004). Regulation of the apoptosis-necrosis switch. *Oncogene,* 23., 2757–2765.

Niso-Santano, M.; Gonzalez-Polo, R.A.; Bravo-San Pedro, J.M.; Gomez-Sanchez, R.; Lastres-Becker, I.; Ortiz-Ortiz, M.A.; Soler, G.; Moran, J.M.; Cuadrado, A. & Fuentes, J.M. (2010). Activation of apoptosis signal-regulating kinase 1 is a key factor in paraquat-induced cell death. Modulation by the Nrf2/Trx axis. *Free Radical Biology and Medicine, 48., 1370-1381.*

Norris, E.H.; Uryu, K.; Leight, S.; Giasson, B.I.; Trojanowski, J.Q. & Lee, V.M. (2007). Pesticide exposure exacerbates alpha-synucleinopathy in an A53T transgenic mouse model. *American Journal of Pathology*, 170., 658–666.

Oehninger, S. (2001). Strategies for the infertile man. *Seminars in Reproductive Medicine,* 19., 231–237.

Okouchi, M. ; Ekshyyan, O.; Maracine, M. & Aw, T.Y. (2007). Neuronal apoptosis in neurodegeneration. *Antioxidants and Redox Signaling* 9., 1059–1096.

Orrenius, S. (1993). Mechanisms of oxidative cell damage. In: *Free Radicals: From Basic Science to Medicine*, Poli, G.; Albano, E. & Dianzani, M.U., pp. 47–64, Birkhauser Verlag, Basel, Switzerland.

Ott, M.; Gogvadze, V.; Orrenius, S. & Zhivotovsky, B. (2007). Mitochondria, oxidative stress and cell death, *Apoptosis,* 12., 913–922.

Palomo, T.; Archer, T.; Beninger, R.J. & Kostrzewa, R.M. (2004). Gene–environment interplay in neurogenesis and neurodegeneration, *Neurotoxicity Research*, 6., 415–434.

Pascussi, J.M., Gerbal-Chaloin, S., Drocourt, L., Assenat, E., Larrey, D., Pichard-Garcia, L., Vilarem, M.J. & Maurel, P. (2004). Cross-talk between xenobiotic detoxication and other signalling pathways: Clinical and toxicological consequences. *Xenobiotica,* 34., 633-664.

Perera, F.P.; Rauh, V.; Whyatt, R.M.; Tang, D.; Tsai, W.Y.; Bernert, J.T.; Tu, Y.H.; Andrews, H.; Barr, D.B.; Camann, D.E.; Diaz, D.; Dietrich, J.; Reyes, A. & Kinney, P.L. (2005). A summary of recent findings on birth outcomes and developmental effects of prenatal ETS, PAH, and pesticide exposures. *Neurotoxicology,* 26., 573–587.

Pérez-Maldonado, I.N.; Athanasiadou, M.; Yáñez, L.; González-Amaro, R.; Bergman, A. & Díaz-Barriga, F. (2006). DDE-induced apoptosis in children exposed to the DDT metabolite. *Science of the Total Environment*, 370., 343–351.

Pérez-Maldonado, I.N.; Díaz-Barriga, F.; De la Fuente, H.; González-Amaro, R.; Calderón, J. & Yañez, L. (2004). DDT induces apoptosis in human mononuclear cells in vitro and is associated with increased apoptosis in exposed children. *Environmental Research*, 94., 38–46.

Pérez-Maldonado, I.N.; Herrera, C.; Batres, L.E.; González-Amaro, R.; Díaz-Barriga, F. & Yañez L. (2005). DDT -Induced oxidative damage in human blood mononuclear cells. *Environmental Research,* 98., 177–184.

Pesticide Action Network (PAN). (2005). List of lists: a catalogue of lists of pesticides identifying those associated with particularly harmful health or environmental impacts.

Ravagnan, L.; Roumier, T. & Kroemer, G. (2002). Mitochondria, the killer organelles and their weapons. *Journal of Cell Physiology*, 192., 131-137.

Reschly, E.J. & Krasowski, M.D. (2006). Evolution and function of the NR1I nuclear hormone receptor subfamily (VDR, PXR, and CAR) with respect to metabolism of xenobiotics and endogenous compounds. *Current Drug Metabolism*, 7., 349-365.

Roos, W.P. & Kaina, B. (2006). DNA damage-induced cell death by apoptosis. *Trends in Molecular Medicine*, 12., 440-450.

Ruch, R.J. & Klaunig, J.E. (1988). Antioxidant prevention of tumor promoter induced inhibition of mouse hepatocyte intercellular communication. *Cancer Letters,* 33., 137-150.

Rüegg, J.; Penttinen-Damdimopoulou, P.; Makela, S; Pongratz, I. & Gustafsson, J.A. (2009). Receptors mediating toxicity and their involvement in endocrine disruption Molecular. Clinical and Environmental Toxicology, In: *Molecular Toxicology*, Luch, A., pp289-323, Birkhäuser Verlag, Switzerland.

Ryter, S.W.; Kim, H.P.; Hoetzel, A.; Park, J.W.; Nakahira, K.; Wang, X. & Choi, A.M. (200 7). Mechanisms of cell death in oxidative stress. *Antioxidants and Redox Signaling*, 9., 49-89.

Ryter, S.W.; Kim, H.P.; Hoetzel, A.; Park, J.W.; Nakahira, K.; Wang, X.& Choi, A.M. (2007). Mechanisms of cell death in oxidative stress. *Antioxidants and Redox Signaling,* 9., 49–89.

Ryu, E.J.; Harding, H.P.; Angelastro, J.M.; Vitolo, O.V.; Ron, D. & Greene, L.A. (2002). Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. *Journal of Neuroscience*, 22., 10690–10698.

Saelens, X.; Festjens, N.; Vande Walle, L.; Van Gurp, M.; G. Van Loo, G. & Vandenabeele, P. (2004). Toxic proteins released from mitochondria in cell death. *Oncogene,* 23., 2861-2874.

Saleh, A.M.; Vijayasarathy, C.; Fernandez-Cabezudo, M.; Taleb, M. & Petroianu, G. (2003). Influence of paraoxon (POX) and parathion (PAT) on apoptosis: a possible mechanism for toxicity in low-dose exposure. *Journal of Applied Toxicology,* 23., 23–29.

Sanderson, T. & Van Den Berg, M. (2003). Interactions of xenobiotics with the steroid hormone biosynthesis pathway. *Pure and Applied Chemistry*, 75., 1957-1971.

Saradha, B.; Vaithinathan, S. & Mathur, P.P. (2009). Lindane induces testicular apoptosis in adult Wistar rats through the involvement of Fas-FasL mitochondria-dependent pathways. *Toxicology,* 255., 131-139.

Sayre, L.M.; Smith, M.A. & Perry, G. (2001). Chemistry and biochemistry of oxidative stress in neurodegenerative disease. *Current Medicinal Chemistry*, 8., 721–738.

Schrader, T.J. & Cooke, G.M. (2000). Examination of selected food additives and organochlorine food contaminants for androgenic activity in vitro.*Toxicological Sciences*, 53., 278-288.

Schreinemachers, D.D. (2003). Birth malformations and other adverse perinatal out comes in four US wheat-producing states. *Environmental Health Perspectives*, 111., 1259-1264.

Scippo, M.L. (2004). Recombinant human estrogen, androgen and progesterone receptors for detection of potential endocrine disruptors. *Analytical and Bioanalytical Chemistry*, 378., 664–669.

Shi, Y.Q.; Song, Y.; Wang, Y.N.; Liang, X.M.; Hu, Y.F.; Guan, X.; Cheng, J. & Yang, K. (2009). P,p′-DDE induces apoptosis of rat Sertoli cells via a FasL-dependent pathway. *Journal of Biomedical Biotechnology*, ID181282 doi: 10.1155/181282.

Shi, Y.Q; Wang,Y.; Song, Y.; Li, H.W.; Liu, C.J.; Wu, Z.G. & Yang, K. (2010). P,p′-DDE induces testicular apoptosis in prepubertal rats via the Fas/FasL pathway. *Toxicology Letters*, 193., 79-85.

Shinomiya, N. & Shinomiya, M. (2003). Dichlorodiphenyltrichloroethane suppresses neurite outgrowth and induces apoptosis in PC12 pheochromocytoma cells. *Toxicology Letters*, 137., 175–183.

Siegel, D. & Ross, D. (2000). Immunodetection of NAD(P)H:quinone oxidoreductase 1(NQO1) in human tissues. *Free Radical Biology and Medicine,* 29., 246–253.

Singer, P.L. (1949). Occupational oligiospermia. *Journal of the American Medical Directors Association,* 140., 1249.

Slater, A.F.G.; Nobel, C.S.I.; Van den Dobbelsteen, D.J. & Orrenius, S. (1995). Signaling mechanisms and oxidative stress in apoptosis. *Toxicology Letters*, 82/83., 149–153.

Soffritti, M.; Belpoggi, F.; Esposti, D.D.; Falcioni, L. & Bua, L. (2008). Consequences of exposure to carcinogens beginning during developmental life. *Basic and Clinical Pharmacology and Toxicology*, 102., 118–124.

Song, Y.; Liang, X., Hu, Y.; Wang, Y.; Haige, Y. & Yang, K. (2008). P,p′-DDE induces mitochondria-mediated apoptosis of cultured rat Sertoli cells. *Toxicology*, 253., 53-61.

Song, Y.; Shi, Y.;Yu, H.; Hu, Y.; Wang, Y. & Yang, K. (2011). p,p′-Dichlorodiphenoxydichloroethylene induced apoptosis of Sertoli cells through

oxidativestress-mediated p38 MAPK and mitochondrial pathway. *Toxicology Letters,* doi:10.1016/j.toxlet.2011.01.020.

Sonnenschein, C. & Soto, A.M. (1998). An updated review of environmental estrogen and androgen mimics and antagonists. *Journal of Steroid Biochemistry and Molecular Biology*, 65., 143–150.

Stevenson, D.E.; Walborg Jr., E.F.; North, D.W.; Sielken Jr., R.L.; Ross, C.E.; Wright, A.S.; Xu, Y. Kamendulis, L.M. & Klaunig, J.E. (1999). Monograph: reassessment of human cancer risk of aldrin/dieldrin. *Toxicology Letters*. 109., 123–186.

Sun, F.; Anantharam, V.; Latchoumycandane, C.; Kanthasamy, A. & Kanthasamy, A.G. (2005). Dieldrin induces ubiquitin-proteasome dysfunction in alphasynuclein overexpressing dopaminergic neuronal cells and enhances susceptibility to apoptotic cell death. *Journal of Pharmacology and Experimental Therapeutics*, 315., 69–79.

Swedenborg, E.; Rüegg, J.; Makela, S.; Pongratz, I. (2009) Endocrine disruptive chemicals: mechanisms of action and involvement in metabolic disorders. *Journal of Molecular Endocrinology,* 43., 1-10.

Szegezdi, E.; Logue, S.E.; Gorman, A.M. & Samali, A. (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. *The European Molecular Biology Organization Reports,* 7., 880-885.

Tebourbi, O., Ben Rhouma, K. & Sakly, M. (1998). DDT induces apoptosis in rat thymocytes. *Bulletin of Environmental Contamination and Toxicology*. 61 216-223.

Tebourbi, O.; Hallegue, D.; Yacoubi, M.T.; Sakly, M. & Ben Rhouma, K. (2010). Subacute toxicity of p,p'-DDT on rat thyroid: Hormonal and histopathological changes. *Environmental Toxicology and Pharmacology*. 29., 271-279.

Tolson, A.H.; Wang. H. (2010). Regulation of drug-metabolizing enzymes by xenobiotic receptors: PXR and CAR Advanced. *Drug Delivery Reviews,* 62., 1238-1249.

Toppari, J. (2008). Environmental endocrine disrupters. *Sexual Development,* 2., 260-267.

Townsend, D.M. (2007). *S*-glutathionylation: indicator of cell stress and regulator of the unfolded protein response, *Molecular Interventions*, 7., 313–324.

Tripathi, R.; Mishra, D.P.; Shaha, C. (2009). Male germ cell development: turning on the apoptotic pathways. *Journal of Reproductive Immunology*, 83., 31–35.

Vaithinathan, S.; Saradha, B. & Mathur, P.P. (2010). Methoxychlor induces apoptosis via mitochondria- and FasL-mediated pathways in adult rat testis. *Chemico-Biological Interactions,* 185., 110-118.

Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M.T.; Mazur, M. & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology*, 39., 44–84.

Vandendobberlsteen, D.J.; Nobel, C.S.I.; Schelegel, J.; Cotgreave, I.A.; Orrenius, S. & Slater, A.F.G. (1996). Rapid and specific efflux of reduced glutathione during apoptosis induced by anti-Fas/APO-1 antibody. *Journal of Biology and Biochemistry,* 271., 15420.

Vogt, M.; Bauer, M.K.; Ferrari, D. & Schulze-Osthoff, K. (1998). Oxidative stress and hypoxia/reoxygenation trigger CD95 (APO-1/Fas) ligand expression in microglial cells. *The Federation of European Biochemical Societies Letters*, 429., 67–72.

Wang, Y. (2008). Bulky DNA lesions induced by reactive oxygen species. *Chemical Research in Toxicology*. 21., 276–281.

West, J.D. & Marnett, L.J. (2006). Endogenous reactive intermediates as modulators of cell signaling and cell death. *Chemical Research in Toxicology*, 19., 173-194.

White, E. (2008). Autophagic cell death unraveled: pharmacological inhibition of apoptosis and autophagy enables necrosis. *Autophagy,* 4, 399-401.

Wijayaratne, A.L. & McDonnell, D.P. (2001). The human estrogen receptor is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. *Journal of Biological Chemistry*, 276: 35684-35692.

Willson, T.M. & Kliewer, S.A. (2002). PXR, CAR and drug metabolism. *Nature Reviews Drug Discovery*, 1., 259-266.

Wood-Kaczmar, A.; Gandhi, S. & Wood, N.W. (2006). Understanding the molecular causes of Parkinson's disease; *Trends in Molecular Medicine* 12., 521–528.

Xu, R.X.; Lambert, M.H.; Wisely, B.B.; Warren, E.N.; Weinert, E.E. & Waitt, G.M. (2004). A structural basis for constitutive activity in the human CAR/RXR alpha heterodimer. *Molecular Cell*, 16., 919-928.

Yang, C. & Chen, S. (1999). Two organochlorine pesticides, toxaphene and chlordane, are antagonists for ERRα-1 orphan receptor. *Cancer Research,* 59., 4519-4524.

Yang, L.Y.; Chen, W.L.; Lin, J.W.; Lee, S.F.; Lee, C.C.; Hung, T.I. & Wei, Y.H. & Shih, C.M. (2005). Differential expression of antioxidant enzymes in various hepatocellular carcinoma cell lines, *Journal of Cellular Biochemistry*, 96., 622–631.

Yang, W. & Tiffany-Castiglioni, E. (2008). Paraquat-induced apoptosis in human neuroblastoma SH-SY5Y cells: involvement of p53 and mitochondria. *Journal of Toxicology and Environmental Health Part* A, 71., 289–299.

Yang, W.; Tiffany-Castiglioni, E.; Koh, H.C. & Son, I.H. (2009). Paraquat activates the IRE1/ASK1/JNK cascade associated with apoptosis in human neuroblastoma SH-SY5Y cells. *Toxicology. Letters*, 191., 203-210.

You, L. (2004). Steroid hormone biotransformation and xenobiotic induction of hepatic steroid metabolizing enzymes. *Chemico-Biological Interactions*. 147., 233-246.

You, L.; Sar, M.; Bartolucci, E.; Ploch, S. & Whitt, M. (2001). Induction of hepatic aromatase by p, p'-DDE in adult male rats. *Molecular and Cellar Endocrinology,* 178., 207-214.

Youle, R.J. & Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. *Nature Reviews Molecular and Cellular Biology*, 9., 47-59.

Zaidi, A.; Fernandes, D.; Bean, J.L. & Michaelis, M.L. (2009). Effects of paraquat-induced oxidative stress on the neuronal plasma membrane Ca(2+)-ATPase. *Free Radical Biology and Medicine*, 47., 1507–1514.

Zala, S. & Penn, D.J. (2004). Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. *Animal Behavior*, 68., 649-664.

Zamzami, N.; Marchetti, P.; Castedo, M.; decaudin, D.; Macho, A.; Hirsch, T.; Susin, S.A.; Petit, P.X.; Mignotte, B. & Kroemer, G. (1995). Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *Journal of Experimental Medicine,* 182., 367-377.

Zhang, J.; Fitsanakis, V.A.; Gu, G.; Jing, D.; Ao, M.; Amarnath, V. & Montine, T.J. (2003). Manganese ethylene-bis-dithiocarbamate and selective dopaminergic neurodegeneration in rat: a link through mitochondrial dysfunction. *Journal of Neurochemistry*, 84., 336–346.

Zhou, Y.; Shie, F.S.; Piccardo, P.; Montine, T.J. & Zhang, J. (2004). Proteasomal inhibition induced by manganese ethylene-bis-dithiocarbamate: relevance to Parkinson's disease. *Neuroscience,* 128., 281–291.

Zucchini-Pascal, N.; De Sousa, G. & Rahmani, R. (2009). Lindane and cell death: At the crossroads between apoptosis, necrosis and autophagy. *Toxicology,* 256., 32–41.

16

Structural and Dynamic Basis of Serine Proteases from Nematophagous Fungi for Cuticle Degradation

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1. Introduction

Serine proteases derived from nematophagous fungi are known as one of the most important virulence determinants during infection processes due to their ability to degrade nematode cuticle, a hard physical barrier protecting nematodes from physical damage or pathogen infections. Understanding the structural and dynamic features of cuticle-degrading serine proteases is fundamental to uncovering and describing the mechanism of nematicide by fungi at molecular level, which in turn will facilitate protein engineering study aimed at improving nematicidal activity of fungi.

In this chapter we describe several three-dimensional structural models of cuticle-degrading serine proteases secreted by different nematophagous fungi. The atomic coordinates of these models were obtained using methods such as the protein comparative modeling or the X-ray crystallography. Detailed comparison between these structures shows that, despite their striking degree of structural similarity, there are subtle differences in size, shape and hydrophathy of substrate-binding pockets S1 and S4 caused by single or multiple amino acid substitutions within the substrate-binding region. This explains why these enzymes have different substrate specificity and catalytic efficiency. Moreover, the amino acid changes in substrate-binding region and in overall enzyme molecule give rise to distinctly different electrostatic surface potential distribution among these proteases, possibly contributing to the distinct ability to attract the nematode cuticle with overall dominantly electronegative charge. The dynamic structural features of a classical serine protease proteinase K (PRK) were also investigated using molecular dynamics (MD) simulation

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technique. The different degree of flexibility of the substrate binding region was proposed to be linked to different substrate affinity and catalytic activity of these proteases; the first few modes of motions of substrate-binding pockets S1-S4, which were obtained using essential dynamics analysis, were proposed to be related to detailed dynamic progresses including substrate binding, positioning, catalysis, and product release.

The difference in thermal stability and nematicidal/catalytic activity between the alkaline serine proteases secreted by nematode-parasitic fungi and neutral proteases derived from nematode-trapping fungi can be attributed to the difference in global structural rigidity and local conformational flexibility (i.e., within the active site) caused by the presence/absence of disulfide bonds in the alkaline/neutral proteases. Of particular interest is that the nematode-parasitic and nematode-trapping fungi have evolved to adopt different strategies to obtain nutrient, i.e., the nematode-parasitic fungi, although have no trap device, produce powerful alkaline proteases (which carry predominant electro-positive surface potential) to diffuse towards and degrade hosts effectively, whereas the nematode-trapping fungi produce neutral proteases that carry predominant electro-negative surface potential and exhibit relatively weak catalytic activity, which can be compensated for by the active prey catch using their trap devices.

Taken together, the differences in dynamic behavior of substrate-binding region between cuticle-degrading proteases, in conjunction with differences in electrostatic surface potential distribution of these enzyme molecules and in architecture and hydrophathic property of substrate-binding pockets, could be used to explain their different catalytic activities towards nematode cuticle, thus facilitating in turn the explanation for different nematicidal activities by different fungi. The structures and dynamics of these cuticle-degrading serine proteases provide a solid basis for exploiting these enzymes from nematicidal fungi as effective bio-control agents.

2. Plant-parasitic nematodes and relevant control strategies

2.1 Description of the plant-parasitic nematodes

Nematodes are unsegmented roundworms and those that attack and parasitize plant are called plant-parasitic nematodes, which are a very important group of agricultural pests. Although the damage the plant-parasitic nematodes cause to plants is often subtle and is easily confused with nutrient problems, their enormous population numbers and broad plant host range can cause very serious damage to farmers and agricultural production, as indicated by the annual loss estimates of >100 billion US dollars throughout the world (Sasser & Freekman, 1987).

Plant nematodes are tiny worms whose body is covered by transparent and colorless cuticle, body length ranges from 0.25 mm to 3 mm and body shape is cylindrical, tapering toward the head and tail. The life cycle of a plant-parasitic nematode has six stages and four molts, i.e., egg, four juvenile stages and adult separated by molts (J1 - M1 - J2 - (Hatch) - M2 - J3- M3 - J4 - M4 - Adult). The first molt (M1) occurs in the egg between the first-stage juvenile (J1) and second-stage juvenile (J2); the emerging J2 is the most common infective stage; and the length of life cycle of plant parasitic nematodes can be anywhere from 20-40 days (on average 25 days at 22 ^{o}C) (Snyder, 2002). Plant parasitic nematodes, whether root-feeders or foliage-feeders, have a spear-like mouthpart that is able to puncture effectively host cells and feed on the cell contents. Such a feeding, accompanied by injection of some enzymes

and toxins into the host cell by nematodes, causes abnormal plant growth, and consequently the plants can be stunted, discolored, or both (Schmitt & Sipes, 1998).

2.2 Nematode control strategies and biological control

The goal of nematode control is to manage their population and reduce their numbers below the damaging levels to plants due to the fact that the thorough elimination of nematodes is not possible. The principle of nematode management is multifaceted, and as a result many conventional methods have been developed including planting resistant crop varieties, rotating crops, incorporating soil amendments, and applying pesticides (Schmitt & Sipes, 1998). Although the management methods involving no pesticides are environmentally friendly, their applications usually fail to keep nematodes below damaging levels for reasons that these methods are all to some extent limited, specific, hard to operate, and of low efficiency. Applications of the chemical nematicides are usually more effective in reducing nematode numbers than traditional non-pesticide methods because of the direct killing action on nematodes. However, most chemical nematicides are highly toxic synthetic pesticides, which are environmentally risky and extremely dangerous to people and other animals if handled carelessly. On the other hand, the abuse of nematicides could induce widespread resistance in field populations of many plant nematodes.

Another interesting nematode control approach is the biological control - using other organisms, i.e., the natural enemies, against the pest organism. The natural enemies of nematodes, which are usually isolated from nematode populations and are able to keep the population of nematode at apparently low levels, are usually some bacteria and fungi called nematophagous bacteria and fungi. Nematophagous bacteria are groups of bacteria that exhibit diverse modes of actions on nematodes, such as emitting potent volatile organic compounds to lure nematodes (Niu et al., 2010), producing toxins to suppress host defense and alter cellular metabolism, and secreting enzymes to degrade host cuticle or intestinal tissue, leading to the parasitization of bacteria and subsequent death of nematodes (Lobna & Zawam, 2010; Tian et al., 2007). Nematophagous fungi are a diverse group of carnivorous fungal species that use their spores or mycelial structures to capture nematodes (which are called nematode-trapping fungi), or use their hyphal tips to parasitize the eggs and cysts of nematodes (Nordbring-Hertz, 2004), or produce toxins/enzymes to attack/degrade nematodes (which are called nematode-parasitic fungi) (T.F. Li et al., 2000; X.Z. Liu et al., 2009). Although under laboratory conditions some nematophagous bacteria and fungi have been shown to reduce populations of some kinds of nematodes, the successes at the full-scale field level have been limited (Schmitt & Sipes, 1998). The possible reasons for this are that i) most organisms that are recognized as biological control agents are highly host-specific, attacking just one or few of the nematode pests; ii) the nematophagous bacteria and fungi are too difficult to culture to obtain sufficient quantities that are useful for field application. Nevertheless, applying biological control as a means to reduce the harm of nematodes to agriculture production has always attracted considerable attention because of the apparent advantages of this method: i) the nematophagous microorganisms are nonpolluting and therefore environmentally acceptable and safe, and as such utilizing such agents can avoid the use of ecologically and environmentally hazardous chemicals, promoting the ecological balance; ii) biological control is considered to be more effective against agricultural pests that are less harmful, that only need to be controlled and suppressed and can not be permanently eliminated (i.e., the nematode) (Writing, 2011), and

therefore the utilization of nematophagous microorganisms is beneficial to increasing biodiversity in soil; iii) biological control has potentially huge economic and commercial value and simultaneously its relevant research, development and application will provide many employment and job opportunities.

During the past decades, the nematode-targeted biological control study is mainly focused on elucidating the pathogenic/infection mechanism of nematophagous microorganisms since this is essential for developing effective biological control agents against nematodes. For example, a "Trojan horse" mechanism of a nematophagous bacterium, *Bacillus nematocida* B16, against nematodes has been reported recently by our lab (Niu et al., 2010), indicating that this bacterium can lure nematodes that are relatively distant to move close to the bacteria through producing volatile organic compounds; and the subsequent uptake of bacteria as food by nematode offers the opportunity for bacteria to secrete proteases with broad substrate specificity to degrade the intestinal wall of nematode. We also sequenced the whole genome of the nematode-trapping fungus *Arthrobotrys oligospora* Fres. (ATCC 24927) to reveal genes likely involved in pathogenesis of this nematophagous fungus; the subsequent proteomic and RT-PCR analyses of protein differential expression induced by the presence of nematode extract revealed that many cellular processes such as energy metabolism, biosynthesis of the cell wall and adhesive proteins, cell division, glycerol accumulation and peroxisome biogenesis, determine, participate in and regulate the formation of the trap device (J. K. Yang et al., 2011). The analysis of the *A. oligospora* genome revealed that this nematophagous species has a larger number of enzyme genes such as those encoding the enzyme families of subtilisin, collagenase, pectinesterase and pectate lyase in comparison with several sequenced model ascomycete fungi (J. K. Yang et al., 2011).

2.3 Nematode cuticle, its components and cuticle-degrading enzymes

Like the majority of pathogens that infect/attack the hosts, the fist step of the infection by nematophagous fungi is to break open the cuticle of the host followed by pathogens entry. The cuticle is a multi-functional exoskeleton and is a highly impervious barrier between the animal (such as nematode and insect) and its environment. Cuticle is also essential for the maintenance of body morphology and integrity, and simultaneously plays a critical role in locomotion via attachments to body-wall muscles (Page & Johnstone, 2007). Since the main structural components of the nematode cuticle are proteins, highly cross-linked collagens, chitin fibrils and specialized insoluble proteins known as "cuticlins", together with glycoproteins and lipids (Page & Johnstone, 2007), it is not surprising that nematophagous fungi need to produce a variety of highly efficient enzymes such as proteases, collagenases, chitinases and lipases to act together to degrade the first defense line - the cuticle of the nematode. Among the above-mentioned enzymes, the proteases are considered to be most important during infection since the protein matrix is the most important component of the nematode cuticle and the quantity and type of proteins vary between species, tissues and growth stages of nematodes (Huang et al., 2004; Jansson & Nordbring-Hertz, 1988).

During the past several years, several cuticle-degrading proteases have been purified and characterized from different nematophagous or entomopathogenic fungi such as *Arthrobotrys oligospora* (Tunlid et al., 1994; Zhao et al., 2004), *Pochonia chlamydospora* (syn. *Verticillium chlamydosporium*) (R. Segers et al., 1994), *Beauveria bassiana* (Joshi et al., 1995), and *Metarhizium anisopliae* (R.J. St Leger et al., 1987); and their catalytic activities towards casein

and cuticle proteins were shown to be high, suggesting their highly nematicidal activities. Cloning and sequencing of the genes coding for these cuticle-degrading proteases (Joshi et al., 1995; R. Segers et al., 1994; R.J. St Leger et al., 1987; Tunlid et al., 1994; Zhao et al., 2004) reveal that these proteases have a high degree of sequence similarity to each other and to the proteinase K (EC 3.4.21.64) derived from *Tritirachium album* Limber (Ebeling et al., 1974), suggesting that these proteases belong to the proteinase K subfamily of the secretory subtilase/subtilisin-like serine protease family, known as the peptidase family S8 according to the classification and nomenclature of peptidases in the MEROPS database (Rawlings et al., 2010). This peptidase family is also the second largest family of serine peptidases, both in terms of number of sequences and characterized peptidases, and distributes broadly from viruses, bacteria, and fungi to plants and animals. Although the physiochemical properties, optimum reaction conditions, substrate specificity, catalytic mechanism, dynamics, and structure-function relationship of the representative enzyme of the peptidase S8 family, the proteinase K, have been extensively studied (Bajorath et al., 1989; Betzel et al., 2001; Betzel et al., 1988; Betzel et al., 1986; Betzel et al., 1993; Ebeling et al., 1974; Hilz et al., 1975; S. Q. Liu et al., 2010, 2011; Müller et al., 1994; Pahler et al., 1984; Tao et al., 2010; Wolf et al., 1991), a thorough understanding of the above characteristics for the cuticle-degrading serine proteases from nematophagous fungi is of crucial importance in uncovering and describing the nematicidal mechanism by fungi at molecular level. In this chapter we describe how the variation in amino acid sequence affect the functional properties (such as substrate specificity and catalytic efficiency) of the cuticle-degrading proteases derived from different fungi through comprehensive investigation into the three-dimensional structural models of these enzymes, which will facilitate the improvement in biological control potential of nematophagous fungi through protein engineering or site-directed mutagenesis.

3. Catalytic mechanism of serine proteases

Proteases are enzymes that break down protein through hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. They occur naturally in all organisms and involve various physiological reactions from simple digestion of proteins in food to highly-regulated cascades. Proteases are currently divided into four major groups according to the character of their catalytic active site and conditions of action: serine proteinases, cysteine (thiol) proteinases, aspartic proteinases, and metalloproteinases. Classification of a protease into a certain group depends on the structure of catalytic site and the essential amino acid acting as a nucleophile. Accordingly, serine proteases (EC 3.4.21) are proteases in which one of the amino acids in the active site of the enzyme is the serine. This group of proteases is present in virtually all organisms and is further classified into two clans: the chymotrypsin-clan and the subtilisin-clan. These two clans are evolutionarily unrelated and have distinct overall folded structures but share the same catalytic mechanism utilizing an identical stereochemistry of the catalytic triad where the serine act as the nucleophile (Blow, 1976; Siezen et al., 1991; Siezen & Leunissen, 1997; Tsukuda & Blow, 1985). This has been regarded as a classic example to illustrate convergent evolution showing the same catalytic mechanism evolved twice independently during evolution. The catalytic triad, located at the active site of protease, is a coordinated structure consisting of three essential amino acids: aspartic acid (Asp39 in proteinase K numbering; hereafter, all numbering uses proteinase K as reference), histidine (His69), and serine (Ser224). These

three key amino acid residues are located adjacent to one another near the heart of the enzyme (Figure 1), each playing an essential role in the cleaving process of the peptide bond (Birktoft & Blow, 1972; Dodson & Wlodawer, 1998; Kraut, 1977; Russell & Fersht, 1987):

- The Ser uses its hydroxyl group (-OH) as the primary nucleophile, which is able to attack the carbonyl carbon (C=O) of the scissile peptide bond (-C(=O)NH-) of the substrate.
- The His plays a dual role as the proton donor and acceptor at different steps in the reaction, i.e., a pair of electrons on the His nitrogen has the ability to accept the hydrogen from the serine hydroxyl group, thus coordinating the attack of the peptide bond.
- The role of Asp is believed to bring the His into the correct orientation by its carboxyl group hydrogen bonding with the His, thus facilitating the nucleophilic attack by the Ser.

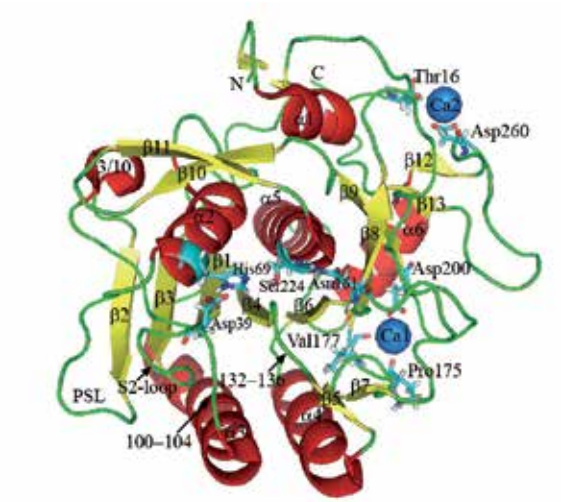

Fig. 1. Ribbon diagram of proteinase K (PDB code 1IC6). The α helices, β strands and loops/links are colored red, yellow and green, respectively. The residues of the catalytic triad (Asp39, His69 and Ser224), oxyanion hole (Asn161), and calcium binding sites (Ca1 site: Pro175, Val177 and Asp200; Ca2 site: Thr16 and Asp260) are shown as stick models with the carbon, nitrogen and oxygen colored cyan, blue and red, respectively. The two bound Ca^{2+} cations, Ca1 and Ca2, are shown as blue spheres. The secondary structure elements, substrate-binding regions of residues 100-104 and 132-132, polar surface loop (PSL loop) and S2-loop are labeled.

The detailed process of the catalytic mechanism can be described as follows (see also Figure 2):

1. After the binding of substrate and the positioning of scissile peptide bond is accomplished, the nitrogen atom of His accepts a hydrogen atom from the hydroxyl group of Ser, followed by a hydroxyl oxygen atom of Ser initiating the attack on the carbonyl carbon atom of the peptide bond, and the subsequent movement of a pair of electrons from the carbonyl double bond to the oxygen, resulting in the formation of a tetrahedral intermediate.
2. The covalent electrons creating the single peptide bond move to attack the hydrogen atom of His, resulting in the breaking of the peptide bond. The electrons on the negative

oxygen move back to recreate carbonyl oxygen double bond, producing an acyl-enzyme intermediate and a new N-terminus of the cleaved peptide chain.

3. A water molecule enters into the reaction through replacing the N-terminus of the cleaved peptide. It attacks the carbonyl carbon atom, followed by electrons moving from the double bond to the oxygen making it negative, resulting in the formation of a bond connecting the oxygen atom of the water and the carbonyl carbon atom. The His nitrogen atom accepts a proton from the water and coordinates the newly formed second tetrahedral intermediate via a hydrogen bond.
4. The final step involves the breaking of the bond formed in the first step between the Ser hydroxyl oxygen atom and the peptide bond carbonyl carbon atom. This is achieved via the attack of the His hydrogen (which is just acquired from the water) on this bond. The resultant electron-deficient carbonyl carbon reforms the double bond with the oxygen, resulting in the final release of the C-terminal product of the peptide.

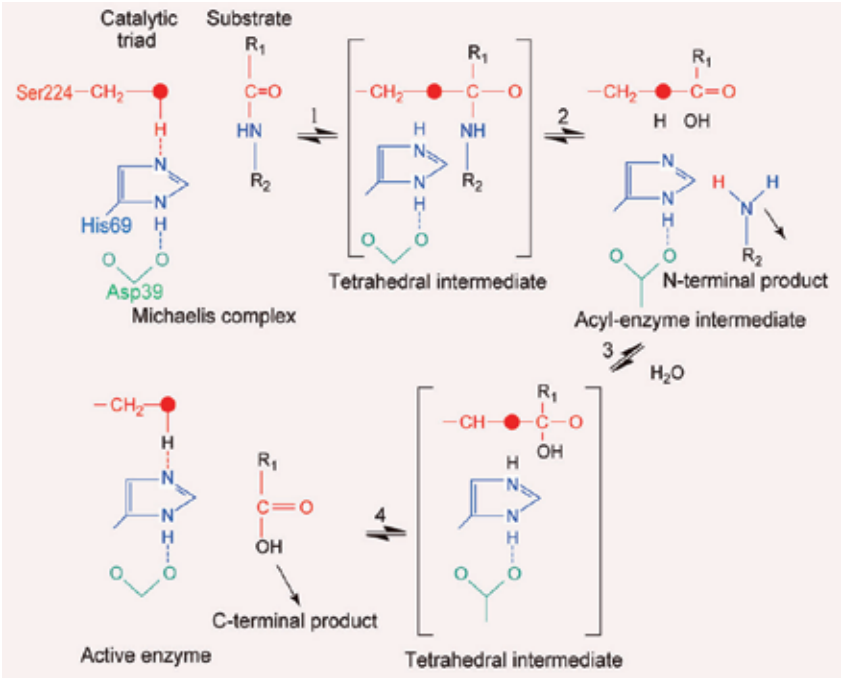

Fig. 2. Steps of the catalytic reaction by the catalytic triad of serine protease proteinase K. The nucleophile residue Ser is shown in red; the red dot indicates the nucleophilic atom hydroxyl oxygen. The His and Asp are shown in blue and green, respectively. This figure is modified from (Dodson & Wlodawer, 1998; Kraut, 1977); see text for details.

During the catalytic reaction process, the resultant negatively charged oxygen ion of the tetrahedral intermediate generated in steps 1 and 3 are believed to be stabilized by the so-called structural component of "oxyanion hole", which is formed by Ser224 (N), Asn161 (N), and Asn161 (N_δ), donating their backbone or side chain hydrogens to hydrogen bond with oxygen ion (Figure 1, 6D, E) (Betzel et al., 1986; Wolf et al., 1991). Like almost all other enzymes, the serine proteases can accelerate reaction because they bind the transition state (here is the tetrahedral intermediate) better than they bind either the Michaelis complex or

the acyl-enzyme intermediate, thus lowering the activation free energy barrier of the reaction.

4. Homology modeling of the cuticle-degrading proteases from fungi

In order to characterize the structural features and functional properties of cuticle-degrading serine proteases from fungi, we have built homology models of several proteases (S.Q. Liu et al., 2007b) based on the X-ray crystallographic structure of proteinase K (Betzel et al., 2001). These are PR1 (GenBank accession number AJ416695) from entomopathogenic fungus *Metarhizium anisopliae* (R.J. St Leger et al., 1987; R.J. St Leger et al., 1993), VCP1 (GenBank accession number AJ427460) from the nematophagous fungus, *Pochonia chlamydosporia* (Syn. *Verticillium chlamydosporium*) (Morton et al., 2003; R. Segers et al., 1996), Ver112 (GenBank accession number AY692148) from the nematophagous fungus *Lecanicillium psalliotae* (J.K. Yang et al., 2005a; J.K. Yang et al., 2005b), and PL646 (GenBank accession number EF094858) (Liang et al., 2010) from a saprobic, filamentous fungus *Paecilomyces lilacinus*, which is found to have a wide range of hosts such as nematodes (Jatala, 1986; Stirling & West, 1991), insects (Fiedler & Sosnowska, 2007; Rombach et al., 1986) and humans (Saberhagen et al., 1997; Westenfeld et al., 1996). *P. lilacinus* has been applied as a biocontrol agent to control the growth of destructive root-knot nematodes, resulting in several successful field trials on a range of crops in different soil types and climates (Jatala, 1986; Jatala et al., 1979). Of these proteases, PR1 is considered as a crucial factor in the infection of the insect in that it was detected with gold-labeled antibodies during the penetration of the insect cuticle by *Metarhizium anisopliae* (Goettel et al., 1989), and the rapid production and high concentration of this enzyme were also observed during infection (R.J. St Leger et al., 1986). The latter three are alkaline serine proteases which exhibit a crucial role in the penetration process of nematode eggs or cuticles.

4.1 Physiochemical properties and optimum reaction conditions

For these four cuticle-degrading proteases and proteinase K, their physiochemical properties were theoretically predicted (i.e., the programs SAPS (Brendel et al., 1992), EXTCOEF (Gill & von Hipple, 1989) and pI web server (Lehninger, 1995) were used for predicting the molecular weight, extinction coefficient (EXTCOEF) at 280 nm, and isoelectric point (pI), respectively) and the optimum reaction conditions were experimentally determined (S.Q. Liu et al., 2007b). The results (Table 1) show that many physiochemical properties among these enzymes are very similar to each other, i.e., the residue number ranges from 279 to 284 and molecular weight is between 28.5 and 28.9 kDa, pI is high with values greater than 7.7, EXTCOEF is comparable at 280 nm, and have common susceptivity to inhibitor PMSF. Enzyme kinetics assays with substrates such as Suc-Ala-Ala-Ala-p-nitroaniline, Suc-Ala-Ala-Pro-Phe-p-nitroaniline and casein showed that these enzymes exhibited almost the same optimum reaction conditions, i.e., the high catalytic efficiencies were found at pH 8-10 and temperatures 50-60 °C (Liang et al., 2010; R. Segers et al., 1996; R.J. St Leger et al., 1987; J.K. Yang et al., 2005b). These highly similar properties can be explained by the high degree of amino acid sequence identity (> 60%) among proteinase K, PR1, VCP1, Ver112 and PL646, suggesting that these five proteases are members of the proteinase K subfamily and all are alkaline serine proteases, in agreement with previous propositions.

In addition, we also used the soluble proteins extracted from *C. elegans* nematode cuticle as substrates to test catalytic efficiencies of Ver112, PL646 and proteinase K. The results

showed that the three proteases commonly had high catalytic activities towards cuticle extract at their optimum reaction conditions (Liang et al., 2010). These three proteases also exhibit similar relatively high catalytic activities towards collagen (Liang et al., 2010).

Protease	Fungus	Host	MW[a] (kDa)	NAA[b]	pI[c]	RK/ DE[d]	EXTCOEF[e] ($M^{-1}cm^{-1}$)	Inhibitor	SI[h] (%)	Optimum[i]	
										pH	T[j](°C)
PRK	*Tritirachium album*	Human	28.9	279	7.71	20/19	33200	PMSF[f] DFP[g]	100	7.5-12	50-60
PR1	*Metarhizium anisopliae*	Insect	28.6	281	8.37	21/18	26800	PMSF	66.4	8-10	50-60
VCP1	*Pochonia chlamydosporia*	Nematode	28.5	281	7.74	20/19	28650	PMSF	62.5	8-10	50-60
Ver112	*Lecanicillium psalliotae*	Insect	28.6	280	8.67	22/18	27370	PMSF	64.1	8-10	50-60
PL646	*Paecilomyces lilacinus*	Nematode /Insect/ Human	28.7	284	8.38	19/16	25760	PMSF	62.0	8-10	50-70

[a] Calculated molecular weight.
[b] Number of amino acids.
[c] Predicted isoelectric point.
[d] Number of positively charged residues/number of negatively charged residues.
[e] Predicted extinction coefficient at 280 nm.
[f] Phenyl methane sulphonyl fluoride.
[g] Diisopropyl fluorophosphates.
[h] Sequence identity with respect to 1IC6.
[i] Optimal reaction conditions.
[j] Temperature.

Table 1. Physiochemical properties and optimal reaction conditions of the proteinase K (PRK) and the four cuticle-degrading enzymes PR1, VCP1, Ver112 and PL646.

The reasons for these proteases exhibiting high catalytic activity at both high temperature and high pH may be explained as follows. The elevated temperature can enhance conformational flexibility of enzyme structures, especially in the substrate-binding regions with relatively few conformational restrictions, thus enhancing the substrate affinity and catalytic efficiency of these enzymes (Gudjonsdottir & Asgeirsson, 2008; S. Q. Liu et al., 2010, 2011; Tao et al., 2010). Because excessively high temperatures will lead to thermal denaturation of the protein structure, the temperature just below the transition temperature is often the optimal reaction temperature of these proteases. The relatively high pH value is beneficial for maintaining the correct hydrogen bonding interaction within the catalytic triad, i.e., the hydrogen bonds His-$N_{\delta1}$-H···$O_{\delta2}$-Asp and Ser-O_{γ}-H···$N_{\varepsilon2}$-His, via proper protonation state for one of the catalytic triad residues His, i.e., protonation on the $N_{\delta1}$ atom but not on $N_{\varepsilon2}$ atom in the imidazole group.

4.2 Description and comparison of structural models

The structural models of PR1, VCP1, Ver112 and PL646 were built based on the high-resolution crystal structure of proteinase K (0.98 Å; PDB code 1IC6; hereafter proteinase K is

also referred to as 1IC6) using homology modeling technique implemented in the software package MODELLER 7v7 (Sali & Blundell, 1993). Because of the high degree of sequence identity (> 60%) of these proteases with respect to their template 1IC6, especially in the loop regions (Figure 3), it is not surprising that these modeled structures are nearly identical, whether to each other or to the template (Figure 4). Commonly, these models show the common α/β scaffold characteristics of the subtilisin-like serine protease, which consist of six α helices, two 3/10 helices, a seven-stranded parallel β sheet, and three two-stranded antiparallel β sheets. The corresponding amino acid sequences of these secondary structure elements are also indicated at the top of the multiple sequence alignment plot (Figure 3).

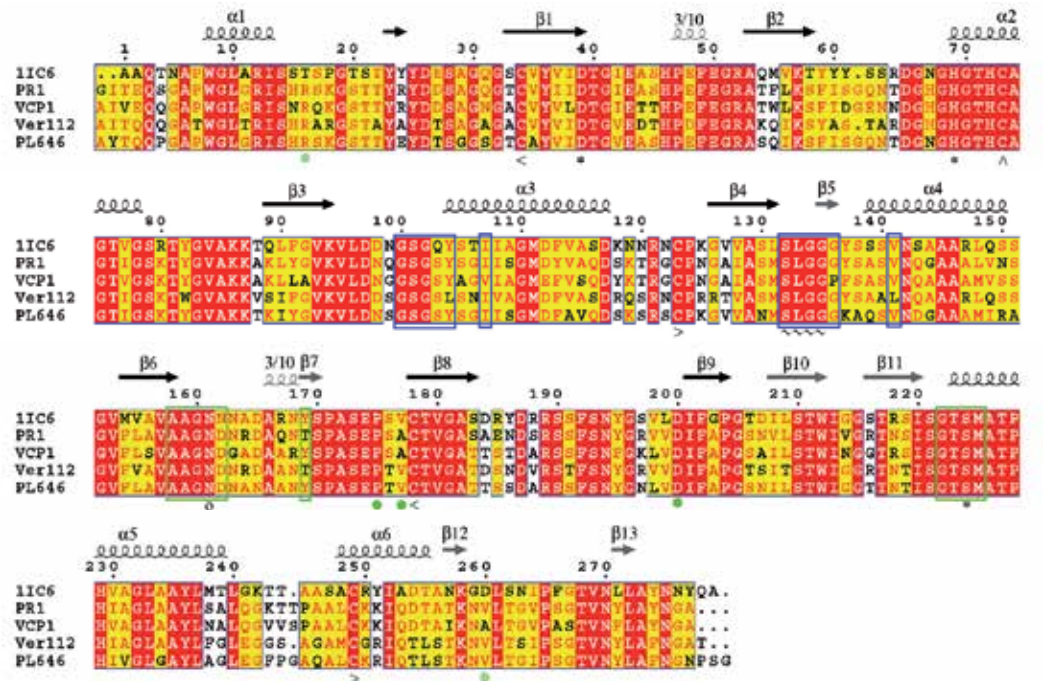

Fig. 3. Multiple sequence alignment of proteinase K (1IC6) and cuticle-degrading proteases PR1, VCP1, Ver112 and PL646. The regions of residues with red background and white foreground are completely conserved; those with yellow background and red foreground are well conserved or similar, and with black foreground are not conserved. The common secondary structure elements are indicated at the top with black arrows for parallel β strands, gray arrows for antiparallel β strands, black curves for α helices, and gray curves for 3/10 helices, respectively. Residues participating in the formation of the S1 site are circled by green frames, and those in the S4 site are circled by blue frames. ~ at the bottom indicates residues that participate in the formation of both S1 and S4 sites. Catalytic triad residues Asp39, His69 and Ser224 (proteinase K numbering) are marked at the bottom with a *. Oxyanion hole residue Asn161 is marked with o. Cysteines involved in the disulfide bridges are marked with < and > (<Cys34-Cys123> and <Cys178-Cys249>). The free Cys73 is with ∧. Residues forming the "strong" calcium binding site (Pro175, Val/Ala177 and Asp200) Ca1 are marked with dark green ball and those forming the weak calcium binding site Ca2 (Thr16 and Asp260) are marked with pale green ball. The Ca2 site is unique for proteinase K.

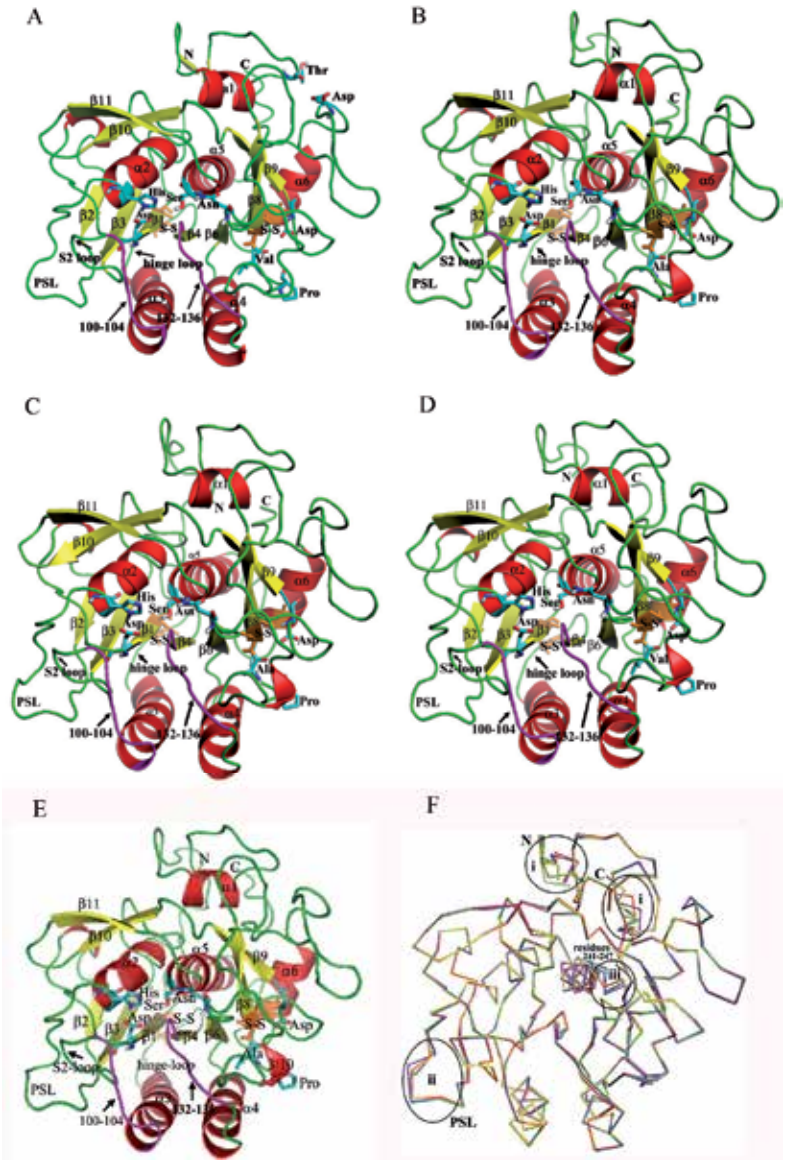

Fig. 4. Structural representations of the homology models of cuticle-degrading proteases. (A) 1IC6, (B) PR1, (C) VCP1, (D) Ver112, (E) PL646 and (F) backbone superposition of the four cuticle-degrading proteases and their template structure proteinase K with PDB code 1IC6. The α helices, β strands and loops are colored red, yellow and green, respectively. Substrate-binding segments 100-104 and 132-136 (1IC6 numbering) are in purple and disulfide bridges in orange. The residues of catalytic triad (Asp39, His69 and Ser224), oxyanion hole (Asn161), disulfide bridges (S-S), and calcium binding sites are rendered as stick models. In plot (F) the backbones of 1IC6, PR1, VCP1, Ver112 and PL646 are colored yellow, blue, green, red and purple; and the structural regions exhibiting relatively large conformational difference are labeled: (i) N- and C-Termini; (ii) surface-exposed polar loop (PSL) located between β2 and α2; and (iii) the loop region (residues 241-247) located between α5 and α6.

Quantitative comparisons between these structural models were performed by using least-square fitting method implemented in Swiss-Pdbviewer (Guex & Peitsch, 1997). The structure backbones were superimposed pairwise onto each other, achieving the backbone root mean deviation (RMSD) values with the range of 0.04-0.80 Å. The order of structural difference between these models is: PR1-VCP1 (0.04 Å) < PR1-PL646 (0.05 Å) < Ver112-PL646 (0.21 Å) < Ver112-PR1 (0.34 Å) = VCP1-PL646 (0.34) < VCP1-Ver112 (0.35 Å) < Ver112-1IC6 (0.58 Å) <1IC6-PR1 (0.75 Å) = 1IC6 -VCP1 (0.75 Å) < 1IC6-PL646 (0.80 Å), suggesting that the cuticle-degrading protease are more similar in structure to each other than to proteinase K. This is in agreement with the postulation that the higher the amino acid residue sequence identity between the proteins, the more similar the structures between them. The sequence identities of PR1-VCP1, PR1-PL646, Ver112-PL646, Ver112-PR1, VCP1-PL646, VCP1-Ver112, Ver112-1IC6, 1IC6-PR1, 1IC6 -VCP1 and 1IC6-PL646 are 78.6%, 74.6%, 71.1%, 69.5%, 68.0%, 64.3%, 64.1%, 66.4%, 62.5% and 62.0%, respectively.

In the crystal structure of proteinase K, four of the five cysteines form two disulfide bonds: Cys34-Cys123 and Cys178-Cys249, whereas Cys73 is free. Interestingly, all the four cuticle-degrading proteases contain the five positionally equivalent cysteines and the two corresponding disulfide bridges are also presented in the resultant structural models of the cuticle-degrading proteases. Close inspection of these models reveals that the free sulfur atom in Cys73 makes close contacts with the active site residues His69(O), Ser224(O), and Ser132(O_γ), for which the function consequence has yet to understand.

The catalytic triad residues Asp39, His69 and Ser224 and the component of the oxyanion hole residue Asn161 are completely conserved among the proteinase K and cuticle-degrading proteases as shown in the multiple sequence alignment plot (Figure 3), resulting in the perfectly structurally conserved architecture of the catalytic triad and oxyanion hole among the structural models of these enzymes (Figure 4). In proteinase K, two polypeptide segments of residues 100-104 and 132-136 constitute the two sides of the substrate-binding channel, where the P4, P3, P2 and P1 residues of the substrate are accommodated as the central strand to form a three-stranded antiparallel β sheet with residues in the S4, S3, S2 and S1 sites of the enzyme (Wolf et al., 1991). Among the four cuticle-degrading proteases, the residues equivalent to substrate-binding regions of proteinase K are well conserved with only subtle amino acid variations being found in the S1 and S4 substrate-binding pockets (Figure 3 and 5).

Despite the essentially global similarity between these structural models, relatively large conformational differences in local structures can still be found upon the superposition of these structural models (Figure 4F). Close inspection of Figure 4F reveals three regions exhibiting large backbone conformational differences: i) the N- and C-terminal regions, which exhibit the largest difference; ii) the surface-exposed loop comprising residues 59-68 with a high percentage of polar residues, and is thus termed polar surface loop (PSL); iii) the surface-exposed loop region (residues 241-247) located between α5 and α6. These large conformational differences are brought about by residue insertions or deletions within these regions as indicated by the sequence alignment (Figure 3). The most insertions or deletions of the target sequence relative to the template 1IC6 are found in the N- and C-termini, thus leading to the largest differences in conformation between these cuticle-degrading proteases. Other insertions are only observed in PSL and in loop region connecting α5 and α6, i.e, in PSL, PR1, VCP1 and PL646 have one residue insertion between positions 61 and 62 relative to the template sequence 1IC6; in loop 241-247 the cuticle-degrading enzymes PR1, VCP1 and PL646 have one insertion between positions 244 and 245. The few insertions or

deletions that are observed merely in loops, in conjunction with the high degree of sequence identity, can be used to explain the virtually identical structural models of these proteases. However, as will be described and discussed later, the subtle differences in architecture of the substrate-binding regions, which arise from variation in amino acid sequence, can be used to predict differences in function properties between these enzymes.

4.3 Factors contributing to the stability of molecular structures

4.3.1 Ca^{2+}-binding sites

Many proteases in the peptide S8 family contain one or more Ca^{2+}-binding sites and binding of calcium cations enhances the thermal stability of the proteases which in turn increase their resistance again proteolysis, either by itself or by other proteases (Bajorath et al., 1989; Betzel et al., 1988; Betzel et al., 1990; Müller et al., 1994). Two Ca^{2+} cations were found in the high resolution crystal structure of proteinase K (Figure 1 and 4A) (Betzel et al., 2001). The first Ca^{2+} is coordinated tightly by the $O_{\delta 1}$ and $O_{\delta 2}$ of Asp200 and the carbonyl oxygen atoms of Pro175 and Val177, and as such this site is termed strong Ca^{2+}-binding site Ca1. Intuitively, the Ca1 is able to stabilize the regions around itself, especially the long loops connecting β7 and β8 and β8 and β9. The second Ca^{2+} is a weakly bound Ca^{2+}, which is coordinated by Ca2 site consisting of the $O_{\delta 1}$ and $O_{\delta 1}$ of Asp260 and the carbonyl oxygen atom of Thr16. The Ca2 stabilizes to some extent the N- and C-terminal regions of the molecular structure. Among the four cuticle-degrading proteases, Ver112 and PL646 completely preserve the Ca1 site, whereas PR1 and VCP1 have residue substitution at position 177, i.e., an Ala in Pr1 and VCP1 substitutes for Val in 1IC6 (Figure 3). Although such substitution may have influence on the calcium affinity, we consider that such effect may be minor because the carbonyl oxygen of Ala177, coupled with carbonyl oxygen of Pro175 and carboxyl oxygens of Asp200, can still coordinate Ca^{2+} in a manner similar to that in 1IC6. In the case of the Ca2 site, the Thr16 in 1IC6 is replaced by an Arg in the four cuticle-degrading enzymes and the Asp260 in 1IC6 is replaced by a Val in PR1, Ver112 and PL646 and by Ala in VCP1, possibly leading to the loss of the ability of these cuticle-degrading enzymes to bind the second calcium cation due to the lack of carboxyl oxygens.

4.3.2 Disulfide bonds

It has been shown that disulfide bonds play an important role in the stability of some proteins by an entropic effect (Matsumura et al., 1989), usually the globular proteins secreted to extracellular medium (Sevier & Kaiser, 2002). As mentioned above, two disulfide bonds equivalent to those in proteinase K were also observed in the homology models of these four cuticle-degrading proteases (Figure 1, 3 and 4). The disulfide bridge Cys34-Cys123 connects the N-terminus of β1 at whose C-terminus lies the catalytic residue Asp39 and the loop (residues 118-126) connecting α3 and β4. Although this loop is located opposite the substrate-binding regions and is relatively far away from the catalytic triad, it links the α3 following the substrate-binding segment residues 100-104 and β4 preceding the other substrate-binding segment residues 132-136. Therefore, this loop can be considered as a "hinge" capable of modulating the orientation of the substrate-binding segments. As a result, the disulfide bond Cys34-Cys123 may participate in such modulation through contributing to the stability of this loop. On the other hand, this disulfide bond may also affect the dynamic behavior of the catalytic residue Asp39 via the rigid strand β1. The other disulfide bridge Cys178-Cys249 connects the N-terminus of β8 (located next to the S1

pocket) and α6, attaching the peripheral α6 to the main body of proteases, contributing to the stability of the C-terminal region. In addition, since β8 is located adjacent to the S1 substrate-binding pocket, Cys178-Cys249 may also influence the orientation of the S1 residues via a loop comprising residues 171-177.

4.3.3 Hydrogen bonds and salt bridges

Hydrogen bonds have been considered to contribute to the overall stability of the protein structure due to their large number and wide distribution (Shirley et al., 1992), whereas salt bridges contribute to a large extent to only the local stability because of its limited number and localized distribution (Arnorsdottir et al., 2005). The numbers of hydrogen bonds/salt bridges in structural models of proteinase K, PR1, VCP1, Ver112 and PL646 are 223/14, 189/12, 189/12, 195/13, and 199/8, respectively (Table 2). Here we mainly focused on the hydrogen bonds and salt bridges that serve to stabilize certain sites of interest. For example, a salt bridge network Arg12:Asp187:Lys/Arg18, which is found in all these four cuticle-degrading proteases, contributes to the stability of the N-terminal region of these proteases. This salt bridge network does not occur in proteinase K due to the lack of positively charged residue at position 18. However, the Ca2 cation, which does not exist in cuticle-degrading proteases, acts as an important factor to stabilize the N-terminus in proteinases K. Another bridge network, Arg52:Glu50:Lyr/Arg80, is found to be conserved throughout the five enzymes, connecting and stabilizing two surface loops between β1 and β2 (residues 40-52) and between α2 and β3 (residues 79-88). A completely conserved salt bridge, Asp117:Arg121, together with the disulfide bridge Cys34-Cys123, anchors and stabilizes the "hinge" loop containing residues 118-126. Additionally, two or more salt bridges are observed between two regions carrying multiple successive negatively or positively charged residues, i.e., Asp26-Glu/Asp27 and Lys86-Lys87, contributing to the stability of the long loop connecting α1 and β1 and of the relatively short loop between α2 and β3, although the stability effect is weaker in Ver112 and PL646 than in 1IC6, PR1 and VCP1 due to the reduction in the number of negatively charged residue in Ver112 and PL646 (replacement of Glu/Asp with Thr). There is a salt bridge, Asp112:Arg147, in the two proteases 1IC6 and Ver112 that is able to bridge α3 and α4, which are located adjacent to the two substrate-binding segments composed of residues 100-104 and 132-136. This salt bridge does not exist in PR1, VCP1 and PL646 due to the replacement of Arg147 with Ala, although the position 112 is occupied by the negatively charged residue (Asp or Glu) throughout the five proteases. In PR1 and VCP1, a salt bridge network, Lys250:Asp254:Lys251, is observed to contribute to the stability of the C-terminal helix α6, while only one salt bridge, Arg250:Asp254, is found in α6 of 1IC6, and no salt bridge in α6 of Ver112 and PL646. Interestingly, another structural factor capable of stabilizing α6, i.e., the disulfide bond Cys178-Cys249 is found in all these four cuticle-degrading proteases and proteinase K.

The region of residues 162-169 is located in the vicinity of S1 pocket and some of its residues form the bottom of the S1 pocket. For all these proteases except for PL646, at least one salt bridge or salt bridge network is found in this region contributing to the stability of this region. For example, 1IC6 contains a salt bridge, Asp165:Arg167, PR1 and Ver112 share a salt bridge, Asp162:Arg164, and VCP1 contains a salt bridge network, Asp162:Arg168:Asp165. Although PL646 contains no salt bridge in this region, a number of hydrogen bonds are observed in this region that can also stabilize the bottom of S1 pocket.

Taken together, the salt bridges coupled with the hydrogen bonds contribute to the stability of the S1 pocket bottom, which may facilitate the precise orientation of the P1 residue of the substrate for nucleophilic attack by the Ser.

Protease	RMSD[a] (Å)	SASA[b] (nm^2)	Rg[c] (nm)	NHB[d]	NNC[e]	NSB[f]	ENE[g]	DIH[h]	SSE[i]		
									α helix	β sheet	Turn
1IC6	0	109.2	1.67	223	134896	14	–9288.2	0	69	66	43
PR1	0.75	113.4	1.67	189	129252	12	–8560.3	0	67	66	42
VCP1	0.75	111.0	1.67	189	128896	12	–8560.0	0	70	64	47
Ver112	0.58	106.6	1.66	195	132572	13	–8732.7	1	70	66	43
PL646	0.80	111.7	1.69	199	130180	8	–8464.9	0	71	66	39

[a] Backbone RMSD with respect to 1IC6.
[b] Total solvent accessible surface area.
[c] Radius of gyration.
[d] Number of hydrogen bonds. A hydrogen bond is considered to exist when the donor-hydrogen-acceptor angle is larger than 120° and the donor-acceptor distance is smaller than 3.5 Å.
[e] Number of native contacts. A native contact is considered to exist if the distance between two atoms is less than 6 Å.
[f] Number of salt bridges. A salt bridge is considered to exist if the distance between two oppositely charged residues is within 6 Å.
[g] Potential energy after energy minimization in the GROMOS96 force field.
[h] Number of residues in the disallowed regions in the Ramachandran plot.
[i] Number of residues in the corresponding secondary structure elements.

Table 2. Geometrical properties of proteinase K crystal structure 1IC6 and structural models of the four cuticle-degrading enzymes PR1, VCP1, Ver112 and PL646.

Charged residue clusters are also found in two surface-exposed loops: the PSL (residues 59-68) and the loop composed of residues 94-101. PSL is located before the catalytic triad residue His69. The loop 95-101 is located in close proximity to the S2 pocket and partially participates in the formation of the S2 pocket, and therefore it is referred to as the S2-loop. A completely conserved salt bridge network Asp65:Lyr94:Asp97 is found in all these five proteases, bridging the PSL and the S2-loop together contributing to the stability of these two loops. Two additional salt bridge networks (Asp65:Lyr94:Asp98 and Arg64:Asp98:Lyr94) capable of enhancing the stability of these two loop are observed in 1IC6 and Ver112. The stability of the S2-loop is further enhanced by an absolutely conserved hydrogen bond network Asp97-[Gly100, S101] in these five proteases. Additionally, a salt bridge Glu43:Arg64, which is found only in proteinase K and Ver112 but not in other proteases, contributes to the stability of the PSL. Another important factor contributing to the stability of the PSL is the hydrogen bond and hydrogen bonding network, i.e., a completely conserved hydrogen bonding network Asp65-[Gly68, Thr71] is observed in all these five enzymes; and the number of hydrogen bonds involved in stabilizing the PSL is 10, 9, 7, 8 and 8 in 1IC6, PR1, VCP1, Ver112 and PL646, respectively. Taken together, we can conclude that a relatively large number of hydrogen bonds in conjunction with a relatively small number of salt bridges constitutes the stability determinants of the PSL. Because of its long loop length, close proximity to activity center and solvent-exposed character, the

stability of the PSL loop is important for the global structural stability or even for the catalytic activity of these proteases. This is reflected by the fact that the calcium cation is observed to stabilize PSL in two members of proteinase K subfamily, the VPRK from *Vibrio* sp. PA44 (PDB code 1SH7) (Arnorsdottir et al., 2005) and the thermitase from *Thermoactinomyces vulgaris* (PDB code 1THM) (Teplyakov et al., 1990).

4.3.4 Aromatic ring stacks

The aromatic ring stacking interaction between aromatic residues can aid in enhancing the structural stability of the protein (Siezen et al., 1991). Two absolutely conserved aromatic ring stacking interactions are found in residue pairs Tyr23-Tyr25 and Tyr/Phe59-Phe/Tyr113. The former contributes to the stability of the N-terminal region; the latter makes contribution to the stability of the PSL and α3. A conserved aromatic ring stacking interaction is observed between residue pair Tyr36-Phe/Tyr91 in 1IC6, PR1, Ver112 and PL646, contributing to the stability of both β1 and β3. Such an aromatic stacking interaction does not exist in VCP1 due to the replacement of the aromatic residue (Phe or Tyr) at position 91 by Leu.

4.4 Substrate-binding region and electrostatic surface potential

4.4.1 Substrate-binding sites/pockets

The substrate-binding region in subtilisin proteases is generally described as a surface channel or crevice that is able to accommodate at least six amino acid residues (P4-P2′) within the substrate such as a polypeptide chain or a pseudo-substrate (i.e., the inhibitor) (Siezen & Leunissen, 1997). In the crystal structure of proteinase K in complex with the inhibitor methoxysuccinyl-Ala-Ala-Pro-Ala-chloromethyl ketone (PDB code 3PRK) (Betzel et al., 1993; Wolf et al., 1991), the P4-P1 residues of the substrate slot in between two extended protease segments of residues 100-104 and 132-136, leading to the formation of a three-stranded antiparallel β sheet (Wolf et al., 1991). The leaving segment P1′-P2′ of the substrate runs along the protease segment of residues 220-222, exhibiting a weak binding to the enzyme. According to the crystal structures of 3PRK and 1IC6, the substrate-binding sites of the cuticle-degrading enzymes are identified (Figure 3 and 5). The S2′ site is a hydrophobic pocket formed primarily by residues 192, 221 and 222. The S1 is a distinct, large and elongated pocket that is primarily formed at the side by residues 132-135 and at the bottom by residues 158-161. This pocket is also surrounded at the rim by residue 162, at the bottom end by residue 169, and at the top by a segment of residues 222-225 at which the nucleophilic residue Ser224 is located. Compared to S1 pocket, the S2 site is a less distinct and relatively smaller pocket, which is bounded at one side by residue 100 and at the other side by the catalytic triad residue His69, at the bottom by the hydrophobic residue Leu96 and the catalytic triad residue Asp39, at the bottom end by residue 40, and at the rim by residue 67, respectively. S3 site is formed by only one residue at position 101, which is not a pocket because the side chain of Ser101 is located at the exterior of the substrate-binding region, forming a convex surface at this site (Figure 5). The residue 100 may also have the potential to interact with the P3 side chain due to their close proximity observed in the proteinase K-inhibitor complex structure. S4 site is located between two segments of residues 100-104 and 132-136, forming a very distinct and large pocket. This pocket can be divided into two subsites, S4a and S4b. S4a is formed by residues 96 and 107 at the bottom, residue 133 at the side, and residue 102 at the rim, respectively. S4b consists of residue 104 at

the side, residue 141 at the bottom, and residues 135-136 at the rim, respectively. It is important to point out that the sizes of the substrate residue-binding pockets are determined by side chains of the pocket-forming residues.

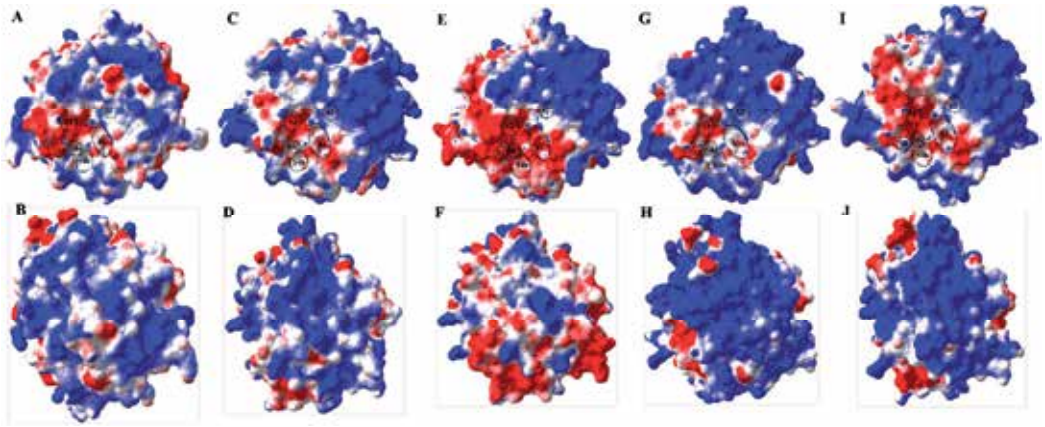

Fig. 5. Electrostatic surface potentials of (A, B) 1IC6, (C, D) PR1, (E, F) VCP1, (G, H) Ver112 and (I, J) PL646. Plots on the top/bottom are the front/back surface of the electrostatic potentials of these proteases, respectively. The positively and negatively charged electrostatic surfaces are colored blue and red, respectively. The approximate locations of the substrate-binding sites and/or pockets, S2′, S1, S2, S3, S4a and S4b are indicated.

We note that the amino acid residues forming the substrate-binding sites S2′ and S3 are completely conserved throughout the proteinase K and the four cuticle-degrading proteases investigated here, while those forming the pockets S1, S2 and S4 are observed to be variable. For example, variable residues within the S1 pocket are found at positions 162 and 169, which, as described above, are residues located at the rim and bottom end of this pocket, respectively. In the four cuticle-degrading proteases, the acidic residue Asp162 substitutes for the neutral Asn162 in the proteinase K. Despite their common side chain conformation, the acidic residue Asp162 make the S1 pocket carry more electro-negative characteristics (Figure 5), which in turn may shift the substrate specificity to the positively charged P1 residues (Siezen et al., 1991; Siezen & Leunissen, 1997). In addition, it has been observed that the electro-negative character can increase flexibility of a protease (Pasternak et al., 1999), especially the flexibility of the active site region (Kumar & Nussinov, 2004). We therefore assume that the Asp162 in the cuticle-degrading enzymes should increase the flexibility of the S1 site, which in turn may enhance the catalytic efficiency of the cuticle-degrading proteases and subsequently the infection virulence by the fungi. Position 169 is occupied by the Tyr in 1IC6, VCP1 and PL646 and by the Thr in PR1 and Ver112. It should be noted that the P1 specificity of subtilisin BPN′ can be significantly affected by residue replacements at this position using site-directed mutagenesis technique (Estell et al., 1986; Wells et al., 1987). Most of the residues within the S2 pocket are conserved throughout the five proteases except for the one at position 67, which is occupied by Asn in 1IC6 and PL646 but by the His in PR1, VCP1 and Ver112, implying a slight preference of the latter three cuticle-degrading proteases at the low pH for the negatively charged P2 residues. The largest number of variable residues in the substrate-binding region is found within the S4 pocket, i.e., there are five positions, 103, 104, 107, 136 and 141, that are occupied by different residues among

these five proteases. For example, Gln103 in proteinase K is replaced with Ser103 in the four cuticle-degrading enzymes, enlarging the S4 pocket of cuticle-degrading proteases due to relatively smaller side chain of Ser than that of the Gln. The Tyr104 in PR1, VCP1, 1IC6 and PL646 is replaced by a Leu in Ver112. Because Tyr104 has been shown to act as a flexible lid to the S4 pocket in the structures of subtilases (Siezen et al., 1991; Siezen & Leunissen, 1997), the Leu104 at the equivalent location broadens the entrance for P4 residues and simultaneously increases the hydrophobic character of the S4 pocket. Two amino acid residue variations, i.e., the substitution of a Val in VCP1 for Ile107 in 1IC6, PR1, Ver112 and PL646, and the substitution of a Leu in Ver112 for Val141 in 1IC6, PR1, VCP1 and PL646, likely have a minor influence on the size and hydrophobic character of the S4 pocket because Ile/Val107 and Val/Leu141 are buried to a large extent within the bottom of S4 pocket and all have strong hydrophobic character. The presence of Pro136 in VCP1 leads to a larger rim of the S4 pocket because of its larger size compared to the equivalent Gly136 presented in 1IC6, PR1, Ver112 and PL646. Interestingly, the amino acid residues at positions 136 and 104 can be seen as two gates for the entrance of the S4 pocket, and as such the presence of larger Pro may affect entry of the P4 residues into the S4 pocket. The hydropathy score of the S4 pocket was calculated approximately as the sum of the hydropathy value (Kyte & Doolittle, 1982) for all the residues within the S4 pocket, yielding values of 13.8, 13.8, 13.5, 18.5 and 13.8 for 1IC6, PR1, VCP1, Ver112 and PL646, respectively, indicating that the S4 pockets of these proteases have strong and comparable hydrophobic character, with the strongest hydrophobicity observed in Ver112.

4.4.2 Electrostatic surface potential

The electrostatic surface potentials of these five proteases were calculated using Poisson-Boltzmann method with Swiss-PdbViewer and the results were shown in Figure 5. Many electrostatic potential features are common to the surfaces of these proteases, i.e., the back surfaces of these proteases are dominated by electro-positive potential except for that of VCP1 (Figure 5F), which exhibits the mixed distribution of positively (blue color) and negatively (red color) charged potentials; the front surfaces of these enzymes exhibit a dual feature of both positively and negatively charged potentials, although the majority of the front surfaces are positively charged. Close inspection of the front surfaces reveals that the electro-negative potentials are mainly concentrated in and/or around the substrate-binding regions of these enzymes, especially those of VCP1 and PL646 (Figure 5E and I). For the other proteases, sporadic distribution of the electro-negative spots can be found outside the substrate-binding sites. The functional significance of the electrostatic surface potential distribution will be discussed in the "Functional implication" section.

4.5 Functional implication

It has been shown that the high-accuracy homology models can be obtained if the sequence identity between the target and template is greater than 50%, with the accuracy of the modeled structures being comparable to that of the medium-resolution NMR or X-ray structures (Baker & Sali, 2001). The high degree of sequence identity of these four cuticle-degrading proteases to the template proteinase K suggests that our structural models are very accurate, as confirmed by comparison of homology models with the X-ray crystallographic structures that we determined later (Liang et al., 2010), which exhibits that for the Ver112 and PL646, the backbone RMSD between the theoretically modeled and experimentally determined structures is 0.57 and 0.64 Å, respectively. Such highly accurate

structural models guarantee the accuracy of prediction for Ca^{2+}-binding site, disulfide bridge, hydrogen bond, salt bridge and aromatic ring stack that contribute to the global or local stability of the enzyme structures, and can also be used to investigate differences in functional properties such as substrate specificity and catalytic activity among these proteases.

As mentioned above, although the four cuticle-degrading enzymes were predicted to contain no weak Ca^{2+} cation Ca2 present in 1IC6, several hydrogen bonds and salt bridge networks were observed within or close to the Ca2-equivalent site to act as stabilizing factors for the N- and C-terminal regions of the cuticle-degrading enzymes (for details see (S.Q. Liu et al., 2007b)). This may explain why the presence of EDTA has a minor effect on the activity of proteinase K and cuticle-degrading proteases (S. Q. Liu et al., 2011; Müller et al., 1994; J.K. Yang et al., 2005b). Of interest is that another Ca^{2+} found in the vicinity of the PSL of proteinase K-like serine protease structures with PDB codes 1SH7 (Arnorsdottir et al., 2005) and 1THM (Teplyakov et al., 1990) does not exist at the equivalent location in the cuticle-degrading proteases investigated here. The abundant hydrogen bonds and salt bridges observed in PSL can contribute to its stability in these proteases.

Our comparative modeling study, together with structural studies using X-ray crystallographic and NMR techniques, indicates that members of the S8 peptidase family have an overall highly rigid globular fold (Betzel et al., 2001; Liang et al., 2010; S. Q. Liu et al., 2010, 2011; Müller et al., 1994; Martin et al., 1997; Siezen et al., 1991; Siezen & Leunissen, 1997). The globular fold can be considered as the consequence of combination of various protein-folding driving forces, including the hydrophobic force that maximizes the entropy of the protein-solvent system through minimizing the solvent-accessible surface area of the protein, making the overall protein shape spherical; and enthalpic contribution through favorable energetic contacts such as van der Waals contacts, electrostatic and hydrogen bonding interactions. It has been proposed that the compact globular packing and high rigidity of the proteinase K-like serine proteases have evolved as the protective measures against autolysis (S. Q. Liu et al., 2010, 2011; Martin et al., 1997). However, a certain degree of flexibility within the substrate-binding regions is required to allow recognition and binding of the substrates with high affinity (Lange et al., 2008; S. Q. Liu et al., 2010, 2011; Perica & Chothia, 2010; Tao et al., 2010). For the substrate-binding regions, the delicate balance between the rigidity and flexibility may play important roles in maintaining structural stability and modulating substrate binding specificity and affinity. The important factors involved in determining rigidity and flexibility of local structural region are hydrogen bonds and salt bridges, especially their number and distribution. An example of crystallographic study on a proteinase K-like protease, SPRK from the psychrotroph *Serratia* species (PDB code 2B6N) shows that a strong hydrogen bond network, Asn97-[S99, S101] brings its S2-loop into a 'hub and spokes' arrangement with high rigidity, which may explain the relatively low binding affinity (higher K_m value) of SPRK towards the synthetic substrate suc-Ala-Ala-Pro-Phe-nitroanilide (Helland et al., 2006). In addition, the tight S2-loop may also influence the substrate specificity profile of SPRK (Helland et al., 2006) because of the conformational selection (Monod et al., 1965; Tobi & Bahar, 2005) and/or induced fit (Koshland, 1958) mechanisms of substrate binding. In the case of the cuticle-degrading proteases studied here, a conserved hydrogen bonding network, Asp97-[Gly100, S101], and a conserved salt bridge network, Asp65:Lyr94:Asp97, are observed to contribute to the stability of the S2-loops. However, for PR1, VCP1, Ver112 and PL646, their number of hydrogen bonds/salt bridges involved in stabilizing the S2-loop is 4/1, 4/1, 5/3 and 7/2,

respectively, implying more rigid S2-loop in Ver112 and PL646 than in the other two enzymes. This leads to the speculation that Ver112 and PL646 may have relatively lower P2 affinity compared to Pr1 and VCP1.

The variation in substrate specificity of subtilisin is most likely to be caused by variable residues within the substrate-binding sites, especially by those whose side chains interact directly with the P1 and P4 residues of the substrate (Siezen et al., 1991; Siezen & Leunissen, 1997). We therefore make some prediction of the variation in substrate specificity among the cuticle-degrading proteases according to this principle. As mentioned above, the Asp162 substitution in the four cuticle-degrading enzymes for Asn in proteinase K could not only enhance the catalytic efficiency but also increase the specificity to the basic P1 residues. The residue 169 is located at the bottom end of the S1 pocket and is either Tyr in proteinase K, VCP1 and PL646 or Thr in PR1 and Ver112. Inspection of the structural models reveals that the bottoms of the S1 pocket of these five proteases remain the same width because the side chains of both Tyr and Thr rotate away from the S1 pocket bottom end (Figure 5), in agreement with the docking data showing that Tyr in 1IC6 does not interact directly with the P1 residue of a substrate (Helland et al., 2006). We therefore consider that this observed residue variation at position 169 may have a minor effect on substrate specificity of these cuticle-degrading enzymes. Five positions with variable residues are found within S4 pocket suggesting that these proteases have most variable specificity for the P4 residues of substrates. For example, the replacement of Gln103 in proteinase K with a Ser in all these four cuticle-degrading proteases increases not only the size but also the hydrophobicity of the S4 pocket to a certain extent, thus broadening the specificity profile of the P4 substrate residues. In Ver112, the Leu substitution at position 104 for Tyr in the other proteases leads to the increased preference of Ver112 for large and hydrophobic P4 residues in comparison with the other proteases. VCP1 may have a preference for relatively small P4 residues compared to the other proteases due to its Pro substitution for Gly at position 136, which narrows the entrance to the S4 pocket. Note that the predicted changes in substrate specificity of these cuticle-degrading proteases based on the structural models need to be verified by experimentally determining the kinetic data of the enzyme-substrate interaction, and this will be further discussed later in this chapter.

The most pronounced feature of the electrostatic surface potential of these cuticle-degrading proteases is that their substrate-binding regions are commonly negatively charged whereas a large fraction of the other surfaces is positively charged. As mentioned above, the anionic feature of the substrate-binding regions is able to increase the local conformational flexibility of these regions, which in turn could increase the substrate affinity and catalytic efficiency. It has been shown that the cuticles of many nematodes and insects contain abundant acidic residues and as such are heavily negatively charged under neutral condition (Bidochka & Khachatourians, 1994; Blaxter et al., 1992; Cox et al., 1981; Himmelhoch & Zuckerman, 1978; Murrell et al., 1983). Therefore, the dominated electro-positive surface outside the substrate-binding regions will facilitate the diffusion and adsorption of the cuticle-degrading proteases to their substrate - the cuticle of nematodes or insects - due to the electrostatic attraction between oppositely charged molecules, thus leading to efficient degradation of cuticle by enzymes. In addition, the large fraction of the positively charged surfaces can also explain why the cuticle-degrading proteases secreted by nematode-parasitic fungi studied here are all alkaline proteases.

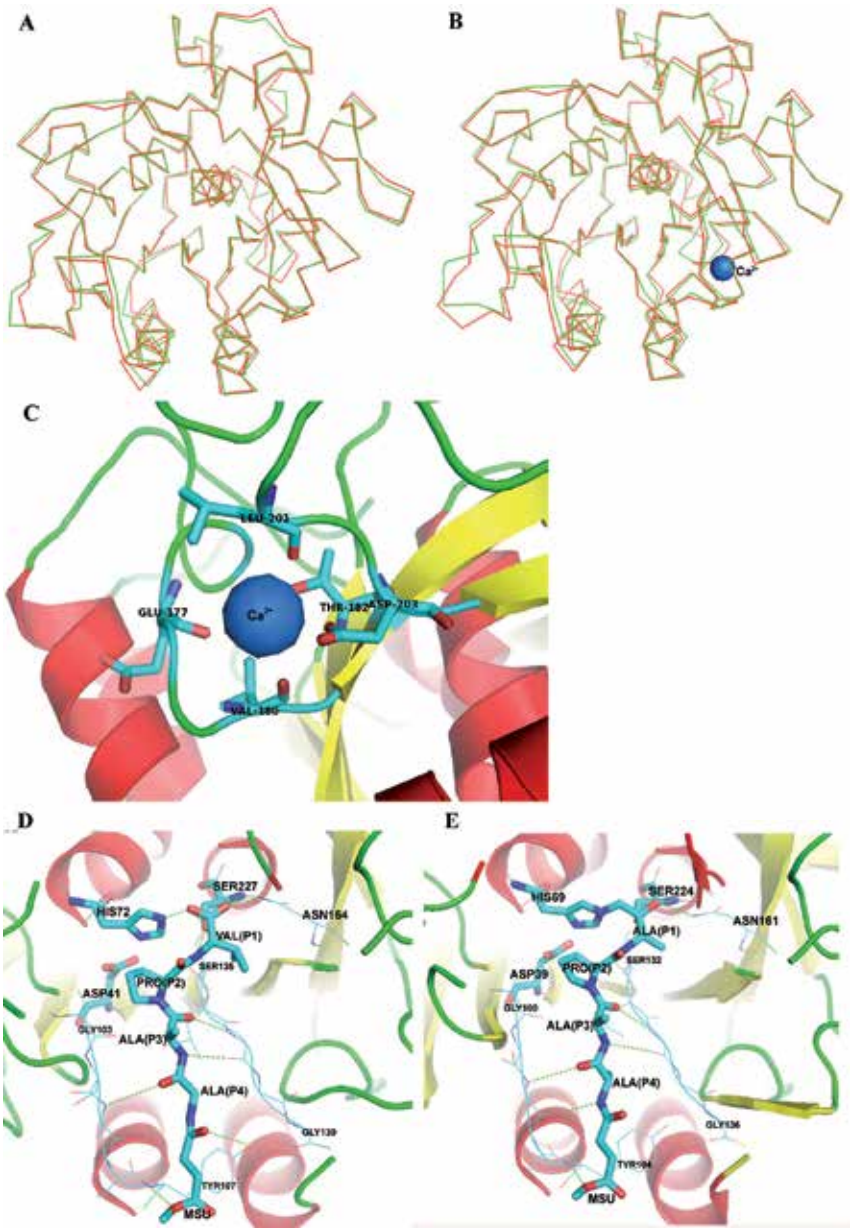

Fig. 6. Crystal structures of Ver112 and PL646 and their comparison with corresponding homology models. (A, B) Backbone superposition of crystal structure and homology model of Ver112 (A) and PL646 (B). Experimental and theoretical structures are shown in red and green, respectively; and calcium cation in PL646 is shown as a blue ball. (C) Residues coordinating with Ca^{2+} (Ca1 site) in PL646. (D, E) Substrate-binding site of PL646 in complex with the inhibitor MSU-AAPV-ketone (D) and of proteinase K in complex with the MSU-AAPA-ketone (E). Residues of the substrate-binding site, catalytic triad, oxyanion hole and substrate P4-P1 are labeled; the carbon, nitrogen and oxygen are shown in cyan, blue and red, respectively; the hydrogen bonds between the substrate and enzyme are denoted by dashed green lines.

5. Crystal structures of two cuticle-degrading proteases and their kinetic data

In order further to investigate the structure-function relationship of the cuticle-degrading proteases and verify the predicting results of some structural and functional properties based on the above modeled homology structures, we select two of the above proteases, Ver112 and PL646, for crystallographic and kinetic studies (Liang et al., 2010).

5.1 Comparison between the crystal structures and homology models

The structures of two cuticle-degrading proteases, Ver112 and PL 646, were solved with X-ray crystallographic method to resolutions of 1.65 Å and 2.1 Å, respectively, which were deposited in the PDB structure database with accession codes of 3F7M for Ver112 and 3F7O for PL646. As expected, structural comparison reveals that the structures of the experimentally determined and theoretically modeled proteases are nearly identical (Figure 6A and B), giving the backbone RMSD values of 0.57 Å (Ver112) and 0.64 Å (PL646), respectively. Consequently, the secondary structural element components, architecture of the catalytic triad, and location of substrate-binding sites observed in the crystal structures are almost the same as those observed in the homology models. Two predicted disulfide bonds, Cys34-Cys123 and Cys178-Cys249 (proteinase K numbering), are also presented in the crystal structures of Ver112 and PL646. One Ca^{2+} cation is found in the crystal structure of PL646, which is coordinated with high affinity by carbonyl oxygens of Glu177, Val180 and Leu201, $O_{\gamma 1}$ of Thr182, and $O_{\delta 2}$ Asp203 (PL646 numbering, Figure 6C), forming a more precise Ca1 site than what is predicted based on the homology model. The weak calcium cation Ca2 present in proteinase K structure with PDB code 1IC6 is not observed in the crystal structure of PL646, in agreement with the prediction based on the homology model. In the case of Ver112, although the amino acid residues forming the Ca1 site are identical to those in PL646 and 1IC6, no Ca^{2+} was observed to bind to this site in the crystal structure. The possible reason for this is that Ver112 was crystallized in a Ca^{2+}-free buffer. Taken together, although the calcium cation has been considered as an important factor in enhancing the thermostability of serine proteases (Bajorath et al., 1989; Betzel et al., 1990; Müller et al., 1994; Siezen et al., 1991), the binding of such cation may not be indispensable for the structural stability, either globally or locally, because the presence or absence of the Ca^{2+} at the same site in different proteases does not affect the overall folding and local architecture of the enzyme structures, and other structural factors like the abundant van der Waals contacts, hydrogen bonds and salt bridges play a dominant role in maintaining the stability of protein structure. However, our molecular dynamics study on proteinase K shows that Ca^{2+} can have an effect on the functional properties of this protease through altering the rigidity/flexibility of the structural regions around the calcium cation (S. Q. Liu et al., 2011), suggesting that the difference in local dynamics can be related to functional difference between members of the same family.

Superposition of the structure of the protease PL646 in complex with the inhibitor MSU-AAPV-ketone (Figure 6D) with that of the proteinase K complexed with MSU-AAPA-ketone (PDB code 3PRK (Wolf et al., 1991); Figure 6E) gives a backbone RMSD of 0.69 Å. In both complex structures, the inhibitor MSU-AAPA-ketone lies well in the substrate channel located between the segments of residues 100-104 (residues 103-107 in PL646) and 132-136 (residues 135-139 in PL646); and the conserved backbone hydrogen bonding interaction between the substrate and the protein residues are also observed, indicating that the predicted substrate-binding pockets shown in Figure 5 are correct. Also, the

electrostatic surface potentials of the two crystal structures were also calculated and compared with those of their corresponding homology models, exhibiting the completely identical electrostatic potential distribution across the molecular surfaces (data not shown).

In the case of the substrate pockets, we have predicted above that the Tyr/Thr alteration at position 169 may not affect the size of the S1 pocket bottom because the side chains of both Tyr and Thr rotate away from the S1 pocket bottom end. In the crystal structure of Ver112, the Thr side chain adopts a similar orientation to that seen in the structural model; and in the crystal structure of PL646 in complex with substrate analogue MSU-AAPV-ketone, the Tyr side chain also rotates away from the S1 pocket bottom end and does not make contact with the P1 residue of a substrate, confirming our prediction and suggesting that residue variation at position 169 unlikely affects the substrate specificity. Further comparison between the crystal structures of Ver112 and PL646 shows that the Ver112 has a wider S4 pocket entrance than PL646 because the position 104 is occupied by residue Leu in Ver112 with smaller side chain compared to that of Tyr in PL646. The bottom of S4 pocket appears flatter and wider in Ver112 than in PL646, which is caused by residue difference at position 141, i.e., Leu/Val in Ver112/PL646, respectively. The relatively larger Leu side chain lies across the bottom of Ver112 S4 pocket, making it flatter and wider than that of PL646.

5.2 Kinetics analysis of Ver112, PL646, and proteinase K

In order to verify the predicted difference in substrate specificity and catalytic activity among cuticle-degrading proteases, the enzyme kinetic parameters of Ver112, PL646 and proteinase K towards several synthesized substrates were determined using a method as described in (DelMar et al., 1979). The polypeptide substrates were designed based on the sequence of the classical protease K inhibitor N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Suc-AAPF-pNA) through altering individual residues, achieving Suc-AAPD-pNA, Suc-NAPF-pNA and Suc-PAPF-pNA. The resultant kinetic data are listed in Table 3 (Liang et al., 2010). The lowest K_m value of 0.093 mM is observed to occur for proteinase K towards the substrate AAPD, exhibiting the strongest proteinase K-AAPD affinity. Cuticle-degrading protease PL646 shows approximately two orders of magnitude weaker affinity towards AAPD (1.21 mM) than proteinase K does (0.093 mM); and Ver112 may not bind AAPD as its K_m can not be determined. It seems likely that it is the electrostatic repulsion between Asp162 in S1 pocket and P1 residue of AAPD that reduces (in PL646) or abolishes (in Ver112) AAPD binding. The kinetic parameters of proteinase K towards NAPF can not be determined, possibly due to the relatively small S4 pocket of proteinase K in comparison to that of Ver112 and PL646. On the contrary, Ver112 and PL646 show both high affinity towards the substrates NAPF and PAPF, reflecting their capability of accommodating peptide substrates with large P1 and P4 residues. Furthermore, Ver112 binds with four-fold/two-fold higher affinity than does PL646 to NAPF/PAPF, respectively. This can be attributed to a wider entrance of S4 pocket in Ver112 than in PL646, allowing highly efficient entry of large P4 residues into S4 pocket of Ver112. PL646 has a relatively smaller S4 pocket than Ver112, possibly making it difficult to adopt appropriate arrangement to interact with P4 Ala of AAPF and AAPD, thus leading to the observed relatively weaker affinity of PL646 towards these two substrates compared to Ver112.

Proteases	AAPF		AAPD		NAPF		PAPF	
	K_m (mM)	K_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m (mM)	K_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m (mM)	K_{cat}/K_m ($s^{-1}mM^{-1}$)	K_m (mM)	K_{cat}/K_m ($s^{-1}mM^{-1}$)
Ver112	0.145	27354	N/A[a]	N/A[a]	0.164	2067	0.226	5206
PL646	1.42	17957	1.21	166	0.695	453	0.472	1576
PRK	0.31	33142	0.093	1229	N/A[a]	N/A[a]	0.111	3558

[a] Not available, meaning that the kinetics parameters can not be determined.

Table 3. Enzyme kinetic data of Ver112, PL646 and proteinase K towards several synthesized substrates. This table is modified from (Liang et al., 2010).

In the case of PL646, its lowest substrate affinity is observed towards AAPF (K_m = 1.42 mM) among the four synthesized substrates. However, this effect is offset by the highest turnover rate (170 1/s) resulting in the highest catalytic efficiency (K_{cat}/K_m) towards AAPF, which is one or two orders of magnitude larger than that towards PAPF, AAPD and NAPF (Table 3). Similarly, Ver112 and proteinase K also have the highest turnover rate towards AAPF, regardless of their high or low affinity towards this substrate, thus leading to the highest catalytic efficiency to the AAPF, explaining why the AAPF has always been used as the canonical substrate for enzyme activity assay. The relatively lower turnover rates of these three proteases towards AAPD, NAPF and PAPF may be caused by the stereochemical clashes while interacting with the large P1 (such as Asp in AAPD) and P4 (such as Pro and Asn in PAPF and NAPF). Out of these three newly designed substrates the PAPF can be considered as the best one for all the three proteases investigated here because of its observed high affinity and turnover rate. Our data show that, like the canonical serine protease proteinase K, the cuticle-degrading proteases also exhibit broad substrate specificity with preference for bulky hydrophobic or aromatic residues at the P1 and P4 positions of the substrate. The intrinsic reason for this is that both the substrate pockets S1 and S4 are large and hydrophobic. Interestingly, for PL646, although its S4 pocket is large to accommodate both large and small P4 substrate residues, its affinities towards AAPF (1.42 mM) and AAPD (1.21 mM) are weaker than those towards NAPF (0.695 mM) and PAPF (0.472 mM). We speculate that the large P4 residues (such as Asn in NAPF and Pro in PAPF) can form more non-bonding contacts, i.e., hydrogen bonding, hydrophobic and van der Waals contacts with S4 pocket than do the small residues (such as Ala in AAPF and AAPD), leading to a tighter association of protease with large residue than with small residue.

6. Dynamic behavior of proteinase K

A complete understanding of the structure-function relationship of a protein requires analysis of its dynamic behavior in addition to the static structure. Even for proteins with highly rigid structure (such as members within the S8 peptidase family), their dynamic behavior and molecular motions are still very important for understanding their function properties. The catalytic reaction of any enzyme is a dynamic process that occurs through a series of dynamic steps such as substrate binding, orientation, catalysis and product release. Detailed information on these dynamic steps as well as on how they are connected and regulated is necessary for a complete understanding of the enzymatic function. Therefore, we selected the classical serine protease proteinase K to investigate deeply into its dynamics using the molecular dynamics (MD) simulation technique (S. Q. Liu et al., 2010, 2011; Tao et

al., 2010). Because of the high degree of sequence and structure similarity between proteinase K and cuticle-degrading proteases, these proteases should also have similar or even identical dynamic features, which can be used to explain experimental data or be linked to functional properties of these enzymes. The content in this section would greatly facilitate the understanding of the structure-function relationship of this class of proteases.

6.1 Rigidity and flexibility of proteinase K and their functional implication

The crystal structure of proteinase K with PDB code 1IC6 (Betzel et al., 2001) was used as a starting model for the MD simulation. The GROMACS molecular dynamics package (Feenstra et al., 1999; Lindahl et al., 2001) was used with the GROMOS96 43a1 force field. The detailed molecular dynamics setup was described in (S. Q. Liu et al., 2010; Tao et al., 2010). The length of the MD simulation is determined by monitoring the convergence of RMSD relative to the starting structure during simulation. The conventional geometrical properties along the MD trajectory such as the number of hydrogen bonds (NHB), number of native contacts (NNC), number of residues in the secondary structure elements (SSE), radius of gyration (Rg), solvent accessible surface area (SASA) and RMSD were calculated using the programs g_hbond, g_mindist, do_dssp (Kabsch & Sander, 1983), g_gyrate, g_sas and g_rms within the GROMACS software package, respectively.

Analyses of the geometrical properties during simulation indicate that the structure of the proteinase K is stable with geometrical properties of NHB, NNC, Rg, SASA and RMSD fluctuating around their respective equilibrium values, and the well-defined global fold maintains well during simulation (data not shown and for details, see (S. Q. Liu et al., 2010)). Furthermore, the calculation of B-factors during simulation shows that proteinase K has a rigid structural core and a limited number of surface loops and links that show high structural flexibility. Among these flexible loops are those regions that participate in the formation of the substrate-binding site (such as segments containing residues 100-104, 132-136 and 160-169) and regions located near or opposite the substrate-binding site (such as segments of residues 59-68, 119-126 and 240-246). The presence of flexibility in the substrate-binding region, which has emerged from studies of many enzymes, supports either the induced-fit (Koshland, 1958) or conformational selection (Monod et al., 1965; Tobi & Bahar, 2005) mechanisms of substrate binding. In the induced-fit mechanism, a certain degree of flexibility should exist in the substrate-binding region for conformational adjustment induced by substrate binding. Under the conformation selection model, the high flexibility of the structure-binding region allows it to exist in an ensemble of conformational substates, one of which can be selectively bound by substrate, lowering the entropy barrier and biasing the equilibrium towards the complex conformation. We consider that these two mechanisms of substrate binding are not two independent and exclusive processes but rather they play a joint role in substrate recognition and binding due to the common prerequisite of conformational flexibility, which are important for both large-scale backbone concerted movement in the conformational selection and side chain adjustment/rotation in the induced-fit process. Accordingly, we believe that it is the high flexibility observed in the substrate-binding region that allows not only for the existence of conformational diversity but also the easy conversion between substates of this region, thus not only facilitating the efficient recognition and binding of substrates but also allowing interactions with different substrates with a variety of sequence and structure motif, broadening the substrate specificity of proteinase K. However, because of the overall structural rigidity of proteinase K, it seems that only the functionally essential flexibility is presented in certain local regions.

Taken together, it can be considered that the serine proteinase family has evolved to be as rigid as possible to resist autolysis or proteolysis by other proteases while remaining as flexible as necessary within certain structural regions to guarantee functional activity.

6.2 Flexibility of the catalytic triad

In order to investigate the dynamic nature of the catalytic triad, we also focus on the interaction strength between Asp39 and His69 and between His69 and Ser224 through calculating the interaction energies. The results show that a stronger electrostatic interaction occurs between His69 and Asp39 (Figure 7B) than between His69 and Ser224 (Figure 7C), resulting in a more stable and tighter association of Asp39-His69 than His69-Ser224 (Figure 7A). This implies that the proton transfer between His69 and Asp39 should be easier and more efficient than that between His69 and Ser224, leading to the speculation that the proton transfer from the $N_{\delta 1}$ of His69 to the carboxyl group of Asp39 may be the prerequisite for the proton transfer from the Ser224 O_{γ} to the His69 $N_{\varepsilon 2}$ (S. Q. Liu et al., 2010). An *ab initio* molecular orbital calculation of the catalytic triad (Nishihira & Tachikawa, 1996) showing a higher hydrogen transfer energy between the serine hydroxyl and the $N_{\varepsilon 2}$ of histidines imidazole than between the $N_{\delta 1}$ of histidine imidazole and carboxyl group of aspartate supports this speculation. In addition, the appropriate orientation of imidazole group within the catalytic triad can be obtained through the strong interaction between His69 and Asp39. Upon orientation, aspartate carboxyl abstracts a proton from the $N_{\delta 1}$ of imidazole group, resulting in an increase in pK_a and alkalinity of the imidazole group (Moult et al., 1985), which in turn is favorable for extraction of proton from the serine hydroxyl. As the consequence of weaker interaction of His69 with Ser224, Ser224 exhibits a higher degree of conformational freedom compared to Asp39 and His69, as indicated by B-

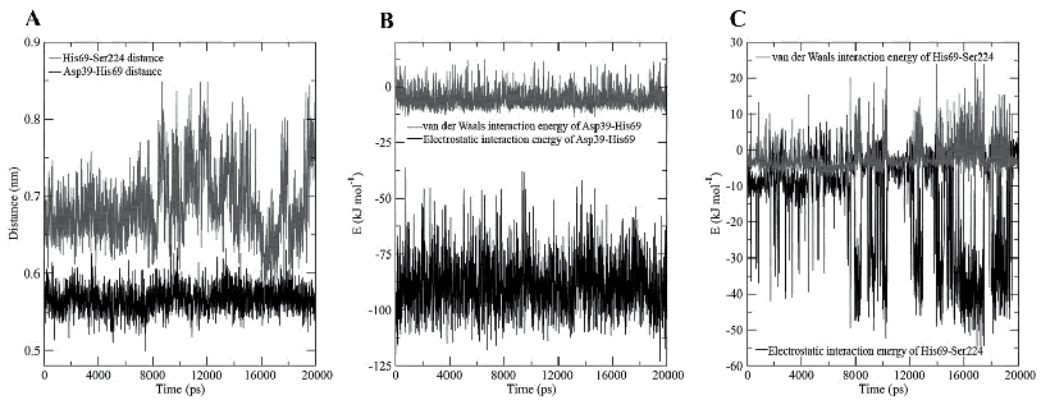

Fig. 7. Distances and interaction energies between residues within the catalytic triad during the molecular dynamics simulation. (A) The distances between the centers of mass of Asp39 and His69 (black line) and between centers of mass of His69 and Ser224 (grey line) as a function of simulation time. (B) The interaction energies between Asp39 and His69 as a function of time. The coulomb's electrostatic and van der Waals interaction energies of Asp39-His69 are shown in black and grey lines, respectively. (C) The interaction energies of His69-Ser224 as a function of time. The coulomb's electrostatic and van der Waals interaction energies of His69-Ser224 are shown in black and grey lines, respectively. This figure is modified from (S. Q. Liu et al., 2010).

factors of 5.5, 6.9 and 15.0 Å^2 for Asp39, His69 and Ser224, respectively. This is consistent with the unfavorable geometry of the Ser O_γ observed in some crystal structures of serine proteases (Dauter et al., 1991; Rypniewski et al., 1995). Functionally, the high flexibility of the serine hydroxyl allows it to adopt different orientations to suit the needs for proton transfer, nucleophilic attack and subsequent release of the cleaved peptide product.

6.3 Large concerted motions of proteinase K

The essential dynamics (ED) technique (Amadei et al., 1993; Balsera et al., 1996) is utilized to investigate large concerted motions of proteinase K. This method is based on the diagonalization of a covariance matrix built from atomic fluctuations in a MD trajectory. The central hypothesis of ED is that only a few eigenvectors with large corresponding eigenvalues are important for describing the overall internal motion of a protein. Fluctuations along the first few eigenvectors are mainly large concerted motions and can often be connected to the functional properties of proteins (Barrett & Noble, 2005; S. Q. Liu et al., 2007a; S. Q. Liu et al., 2008; Mello et al., 2002).

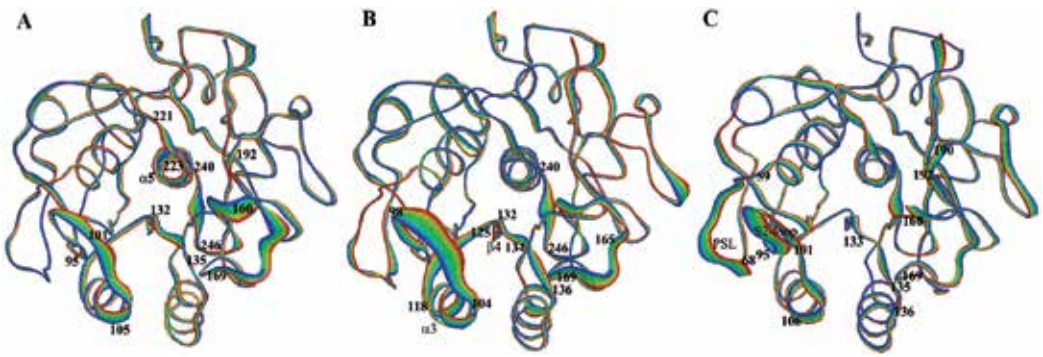

Fig. 8. The first three large concerted motions of proteinase K derived from essential dynamics analysis of the molecular dynamics simulation trajectory. (A) The most significant motion described by eigenvector 1, (B) Motion along eigenvector 2 and (C) motion along eigenvector 3. For each eigenvector, two extreme structures, which correspond to the largest and smallest projection values, respectively, were extracted from the eigenvector projection; and the intermediate frames were produced by simple interpolation between these two extremes. The linear interpolations between the two extremes are colored from blue to red to stress the structural differences between the two extremes but do not represent the transition pathway. This figure is modified from (S. Q. Liu et al., 2010).

For proteinase K, the large concerted motions described by the first three eigenvectors are shown in Figure 8. Visualization of motion along eigenvectors 1, 2 and 3 reveals that the structural changes originate mainly from displacements of the structural regions located within/around or opposite the substrate-binding site, i.e., residues100-105 (which are a part of the substrate-binding pocket S4), residues 118-125 (which are located opposite the S4 pocket), residues 160-169 and 221-223 (which are within the substrate-binding pocket S1), and residues 240-246 (which is located opposite the S1 pocket). The direct consequence of motions of the substrate-binding regions is dynamic variations of the substrate-binding pockets, i.e., the openings or closings of the S1 and S4 pockets, which may facilitate the binding/release of the peptide substrate/product into/out of the substrate-binding cleft.

Another interesting effect is that the large displacements in the regions located opposite or near the substrate-binding sites can mediate/modulate dynamics of substrate-binding regions. In the case of eigenvector 1, there are two segments containing residues 221-223 and 240-246 that exhibit large concerted displacements. As shown in Figure 8A, these two segments are separated by a helix α5 and lie opposite to each other. Since the α5 is rigid and shows only small fluctuation, we consider that it may act as the hinge region, allowing the large conformational displacements of segment 221-223 to be mediated by structural changes in the loop 240-246, suggesting that large concerted motions occurring in regions opposite the active Ser224 and S1 pocket can have effect on the dynamics of the active site. In the case of motions along eigenvector 2 (Figure 8B), a large displacement is observed in the loop region 118-125, which is located between the α3 following the substrate-binding residues 100-104 and the β4 preceding substrate-binding residues 132-136. Both α3 and β4 appear to be rigid and show small fluctuations, indicating that the dynamics of the substrate-binding segments 100-104 and 132-136 may be modulated by dynamic behavior of the opposite loop region containing residues 118-125. Here the rigid secondary structural elements linking the substrate-binding regions and the opposite loops may serve as hinge segments, allowing the transmission and communication of the conformational changes between these segments. In the case of eigenvector 3 (Figure 8C), an apparent large concerted motion is observed occurring between PSL (residues 59-68) and S2-loop (residues 95-101), which could, on the one hand, avoid steric clashes between these two loops because of their close locations in space; on the other hand, modulate the flexibility and orientation of the S2-loop, which, as described above (see section 4.5), plays a critical role in recognition and binding of the P2 and P3 residues of the substrate.

7. Substrate-induced change in molecular motions of proteinase K

In order further to investigate changes in dynamics of proteinase K upon the substrate binding, MD simulation of the structure of proteinase K in complex with the peptide substrate AAPA (Figure 6E; PDB code 3PRK (Wolf et al., 1991) was also performed (Tao et al., 2010). In this section, changes in molecular motions upon substrate binding and the dynamic behavior of substrate-binding pockets in the complexed proteinase K are examined. In addition, the functional implication of the changes in dynamics upon substrate binding and the mechanism underlying the conformational changes of substrate-binding regions are also discussed.

7.1 Changes in molecular motions of proteinase K upon substrate binding

Comparison of geometrical properties such as SASA, NNC, Rg and RMSD between the substrate-free and substrate-complexed proteinases K during MD simulations suggests that the enzyme structure is on average in a more compact and stable conformational state when the peptide substrate is present (data not shown; and for details see (Tao et al., 2010)).

Change in molecular motions of proteinase K upon substrate binding was investigated by using a method termed combined ED analysis. In this method, the MD trajectories of the free- and complexed-proteinases K were concatenated and the covariance matrix was constructed and diagonalized; the trajectories were then projected onto the combined eigenvectors and the properties of these projections were analyzed and compared. Figure 9A shows the comparison in the average values of the first 30 eigenvector projections between the two forms of proteinases K. The largest difference in the average of projection is

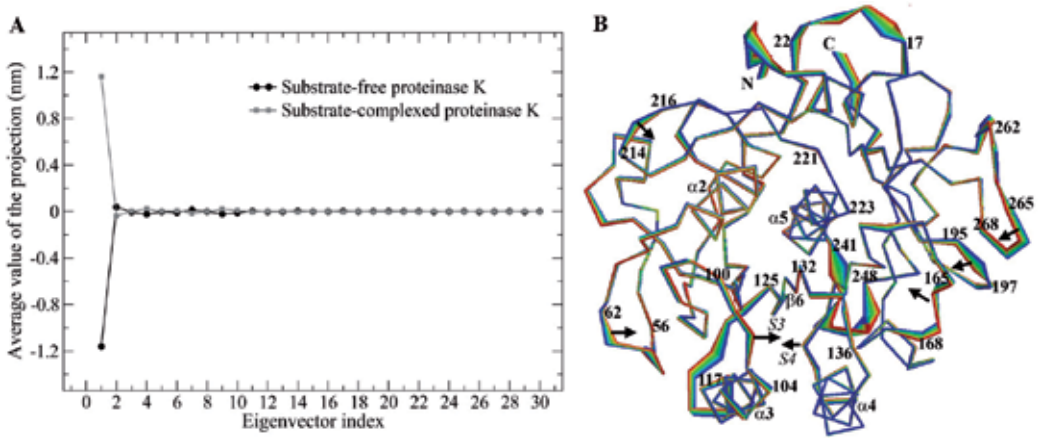

Fig. 9. Change in equilibrium conformation (molecular motion) of proteinase K upon substrate binding. (A) Comparison of the average values of projections of molecular dynamics simulation trajectories onto the combined eigenvectors as a function of eigenvector index. The substrate-free and substrate-complexed proteinase K trajectories were projected separately onto the combined eigenvectors followed by calculation of the average value of each projection. The values of the free and complexed proteinases K are indicated by black circles and grey squares, respectively. (B) The extreme structures extracted from the projection of the merged trajectory onto the first "combined" eigenvector. The linear interpolations between these two extremes are colored from blue (the free proteinase K) to red (the complexed proteinase K) to highlight the structural differences between these two states but do not represent the transition pathway. This figure is modified from (Tao et al., 2010).

observed between the first eigenvectors, indicating that the most significant change in molecular motions upon substrate binding is characterized by eigenvector 1. Similar or almost identical average values are observed for the eigenvectors with index greater than 2, suggesting that the molecular motions described by these eigenvectors are similar between these two states. Figure 9B shows that most of the structure exhibits minor conformational change upon substrate binding with the exception of the structural regions such as the N- and C-termini and some surface-exposed loops containing residues 56-62, 117-125, 162-168, 241-247 and 265-268, suggesting that substrate binding has only a minor influence on dynamics of the protein internal rigid core but a large effect on the external loops, in particular those located within or in the vicinity of the substrate binding site. Visualization of the motions along eigenvector 1 reveals that upon substrate binding, the large concerted motions originate mainly from displacements of residues 100-104 (which participate in the formation of the substrate-binding subsites S2-S4) and 165-168 (which are part of pocket S1), moving towards the peptide substrate leading to the closing of these substrate-binding pockets/sites. These are accompanied by motions of the surface-exposed loop residues 195-197 and 265-268 (which are located spatially close to residues 165-168) and 56-62 (which lie spatially adjacent to residues 100-104), moving concertedly to the direction of the substrate-binding groove resulting in the contractions of these surface loops. The most pronounced structural displacement upon substrate binding is observed in the loop composed of residues 241-248, which is located opposite the substrate-binding pocket S1 and connected to the surface-exposed loop residues 262-268 via helix α6. The small fluctuations of α6

indicate that the displacement of the segment 241-248 is capable of mediating structural change of the loop region 262-268, which in turn can modulate structural change of the loop residues 165-168 via the segment 195-197 to accommodate the C-terminal portion of the incoming substrate. Additionally, the displacement of the residues 241-248 also has an effect, similar to that observed in the substrate-free proteinase K, on dynamics of the segment 221-223. Another large displacement upon substrate binding is observed in the loop region comprising residues 117-125. Like also what is seen in the free proteinase K, the dynamic behavior of this loop can modulate the dynamics of the segments 100-104 and 132-136. Since the two segments 100-104 and 132-136 form the majority of the substrate-binding subsites/pockets S1-S4, their dynamic behaviors are of high importance in substrate recognition and binding and product release

7.2 Dynamic pockets of the substrate-complexed proteinase K

In the proteinase K-substrate complex, the large concerted motions of the substrate binding regions lead to the dynamic variations of the substrate-binding subsites/pockets,

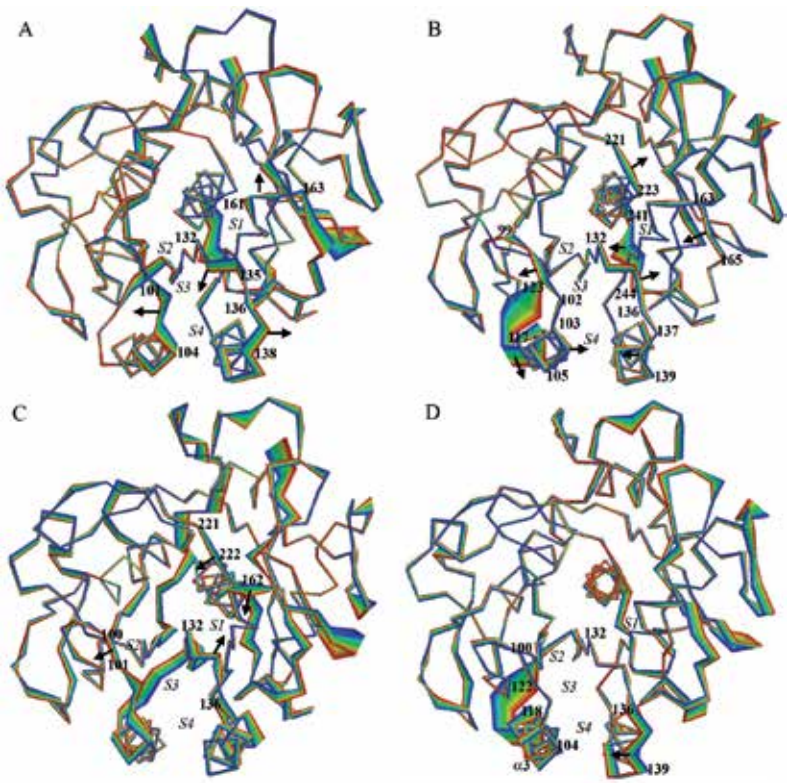

Fig. 10. The first four large concerted motions of the substrate-complexed proteinase K occurring along (A) eigenvector 1, (B) eigenvector 2, (C) eigenvector 3, and (D) eigenvector 4. The linear interpolations between the two extremes extracted from the projections of trajectory onto the first four eigenvectors are colored from blue to red to highlight the structural differences between them. The regions involving large concerted motions that cause dynamic variations of the substrate-binding pockets/subsites are labeled. This figure is modified from (Tao et al., 2010).

which are also observed in the free proteinase K as described in section 6.3. In the case of eigenvector 1, the concerted outward movements of the segments around S1 and S4 pockets, i.e., the segments 101-104 and 136-138 that form the two sides of the S4 pocket; and the segments 132-135 and 161-163 that form respectively the side and bottom of the S1 pocket, result in the enlargement of the these two pockets S1 and S4 (Figure 10A). In addition, the upper portion of the groove, where the subsites S2 and S3 reside, becomes narrowed due to the large inward displacement of the segment 132-134. In the case of eigenvector 2 (Figure 10B), loop 117-123 shows the largest downward displacement, which causes the segments 99-102 and 132-136 to move away from each other resulting in the opening of the upper portion of the binding groove. However, the lower portion of the binding groove (where the S4 pocket resides) becomes slightly narrowed due to the concerted inward movements of the segment 103-105 and 137-139. Simultaneously, the large leftward displacement of the segment 240-246 causes the segment 221-223 to move outwards, thus opening up the lid of the S1 pocket, while the inward movement of the segments 132-135 and 163-165 reduce the size of the bottom of the S1 pocket. The large concerted motions along eigenvector 3 lead to a twist of the binding groove and closure of the S1 pocket (Figure 10C). The obvious outward displacement of residues 100-101 widens the S2 subsite and provides more space for the P2 residue, Pro282. In the case of eigenvector 4 (Figure 10D), no apparent displacement is observed in segments of residues 100-104 and 132-136 whereas the largest displacement is observed in the segment 118-123, which mediates the segment 136-139 to move towards the helix α3, resulting in the reduction in size of the S4 pocket bottom. These complicated dynamic variations of the substrate-binding subsites/pockets reflect the rough free energy surface with several minima of the energy landscape of proteinase K, which can be related to dynamic catalytic processes of proteinase K and this, will be discussed below.

7.3 Functional implication of the dynamic pockets

In the case of the substrate-complexed proteinase K, the large concerted motions along the first eigenvector enlarge the pockets S1 and S4, providing more space for the P1 and P4 substrate residues for accommodation and subsequent orientation of them. The simultaneous closing of the subsites S2 and S3 could grip the corresponding substrate residues P2 and P3 to prevent release of the peptide substrate prior to catalysis. Because the motions along the eigenvector 2 lead to the opening of the lids and reduction of the bottoms of both S1 and S4 pockets, together with the opening of the upper portion (where the subsites S2 and S3 reside) of the binding groove, we may conclude that these motions possibly facilitate the substrate release. The twist motion of the binding groove along eigenvector 3 could facilitate the precise orientation of the P2-P4 residues. Interestingly, the observed closing of the S1 pocket in this subspace could be related to the precise positioning of the P1 substrate residue, a prerequisite for the nucleophilic attack to take place. Also worth noting is the approach of Ser224 to the P1 substrate residue, suggesting that this motional mode may be in the stage ready for the nucleophilic attack. The large concerted motions described by eigenvector 4 reduce the size of S4 pocket bottom and as such can be linked to the release of the S4 substrate residue. Among the first four motional modes, two modes, i.e., those along eigenvectors 2 and 4, lead to reduced size of the bottom of the S4 pocket, implying that the release of the cleaved C-terminal peptide product is possibly initiated by the dissociation of the P4 from the enzyme. In summary, the catalytic processes of the substrate binding, orientation, catalysis and product release occur at distinct modes of molecular flexibility, which correspond to different conformational substates located within

the wells of local free energy minima of the free energy landscape. The relatively small energy barriers between these minima allow the molecular motions and conformational transition to occur among them, thus realizing the catalytic function of the protease.

8. Difference in structural features between cuticle-degrading proteases secreted by nematode-parasitic and nematode-trapping fungi

8.1 Functional differences between alkaline and neutral cuticle-degrading proteases

The classical serine protease proteinase K and cuticle-degrading proteases such as PR1, VCP1, Ver112 and PL646 investigated above are all alkaline proteases because they work best in the pH range of 8 to 11. Another feature of alkaline proteases is that they show both high structural stability and high catalytic activity at high temperature, with a typical range of optimal reaction temperature of 50-60 °C. Interestingly, all the alkaline cuticle-degrading proteases that have been identified and characterized to date are secreted by nematode-parasitic fungi (J. Li et al., 2010), one type of nematophagous fungi that exist as conidia in the environment and infect nematodes by either adhering to the surface of the prey or by direct ingesting (X.Z. Liu et al., 2009). The conidia of the nematode-parasitic fungi can germinate rapidly and invade the entire nematode with assimilative hyphae absorbing all the body contents (Gray, 1987). Another type of nematophagous fungi is the nematode-trapping fungi, which can catch and hold actively the live nematodes using their trapping devices, i.e., the hyphal network, constricting rings and adhesive knobs that are formed by an extensive hyphal system (X.Z. Liu et al., 2009). The captured victim is then penetrated, with its entire body contents being consumed rapidly (Barron, 1977). During such a process, nematode-trapping fungi can also secrete enzymes to degrade the cuticle and body contents of the nematodes. Most of these enzymes were identified to be serine proteases, which, like those secreted by nematode-parasitic fungi, belong also to S8 peptidase family (Huang et al., 2004; J. K. Yang et al., 2007). Phylogenetic analysis of these serine proteases indicates that, despite belonging to the same family, they can be divided into two groups (Gray, 1987), i.e., the alkaline serine proteases derived from nematode-parasitic fungi and the neutral proteases derive from the nematode-trapping fungi. Compared to the alkaline serine proteases, the neutral cuticle-degrading proteases is optimally active at neutral pH and at relative low temperatures ranging from 30 to 40 °C. Furthermore, neutral protease exhibits relatively low structural stability and catalytic activity against nematode cuticle extract in comparison to alkaline protease (Liang et al., 2011).

Here in this section, we selected two cuticle-degrading proteases, i.e., Ver112 derived from nematode-parasitic fungus *L. psalliotae* and PII from nematode-trapping fungus *A. oligospora*, as representatives of the alkaline and neutral proteases, respectively, to identify structural factors responsible for different thermal stability and catalytic activity against nematode cuticle. The correlation between the electrostatic surface potential and phylogenetic relationship of alkaline and neutral proteases was investigated to probe the mechanism responsible for the distinct infectious virulence by different fungal species.

8.2 Structural comparison between Ver112 and PII

The amino acid sequence length of the alkaline protease Ver112 and neutral protease PII is 280 and 286, respectively; and the sequence identity between them is 46.6%. The three-dimensional structure of PII was modeled based on templates of the crystal structures of Ver112 (PDB code 3F7M (Liang et al., 2010)) and proteinase K (PDB code 1IC6) using

homology modeling software package MODELLER (Sali & Blundell, 1993). Because of their high degree of sequence identity, it is not surprising that the structural model of PII is highly similar to the crystal structure of Ver112, with backbone RMSD between them being ~ 0.65 Å. Their structural superposition shows that the structure core composed of the major secondary structure elements are well matched, while some surface-exposed turns and loops exhibiting relatively large structural deviations (Figure 11B). We consider that it is the highly conserved amino acids within the regions of secondary structure elements and more amino acid insertions and deletions in regions of loops/turns than in secondary structural regions that give rise to these differences. The catalytic triad Asp-His-Ser and oxyanion hole are conserved between proteases Ver112 and PII, either in sequence or structurally.

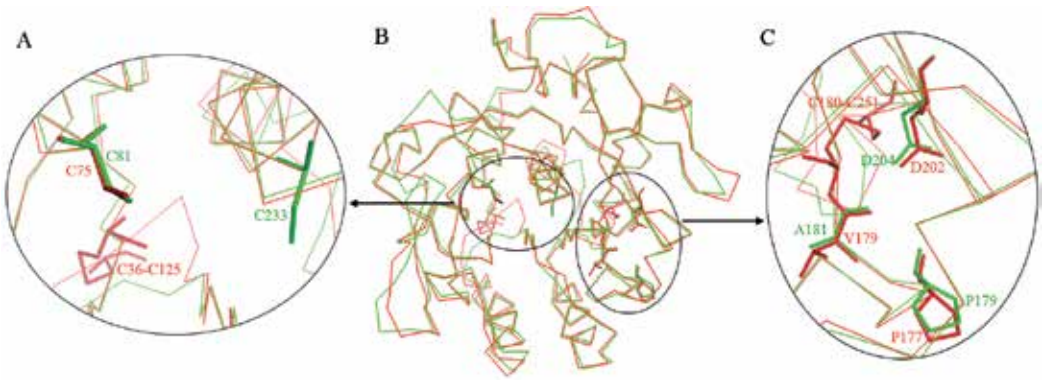

Fig. 11. Structural comparison between the experimentally determined crystal structure of Ver112 (PDB code 3F7M) and theoretically modeled structure of PII. (A, C) Locally enlarged structural regions showing disulfide bond, free cysteine and calcium binding site. The residues forming the disulfide bonds and Ca1 sites and the free cysteines are shown as stick models and labeled with red for Ver112 and green for PII. (B) The superposition of backbone of Ver112 (red) and PII (green).

The strong calcium cation Ca1 may also present in the structure of PII because of the presence of the Ca1 site residues (i.e., Pro179, Ala181 and Asp204 shown in Figure 11C) positionally equivalent to those in PL646 (PDB code 3F7O) and proteinase K (PDB code 1IC6), contributing to some extent to the stability of PII structure. However, the weak calcium cation Ca2 present in 1IC6 may not exist in PII due to the lack of equivalent Ca2 site residues.

As described in sections 4.2 and 4.3.2, the five completely conserved cysteines (at positions 34, 73, 123, 178 and 249; proteinase K numbering) among the alkaline proteases are predicted to form two disulfide bonds, i.e., Cys34-Cys123 and Cys178-Cys249, based on the homology models; and this is verified by the subsequently determined high-resolution crystal structure of Ver112 (PDB code 3F7M; Figure 11) as described in section 5.1, showing the presence of two disulfide bonds Cys36-Cys125 and Cys180-Cys251; and that the Cys75 is free. However, there is no cysteine found at the positions 34, 123, 178 and 249 (proteinase K numbering) in the neutral protease PII; and the disulfide bond can not be formed between the two mere cysteines Cys81 (equivalent to the free Cys73 in 1IC6 and free Cys75 in Ver112) and Cys233 in PII due to the large spatial separation between them (Figure 11A, B). Summarily, the alkaline protease Ver112 has two disulfide bonds, Cys36-C125 and C180-

C251 (Ver112 numbering) whereas the neutral protease PII contains no disulfide bond; and Ver112 displays higher thermal stability and stronger nematicidal/catalytic activity than PII does (Liang et al., 2011).

8.3 Role of disulfide bond in structural stability and flexibility

In order to investigate how the disulfide bonds influence structural stability and flexibility of these two proteases, MD simulations on their structures of wild-type and disulfide bond-disrupted mutant (Ver112_123C/A, Ver112_178C/A, and Ver112_123C/A_178C/A; proteinase K numbering) were performed at temperatures 300 K and 400 K, respectively. Analyses of the geometrical properties along the 300 K MD trajectories indicate that PII has higher average values of C_α RMSD and SASA while lower average values of NNH and NNC, suggesting a higher flexibility and less compact equilibrium structure of PII in comparison with Ver112. This may be caused by the lack of equivalent disulfide bonds in PII.

The geometrical properties of Ver112 are similar on average to those of its three mutants during MD simulations at 300 K, whereas at 400 K the wild-type Ver112 presents more NHB and NNC and less SASA than its mutants, suggesting that disulfide bonds contribute to the global stability of Ver112 at high temperature. Additionally, the stability of local structures within 5 Å of the two disulfide bonds Cys34-C123 and Cys178-Cys249 was also enhanced by disulfide bonds, as demonstrated by their increased RMSF and decreased NNC values in mutants with disrupted disulfide bonds compared to the wild-type structure. Analyses of the average RMSF values of the S1 and S4 substrate-binding pockets show that upon disruption of Cys34-Cys123, RMSF of S1 pocket decreased by 21.2% while that of S4 pocket showed almost no change; upon disruption of Cys178-Cys249, the relatively large reduction in flexibility of both S1 and S4 pockets was observed; and the most pronounced reduction (30.7% and 17.2%) was observed when both disulfide bonds were broken.

We can conclude based on these results: i) the presence of disulfide bonds enhances not only the local but also the global stability of the protease, thus explaining the higher thermal stability of the alkaline protease Ver112 compared to that of the neutral protease PII; ii) the presence of disulfide bonds increases the flexibility of substrate-binding pockets located relatively far from disulfide bonds, thus explaining why alkaline proteases have higher substrate affinity and catalytic activity (S. Q. Liu et al., 2011; Tao et al., 2010) than neutral proteases.

8.4 The correlation between electrostatic surface potential and phylogenetic relationship

Furthermore, the correlation between electrostatic surface potential and phylogenetic relationship of the available cuticle-degrading proteases were investigated. These include 14 neutral proteases derived from nematode-trapping fungi and four alkaline proteases from nematode-parasitic fungi. The amino acid sequences of the mature neutral proteases were obtained from GeneBank database with accession numbers/species sources of AB120125/*M.megalosporum,* AF516146 (Aoz1)/*A.oligospora,* AY859780/*M.cystosporum,* AY859781/*M.elegans,* AY859782/*A.Conoides,* DQ531603/*D.varietas,* EF055263/*A.multisecundaria,* EF113088/*A.Musiformis,* EF113089/*A.yunnanensis,* EF113090/*M.psychrophilum,* EF113091/*M.Coelobrochum,* EF113092/*D.shizishanna,* EF681769/*M.haptotylum,* X94121 (PII)/*A.oligospora*. The structural models of these 14 proteases were constructed using homology modeling method based on template structures

Ver112 (PDB code 3F7M) and proteinase K (PDB code 1IC6) as described before (Liang et al., 2011; S.Q. Liu et al., 2007b). The structures of the four alkaline proteases PR1 (GeneBank accession number AJ416695), VCP1 (AJ427460), Ver112 (AY692148) and PL646 (EF094858) were either determined by X-ray crystallographic technique (for Ver112 and PL646) (Liang et al., 2010) or modeled by homology modeling method (for PR1 and VCP1) (S.Q. Liu et al., 2007b). The electrostatic surface potentials of these 18 cuticle-degrading proteases were then calculated with the Poisson-Boltzmann method using Swiss-PdbViewer (Guex & Peitsch, 1997); the phylogenetic tree of these proteases was constructed with neighbor-joining (NJ) method using the MEGA program (Tamura et al., 2007); and finally each electrostatic surface potential plot of these proteases was placed corresponding to its branch in the phylogenetic tree (Figure 12).

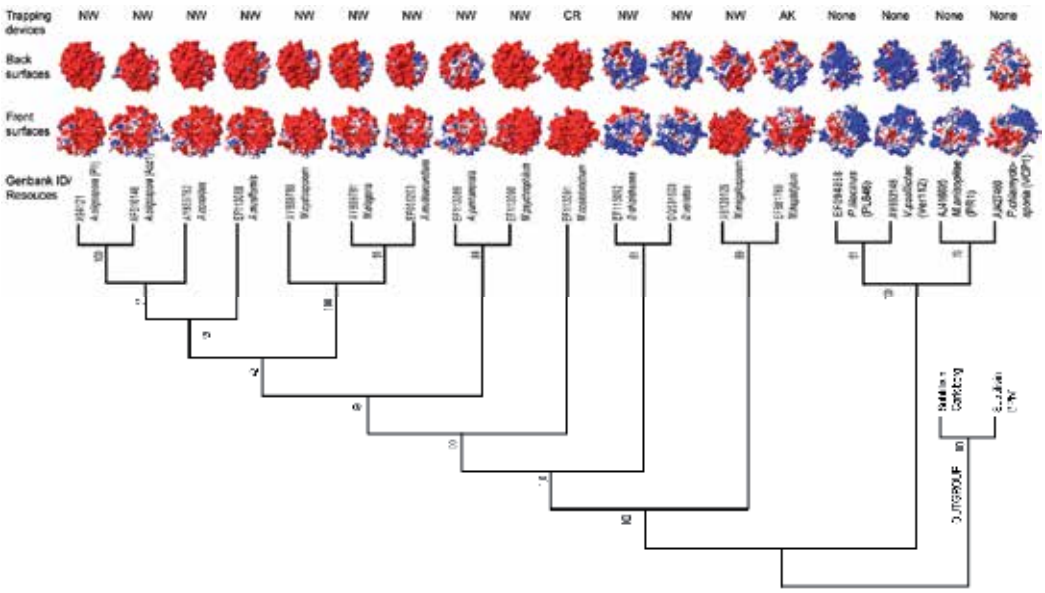

Fig. 12. Correlation between molecular electrostatic surface potentials of cuticle-degrading proteases and their phylogenetic relationship. The molecular electrostatic surfaces of these 18 proteases were calculated using Poisson-Boltzmann method and the phylogenetic tree was constructed using NJ method; and finally the electrostatic surface potential plots of each protease were placed at the position corresponding to its branch in the phylogenetic tree. Trapping devices of networks, constricting rings and adhesive knobs in the nematode-trapping fungi are abbreviated as NW, CR and AK, respectively. The nematode-parasitic fungi PL646, Ver112, PR1 and VCP1 contain no trapping device (None). Front surfaces are defined as molecular surfaces containing active site at its center; back surfaces are opposite to front surfaces. The positively and negatively charged electrostatic surfaces are colored blue and red, respectively. This figure is modified from (Liang et al., 2011).

Close inspection of Figure 12 reveals a good qualitative correlation between electrostatic surface potential and phylogenetic relationship of these proteases. Briefly, if proteases are phylogenetically more similar, they also exhibit more similar electrostatic potential distribution on the molecular surfaces, including both the front and back surfaces. For example, the neutral proteases PII and Aoz1 are clustered into the same small clade and

both exhibit overwhelming electro-negative potential distribution over all their protein surfaces. This can be interpreted by the highly similar amino acid sequence between these two proteases and the almost identical location of the equivalent amino acids in their three dimensional structures, leading to highly similar distribution of the partial charges between molecular surfaces of these two proteases. As expected, all the 14 neutral proteases derived from fungi possessing trap devices are grouped into one large clade; and the other four alkaline proteases derived from fungi without trap device form the another clade. A common feature of the neutral proteases is that they exhibit a predominant electro-negative potential distribution both on the front surface (except for EF113092 and DQ531603 located in the same branch) and on the back-side surface (except for EF113092 and DQ531603 in the same branch, and AB120125 and EF681769 in the same branch), while for the alkaline proteases their back surfaces are predominantly positively charged (except for AJ427460/VCP1) and their front surfaces display dual character of electrostatic potential distribution: the electro-negative potential (red color) is mainly concentrated around the surface of substrate-binding channel, and the other regions of the front surface are largely positively charged (blue color) or exhibit electro-neutrality (white color). As described and discussed in earlier section, the anionic feature of the substrate-binding regions is able to increase the local conformational flexibility of binding pockets/sites, which in turn facilitates substrate binding and even enhances catalytic efficiency of proteases. This may explain the maintenance of electro-negative potential around the substrate-binding groove surface of these proteases, in particular around that of the alkaline cuticle-degrading proteases with dominant positive charges. Interestingly, the dominant distribution of electro-positive potential on the surface of the alkaline proteases would undoubtedly increase efficiency of diffusion and subsequent adsorption of proteases to the negatively charged surface of nematode cuticle due to electrostatic attraction force between the oppositely charged proteases and the cuticle. On the contrary, the diffusion of the neutral proteases towards nematode cuticle seems to be suppressed due to the electrostatic repulsion between the heavily negatively charged proteases and the negatively charged cuticle. However, the diffusion of proteases towards the host cuticle is unlikely the bottleneck of infection by the nematode-trapping fungi because the active capture of the prey with trap devices makes it easy for the directly full contact between the prey and the proteases, and therefore the secreted neutral proteases can degrade the captured nematode "unhurriedly". We consider that the trap devices may eliminate the constraints faced by nematode-parasitic fungi and, with the aid of the traps, the nematode-trapping fungi's neutral cuticle-degrading proteases may be subjected to a low selective pressure, whereas the nematode-parasitic fungi have to evolve to produce more powerful alkaline proteases to acquire nutrients due to the lack of trap device (J. Li et al., 2010; Liang et al., 2011).

9. Conclusion

Because of their essential role in the breakdown of the cuticles of nematodes or insects, a thorough understanding of the structure-function relationship of the cuticle-degrading proteases is of crucial importance for improving infectious virulence of nematophagous fungi through engineering modifications, which will facilitate in turn the development and exploitation of various nematophagous fungi as effective bio-control agents against nematodes. Here in this chapter a number of cuticle-degrading proteases produced by different fungi were studied in detail in terms of physicochemical properties, structural

features, catalytic kinetics, structural dynamics and electrostatic surface potential-phylogeny relationship. The high degree of sequence identity among these proteases determines their very similar three-dimensional structures and their category to belong to the S8 peptidase family. A common feature of the cuticle-degrading serine proteases is their broad substrate specificity and high catalytic activity, allowing nematophagous fungi to utilize maximally different kinds of cuticle protein components to acquire nutrients. The overall structures of these proteases present a well-defined globular fold with high rigidity that is maintained by multiple structural factors such van der Waal contacts, hydrogen bonds, salt bridges, disulfide bonds, Ca^{2+} binding and aromatic ring stacking. It is the high rigidity of the overall structures that prevents the autolysis or proteolysis of the cuticle-degrading proteases. However, a certain degree of flexibility in some surface-exposed loops, especially those around the substrate-binding sites, is necessary and even crucial for substrate binding and enzyme turnover. Like almost all the other enzymes, the catalytic reaction of cuticle-degrading proteases can be considered to consist of the following consecutive dynamic steps: i) diffusion of the substrate to the entrance to the substrate-binding site; ii) initial contacts of the substrate with substrate-binding site; iii) rearrangement of the substrate at the binding site into its bound orientation which is ready for nucleophilic attack; iv) enzyme catalysis and the formation of tetrahedral complex; v) rearrangement of the cleaved substrate to be ready for release; and vi) product release. All these steps involve mutual conformational adaption of the enzyme and the substrate, which is facilitated by dynamic variation of substrate-binding sites/pockets resulting from either the large concerted motions of enzyme backbone or the small side chain rearrangements/rotations. Despite the highly similar sequences and structures among the cuticle-degrading serine proteases derived from different nematophagous fungi, they can be divided into two groups of neutral and alkaline proteases, which are produced by nematode-trapping fungi and nematode-parasitic fungi, respectively. The differences in optimal reaction temperature, thermal stability, electrostatic surface potential, substrate-binding affinity and catalytic activity among these proteases can be considered to be the consequence of the subtle differences in amino acid components of these proteases, particularly those located in the substrate-binding regions, resulting in the changes in local and/or global structural dynamics, distribution of partial charges and in architecture and hydrophathy of substrate-binding pockets. Our results also suggest that nematode-parasitic and nematode-trapping fungi have evolved to adopt different strategy to infect nematode as reflected by different properties of the secreted cuticle-degrading proteases.

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11. References

Amadei, A.; Linssen, A.B.M. & Berendsen, H.J.C. (1993). Essential dynamics of proteins. *Proteins,* 17, 412-425.

Arnorsdottir, J.; Kristjansson, M.M. & Ficner, R. (2005). Crystal structure of a subtilisin-like serine proteinase from a psychrotrophic *Vibrio* species reveals structural aspects of cold adaptation. *Febs J,* 272, 832-845.

Bajorath, J.; Raghunathan, S.; Hinricks, W. & Saenger, W. (1989). Long-range structural changes in proteinase K triggered by calcium ion removal. *Nature,* 337, 481-484.

Baker, D. & Sali, A. (2001). Protein structure prediction and structural genomics. *Science,* 294, 93-96.

Balsera, M.A.; Wriggers, W.; Oono, Y. & Schulten, K. (1996). Principal component analysis and long time protein dynamics. *J Phys Chem,* 100, 2567-2572.

Barrett, C.P. & Noble, M.E. (2005). Molecular motions of human cyclin-dependent kinase 2. *J Biol Chem,* 280, 13993-14005.

Barron, G.L. (1977). The nematode-destroying fungi. In: *Topics in mycobiology,* pp. 1-140, Canadian Biological Publications, Guelph.

Betzel, C.; Pal, G.P.; Struck, M.; Jany, K.D. & Saenger, W. (1986). Active-site geometry of proteinase K: crystallographic study of its complex with a dipeptide chloromethyl ketone inhibitor. *FEBS Lett,* 197, 105-110.

Betzel, C.; Pal, G.P. & Saenger, W. (1988). Three dimensional structure of proteinase K at 0.15 nm resolution. *Eur J Biochem,* 178, 155-171.

Betzel, C.; Teplyakov, A.V.; Harutyunyan, E.H.; Saenger, W. & Wilson, K.S. (1990). Thermitase and proteinase K: a comparison of the refined three-dimensional structures of the native enzymes. *Protein Eng,* 3, 161-172.

Betzel, C.; Singh, T.P.; Visanji, M.; Peters, K.; Fittkau, S.; Saenger, W. & Wilson, K.S. (1993). Structure of the complex of proteinase K with a substrate analogue hexapeptide inhibitor at 2.2-A resolution. *J Biol Chem,* 268, 15854-15858.

Betzel, C.; Gourinath, S.; Kumar, P.; Kaur, P.; Perbandt, M.; Eschenburg, S. & Singh, T.P. (2001). Structure of a serine protease proteinase K from *Tritirachium album* limber at 0.98 Å resolution. *Biochemistry,* 40, 3080-3088.

Bidochka, M.J. & Khachatourians, G.G. (1994). Basic proteases of entomopathogenic fungi differ in their adsorption properties to insect cuticle. *J Invertebr Pathol,* 64, 26-32.

Birktoft, J.J. & Blow, D.M. (1972). Structure of alpha-chymotrypsin. *J Mol Biol,* l68, 187-240.

Blaxter, M.L.; Page, A.P.; Rudin, W. & Maizels, R.M. (1992). Nematode surface coats: actively evading immunity. *Parasitol Today,* 8, 243-247.

Blow, D.M. (1976). Structure and mechanism of chymotrypsin. *Acc Chem Res,* 9, 145-152.

Brendel, V.; Bucher, P.; Nourbakhsh, I.; Blaisdell, B.E. & Karlin, S. (1992). Methods and algorithms for statistical analysis of protein sequences. *Proc Natl Acad Sci USA,* 89, 2002-2006.

Cox, G.; Kusch, M. & Edgar, R. (1981). Cuticle of Caenorhabditis elegans: its isolation and partial characterization. *J Cell Biol,* 90, 7-17.

Dauter, Z.; Betzel, C.; Genov, N.; Pipon, N. & Wilson, K.S. (1991). Complex between the subtilisin from a mesophilic bacterium and the Leech inhibitor eglin-C. *Acta Cryst B,* 47, 707-730.

DelMar, E.; Largman, C.; Brodrick, J. & Geokas, M. (1979). A sensitive new substrate for chymotrypsin. *Anal. Biochem,* 99, 316–320.

Dodson, G. & Wlodawer, A. (1998). Catalytic triads and their relatives. *Trends Biochem Sci,* 23, 347-352.

Ebeling, W.; Hennrich, N.; Klockow, M.; Metz, H.; Orth, H.D. & Lang, H. (1974). Proteinase K from *Tritirachium album* limber. *Eur J Biochem,* 47, 91-97.

Estell, D.A.; Graycar, T.P.; Miller, J.V.; Powers, D.B.; Burnier, J.P.; Ng, P.G. & Wells, J.A. (1986). Probing steric and hydrophobic effects on enzyme interactions by protein engineering. *Science,* 233, 659-663.

Feenstra, K.A.; Hess, B. & Berendsen, H.J.C. (1999). Improving efficiency of large time-scale molecular dynamics simulations of hydrogen-rich systems. *J Comp Chem,* 20, 786-798.

Fiedler, Z. & Sosnowska, D. (2007). Nematophagous fungus *Paecilomyces lilacinus* (Thom) Samson is also a biological agent for control of greenhouse insects and mite pests. *BioControl,* 52, 547-558.

Gill, S.C. & von Hipple, P. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem,* 182, 319-326.

Goettel, M.S.; St Leger, R.J.; Rizzo, N.W.; Staples, R.C. & Roberts, D.W. (1989). Ultrastructural localization of a cuticle-degrading protease produced by the entomopathogenic fungus *Metarhizium anisopliae* during penetration of host (Manduca sexta) cuticle. *J Gen Microbiol,* 135, 2233-2239.

Gray, N.F. (1987). Nematophagous fungi with particular reference to their ecology. *Biol Rev,* 62, 245–304.

Gudjonsdottir, K. & Asgeirsson, B. (2008). Effects of replacing active site residues in a cold-active alkaline phosphatase with those found in its mesophilic counterpart from Escherichia coli. *Febs J,* 275, 117-127.

Guex, N. & Peitsch, M.C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis,* 18, 2714-2723.

Helland, R.; Larsen, A.N.; Smalas, A.O. & Willassen, N.P. (2006). The 1.8 Å crystal structure of a proteinase K-like enzyme from a psychrotroph Serratia species. *Febs J,* 273, 61-71.

Hilz, H.; Wiegers, U. & Adamietz, P. (1975). Stimulation of proteinase K action by denaturing agents: application to the isolation of nucleic acids and the degradation of 'masked' proteins. *Eur J Biochem,* 56, 103-108.

Himmelhoch, S. & Zuckerman, B.M. (1978). Caenorhabditis briggsae: aging and the structural turnover of the outer cuticle surface and the intestine. *Exp Parasitol,* 45, 208-214.

Huang, X.W.; Zhao, N.H. & Zhang, K.Q. (2004). Extracellular enzymes serving as virulence factors in nematophagous fungi involved in infection of the host. *Res. Microbiol,* 155, 811-816.

Jansson, H.B. & Nordbring-Hertz, B. (1988). Infection mechanism in the fungus-nematode system. In: *Diseases of Nematodes*, G.J. Poinar, & H.B. Jansson, (Eds.), Vol. 2, pp. 59-72, CRC Press, Boca Raton Fla.

Jatala, P.; Kaltenbach, R. & Bocangel, M. (1979). Biological control of Meloidogyne incognita acrita and Globodera pallida on potatoes. *J Nematol,* 11, 303.

Jatala, P. (1986). Biological control of plant-parasitic nematodes. *Annu Rev Phytopathol,* 24, 453-489.

Joshi, L.; St Leger, R.J. & Bidochka, M.J. (1995). Cloning of a cuticle-degrading protease from the entomopathogenic fungus, *Beauveria bassiana*. *FEMS Microbiol Lett,* 125, 211-217.

Kabsch, W. & Sander, C. (1983). Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers,* 22, 2577-2637.

Koshland, D.E.J. (1958). Application of a theory of enzyme specificity to protein synthesis. *Proc Natl Acad Sci USA,* 44, 98–104.

Kraut, J. (1977). Serine proteases: structure and mechanism of catalysis. *Ann Rev Biochem,* 46, 331-358.

Kumar, S. & Nussinov, R. (2004). Different roles of electrostatics in heat and in cold: adaptation by citrate synthase. *Chembiochem,* 5, 280-290.

Kyte, J. & Doolittle, R. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol,* 157, 105-132.

Lange, O.F.; Lakomek, N.A.; Fares, C.; Schroder, G.F.; Walter, K.F.A.; Becker, S.; Meiler, J.; Grubmuller, H.; Griesinger, C. & de Groot, B.L. (2008). Recognition dynamics up to microseconds revealed from an RDC-derived ubiquitin ensemble in solution. *Science,* 320, 1471-1475.

Lehninger, A.L. (1995). EMBL WWW Gateway to Isoelectric Point Service, In: *European Molecular Biology Laboratory,* Accessed Mar. 1, 2011, Available from: http://www.embl-heidelberg.de/cgi/pi-wrapper.pl

Li, J.; Yu, L.; Yang, J.K.; Dong, L.Q.; Tian, B.Y.; Yu, Z.F.; Liang, L.M.; Zhang, Y.; Wang, X. & Zhang, K. (2010). New insights into the evolution of subtilisin-like serine protease genes in Pezizomycotina. *BMC Evol Biol,* 10, 68.

Li, T.F.; Zhang, K.Q. & Liu, X.Z. (2000). *Taxonomy of nematophagous fungi*, Chinese Science and Technical Publishing, Beijing.

Liang, L.M.; Lou, Z.Y.; Ye, F.P.; Yang, J.K.; Liu, S.Q.; SunY.N; Guo, Y.; Mi, Q.L.; Huang, X.W.; Zou, C.G.; Meng, Z.H.; Rao, Z.H. & Zhang, K.Q. (2010). The crystal structures of two cuticle-degrading proteases from nematophagous fungi and their contribution to infection against nematodes. *Faseb J,* 24, 1391-1400.

Liang, L.M.; Liu, S.Q.; Yang, J.K.; Meng, Z.H.; Lei, L.P. & Zhang, K.Q. (2011). Comparison of homology models and crystal structures of cuticle-degrading proteases from nematophagous fungi: assessing the structural basis of nematicidal activity. *Faseb J,* Epub ahead of print. doi: 10.1096/fj.10-175653 fj.10-175653

Lindahl, E.; Hess, B. & van der Spoel, D. (2001). GROMACS 3.0: A package for molecular simulation and trajectory analysis. *J Mol Model,* 7, 306-317.

Liu, S.Q.; Liu, C.Q. & Fu, Y.X. (2007a). Molecular motions in HIV-1 gp120 mutants reveal their preferences for different conformations. *J Mol Graphics Modell,* 26, 306-318.

Liu, S.Q.; Meng, Z.H.; Yang, J.K.; Fu, Y.X. & Zhang, K.Q. (2007b). Characterizing structural features of cuticle-degrading proteases from fungi by molecular modeling. *BMC Struct Biol,* 7, 33.

Liu, S.Q.; Liu, S.X. & Fu, Y.X. (2008). Molecular Motions of Human HIV-1 gp120 Envelope Glycoproteins. *J Mol Model,* 14, 857-870.

Liu, S.Q.; Meng, Z.H.; Fu, Y.X. & Zhang, K.Q. (2010). Insights derived from molecular dynamics simulation into the molecular motions of serine protease proteinase K. *J Mol Model,* 16, 17-28.

Liu, S.Q.; Meng, Z.H.; Fu, Y.X. & Zhang, K.Q. (2011). The effect of calciums on the molecular motions of proteinase K. *J Mol Model,* 17, 289-300.

Liu, X.Z.; Xiang, M.C. & Che, Y.S. (2009). The living strategy of nematophagous fungi. *Mycoscience,* 50, 20-25.

Lobna, M. & Zawam, H. (2010). Efficacy of some biocontrol agents on reproduction and development of meloidogyne incognita infecting tomato. *Journal of American Science,* 6, 495-509.

Müller, A.; Hinrichs, W.; Wolf, W.M. & Saenger, W. (1994). Crystal structure of calcium-free proteinase K at 1.5-Å resolution. *J Biol Chem,* 269, 23108-23111.

Martin, J.R.; Mulder, F.A.A.; Karimi-Nejad, Y.; van der Zwan, J.; Mariani, M.; Schipper, D. & Boelens, R. (1997). The solution structure of serine protease PB92 from *bacillus alcalophilus*. *Structure,* 5, 521–532.

Matsumura, M.; Signor, G. & Matthews, B.W. (1989). Substantial increase of protein stability by multiple disulfide bonds. *Nature,* 342, 291-293.

Mello, L.V.; de Groot, B.L.; Li, S. & Jedrzejas, M.J. (2002). Structure and flexibility of Streptococcus agalactiae hyaluronate lyase complex with its substrate: insights into the mechanism of processive degradation of hyaluronan. *J Biol Chem,* 277, 36678-36688.

Monod, J.; Wyman, J. & Changeux, J.P. (1965). On the nature of allosteric transitions: a plausible model. *J Mol Biol,* 12, 88-118.

Morton, C.O.; Hirsch, P.R.; Peberdy, J.P. & Kerry, B.R. (2003). Cloning of and genetic variation in protease VCP1 from the nematophagous fungus *Pochonia chlamydosporia*. *Mycol Res,* 107, 38-46.

Moult, J.; Sussman, F. & James, M.N.G. (1985). Electron density calculations as an extension of protein structure refinement: SGPA at 1.5 Å resolution. *J Mol Biol,* 182, 555-566.

Murrell, K.D.; Graham, C.E. & McGreevy, M. (1983). Strongyloides ratti and Trichinella spiralis: net charge of epicuticle. *Exp Parasitol,* 55, 331-339.

Nishihira, J. & Tachikawa, H. (1996). Theoretical evaluation of a model of the catalytic triads of serine and cysteine proteases by ab initio molecular orbital calculation. *J Theor Biol,* 196, 513-519.

Niu, Q.H.; Huang, X.W.; Zhang, L.; Xu, J.P.; Yang, D.M.; Wei, K.B.; Niu, X.M.; An, Z.Q.; Bennett, J.W.; Zou, C.G.; Yang, J.K. & Zhang, K.Q. (2010). A Trojan horse mechanism of bacterial pathogenesis against nematodes. *Proc Natl Acad Sci USA,* 107, 16631-16636.

Nordbring-Hertz, B. (2004). Morphogenesis in the nematode-trapping fungus Arthrobotrys oligospora: an extensive plasticity of infection structures. *Mycologist,* 18, 125-133.

Page, A.P. & Johnstone, I.L. (2007). The cuticle. In: *WormBook,* J.M. Kramer, & D.G. Moerman, (Eds.), The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.138.1.

Pahler, A.; Banerjee, A.; Dattagupta, J.K.; Fujiwara, T.; Lindner, K.; Pal, G.P.; Suck, D. & Saenger, W. (1984). Three-dimensional structure of fungal proteinase K reveals similarity to bacterial subtilisin. *Embo J,* 3, 1311-1314.

Pasternak, A.; Ringe, D. & Hedstrom, L. (1999). Comparison of anionic and cationic trypsinogens: the anionic activation domain is more flexible in solution and differs in its mode of BPTI binding in the crystal structure. *Protein Sci,* 8, 253-258.

Perica, T. & Chothia, C. (2010). Ubiquitin - molecular mechanisms for recognition of different structures. *Curr Opin Struct Biol,* 20, 367-376.

Rawlings, N.D.; Barrett, A.J. & Bateman, A. (2010). MEROPS: the peptidase database. *Nucleic Acids Res,* 38, D227-D233.

Rombach, M.C.; Aguda, R.M.; Shepard, B.M. & Roberts, D.W. (1986). Infection of rice brown planthopper, Nilaparvata lugens (Homoptera: Delphacidae), by field application of entomopathogenic hyphomycetes (Deuteromycotina). *Environ Entomol,* 15, 1070-1073.

Russell, A.J. & Fersht, A.R. (1987). Rational modification of enzyme catalysis by engineering surface charge. *Nature,* 328, 496-500.

Rypniewski, W.R.; Dambmann, C.; von der Osten, C.; Dauter, M. & Wilson, K.S. (1995). Structure of inhibited trypsin from *Fusarium oxysporum* at 1.55 Å. *Acta Cryst D,* 51, 73-84.

Saberhagen, C.; Klotz, S.A.; Bartholomew, W.; Drews, D. & Dixon, A. (1997). Infection due to *Paecilomyces lilacinus*: a challenging clinical identification. *Clin Infect Dis,* 25, 1411-1413.

Sali, A. & Blundell, T.L. (1993). Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol,* 234, 779-815.

Sasser, J. & Freekman, D. (1987). A world perspective on nematology: the role of the society. In: *Vistas on Nematology*, J.A. Veech, & D.W. Dickson, (Eds.), Society of Nematologists, Hyattsville, MD, USA.

Schmitt, D.P. & Sipes, B.S. (1998). *Plant-parasitic nematodes and their management*. Cooperative extension service, college of tropical agriculture and human resources, University of Hawaii at Manoa, Retrieved from http://www.ctahr.hawaii.edu/oc/freepubs/pdf/PD-15.pdf

Segers, R.; Butt, T.; Kerry, B. & Peberdy, J. (1994). The nematophagous fungus *Verticillium chlamydosporium* produces a chymoelastase-like protease which hydrolyses host nematode proteins in situ. *Microbiology,* 140, 2715-2723.

Segers, R.; Butt, T.M.; Kerry, B.R.; Beckett, A. & Peberdy, J.F. (1996). The role of the proteinase VCP1 produced by the nematophagous *Verticillium chlamydosporium* in the infection process of nematode eggs. *Mycol Res,* 100, 421-428.

Sevier, C.S. & Kaiser, C.A. (2002). Formation and transfer of disulphide bonds in living cells. *Nat Rev Mol Cell Biol,* 3, 836-847.

Shirley, B.A.; Stanssens, P.; Hahn, U. & Pace, C.N. (1992). Contribution of hydrogen bonding to the conformational stability of ribonuclease T1. *Biochemistry,* 31, 725-732.

Siezen, R.J.; de Vos, W.M.; Leunissen, J.A. & Dijkstra, B.W. (1991). Homology modelling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. *Protein Eng,* 4, 719-737.

Siezen, R.J. & Leunissen, J.A.M. (1997). Subtilases: the superfamily of subtilisin-like serine proteases. *Protein Sci,* 6, 501-523.

Snyder, D. (2002). Plant parasitic nematodes-an introduction, In: *North Crolina State University,* Accessed Mar. 10, 2011, Available from: http://www.cals.ncsu.edu/pgg/dan_webpage/index.htm

St Leger, R.J.; Cooper, R.M. & Charnley, A.K. (1986). Cuticle-degrading enzymes of entomopathogenic fungi: degradation in vitro by enzymes from entomopathogens. *J Invertebr Pathol,* 48, 167-177.

St Leger, R.J.; Charnley, A.K. & Cooper, R.M. (1987). Characterization of cuticle-degrading proteases produced by the entomopathogen *Metarhizium anisopliae. Arch Biochem Biophys,* 253, 221-232.

St Leger, R.J.; Frank, D.C.; Roberts, D.W. & Staples, R.C. (1993). Molecular cloning and regulatory analysis of the cuticle degrading protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*. *Eur J Biochem,* 204, 991-1001.

Stirling, G.R. & West, L.M. (1991). Fungal parasites of root-knot nematode eggs from tropical and sub-tropical regions of Australia. *Australas Plant Path,* 20, 149-154.

Tamura, K.; Dudley, J.; Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol,* 24, 1596-1599.

Tao, Y.; Rao, Z.H. & Liu, S.Q. (2010). Insight derived from molecular dynamics simulation into substrate-induced changes in protein motions of proteinase K. *J Biomol Struct Dyn,* 28, 143-157.

Teplyakov, A.V.; Kuranova, I.P.; Harutyunyan, E.H.; Vainstein, B.K.; Frommel, C.; Hohne, W.E. & Wilson, K.S. (1990). Crystal structure of thermitase at 1.4 Å. *J Mol Biol,* 214, 261-279.

Tian, B.; Yang, J.K. & Zhang, K.Q. (2007). Bacteria used in the biological control of plant-parasitic nematodes: populations, mechanisms of action and future prospects. *FEMS Microbiol Ecol,* 61, 197-213.

Tobi, D. & Bahar, I. (2005). Structural changes involved in protein folding correlate with intrinsic motions of proteins in the unbound state. *Proc Natl Acad Sci USA,* 102, 18908-18913.

Tsukuda, H. & Blow, D.M. (1985). Structure of alpha-chymotrypsin refined at 1.68 Å resolution. *J Mol Biol,* 184, 703-711.

Tunlid, A.; Rosen, S.; Ek, B. & Rask, L. (1994). Purification and characterization of an extracellular serine protease from the nematode-trapping fungus Arthrobotrys oligospora. *Microbiology,* 140, 1687-1695.

Wells, J.A.; Powers, D.B.; Bott, R.R.; Graycar, T.P. & Estell, D.A. (1987). Designing substrate specificity by protein engineering of electrostatic interactions. *Proc Natl Acad Sci USA,* 84, 1219-1223.

Westenfeld, F.; Alston, W.K. & Winn, W.C. (1996). Complicated soft tissue infection with prepatellar bursitis caused by *Paecilomyces lilacinus* in an immunocompetent host: case report and review. *J Clin Microbiol,* 34, 1559-1562.

Wolf, W.M.; Bajorath, J.; Müller, A.; Raghunathan, S.; Singh, T.P.; Hinrichs, W. & Saenger, W. (1991). Inhibition of proteinase K by methoxysuccinyl-Ala-Ala-Pro-Ala-chloromethyl ketone: an X-ray study at 2.2-Å resolution. *J Biol Chem,* 266, 17695-17699.

Writing, A. (2011). The advantages of biological pest control, In: *ehow.com,* Accessed Mar. 12, 2011, Available from: http://www.ehow.com/list_5985349_advantages-biological-pest-control.html

Yang, J.K.; Huang, X.W.; Tian, B.Y.; Sun, H.; Duan, J.X.; Wu, W.P. & Zhang, K.Q. (2005a). Characterization of an extracellular serine protease gene from the nematophagous fungus *Lecanicillium psalliotae*. *Biotechnol Lett,* 27, 1329-1334.

Yang, J.K.; Huang, X.W.; Tian, B.Y.; Wang, M.; Niu, Q.H. & Zhang, K.Q. (2005b). Isolation and characterization of a serine protease from the nematophagous fungus, *Lecanicillium psalliotae*, displaying nematicidal activity. *Biotechnol Lett,* 27, 1123-1128.

Yang, J.K.; Tian, B.Y.; Liang, L.M. & Zhang, K.Q. (2007). Extracellular enzymes and the pathogenesis of nematophagous fungi. *Appl Microbiol Biotechnol,* 75, 21-31.

Yang, J.K.; Wang, L.; Ji, X.L.; Feng, Y.; Li, X.M.; Zou, C.G.; Xu, J.P.; Ren, Y.; Mi, Q.L.; Wu, J.L.; Liu, S.Q.; Liu, Y.; Huang, X.W.; Wang, H.Y.; Niu, X.M.; Li, J.; Liang, L.M.; Ji, K.F.; Zhou, W.; Yu, Z.F.; Li, G.H.; Liu, Y.J.; Li, L.; Qiao, M.; Feng, L. & Zhang, K.Q. (2011). Genomic and proteomic analyses of the fungus Arthrobotrys oligospora provide insights into nematode-trap formation. *Plos Pathog,* submitted.

Zhao, M.L.; Mo, M.H. & Zhang, K.Q. (2004). Characterization of a neutral serine protease and its full-length cDNA from the nematode-trapping fungus *Arthrobotrys oligospora*. *Mycologia,* 96, 16-22.

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The Effects of Pyrethroid and Triazine Pesticides on Fish Physiology

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1. Introduction

Worldwide pesticide usage has increased dramatically during the past two decades, coinciding with changes in farming practices and increasingly intensive agriculture. Environmental pollution caused by pesticides, especially in aquatic ecosystems, has become a serious problem. Contamination of water by pesticides, either directly or indirectly, can lead to fish kills, reduced fish productivity, or elevated concentrations of undesirable chemicals in edible fish tissue which can affect the health of humans consuming these fish. Residual amounts of pesticides and their metabolites have been found in drinking water and foods, increasing concern for the possible threats to human health posed by exposure to these chemicals. Contamination of surface waters has been well documented worldwide and constitutes a major issue at local, regional, national, and global levels (Cerejeira et al., 2003; Spalding et al., 2003).

Synthetic analogues of the pyrethrins, extracts from the ornamental *Chrysanthemum cinerariaefolium*, have been developed to circumvent the rapid photodegradation problem encountered with the natural insecticidal pyrethrins. The widespread use of these insecticides leads to the exposure of manufacturing workers, field applicators, the ecosystem, and the public to their possible toxic effects (Solomon et al., 2001). During investigations to modify the chemical structure of natural pyrethrins, a number of synthetic pyrethroids were produced with improved physical (involatility, lipophilicity) properties and greater insecticidal activity (knockdown). Several of the earlier synthetic pyrethroids have been successfully adapted for commercial use, mainly for the control of household insects. Other more recently developed pyrethroids have been introduced as agricultural insecticides because of their effectiveness against a wide range of insect pests and their non-persistence in the environment. Synthetic pyrethroids are fairly rapidly degraded in soil and in plants. Ester hydrolysis and oxidation at various sites on the molecule are the major degradation processes. Pyrethroids are strongly adsorbed on soil and sediments, and minimally eluted with water. There is little tendency for bioaccumulation in organisms (Haya, 1989). More than 1,000 pyrethroids have been synthesized since 1973. Their toxicity for non-target organisms is in the parts per billion (Bradbury & Coast, 1989).

Synthetic pyrethroids are non-systemic insecticides. Type I pyrethroids (e.g. bifenthrin, permethrin) block sodium channels in nerve filaments and cause the 'T-syndrome' in

mammals. Type II pyrethroids (e.g. cypermethrin, deltamethrin) act by blocking sodium channels and affecting the function of GABA-receptors in nerve filaments. In mammals, type II pyrethroids trigger clinical symptoms known as the 'CS-syndrome' (Roberts & Hudson, 1999).

Deltamethrin [(S)-a-cyano-3-phenoxybenzyl(1R,3R)-3-(2,2-dibromvinyl)-2,2-dimethylcyclo propan-carboxylate], a widely used pesticide, is among the most effective pyrethroid preparations (Bradbury & Coast, 1989). Deltamethrin was synthesised in 1974 and first marketed in 1977. It works by paralysing the insect nervous system, giving a quick knockdown effect following surface contact or ingestion. It is commonly used to control caterpillars on apples, pears, and hops, and for the control of aphids, mealy bugs, scale insects, and whiteflies on greenhouse cucumbers, tomatoes, potted plants, and ornamentals. Deltamethrin is the active ingredient in Butoflin, Butoss, Butox, Cislin, Crackdown, Cresus, Decis, Decis-Prime, K-Othrin, and K-Otek. It is the first potent and photostable insecticide belonging to the type II pyrethroid group. In the summers of 1991 and 1995, the pesticide caused massive eel (*Anguilla anguilla*) kills in Lake Balaton, Hungary following application for mosquito control. In 1995, the presence of deltamethrin was demonstrated in several other fish species and in sediment samples taken from the lake (Balint et al., 1995). The mechanism of its toxicity in fish is the same as that of other pyrethroids containing -cyano-3-phenoxybenzyl groups. They block the sodium channels of nerve filaments, lengthening the depolarisation phase. They also affect the GABA receptors in the nerve filaments (Eshleman & Murray, 1991).

Cypermethrin [(RS)-α-cyano-3-phenoxybenzyl(1RS)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate], another widely used pyrethroid pesticide, is among the most effective pyrethroid preparations (Bradbury & Coats, 1989). Cypermethrin is the active ingredient in Ammo, Arrivo, Barricade, Basathrin, Cymbush, Cymperator, Cynoff, Cypercopal, Cyperguard, Cyperhard, Cyperkill, Cypermar, Demon, Flectron, Fligene, Kafil, Polytrin, Siperin, and Super. The mechanism of its toxicity in fish is the same as that of other type II pyrethroids (Hayes, 1994). Cypermethrin is a synthetic pyrethroid used for the control of ectoparasites infesting cattle, sheep, poultry, and some companion animals. Recently, the compound has been used for the control of ectoparasite infestations (*Lepeophtheirus salmonis* and *Caligus elongatus)* in marine cage culture of Atlantic salmon, *Salmo salar* (Treasurer & Wadsworth, 2004).

Bifenthrin [2-methylbiphenyl-3-ylmethyl (Z)-(1RS, 3RS)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropane-carboxylate], a newer member of the synthetic pyrethroid family, is a contact insecticide and acaricide used on a variety of crops, on stored grain, and as a preconstruction termite barrier. Bifenthrin is the active ingredient in Talstar, Bifenthrine, Brigade, Capture, Torant, and Zipak. It is a type I pyrethroid (Shan et al., 1997), and has some structural similarities to cypermethrin, tetramethrin, and permethrin but is characterised by greater photostability and insecticidal activity than earlier pyrethroids (Yadav et al., 2003). It is effective as a gut or contact insecticide that affects the nervous system of vertebrates and invertebrates. Bifenthrin acts on sodium channels at the nerve cell endings to depolarize the presynaptic terminals. It also affects cellular ATPase production (Roberts and Hutson, 1999).

Triazines (a six-membered ring containing three carbon and three nitrogen atoms) are some of the oldest herbicides, with research initiated on weed control properties during the early 1950s. Triazine herbicides are categorized into two groups, the asymmetrical triazines, such as metribuzin, and the symmetrical triazines. The major commercially used symmetrical

triazines are simazine, atrazine, propazine, cyanazine, ametryn, prometryn, prometon, and terbutryn. As a chemical family, the triazines are a group of pesticides with a wide range of uses. Most are used in selective weed control programs, others, such as prometon, have no selective properties, which makes them suitable for use on industrial sites (Fan et al., 2007). A unique member of this family is cyromazine, which is an insect growth regulator useful in livestock, vegetable, and ornamental plant applications through interference with insect moulting and pupation. As herbicides, the triazines may be used alone or in combination with other herbicide active ingredients to increase the weed control spectrum (Solomon et al., 1996). Triazine's herbicidal activity is mediated through the inhibition of photosynthesis (Das et al., 2000) by blocking electron transport during the Hill reaction of photosystem-II (DeLorenzo et al., 2001). It binds to a plastoquinine-binding niche on D1, a 32-kD protein encoded by the psbA gene of the photosystem-II reaction complex (Das et al., 2000). In plants it is metabolised by oxidation to 2-hydroxy derivates and by side-chain de-alkylation (Roberts et al., 1998).

Terbutryn [(*N*2-*tert*-butyl-*N*4-ethyl-6-methylthio-1,3,5-triazine-2,4-diamine] is used as a selective pre- and early post- emergence control agent of most grasses and many annual broadleaf weeds on a variety of crops, such as cereals, legumes, and tree fruits. It is also used as an herbicide for control of submerged and free-floating weeds and algae in water courses, reservoirs, and fish ponds (Tomlin, 2003). Terbutryn is the active ingredient in Prebane, Igran, Shortstop, Clarosan, GS 14260, Plantonit, Gesaprim Combi (with Atrazine 1:1), Senate (with trietazine), and Igrater 50WP (with metobromuron 1:1). Terbutryn is moderately toxic to fish (Meister, 1992). Kidd & James (1991) reported the mean lethal toxicity of terbutryn (96 h LC50) 4 mg/L for common carp and 3 mg/L for rainbow trout. Large quantities of terbutryn have been used since the mid-1980s (Larsen et al., 2000). Terbutryn degrades slowly, with a half-life of 240 and 180 days in pond and river sediments, respectively (Muir et al., 1980). Its tendency to move from treated soils into water compartments through runoff and leaching has been demonstrated, and residual amounts of terbutryn and its metabolites have been found in drinking water and industrial food products long after application (Konstantinov et al., 2006). The application of terbutryn has been banned in many countries because it has the potential to bioaccumulate in organisms, but is still present in waters (Rioboo et al., 2007). Preparations containing terbutryn have not been registered in the Czech Republic since 2005, but its presence can be still detected in the environment. The highest concentration reported in surface water in the Czech Republic is 0.02 μg/L (Velisek et al., 2010). In Europe terbutryn levels can reach values up to 5.6 μg/L (Quednow & Puttmann, 2007).

Simazine (6-chloro-*N*,*N*'-diethyl-1,3,5-triazine-2,4-diamine) was introduced by the Swiss company J. R. Geigy (Cremlyn, 1990) in 1956 and is also a member of the triazine family of compounds. It is a selective herbicide used for control of annual broadleaf and grass weeds in raspberries, loganberries, highbush blueberries, apples, asparagus, and ornamentals. Non-crop uses include total weed control in industrial areas, at airports, along shelterbelts and rights-of-way, and for aquatic weed control in ditches, farm ponds, fish hatcheries, aquaria, and fountains (Arufe et al., 2004). Simazine is the active ingredient in Princep Caliber 90, Princep Liquid, Caliber, Cekusan, Cekusima, Framed, Gesatop, Simadex, Simanex, Simtrol, Tanzine, Totazine, and other trade name herbicides as well as in the algicide Aquazine. Simazine is slightly toxic to fish. Hashimoto & Nishiuchi (1981) give a value of 40 mg/L for 48hLC50 for common carp (*Cyprinus carpio* L.) and goldfish (*Carassius auratus*). Simazine degrades slowly, with an aerobic soil half-life of 91 days and an anaerobic aquatic half-life of 664 days. It is expected to be persistent in the environment, resulting in

the potential for this chemical to reach the aquatic environment through run-off. Simazine may also be expected to leach into groundwater systems due to high mobility in soil. It has been found to photo-degrade in soil, but to be resistant to aqueous abiotic reactions. Based on a low vapour pressure, volatilization is not expected to be an important process for simazine (Wackett et al., 2002). Simazine does not biomagnify in the food web, and its bioaccumulation potential is low, with bioconcentration factors (BCF) < 100. The depuration half-life in fish is < 7 days if the organism is transferred to uncontaminated water following exposure, indicating that simazine is rapidly excreted or metabolized (Niimi, 1987). Simazine is the second most commonly detected pesticide in surface and ground waters in the U.S., Europe, and Australia, presumably due to relatively high persistence (Inoue et al., 2006). Its degradation products are detected less frequently than atrazine and other triazine pesticides in the aquatic environment. The highest concentration reported in surface water in the Czech Republic is 0.06 μg/L (Velisek et al., 2009a). In Europe simazine levels reach values up to 5 μg/L (Belmonte et al., 2005). Simazine was the most frequently detected pesticide in 20 counties in California, USA with concentrations ranging from 0.02 to 49.2 μg/L (US Environmental Protection Agency [US EPA], 1994).
The following is an overview of the ecotoxicological risks presented by pesticides to ecosystems, based on data of the toxicity and effects of pesticide preparations to non-target organisms, especially fish. The objective is to describe whether and how pyrethroids and triazines might affect fish physiology. These two groups of pesticides were chosen because 1) the major negative impact of pyrethroids is their high toxicity to fish (e.g. fish mortality in Balaton in 1991 and 1995) combined with their use for control of some parasitic diseases in fish, e.g. *Lepeophtherius salmonis* in salmon farming; and 2) Triazine residues accumulate in fish tissue and s-triazines have been identified as relevant in a study on the prioritizing of substances dangerous to the aquatic environment in the member states of the European Community and are included in the EU Priority Pollutants List and the US EPA priority pollutants list.

2. Experimentation

2.1 Pyrethroids

The toxic effect was assessed by the results of acute toxicity tests and results of haematological, biochemical, and histological examination of rainbow trout and common carp after exposure to selected pyrethroid pesticides. We selected three active pyrethroid-based substances: deltamethrin (ingredient of Decis EW 50 - 50 g/L deltamethrin), cypermethrin (ingredient of Alimetrin 10 EM - 100 g/L cypermethrin), and bifenthrin (ingredient of Talstar EC 10 - 100 g/L bifrenthrin).

2.1.1 Acute toxicity test

The acute toxicity test on rainbow trout and common carp with selected pyrethroid pesticides followed the OECD Directive No. 203 'Fish, acute toxicity test.' Seven concentrations and a control were used in the basic test. Ten fish were used for each concentration and for the control group. The test was conducted semi-statically for 96 h. Fish mortalities were recorded at 24, 48, 72, and 96 h. Fish status and behaviour, along with water temperature, pH, and oxygen saturation were monitored throughout the test. The bath was changed every 12 h. The LC50, LC0, and LC100 values for the respective time intervals were determined by probit analysis (EKO-TOX 5.1 software).

2.1.2 Biochemical, haematological profile, and histopathological examination following pyrethroid exposure

Examinations were performed the 96 h exposure period with deltamethrin, cypermethrin, and bifenthrin at an exposure level of 96hLC50. Rainbow trout or carp, respectively in the control group were monitored concurrently. The test was performed in sixteen 300 l tanks. Each tank contained 20 rainbow trout or common carp, i.e. six tanks with 96hLC50 of deltamethrin, cypermethrin, or bifenthrin, and one control tank with rainbow trout and one control tank with carp. Tanks for all treated fish and controls were replicated, Presence of the tested substance (above 80% of the nominal concentration) was ensured through a 12 h exchange of the water bath. Determination of pyrethroid concentration in water was measured using gas chromatography (Mekebri et al., 2008).

Forty-eight experimental (8 fish from each pesticide duplicated) carp or rainbow trout and sixteen control carp or rainbow trout were selected at random and used for haematological, biochemical, and histological examination at the end of the 96 h exposure. Blood was sampled from the *vena caudalis*, using an 18G × 1 1/2 in syringe. Fish were not anaesthetized prior to blood sampling, as they were calm due to low water temperature and there was no danger of tissue trauma or handling stress. Heparin was used as an anticoagulant (Heparin inj., Leciva, Czech Republic) at a concentration of 40 I.U. heparin sodium salt in 1 ml blood. The indices used to evaluate the haematological profile included erythrocyte count (RBC), haemoglobin concentration (Hb), haematocrit (PCV), mean erythrocyte volume (MCV), mean colour concentration (MCHC), erythrocyte haemoglobin (MCH), leukocyte count (Leuko), and the differential leukocyte count (Leukogram). The procedures were based on unified methods for haematological examination of fish (Svobodova et al., 1991).

Blood was sampled by *v. caudalis* as mentioned above. Plasma was obtained by centrifuging blood samples in a cooled centrifuge (4 °C, 837×g). Plasma samples were held at -80 °C until analysis. Biochemical indices included glucose (GLU), total proteins (TP), albumins (ALB), total globulins (GLOB), ammonia (NH_3), tricylglycerols (TAG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), gama-glutamyl-transferase (GGT), creatine kinase (CK), lactate (LACT), alkaline phosphatase (ALP), calcium (Ca^{2+}), magnesium (Mg), and inorganic phosphate (PHOS). For the biochemical analysis of blood plasma, the VETTEST 8008 analyzer (IDEXX Laboratories Inc., Maine, USA) was used.

For histological studies, gill, skin, liver, cranial and caudal kidney, and spleen were fixed in a solution containing ethanol, formalin, and acetic acid (ALFAC) and stored in 70% ethanol. Tissues were embedded in paraffin, sectioned (5 μm), and the slides stained with haematoxylin and eosin (H&E). The sections were examined by light microscopy, using as reference Takashima & Hibiya (1995), and photographed using a digital camera.

2.2 Triazines

The effect of triazines was assessed by the results of biometric, haematological, biochemical, liver biomarker, and histological examination of common carp following long-term (chronic) exposure to selected triazine pesticides. From triazine we selected two active substances: terbutryn (Sigma Aldrich, Czech Republic, chemical purity 99.2%) and simazine (Sigma Aldrich, Czech Republic, chemical purity 99.5%).

Fish were acclimatized for 10 days before the beginning of the experiment and fed commercial food. The experiment was a semi-static assay conducted over 90 days. One hundred sixty one-year-old common carp were allocated, in groups of 10, to one of three experimental regimes or to an untreated control group for each active substance. The

conditions were duplicated for a total of sixteen groups, each held in an aquarium containing 200 L water. Aquaria for all treated fish and controls were replicated, and fish were transferred daily to the replicate aquarium containing freshly diluted terbutryn or simazine at the appropriate concentration, or freshwater for the controls. Fish were fed commercial fish pellets at about 1% body weight per day in two feedings. Terbutryn and simazine concentrations were checked daily by high performance liquid chromatography HPLC (Katsumata et al., 2005; Richter & Nagel, 2007).
The experimental fish were exposed to terbutryn at the following concentrations in water: Group 1, 0.02 μg/L (reported environmental concentration in Czech rivers); Group 2, 0.2 μg/L; and Group 3, 2 μg/L. The terbutryn concentrations of 0.2 μg/L and 2 μg/L corresponded to the 0.0005% 96hLC50 and 0.005% 96hLC50 determined for carp. The experimental fish were exposed to simazine at the following concentrations in water: Group 1, 0.06 μg/L (reported environmental concentration in Czech rivers); Group 2, 2 μg/L; and Group 3, 4 μg/L. The simazine concentrations of 2 μg/L and 4 μg/L corresponded to the 0.5% 48hLC50 and 1% 48hLC50 for carp.

2.2.1 Biochemical, haematological profile, liver biomarkers, and histopathological examination after triazine exposure

After 90 days exposure, the fish were individually sampled and weighed. Eight fish from each replicate of each group were examined to determine biometric parameters, and haematological, biochemical, and histopathological profiles.
Blood was drawn from the *v. caudalis* and samples stabilized with 50 IU sodium heparin 1 mL blood. Erythrocyte count, haemoglobin concentration, haematocrit, mean erythrocyte volume, mean colour concentration, erythrocyte haemoglobin, leukocyte count, and the differential leukocyte count, were determined (Svobodova et al., 1991).
Blood was separated by centrifugation at 12 000 x g for 10 min at 4 °C. Plasma samples were held at -80 °C until analysis. Biochemical indices evaluated included glucose, total protein, albumin, total globulins, ammonia, triacylglycerols, aspartate aminotransferase, alanine aminotransferase, gama-glutamyl-transferase, lactate dehydrogenase, creatine kinase, creatine (CREA), lactate, amylase (AMYL), lipase (LIPA), alkaline phosphatase, calcium, magnesium, and inorganic phosphate. For the biochemical analysis of plasma, the VETTEST 8008 analyzer. Vitellogenin (VTG) and 11-ketotestosterone (KT) in plasma was measured using pre-coated ELISA kits (Biosense Laboratories Norway) according to the manufacturer's instructions. The use of carp VTG ELISA for determination of vitellogenin in carp was validated by Flammarion et al. (2000). Absorbance was measured using an SLT Spectra (A5082) set at 492 nm for VTG and at 420 nm for KT detection.
After blood sampling, body weight (BW) and standard length (SL) were recorded. Condition factor (CF) of each fish was calculated according to the formula CF = BW (g)/SL $(cm)^3$ x 100. Liver weight (LW) and spleen weight (SW) were determined, and a hepatosomatic index (HSI) for each fish was calculated (HSI = LW/BW x 100).
Samples of liver were taken for biomarker examinations. The tissue was quickly removed, immediately frozen, and stored at -80 °C until analysis. Liver samples were homogenized in buffer (0.25 M saccharose, 0.01 M TRIS and 0.1 mM EDTA) and centrifuged at 10 000 x g for 15 min at 4 °C. The supernatant was transferred to ultracentrifugation tubes and centrifuged again at 100 000 x *g* for 1 h at 4 °C. The supernatant was drained, and pellets were washed with buffer and resuspended in buffer. This suspension was put into individual Eppendorf tubes. Before the enzymes were assayed, microsomal protein concentrations were

determined by the Lowry method (Lowry et al., 1951). The hepatic ethoxyresorufin-O-deethylase (EROD) activity was determined spectrofluorometrically. In the presence of NADPH, EROD activity converts the substrate ethoxyresorufin, which is a fluorescent product. Standard phosphate buffer, NADPH, and suspension adequate for 0.2 mg/mL protein were put into a cell. Ethoxyresorufin was added, and the increase in fluorescence was monitored for 5 min (excitation/emission wavelengths were 535/585 nm). The EROD activity was subsequently calculated based on comparison with fluorescence of the standard (resorufin) of known concentration. Total cytochrome P450 (Cyt P450) was determined by visible light spectrophotometry at 400-490 nm on the basis of the difference between absorbance readings at 450 and 490 nm, and the values were transformed to final concentrations. Measurements were made after cytochrome reduction by sodium dithionite and after the complex with carbon oxide was formed (Siroka et al., 2005).
Thawed liver samples were extracted with phosphate buffer (pH 7.2) and homogenized. The homogenates were centrifuged (10 000 x g for 10 min, at 4 °C) and supernatants used for determination of GST, GSH, and protein concentration. Glutathione-S-transferase activity was measured spectrophotometrically using 25 mM 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate and 10 mM GSH in phosphate buffer saline (pH 7.2) (Habig et al., 1974). Reduced glutathione was determined according to the method of Ellman (1959) using 1 mM 5,50-dithiobis-2-nitrobenzoic acid (DTNB) as a substrate. Absorbance of GSH-DTNB conjugate was assessed at 412 nm, and GSH concentrations were calculated according to the standard calibration. Protein concentration was quantified with the Bicinchoninic Acid Protein Assay Kit (Sigma–Aldrich) using bovine serum albumin as standard.
For histological studies, the liver, spleen, cranial and caudal kidney were fixed in a solution containing ethanol, formalin, and acetic acid, and stored in 70% ethanol. Tissues were embedded in paraffin, sectioned (5 μm), and the slides stained with H&E. The sections were examined by light microscopy, and photographed using a digital camera.

2.3 Statistical analysis

Statistical analysis was carried out using Statistica software 8.0 for Windows (StatSoft). Data were first tested for normality (Kolmogorov-Smirnov test) and homoskedasticity of variance (Bartlett's test). If those conditions were satisfied, one-way analysis of variance (ANOVA) was employed to determine whether there were any significant differences in measured variables between control and experimental groups. When a difference was detected ($P < 0.05$), Tukey's multiple comparison test was applied to identify which treatments were significantly different. If the conditions for ANOVA were not satisfied, the non-parametric Kruskal-Wallis test was used.

3. Results

3.1 Pyrethroids

For the acute test with deltamethrin, cypermethrin, and bifenthrin, juvenile rainbow trout (Kamloops), 4.10-26.50 g body weight and 65.10-154.00 mm body length and juvenile common carp (mirror carp M 72), 9.90-15.30 g body weight and 59.00-75.20 mm body length, were used.
For examinations of pyrethroid effects on haematological, biochemical, and histological profiles, one-to-two-year old rainbow trout of 115.49-309.18 g weight and 241-307 mm body length, and one-to-two-year old common carp of 115.49-832.80 g body weight and 184-366 mm average body length were used.

3.1.1 Acute toxicity of pyrethroids

The LC0, LC50, and LC100 values of deltamethrin, cypermethrin, and bifenthrin for rainbow trout and common carp juveniles are given in Table 1.

	rainbow trout			common carp		
	LC0	LC50	LC100	LC0	LC50	LC100
deltamethrin	0.50	1.00	2.50	2.14	3.25	6.08
cypermethrin	1.98	3.14	4.96	1.82	2.91	4.64
bifenthrin	1.04	1.47	2.09	2.15	5.75	10.51

Table 1. Acute toxicity of pyrethroids in rainbow trout and common carp juveniles (value µg/L) (Dobsikova et al., 2006; Velisek et al., 2006a,b, 2007, 2009b,c).

In the course of acute exposure to pyrethroid pesticides, the following clinical symptoms were observed: increased respiration, loss of coordination, and fish lying on their flank and moving in this orientation. Subsequent short excitation stages with convulsions, jumping above the water surface, and moving in circles alternated with resting. Necropsy performed after the acute toxicity test revealed increased watery mucus on body surfaces. The body cavity contained excess fluid and showed congestion of visceral vessels.

3.1.2 Biochemical examination after pyrethroid exposure

Acute exposure to deltamethrin in rainbow trout was associated with a significantly ($P < 0.05$) lower concentration of GLU, ALT, and significantly ($P < 0.05$) greater of TP, ALB, NH_3, AST, and Ca^{2+} compared to controls. The common carp exposed to deltamthrin exhibited significantly higher ($P < 0.05$) value of NH_3, AST, and ALT compared to controls.

Acute exposure to cypermethrin resulted in a significantly ($P < 0.01$) lower concentration of ALP and significantly ($P < 0.01$) higher concentration of NH_3, AST, LDH, CK, and LACT in rainbow trout compared to controls fish. In common carp cypermethrin resulted in a significant ($P < 0.01$) lower in TP, ALB, GLOB, NH_3, LDH, and ALP, and a significant ($P < 0.01$) higher in GLU, LACT, and CK levels compared to controls.

Acute exposure to bifenthrin resulted in significantly ($P < 0.01$) lower NH_3 and significantly ($P < 0.01$) higher concentrations of GLU, LDH, ALP, and CK in rainbow trout compared to control trout. Common carp exposed to bifenthrin showed significantly ($P < 0.01$) higher levels of GLU, NH_3, AST and CK compared to controls.

3.1.3 Haematological examination after pyrethroid exposure

Acute exposure of rainbow trout to deltamethrin was associated with significantly higher ($P < 0.05$) erythrocyte count, haemoglobin content, and haematocrit than in the control group. On the other hand, deltamethrin exposure in common carp led to significantly lower values ($P < 0.01$) of RBC, Hb and PCV compared to controls.

Rainbow trout acute exposed to cypermethrin exhibited significantly lower ($P < 0.05$) numbers of developmental forms of myeloid sequence and segmented neutrophilic granulocytes than did untreated fish. Moreover, cypermethrin exposure in common carp resulted in significantly ($P < 0.01$) higher values of RBC, MCV, MCH, and lymphocyte count ($P < 0.01$) compared to controls.

Acute exposure of rainbow trout to bifenthrin caused significantly higher ($P < 0.01$) values of MCV, MCH, and neutrophil granulocyte count compared to controls. In common carp

bifenthrin was associated only with significantly higher ($P < 0.01$) of monocyte counts compared to control fish.

3.1.4 Histopathological examination after pyrethroids exposure

Acute exposure of deltamethrin did not cause histopathological changes in gills, skin, liver, spleen, cranial and caudal kidney of rainbow trout and common carp.

Acute toxicity exposure (96 h) of cypermethrin caused severe teleagioectasia in the secondary lamellae of gills with the rupture of pillar cells (Fig. 1) and degeneration of hepatocytes, especially in the periportal zones in rainbow tout. Affected hepatocytes showed pycnotic nuclei and many small vacuoles or one large vacuole in the cytoplasm. The shape of vacuoles was typical for fatty degeneration of liver.

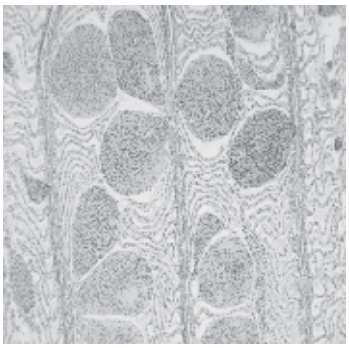

Fig. 1. Gill of rainbow trout with teleangioectasia in the secondary lamellae after acute cypermethrin exposure; H&E, x100 (from Velisek et al., 2006a).

In carp, acute exposure to cypermethrin resulted in hyperaemia and perivascular lymphocyte infiltration in skin, mild hyperplasia of respiratory epithelium chloride cell activation in the gills (Fig. 2), and vacuolisation of pancreas exocrine cells (Fig. 3).

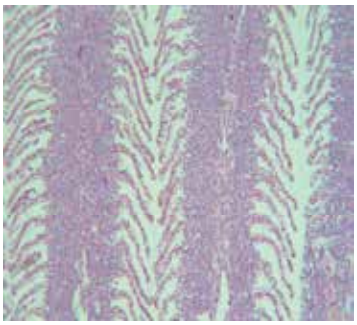

Fig. 2. Mild hyperplasia of respiratory epithelium and activation of chloride cells in carp gills after acute cypermethrin exposure (from Dobsikova et al., 2006).

Acute exposure to bifenthrin in rainbow trout and common carp was associated with degeneration of hepatocytes (Fig. 4), especially in the periportal zones. Affected hepatocytes showed pycnotic nuclei and many small vacuoles or one large vacuole in the cytoplasm. Vacuole shape was typical of fatty degeneration of the liver. Moreover bifenthrin in common carp caused severe teleangioectasia in the secondary lamellae of gills, with the rupture of pillar cells.

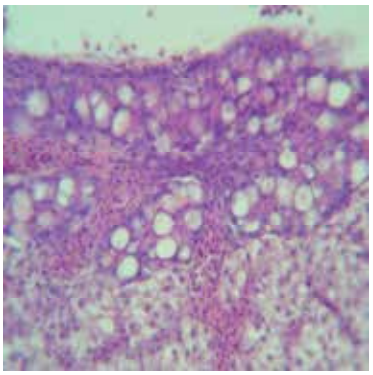

Fig. 3. Vacuoles in pancreatic exocrine carp cells after acute cypermethrin exposure (from Dobsikova et al., 2006).

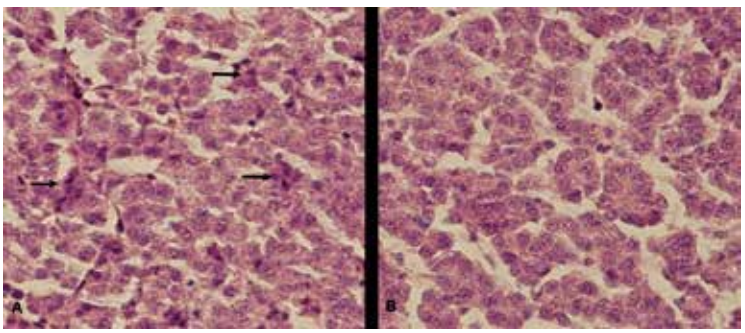

Fig. 4. Liver of rainbow trout after acute exposure to bifenthrin (A) and control (B); H&E, x100. Note degenerated hepatic cells with pycnotic nuclei (arrows) (from Velisek et al., 2009b).

3.2 Triazines

For the long-term test with terbutryn, common carp, 297.38 ± 55.58 g mean body weight and 222.3 ± 18.8 mm mean total body length, were used. For the long-term test with simazine, common carp, 353.24 ± 81.67 g mean body weight and 258.4 ± 19.6 mm mean total body length, were used. Common carp were obtained from a commercial hatchery (Vodnany, Czech Republic).

3.2.1 Fish behaviour after long-term triazine exposure

During the experiment with terbutryn and simazine both control and exposed common carp showed normal feeding behaviour. There were no signs of respiratory distress such as rapid ventilation, increased rate of gill opercular movements, or floating at the surface of water. There were no mortalities during the experiment.

3.2.2 Biometric parameters after long-term triazine exposure

Long-term exposure of terbutryn at concentrations of 0.02 μg/L, 0.2 μg/L, and 2 μg/L had no significant effects on biometric parameters (SL, BW, LW, SW, CF, and HSI) of experimental common carp.

Biometric parameters of common carp exposed to simazine at the recorded environmental concentration of 0.06 μg/L showed no differences from untreated fish. Long-term exposure to

simazine at concentrations of 2 μg/L and 4 μg/L caused significant increases ($P < 0.01$) in HSI relative to controls. No differences in the remaining parameters (SL, BW, LW, SW, and CF) investigated were found among any groups (Kruskal-Wallis test for all comparisons $P > 0.05$).

3.2.3 Biochemical results of long-term triazine exposure

Results of biochemical profiling after terbutryn exposure are given in Table 2. Biochemical profiles of common carp exposed to terbutryn at the recorded environmental concentration of 0.02 μg/L showed no differences from untreated fish. In fish exposed to terbutryn at concentrations of 0.2 and 2 μg/L, significant ($P < 0.01$) decreases in the level of CREA and Mg and a significant ($P < 0.01$) increase in GLU, AST, LDH, and LACT levels in plasma were observed compared with controls. The remaining indices: TP, ALB, GLOB, NH_3, TAG, ALT, GGT, CK, AMYL, LIPA, ALP, Ca^{+2}, PHOS, VTG, and KT were similar in all groups.

Fish Group	Control	1	2	3
Terbutryn (μg/L)	-	0.02	0.2	2
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
GLU (mmol/L)	4.35 ± 0.76	4.48 ± 0.43	9.62 ± 1.15*	8.54 ± 1.35*
AST (μkat/L)	1.12 ± 0.26	1.36 ± 0.19	3.11 ± 0.25*	3.36 ± 0.20*
LDH (μkat/L)	13.25 ± 1.28	14.11 ± 2.35	20.65 ± 3.62*	21.08 ± 3.01*
CREA (mmol/L)	50.95 ± 5.67	48.63 ± 4.12	30.15 ± 2.54*	29.78 ± 3.14*
LACT (mmol/L)	1.28 ± 0.28	1.02 ± 0.14	2.63 ± 0.58*	2.80 ± 0.36*
Mg (mmol/L)	1.12 ± 0.11	1.18 ± 0.12	0.57 ± 0.10*	0.48 ± 0.13*

Table 2. Derived biochemical parameters in common carp following long-term exposure to terbutryn ($n = 16$) (from Velisek et a., 2011a). *Experimental groups are significantly ($P < 0.01$) different from the control.

Biochemical profiles after simazine exposure are given in Table 3. Biochemical profiles of common carp exposed to simazine at the recorded environmental concentration of 0.06 μg/L showed significantly ($P < 0.01$) higher of ALP activity compared to controls.

Fish Group	Control	1	2	3
Simazin (μg/L)	-	0.06	2	4
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
TP (g/L)	34.88 ± 5.58	33.75 ± 3.63	33.75 ± 2.44	24.50 ± 5.36*
ALB (g/L)	5.38 ± 2.29	4.31 ± 1.62	5.50 ± 1.22	1.23 ± 1.65*
ALT (μkat/L)	0.26 ± 0.08	0.23 ± 0.09	0.05 ± 0.02*	0.05 ± 0.02*
ALP (μkat/L)	0.65 ± 0.04	0.21 ± 0.09**	0.23 ± 0.11**	0.17 ± 0.13**

Table 3. Derived biochemical parameters in common carp following long-term exposure to simazine ($n = 16$). Experimental groups are significantly **$P < 0.01$ and *$P < 0.05$ different from controls (from Velisek et al., 2011b).

Biochemical profiles of carp exposed to simazine at the concentration of 2 μg/L showed significantly higher activity of ALP ($P < 0.01$) and ALT ($P < 0.05$) than controls carp. In carp, simazine at a concentration of 4 μg/L caused a significant increase in TP ($P < 0.05$), ALB ($P < 0.05$), ALP ($P < 0.01$) and ALT activity ($P < 0.05$) compared to controls.

The remaining indices, GLU, GLOB, NH_3, TAG, AST, GGT, LDH, CK, CREA, LACT, AMYL, LIPA, Ca^{+2}, Mg, PHOS, VTG, and KT were similar in all groups.

3.2.4 Haematological results of long-term triazine exposure

Haematological profiles following terbutryn exposure are given in Table 4. Haematological profiles of common carp exposed to terbutryn at the recorded environmental concentration of 0.02 μg/L showed no differences from untreated fish. In fish exposed of terbutryn at concentrations of 0.2 and 2 μg/L, RBC, lymphocyte counts, and mean corpuscular haemoglobin concentrations increased significantly ($P < 0.01$), and Leuko, neutrophil granulocyte bands, and MCV decreased significantly ($P < 0.01$) relative to controls. The values of Hb, PCV, and MCH were similar among all groups.

Fish Group	Control	1	2	3
Terbutryn (μg/L)	-	0.02	0.2	2
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
RBC (T/L)	1.36 ± 0.27	1.25 ± 0.16	1.89 ± 0.11*	1.84 ± 0.10*
MCV (fl)	187.38 ± 17.67	188.45 ± 23.84	126.63 ± 21.70*	132.30 ± 16.22*
MCHC (g/L)	254.82 ± 13.24	261.49 ± 12.30	298.19 ± 17.83*	294.11 ± 10.37*
Leuko (G/L)	97.00 ± 42.16	112.19 ± 57.88	31.06 ± 13.43*	28.32 ± 10.15*
Lymphocytes (G/L)	86.99 ± 6.86	104.06 ± 3.29	12.59 ± 4.16*	15.86 ± 3.26*
Neutrophil granulocytes bands (G/L)	6.12 ± 2.44	4.98 ± 2.62	16.50 ± 4.62*	10.97 ± 2.21*

Table 4. Derived haematological parameters in common carp following long-term exposure to terbutryn ($n = 16$). *Experimental groups are significantly ($P < 0.01$) different from the control (from Velisek et al., 2011b).

Simazine at concentrations of 0.06 μg/L, 2 μg/L, and 4 μg/L led to significant ($P < 0.01$) decrease in Leuko relative to controls after 90 days exposure. The values for Hb, PCV, MCH, MCV, MCHC, and the Leukogram were similar among all groups.

3.2.5 Liver biomarkers after long-term triazine exposure

Long-term exposure to terbutryn at concentrations of 0.02 μg/L (reported environmental concentration in Czech rivers), 0.2 μg/L, 2 μg/L, and simazine at concentrations of 0.06 μg/L (reported environmental concentration in Czech rivers), 2 μg/L, and 4 μg/L had no significant effects on the activity of phase I detoxification enzymes (CYP 450, EROD) and phase II detoxification enzymes (GST, GSH) in liver.

3.2.6 Histopathology following long-term triazine exposure

No histopathological anomalies were demonstrated in liver, spleen, or cranial and caudal kidney of carp following long-term exposure to terbutryn at concentrations of 0.02, 0.2, and 2 μg/L.

Long-term exposure to simazine at concentrations of 0.06 μg/L, 2 μg/L, and 4 μg/L caused severe hyaline degeneration of the epithelial cells of renal tubules of the caudal kidney (Fig. 5); while, in the control fish, the caudal kidney parenchyma was intact. The altered tubular

epithelium was atrophic in tubules, with and without casts. Some tubules appear expanded, but, if they did not contain casts, were small with a thickened basement membrane. No histopathological changes were demonstrated in liver, spleen, or cranial kidney following long-term exposure to simazine.

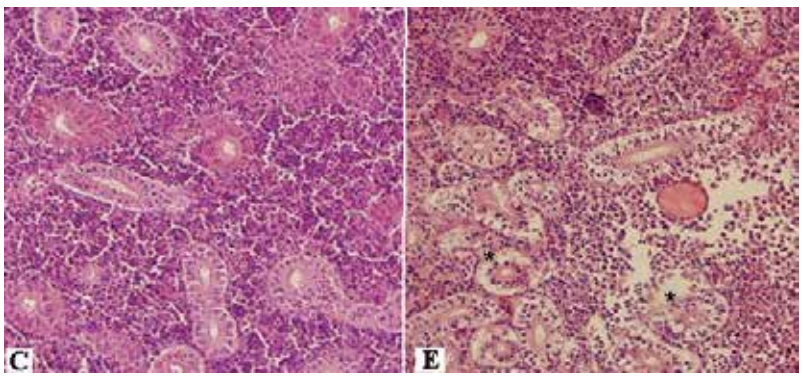

Fig. 5. Caudal kidney of common carp following exposure to simazine in a 90 day trial. H&E, x200. C=control, G=group 4 (concentration 4 µg/L of simazine). The asterisks indicate areas of tubule destruction (from Velisek et al., 2011b).

4. Discussion

Laboratory studies of physiological responses in fish exposed to pesticides can provide information on, and help to elucidate the mechanisms of, the impact of pesticides on fish. The results reported here provide further data on acute exposure to pyrethroids and chronic exposure to triazine pesticides for consideration in risk assessment.

4.1 Pyrethroids

In recent years, awareness of residual pyrethroid pesticides in the aquatic environment is growing as investigations increase and analytical detection techniques improve. Fish exposed to insecticides exhibit a variety of physiological responses, including blood balance disturbances. Laboratory studies of biochemical responses in tissues of fish exposed to insecticides can help to elucidate the mechanism, and provide information on the impact of residual environmental insecticides on fish.

In our study, the 96hLC50 of tested pyrethroid pesticides (deltamethrin, cypermethrin and bifenthrin) was found to be less than 6 µg/L. In view of this, deltamethrin, cypermethrin, and bifenthrin were included in the group of substances strongly toxic to fish. Bifenthrin is more toxic at cooler temperatures, and thus more toxic to cold water fish than to warm water species, but the toxicity of pyrethroids is little affected by pH or water hardness (Mauck et al., 1976). Pyrethroids are more toxic to smaller fish than larger ones (Baser et al., 2003). The values found in the studies were in agreement with data reported by other authors who determined the toxicity of pyrethroid pesticides for various species of fish. Liu et al. (2005) report a 96hLC50 value of 2.08 µg/L and 0.80 µg/L for common carp and tilapia (*Tilapia* spp.), respectively. Bradbury & Coats (1989) report mean lethal toxicity of cypermethrin to various fish species in laboratory conditions to be below 10 µg/L. Shires (1985) reported the 96hLC50 value of cypermethrin for rainbow trout to be 2.57 µg/L.

Gangolli (1999) reports the values of 96hLC50 of deltamethrin in common carp and rainbow trout as ranging from 0.0005 to 0.0018 mg/L.

Behaviour observation is considered a promising tool in ecotoxicology, and these studies are becoming prominent in toxicity assessments in many species, including fish. Since behaviour is not a random process, but rather a selective response that is constantly adapting through direct interaction with physical, chemical, social, and physiological aspects of the environment, behavioural endpoints serve as valuable tools to discern and evaluate effects of exposure to environmental stressors, and fish behavioural alterations can provide important indices for ecosystem assessment (Kane et al., 2005). Clinical symptoms (e.g. accelerated respiration, loss of movement and coordination, fish lying at the tank bottom and moving in one spot, subsequent short excitation periods with convulsions and movement in circles) observed during acute exposure of rainbow trout and common carp to pyrethroids (deltamethrin, cypermethrin and bifenthrin) correspond to observations by other authors reporting on the toxicity of pyrethroid pesticides (Dobsikova et al., 2006; Velisek et al., 2006a). Bradbury & Coats (1989) reported signs of fenvalerate poisoning in fish, that included loss of schooling behaviour, swimming near the water surface, hyperactivity, erratic swimming, seizures, loss of buoyancy, increased cough rate, increased gill mucus secretions, flaring of the gill arches, head shaking, and listlessness before death.

The main acute haematological response of rainbow trout and common carp to the effects of pyrethroid was a significant change in the RBC, Hb, MCV, MCHC, lymphocyte, and segmented neutrophilic granulocyte counts. The reduction in RBC count and PCV value and the higher erythrocyte haemoglobin of fish can be attributed to haemodilution due to damage of organs and changes in the haematological parameters PCV, RBC, and Hb, which can be interpreted as a compensatory response to increase the O_2 carrying capacity of the blood to maintain gas transfer, also indicating a change of the water-blood barrier for gas exchange in gill lamellae. Haematological results indicated decrease in nonspecific immunity. A decrease in PCV, Hb, Leuko and RBC has been reported in carp after poisoning with cypermethrin (Dorucu & Girgin, 2001), and a decrease in total leukocyte count and neutrophil granulocyte count was observed in carp following acute poisoning with permethrin (Sopinska & Guz, 1998).

The change in blood GLU concentration after pyrethroid exposure demonstrated the response of exposed fish to metabolic stress. Cypermethrin caused an increase in plasma NH_3 levels, presumably due to an increase in amino acid catabolism and a failure of ammonia excretion mechanisms. Increased NH_3 concentration indicates organism inability to convert the toxic ammonia to less harmful substances. An enhanced energy demand caused by short-term pyrethroid stress stimulates the activity of glutamate dehydrogenase (GDH) which induces glutamate fission into ammonia and α-ketoglutaric acid utilized in the TCA cycle. The enzymes used for the purpose are LDH, CK, and transaminases AST and ALT. A significant increase in the activity of the above mentioned plasma enzymes indicates stress-related tissue impairment. Increased activity of transaminases indicated amplified transamination processes. An increase in transamination occurs with amino acid input into the TCA cycle to cope with the energy crisis during pyrethroid induced stress (Philip et al., 1995). The changes in LDH level indicated metabolic changes, i.e. glycogen catabolism and glucose shift to the formation of lactate in stressed fish, primarily in the muscle tissue. Jee et al. (2005) found an increase in levels of serum glutamic-acid-oxylacetic-acid-transaminase, glutamic-acid-pyruvic-acid-transaminase, GLU, and ALP and a decrease in the concentration of plasma TP, ALB, cholesterol, and lysozyme in Korean rockfish (*Sebastes*

schlegeli) exposed to cypermethrin. Balint et al. (1995) observed an increase of GLU in common carp (*Cyprinus carpio*) after exposure to deltamethrin. Atamanalp et al. (2002) found changes in the concentration of Ca^{2+} and phosphorus in rainbow trout following cypermethrin exposure.

We observed teleangioectasia of secondary lamellae of the gills and degeneration of hepatocytes in periportal zones in rainbow trout after cypermethrin exposure. Teleangioectasia indicate acute respiratory distress. Sarkar et al. (2005) found significant changes such as hyperplasia, disintegration of hepatic mass, and focal coagulative necrosis in *Labeo rohita* exposed to cypermethrin. Edwards et al. (1986) reported acute toxicity symptoms of cypermethrin in rainbow trout including gill flailing, hyperactivity, loss of buoyancy, and inability to remain upright. Cengiz (2006) observed histopathological effects of deltamethrin on the gill (desquamation, necrosis, aneurysm in secondary lamellae, lifting of the lamellar epithelium, oedema, epithelial hyperplasia, and fusion of the secondary lamellae) of common carp after acute exposure in concentration of 0.029 and 0.041 mg/L. Acute effects of pyrethroid pesticides in fish include damage of gills and behavioural changes. Because they are highly lipophilic, pyrethroids are likely to be strongly absorbed by the gills, even from water containing low levels of pyrethroids. Degeneration of hepatocytes in periportal zones can imply the influence of toxic compounds in the digestive tract. The biochemical changes in liver profile may also be related to hepatocyte damage. Significant changes such as hyperplasia, disintegration of hepatic mass, and focal coagulative necrosis were found in *Labeo rohita* exposed to cypermethrin (Jee et al., 2005).

4.2 Triazines

In order to make an accurate assessment of the hazards that a contaminant may pose to a natural system, behavioural indices selected for monitoring must reflect the organism's behaviour in the field. Repeated opening and closing of the mouth and opercular movements are obvious indicators of a toxicant's effect in fish. During the laboratory toxicity test, common carp exposed to terbutryn and simazine showed normal feeding habits and exhibited no abnormal behaviour. Velisek et al. (2009b) reported accelerated respiration and loss of movement coordination in rainbow trout and carp following acute poisoning with metribuzin. These characteristics have also been reported in *Oreochromis niloticus* and *Chrysichthyes auratus* (Hussein et al., 1996) and in *Carassius auratus* by Saglio & Trijasse (1998) following acute poisoning with atrazine. Movement imbalance in freshwater fish (*Labeo rohita, Mystus vittatus*, and *Cirrhinus mrigala)* exposed to simazine and cyanazine has been reported by Dad and Tripathi (1980). Oropesa et al. (2009) reported respiratory distress such as rapid ventilation, increased rate of gill cover movements, and floating at the surface of water in common carp after exposure to simazine. Our results differ from these, as, during the assay, both control and exposed carp behaved normally. However, different exposure regimes as well as different fish species were used in our study.

Biometric parameters of common carp exposed to terbutryn or simazine at the recorded environmental concentration had no effects on biometric parameters. Only simazine in concentrations of 2 and 4 μg/L showed increases in HSI relative to controls. Biometric parameters are regarded as general indicators of fish health and the quality of the aquatic environment. The hepatosomatic index is a non-specific biomarker influenced by factors such sex, season, disease, and nutritional level. Dewey (1986) reported reduction in body weight and length and decrease of condition in brook trout (*Salvelinus fontinalis*) exposed 306 days to atrazine at a concentration of 120 μg/L. Davies et al. (1994) observed growth rate reduction in

the inanga (*Galaxias maculatus*) following exposure to low concentrations of atrazine. Atrazine, at doses of 100 and 1000 μg/L, showed no dose- or time-related effects on gonad growth (GSI) in either males or females over a 21-day study period (Spano et al., 2004).

Biochemical profiles of blood can provide important information about the internal environment of the organism (Masopust, 2000). Biochemical alterations are usually the first detectable and quantifiable responses to environmental change. Chronic exposure to terbutryn at 0.2 and 2 μg/L resulted in a significant increased in plasma GLU concentration, demonstrating the response of exposed fish to metabolic stress. Mekkawy et al. (1996) observed increases in GLU levels in Nile tilapia (*Oreochromis niloticus*) and catfish (*Chrysichtheys auratus*) after atrazine exposure at 3 mg/L. Chronic exposure to simazine at 0.06, 2, and 4 μg/L resulted in a significant decrease in plasma ALP activity. The source of ALP includes synthesis in the intestinal epithelium, kidney, and liver and is often increased in response to a biliary obstruction. Velisek et al. (2008) also reported decreased ALP in rainbow trout after acute exposure to metribuzin. Chronic exposure to terbutryn at 0.2 and 2 μg/L resulted in significant increase in plasma AST and LDH activity. Chronic exposure to simazine at 2 and 4 μg/L resulted in a significant decrease in plasma ALT activity. LDH is the terminal enzyme of anaerobic glycolysis and therefore of crucial importance in muscle physiology, particularly in conditions of chemical stress when high levels of energy may be required for a short period of time (Monteiro et al., 2007). The increase in LDH level indicated metabolic changes, i.e. glycogen catabolism and a glucose shift towards the formation of lactate, primarily in muscle. A significant change in the activity of plasma enzymes LDH and the transaminases ALT and AST indicates stress-based tissue impairment. Change in activity of transaminases indicates amplified transamination processes. An increase in transamination occurs with amino acid input into the TCA cycle to cope with the energy crisis during pesticide stress. It has been suggested that, in general, stress induces elevation of the transamination pathway and is likely to have contributed to toxic effects induced by terbutryn and simazine and the altered transaminase activity observed in the present study. Chronic exposure to simazine at 4 μg/L resulted in significant decrease in plasma TP and ALB concentration. In these circumstances, changes in serum protein concentration might arise from protein leakage from damaged tissue. In the present study, the reduction of plasma protein and albumins with chronic exposure confirms the toxic effects of simazine on the immune system and/or the haemodilution effect, and may account for the pathological effects on caudal kidney. These results agree with Hussein et al. (1996) and Mekkawy et al. (1996) who reported a decrease of TP in atrazine exposed Nile tilapia and catfish. Davies et al. (1994) also observed a decrease in TP in rainbow trout after acute exposure to atrazine at a concentration of 50 μg/L. Other authors found changes in biochemical profiles of fish following triazine exposure. Velisek et al. (2009a) found a decrease in the activity of AST and an increase in GLU, NH_3, LDH, CK, and CREA levels in common carp after subchronic exposure to simazine. The biochemical profiles determined in the present chapter suggest that internal organs and tissue of common carp were slightly altered with exposure to terbutryn and simazine.

The evaluation of haematological characteristics of fish has become an important means of understanding normal and pathological processes and toxicological impacts. Haematological alterations are usually the first detectable and quantifiable responses to environmental change (Wendelaar Bonga, 1997). In our study, simazine was associated with decreased leukocyte count relative to controls. Leukocytes are involved in the regulation of immunological function and a protective response to stress in fish. The reduction in

leukocyte count occurs through an alteration in lymphopoesis and/or altered release of lymphocytes from lymphoid tissues. The decrease in leukocyte count in the present study indicates the stress condition of the fish subsequent to simazine exposure, which may have produced hypoxia and kidney damage. The response to environmental challenges often leads to leucopenia with lymphopenia and sometimes neutrophilia, which is similar to the classic leukocytic response to stress in mammals (Ainsworth, 1992). In the present study, the decrease in leukocyte count and the lymphopenia in carp exposed to terbutryn indicated a reduction in non-specific immunity. Prolonged stress may have caused disruption of leukopoiesis, resulting in reduction in the total leukocyte count. Exposure to terbutryn was associated with the highest RBC value in fish exposed to the higher concentrations, when stress-induced RBC release from spleen to blood circulation was reported (Tort et al., 2002).. Haematological changes may result from the release of immature erythrocytes from the spleen and could be an immediate response to acute stress mediated by catecholamines. Oropesa et al. (2009) reported no effect on the haematological profiles of common carp exposed to 45 μg/L simazine.

The present experiments attempted to evaluate xenoestrogenic potency of triazine pesticide using vitellogenin and 11-ketotestosterone as a biomarker of exposure to (xeno)oestrogens. The monitoring of VTG and KT are proving to be useful tools for study of the effects of endocrine disrupting chemicals in fish. Synthesis of VTG, a lipophosphoprotein, is induced by oestradiol in the liver of female fish. Its presence in male and juvenile fish indicates contact with xenoestrogenic compounds, since VTG synthesis is oestrogen-dependent. In the presence of substances with oestrogenic effects, synthesis of VTG is carried out in the liver of male fish, which may lead to degenerative alterations of male gonads, reproductive breakdown, and, in extreme cases, sex reversal. Crain et al. (1997) showed that chloro-s-triazine herbicides have the ability to stimulate production of the enzyme aromatase, which converts androgens to oestrogens, and presumably could interfere with sex differentiation and development. However, terbutryn and simazine did not further influence plasma VTG and KT levels in the exposed fish after 90 days. Moore & Waring (1998) observed that an atrazine concentration of 3.6 μg/L altered plasma testosterone and, at 6.0 μg/L, affected KT in Atlantic salmon (*Salmo salar*). Tennant et al. (1994), working with rats, concluded that, while the chloro-s-triazine herbicides atrazine and simazine did not possess any intrinsic oestrogenic activity, these two compounds were capable of weak inhibition of oestrogen-stimulated responses in the rat uterus (i.e., effect on progesterone receptor binding and thymidine incorporation into uterine DNA).

Determination of enzymes and cofactors involved in xenobiotic biotransformation is widely practiced for assessment of exposure to pollutants. As compared with phase I systems, the induction responses of phase II enzymes are generally less pronounced. Cytochrome P450, members of a large family of heme proteins, are membrane-bound proteins which are predominantly located in the endoplasmic reticulum of the liver. The CYP450 reactions can be grouped according to the type of substrate and separated into the synthesis and degradation of endogenous substrates and the metabolism of xenobiotic substrates. The presence of the CYP450 1A isoform is expressed as EROD activity. The EROD activity may be indicative of the cytochrome P4501A1 enzyme system function. Induction of EROD is commonly observed in fish and other vertebrates exposed to Ah-receptor agonists (i.e. dioxins, polychlorinated biphenyls, polyaromatic hydrocarbons, pesticides) (reviewed in van der Oost et al., 2003). The liver is probably the most commonly studied organ in preclinical toxicology as a detoxification organ essential for the excretion of toxic substances

in animals. The major site of cytochrome P450 expression in teleost fish is the liver. Tripeptide glutathione (L-γ-glutamyl-cysteinyl-glycine; GSH) is a major component of cellular antioxidant defences and a key conjugate of electrophilic intermediates in phase II metabolism. The conjugation reaction is mediated principally via GST. Another function of the GST family is the transport of endogenous hydrophobic compounds, such as steroids, bilirubin, heme, and bile salts, as well as the prevention of lipid peroxidation. It has been reported that terbutryn is able to induce EROD activity in rainbow trout (Tarja et al., 2003). Dong et al. (2009) reported that P450 content in zebrafish (*D*anio *rerio*) (both male and female) was induced by atrazine, even at a dose of 0.01 mg/L. Exposure to terbutryn and simazine did not influence CYP concentration or EROD, GST, or GSH activity, suggesting that a role for CYP450 1A in the metabolism of terbutryn and simazine in common carp can be ruled out. In rodents, the dominant phase I metabolic reaction for triazine pesticides is cytochrome P450-mediated N-dealkylation (Hanioka et al., 1999 a,b).

Triazine pesticides have a direct effect on kidney structure and function in freshwater fish (Velisek et al., 2008, 2009b). In our experiment, the caudal kidney of carp with chronic exposure to simazine showed destruction of the tubules, although chronic exposure to terbutryn had no effect on caudal kidney. The kidney is important for the maintenance of a stable internal environment with respect to water and salt, excretion, and, partially, for the metabolism of xenobiotics. In fish, this organ receives the largest proportion of postbranchial blood.

The uptake of triazine via gill seems to be of major significance; therefore renal lesions might be expected to be good indicators of environmental pollution. In addition, this is a target organ of certain toxicants, since it is a major route for the excretion of foreign chemicals. It has been reported that acute and subchronic exposures to triazine result in lesions in kidneys and liver in fish (Velisek et al., 2008, 2009b). Simazine showed no effect on liver of common carp. On the other hand, Velisek et al. (2010) found cell shape changes and lipid inclusions in hepatocytes of common carp with subchronic terbutryn exposure in concentrations of 4, 20, and 40 µg/L. Similar alterations in liver were observed by Arufe et al. (2004), who exposed the larvae of gilthead sea bream (*Sparus aurata*) to terbutryn-triasulfuron at a concentration of 2.5 mg/L for 72 h. Steatosis in liver of grey mullet (*Liza ramada*) (Biagianti-Risbourg & Bastide, 1995) has been observed after atrazine exposure and has been proposed as a mechanism of sequestration of the pesticide molecules, protecting fish from toxic effects. Changes in metabolism of hepatic lipids and vacuolar degeneration of hepatocytes have been observed in various fish species exposed to herbicide such as clomazone (Crestani et al., 2007).

5. Conclusion

Toxicological and environmental problems resulting from the widespread use of pesticides in agriculture have raised concerns, particularly with respect to the potential toxic effects in humans and animals. The acute exposure of rainbow trout and common carp to the pyrethroids deltamethrin, cypermathrin, and bifenthrin were associated with alterations in haematological and biochemical indices as well as in tissue enzymes, resulting in stress to the organism. These pyrethroids are therefore classified as belonging to substances strongly toxic for fish. Long-term exposure to triazines terbutryn and simazine in environmental concentrations can affect the biochemical, haematological, and biometric profiles of common carp. Some changes were observed only with the higher

exposures. These results suggest that biometric, blood, liver biomarkers, and histopathological responses could be used as potential biomarkers for monitoring residual pesticides present in aquatic environments and provide useful parameters for evaluating physiological effects in fish, but the application of these findings will need more detailed laboratory study before they can be established as special biomarkers for monitoring the aquatic environment. Other classical morphologic indices (e.g. condition factor and hepatosomatic index) in fish could provide useful information for evaluating environmental stress. It is not clear that whether these pesticide-induced responses in fish were related to the level of stress hormones (especially catecholamines and cortisol), enzymatic kinetics, and molecular mechanisms, which need further investigation. Research should be focused not only on the effects of pesticides alone, but also on interactions of pesticides with other pollutants in environmental concentrations with long-term exposure, since the aquatic environment may be polluted by many substances, the effects of which can be potentiated with concurrent exposures.

6. Acknowledgment

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7. References

Ainsworth, A.J. (1992). Fish granulocytes: morphology, distribution, and function. *Annual Review of Fish Diseases*, Vol. 2, No. 2, (June 1992), pp. 123-148, ISSN 0959-8030

Arufe, M.I.; Arellano, J.; Moreno, M.J. & Sarasquetec, C. (2004) Toxicity of a commercial herbicide containing terbutryn and triasulfuron to seabream (*Sparus aurata* L.) larvae: a comparison with the Microtox test. *Ecotoxicology and Environmental Safety*, Vol. 59, No. 2, (October 2004), pp. 209–216, ISSN 0147-6513

Atamanalp, M.; Keles, M.S.; Haliloglu, H.I. & Aras, M.S. (2002):.The effects of cypermethrin (a synthetic pyrethroid) on some biochemical parameters (Ca, P, Na and TP) of rainbow trout (*Oncorhynchus mykiss*). *Turkish Journal of Veterinary Animal Sciences*, Vol. 26, No. 5, (October 2002), pp. 1157–1160, ISSN 1300-0128

Balint, T.; Szegletes, T.; Szegletes, Z.; Halasy, K. & Nemcsok, J. (1995). Biochemical and subcellular changes in carp exposed to the organophosphorus methidathion and the pyrethroid deltamethrin. *Aquatic Toxicology*, Vol.33, No. 3-4, (October 1995) , pp. 279-295, ISSN 0166-445X

Baser, S.; Erkoc, F.; Selvi, M. & Kocak, O. (2003). Investigation of acute toxicity of permethrin on guppies *Poecilia reticulata*. *Chemosphere*, Vol. 51, No. 6, (May 2003), pp. 469-474, ISSN 0045-6535

Belmonte, A.; Garrido, A. & Martinez, J.L. (2005). Monitoring of pesticides in agricultural water and soil samples from Andalusia by liquid chromatography coupled to mass spectrometry. *Analytica Chimica Acta*, Vol. 538, No. 1-4, (May 2005), pp. 117–127, ISSN 0003-2670

Biagianti-Risbourg, S. & Bastide, J. (1995). Hepatic perturbations induced by a herbicide (atrazine) in juvenile grey mullet *Liza ramada* (Mugilidae, teleostei): an

ultrastructural study. *Aquatic Toxicology*, Vol. 31, No. 3, (March 1995), pp. 217–229, ISSN 0166-445X

Bradbury, S.P. & Coats, J.R. (1989). Toxicokinetics and toxicodynamics of pyrethroid insecticides in fish. *Environmental Toxicology and Chemistry*, Vol. 8, No. 5, (May 1989), pp. 373–380, ISSN 0730-7268

Cengiz, E.I. (2006). Gill and kidney histopathology in the freshwater fish *Cyprinus carpio* after acute exposure to deltamethrin. *Environmental Toxicology and Pharmacology*, Vol. 21, No. 2, (February 2006), pp. 1093-1096, ISSN 1382-6689

Cerejeira, M.J.; Viana, P.; Batista, S.; Pereira, T.; Silva, E.; Valerio, M.J.; Silva, A.; Ferreira, M. & Silva-Fernandes, A.M. (2003). Pesticides in Portuguese surface and ground waters. *Water Research*, Vol. 37, No. 5, (March 2003), pp. 1055-1063, ISSN 0043-1354

Crain, D.A.; Guillette, L.J.; Rooney, A.A. & Pickford, D.B. (1997). Alterations in steroidogenesis in alligators (*Alligator mississippiensis*) exposed naturally and experimentally to environmental contaminants. *Environmental Health Perspectives*, Vol. 105, No.5 , (May 1997), pp. 528-533, ISSN 0091-6765

Cremlyn, R.J. (1990). *Agrochemicals; Preparation and mode of action*. John Wiley & Sons Ltd., ISBN 0471929921, West Sussex UK.

Crestani, M.; Menezes, C.; Glusczak, L.; Miron, D.D.; Spanevello, R.; Silveira, A.; Goncalves, F.F.; Zanella, R. & Loro, V.L. (2007). Effect of clomazone herbicide on biochemical and histological aspects of silver catfish (*Rhamdia quelen*) and recovery pattern. *Chemosphere*, Vol. 67, No. 11, (May 2007), pp. 2305-2311, ISSN 0045-6535.

Dad, N.K. & Tripathi, P.S. (1980). Acute toxicity of herbicides to freshwater fish and midge larvae, *Chironomus tentans*. *Environment International*, Vol. 4, No. 5-6, (December 1980), pp. 435-437, ISSN 0160-4120

Das, P.C.; McElroy, W.K. & Cooper, R.L. (2000). Differential modulation of catecholamines by chlorotriazine herbicides in pheochromocytoma (PC12) cells in vitro. *Toxicological Sciences*, Vol. 56, No. 2, (August 2000), pp. 324-331, ISSN 1096-6080

Davies, P.E.; Cook, L.S.J. & Goenarso, D. (1994). Sublethal responses to pesticides of several species of Australian freshwater fish and crustaceans and rainbow trout. *Environmental Toxicology and Chemistry*, Vol. 13, No. 8, (August 1994), pp. 1341-1354, ISSN 0730-7268

DeLorenzo, M.E.; Scott, G.I. & Ross, P.E. (2001). Toxicity of pesticides to aquatic microorganisms: A review. *Environmental Toxicology and Chemistry*, *Vol.* 20, *No. 1, (January 2001), pp.* 84-98, ISSN 0730-7268

Dewey, S.L. (1986). Effects of the herbicide atrazine on aquatic insect community structure and emergence. *Ecology*, Vol. 67, No. 1, (February 1986), pp. 148-162, ISSN 0012-9658

Dobsikova, R.; Velisek, J.; Wlasow, T.; Gomulka, P.; Svobodova, Z. & Novotny, L. (2006). Effects of cypermethrin on some haematological, biochemical and histopathological parameters of common carp (*Cyprinus carpio* L.). *Neuroendocrinology Letters*, Vol. 27, No. suppl. 2, (December 2006), pp. 101-105, ISSN 0172-780X

Dong, X.; Zhu, L.; Wang, J.; Wang, J.; Xie, H.; Hou, X. & Jia, W. (2009). Effects of atrazine on cytochrome P450 enzymes of zebrafish (*Danio rerio*). *Chemosphere*, Vol. 77, No. 3, (October 2009), pp. 404-412, ISSN 0045-6535

Dorucu, M. & Girgin, A. (2001). The effects of cypermethrin on some haematological parameters of *Cyprinus carpio*. *Aquaculture International, Vol.* 9, No. 2, (March 2002), pp. 183-187, ISSN 0967-6120

Edwards, R.; Millburn, P. & Hutson, D.H. (1986). Comparative toxicity of cys-cypermethrin in rainbow trout, frog, mouse and quail. *Toxicology and Applied Pharmacology*, Vol. 84, No. 3, (December 1986), pp. 512-522, ISSN 0041-008X

Ellman, G.L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, Vol. 82, No. 1, (May 1959), pp. 70-77, ISSN 0003-9861

Eshleman, A.J. & Murray, T.F. (1991). Pyrethroid insecticides indirectly inhibit GABA-dependent 36Cl-influx in synaptoneurosomes from the trout brain. *Neuropharmacology*, Vol. 30, No. 12, (December 1991), pp. 1333–1341, ISSN 0028-3908

European Commission (1999). Study on the prioritisation of substances dangerous to the aquatic environment. Office for Official Publications of the European Communities, Luxembourg 120.

Fan, W.Q.; Yanase, T.; Morinaga, H.; Gondo, S.; Okabe, T.; Nomura, M.; Komatsu, T.; Morohashi, K.I.; Hayes, T.B.; Takayanagi, R. & Nawata, H. (2007). Atrazine-Induced aromatase expression Is SF-1 dependent: implications for endocrine disruption in wildlife and reproductive cancers in humans. *Environmental Health Perspectives*, Vo. 115, No. 5, (May 2007), pp. 720-727, ISSN 0091-6765

Flammarion, P.; Brion, F.; Babut, M.; Garric, J.; Migeon, B.; Noury, P. & Thybaud, E. (2000). Induction of fish vitaellogenin and alterations intesticular structure: preliminary results of estrogenic effects in chub (*Leuciscus cephalus*). *Ecotoxicology*, Vol. 9, No. 1-2, (April 2000), pp. 127–135, ISSN 0963-9292

Gangolli, E.D. (1999). *The dictionary of substances and their effects.* (2nd ed.), Royal of Society of Chemistry, ISBN 0-85404-833-2, Cambridge, UK

Habig, W.H.; Pabst, M.J. & Jakobi, W.B. (1974). Glutathione-S-transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biological and Chemistry*, Vol. 249, No. 22, (November 1974), pp. 7130–7139, ISSN 0949-8257

Hashimoto, Y. & Nishiuchi, Y. (1981). Establishment of bioassay methods for the evaluation of acute toxicity of pesticides to aquatic organisms. *Journal of Pesticide Science*, Vol. 6, No. 2, (May 1981), pp. 257-264, ISSN 0385-1559

Haya, K. (1989). Toxicity of pyrethroid insecticides to fish. *Environmental Toxicology and Chemistry*, Vol. 8, No. 5, (May 1989), pp. 381–391, ISSN 0730-7268

Hayes, A.W. (1994). *Principles and methods of toxicology*. Raven Press, ISBN 0-7817-0131-7, New York, NY.

Hanioka, N.; Jinno, H.; Tanaka-Kagawa, T.; Nishimura, T. & Ando, M. (1999a). In vitro metabolism of chlorotiazines: characterization of simazine, atrazine, and propazine metabolism using liver microsomes from rats with various cytochrome P450 inducers. *Toxicology and Applied Pharmacology*, Vol. 156, No. 3, (May 1999), pp. 195–205, ISSN 0041-008X

Hanioka, N.; Jinno, H.; Tanaka-Kagawa, T.; Nishimura, T. & Ando, M. (1999b). In vitro metabolism of simazine, atrazine, and propazine by hepatic cytochrome P450 enzymes of rat, mouse and guinea pig and oestrogenic activity of chlorotriazines and their main metabolites. *Xenobiotica*, Vol. 29, No. 12, (December 1999), pp. 1213–1226, ISSN 0049-8254

Hussein, S.Y.; El-Nasser, M.A. & Ahmed, S.M. (1996). Comparative studies on the effects of herbicide atrazine on fresh water fish *Oreochromis niloticus* and *Chrysichthyes auratus* at Assiut, Egypt. *Bulletin of Environmental Contamination and Toxicology*, Vol. 57, No. 3, (September 1996), pp. 503-510, ISSN 0007-4861

Inoue, M.H.; Oliveira, R.S.; Regitano, J.B.; Tormena, C.A.; Constantin, J. & Tornisielo, V.L. (2006). Sorption-desorption of atrazine and diuron in soils from southern Brazil. Journal *of* Environmental Science *and* Health *Part* B*, Vol.* 41, No. 5, (June 2006), pp. 605–621, ISSN 0360-1234

Jee, L.H.; Masroor, F. & Kang, J.C. (2005). Responses of cypermethrin-induced stress in haematological parameters of Korean rockfish, *Sebastes schlegeli* (Hilgendorf). *Aquaculture Research*, Vol. 36, No. 9, (June 2005), pp. 898–905, ISSN 1365-2109

Katsumata, H.; Fujii, A.; Kaneco, S.; Suzuki, T. & Ohta, K. (2005). Determination of simazine in water samples by HPLC after preconcentration with diatomaceous earth. *Talanta*, Vol. 65, No. 1, (January 2005), pp. 129–134, ISSN 0039-9140

Kane, A.S.; Salierno, J.D. & Brewer, S.K. (2005). Fish models in behavioral toxicology: Automated techniques, updates and perspectives. In: *Methods in Aquatic Toxicology*, Ostrander, GK, pp. 559-590, Lewis Publishers, ISBN 978-1-56670-664-3, Boca Raton, FL

Kidd, H. & James, D.R. (1991). *The Agrochemicals Handbook*. (3rd ed.), Royal Society of Chemistry Information Services, ISBN 0 85186 416 3, Cambridge, UK.

Konstantinou, I.K.; Hela, D.G. & Albanis, T.A. (2006). The status of pesticide pollution in surface waters (rivers and lakes) of Greece. Part I. Review on occurrence and levels. *Environmental Pollution*, Vol. 141, No. 3, (June 2006), pp. 555-570, ISSN 0269-7491

Larsen, L.; Sorensen, S.R. & Aamand, J. (2000). Mecroprop, isoproturon, and atrazine in and above a sandy aquifer: vertical distribution of mineralization potential. *Environmental Science and Technology*, Vol. 34, No. 12, (May 2000), pp. 2426–2430, ISSN 0013-936X

Liu, T.L.; Wang, Y.S. & Yen, J.H. (2005). Separation of bifenthrin enantiomers by chiral HPLC and determination of their toxicity to aquatic organism. *Journal of Food and Drug Analysis*, Vol. 12, No. 3, (September 2005), pp. 357-360, ISSN 1021-9498

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological and Chemistry*, Vol. 193, No. 1, (November 1951), pp. 265-275, ISSN 0949-8257

Mauck, W.L.; Olson, L.E., & Marking, L.L. (1976). Toxicity of natural pyrethrins and five pyrethroids to fish. *Archives of Environmental Contamination and Toxicology*, Vol. 4, No. 1, (February 1976), pp. 18-29, ISSN 0090-4341

Masopust, J. (2000). *Clinical biochemistry*. Karolinum, ISBN 8071846493, Praha, (In Czech).

Meister, R.T. (1992). *Farm Chemicals Handbook*. Meister Publishing Company, Willoughby

Mekebri, A.; Crane, D.B.; Blondina, G.J.; Oros, D.R. & Rocca, J.L. (2008). Extraction and analysis methods for the determination of pyrethroid insecticides in surface water, sediments and biological tissues at environmentally relevant concentrations. *Bulletin of Environmental Contamination and Toxicology*, Vol. 80, No. 5, (May 2008), pp. 455-460, ISSN 0007-4861

Mekkawy, A.A.; Hussain, S.Y. & Ahmed, S.M. (1996). Comparative studies on the effects of herbicide atrazine on some blood constituents and protein electrophoretic patterns of *Oreochromis niloticus* and *Chrysichthyes auratus* at Assiut, Egypt. *Journal of the Egyptian German Society of Zoology*, Vol. 19, No. 4, (January 1996), pp. 283-319, ISSN 1110-5321

Monteiro, M.; Quintaneiro, C.; Noqueira, A.J.A.; Morgado, F.; Soares, A.M.V.M. & Guilhermino, L. (2007). Impact of chemical exposure on the fish *Pomatoschistus microps Krøyer* (1838) in estuaries of the Portuguese Northwest coast. *Chemosphere*, Vol. 66, No. 3, (January 2007), pp. 514-522, ISSN 0045-6535

Moore, A. & Waring, C.P. (1998). Mechanistic effects of a triazine pesticide on reproductive endocrine function in mature male Atlantic salmon (*Salmo salar* L.) parr. *Pesticide Biochemistry and Physiology*, Vol. 62, No. 1, (October 1998), pp. 41-50, ISSN 0048-3575

Muir, D.C. (1980). Determination of terbutryn and its degradation products in water sediments, aquatic plants, and fish. *Journal of Agricultural Food Chemistry*, Vol. 28, No. 4, (July 1980), pp. 714-719, ISSN 0021-8561

Niimi, A.J. (1987). Biological half-lives of chemicals in fishes. *Reviews Environmental Contamination and Toxicology*, Vol. 99, No. 1, (January 1987), pp. 1-46, ISSN 0179-5953

Ormad, M.P.; Miguel, N.; Claver, A.; Matesanz, J.M. & Ovelleiro, J.L. (2008). Pesticides removal in the process of drinking water production. *Chemosphere*, Vol. 71, No. 1, (March 2008), pp. 97–106, ISSN 0045-6535

Oropesa, A.L.; Garcia-Cambero, J.P.; Gomez, L.; Roncero, V. & Soler, F. (2009). Effect of long-term exposure to simazine on histopathology, hematological, and biochemical parameters in *Cyprinus carpio*. *Environmental Toxicology*, Vol. 24, No. 2, (April 2009), pp. 187-99, ISSN 1522-7278

Philip, G.H.; Reddy, P.M. & Sridevi, G. (1995). Cypermethrin-induced *in vivo* alterations in the carbohydrate metabolism of freshwater fish, *Labeo rohita*. *Ecotoxicology and Environmental Safety*, Vol. 31, No. 2, (July 1995), pp. 173-178, ISSN 0147-651

Rioboo, C.; Prado, R.; Herrero, C. & Cid, A. (2007). Population growth study of the rotifer Brachionus sp. Fed with triazine-exposed microalgae. *Aquatic Toxicology*, Vol. 83, No. 4, (August 2007), pp. 247–253, ISSN 0166-445X

Richter, S. & Nagel, R. (2007). Bioconcentration, biomagnification and metabolism of 14C-terbutryn and 14C-benzo[a]pyrene in *Gammarus fossarum* and *Asellus aquaticus*. *Chemosphere*, Vol. 66, No. 4, (June 2007), pp. 603–610, ISSN 0045-6535

Roberts, T. & Hudson, D. (1999). *Metabolic pathway of agrochemicals. Part 2: insecticides and fungicides* (1st ed.), The Royal Society of Chemistry, ISBN 085404499X, Cambridge

Roberts, T.R.; Hutson, D.H.; Lee, P.W.; Nicholls, P.H. & Plimmer, J.R. (1998). *Metabolic Pathways of Agrochemicals. Part 1: Herbicides and Plant growth regulators.* (1st ed.) The Royal Society of Chemistry, ISBN 0854044949, Cambridge, UK

Sarkar, B.; Chatterjee, A.; Adhikari, S. & Ayyappan, S. (2005). Carbofuran- and cypermethrin-induced histopathological alterations in the liver of *Labeo rohita* (Hamilton) and its recovery. *Journal of Applied Ichthyology*, Vol. 21, No. 2, (April 2005), pp. 131–135, ISSN 1439-0426

Shan, G.; Hammer, R.P. & Ottea, J.A. (1997). Biological activity of pyrethroid analogs in pyrethroid-susceptible and –resistant tobacco budworms, *Heliothis virescens* (F.). *Journal of Agricultural Food and Chemistry*, Vol. 45, No. 11, (November 1997), pp. 4466–4473, ISSN 0021-8561

Shires S.W. (1985): Toxicity of a new pyrethroid insecticide, WL 85871, to rainbow trout. *Bulletin of Environmental Contamination and Toxicology*, Vol. 34, No. 2, (February 1985), pp. 134-137, ISSN 0007-4861

Siroka, Z.; Krijt, J.; Randak, T.; Svobodova, Z.; Peskova, G.; Fuksa, J.; Hajslova, J.; Jarkovsky, J. & Janska, M. (2005). Organic pollutant contamination of the River Elbe as assessed by biochemical markers. *Acta Veterinaria Brno*, Vo. 74, No. 2, (June 2005), pp. 293–303, ISSN 0001-7213

Solomon, K.R.; Baker, D.B.; Richards, R.P.; Dixon, K.R.; Klaine; S.J.; Point, T.W.; Kendall, J.; Weisskopf, C.P.; Giddings, J.M.; Giesy, J.P.; Hall, L.W. & Williams, W.M. (1996). Ecological risk assesment of atrazine in North American surface waters. *Environmental Toxicology and Chemistry*, Vol. 15, No. 1, (January 1996), pp. 31-76, ISSN 0730-7268

Solomon, K.R.; Giddings, J.M. & Maund, S.J. (2001). Probabilistic risk assessment of cotton pyrethroids: I. Distributional analysis of laboratory aquatic toxicity data. *Environmental Toxicology and Chemistry*, Vol. 20, No. 3, (March 2001), pp. 652–659, ISSN 0730-7268

Sopinska, A. & Guz, L. (1998). Influence of permethrin on phagocytic activity of carp. *Medycyna Weterynaryjna,* Vol. 54, No. 2, (February 1998), pp. 126-128, ISSN 0025-8628

Spalding, R.F.; Exner, M.E.; Snow, D.D.; Cassada, D.A.; Burbach, M.E. & Monson, S.J. (2003). Herbicides in ground water beneath Nebraska's management systems evaluation area, *Journal of Environmental Quality*, Vol. 32, No. 1, (January 2003), pp. 92-98, ISSN 0047-2452

Spano, L.; Tyler, C.R.; van Aerle, R.; Devos, P.; Mandiki, S.N.M.; Silvestre, F.; Thomec, J.P. & Kestemont, P. (2004). Effects of atrazine on sex steroid dynamics, plasma vitellogenin concentration and gonad development in adult goldfish (*Carassius auratus*). *Aquatic Toxicology*, Vol. 66, No. 4, (March 2004), pp. 369-379, ISSN 0166-445X

Svobodova, Z.; Pravda, D. & Palackova, J. (1991). Unified methods of haematological examination of fish. *Research Institute of Fish Culture and Hydrobiology, Vodnany*, Methods No. 20, pp. 31

Takashima, F. & Hibiya, T. (1995). *An atlas of fish histology: Normal and pathological features*, (2nd ed.), Kodansha Ltd., ISBN 987-3437307829, Tokyo

Tarja, N.; Kirsti, E.; Marja, L. & Kari, E. (2003). Thermal and metabolic factors affecting bioaccumulation of triazine herbicides by rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology*, Vol. 18, No. 4, (July 2003), pp. 219-226, ISSN 1522-7278

Tennant, M.K.; Hill, D.S.; Eldridge, J.C.; Wetze, L.T.; Breckenridge, C.B. & Stevens, J.T. (1994). Possible antiestrogenic properties of chloro-s-trianzines in rat uterus. *Journal of Toxicology and Environmetal Health*, Vol. 43, No.2 , (October 1994), pp. 183-196, ISSN 1528-7394

Tomlin, C. (2003). *The Pesticide Manual: A World Compendium*. (13th ed.), British Crop Protection Council, ISBN 0901436445, Hampshire, UK

Tort, L.; Puigcerver, M.; Crespo, S. & Padros, F. (2002). Cortisol and haematological response in sea bream and trout subjected to the anesthetics clove oil and 2-phenoxyethanol. *Aquaculture Research*, Vol. 33, No. 11, (September 2002), pp. 907–910, ISSN 1365-2109

Treasurer, J.W. & Wadsworth, S.L. (2004). Interspecific comparison of experimental and natural routes of *Lepeophtheirus salmonis* and *Caligus elongatus* challenge and consequences for distribution of chalimus on salmonids and therapeutant screening. *Aquaculture Research*, Vol. 35, No. 8, (July 2004), pp. 773–83, ISSN 1365-2109

U.S. EPA - Environmental protection agency (1994). Atrazine, simazine and cyanazine: notice of initiation of special review. Federal register 59 FR60412, Vol. 59, No. 225, 30000–60.

van der Oost, R.; Beyer, J. & Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, Vol. 13, No. 2, (February 2003), pp. 57-149, ISSN 1382-6689

Velisek, J., Sudova, E., Zlabek, V., Kolarova, J., Svobodova, Z. (2011a). Effects of chronic exposure to terbutryn in common carp (*Cyprinus carpio* L.). *Veterinarni Medicina*, in press, ISSN 0375-8427

Velisek, J., Stara, A., Machova, J., Svobodova, Z. (2011b). Effects of long-term exposure to simazine in real concetration on common carp (*Cyprinus carpio* L.). *Ecotoxicology and Environmental Safety*, in press, ISSN 0147-6513

Velisek, J.; Sudova, E.; Machova, J. & Svobodova, Z. (2010). Effects of sub-chronic exposure to terbutryn in common carp (*Cyprinus carpio* L.). *Ecotoxicology and Environmental Safety*, Vol. 73, No. 3, (March 2010), pp. 384-390, ISSN 0147-6513

Velisek, J.; Stastna, K.; Sudova, E.; Turek, J. & Svobodova, Z. (2009a). Effects of subchronic simazine exposure on some biometric, biochemical, hematological and histopatological parameters of the common carp (*Cyprinus carpio* L.). *Neuroendocrinology Letters*, Vol. 30, No. Suppl. 1, (December 2009), pp. 236-241, ISSN 0172-780X

Velisek, J.; Svobodova, Z. & Piackova, V. (2009b). Effects of acute exposure to bifenthrin on some haematological, biochemical and histopathological parameters of rainbow trout (*Oncorhynchus mykiss*). *Veterinarni Medicina*, Vol. 54, No. 3, (March 2009), pp. 131-137, ISSN 0375-8427

Velisek, J., Svobodova, Z., Machova, J., (2009c). Effects of bifenthrinu on some haematological, biochemical and histopathological parameters of common carp

(*Cyprinus carpio* L.). *Fish Physiology and Biochemistry*, Vol. 35, No. 4, (November 2009), pp. 583-590, ISSN 09201742

Velisek, J.; Svobodova, Z.; Piackova, V.; Novotny, L.; Blahova, J.; Sudova, E. & Maly, V. (2008). Effects of metribuzin on rainbow trout (*Oncorhynchus mykiss*). *Veterinarni Medicina*, Vol. 53, No. 6, (July 2008), pp. 324-332, ISSN 0375-8427

Velisek, J., Jurcikova J., Dobsikova, R., Svobodova, Z., Piackova, V., Machova, J., Novotny, L. (2007). Effects of deltamethrin on rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Pharmacology,* Vol. 23, No. 3, (May 2007), pp. 297-301, ISSN 1382-6689

Velisek, J.; Wlasow, T.; Gomulka, P.; Svobodova, Z.; Dobsikova, R.; Novotny, L. & Dudzik, M. (2006a). Effects of cypermethrin on rainbow trout (*Oncorhynchus mykiss*). *Veterinarni Medicina*, Vol. 51, No. 10, (October 2006), pp. 469-476, ISSN 0375-8427

Velisek, J., Dobsikova, R., Svobodova, Z., Modra, H., Luskova, V. (2006b). Effect of deltamethrin on the biochemical profile of common carp (*Cyprinus carpio* L.). *Bulletin of Environmental Contamination and Toxicology*, Vol. 76, No. 6, (June 2006), pp. 992-998, ISSN 0007-4861

Wackett, L.P.; Sadowsky, M.J.; Martinez, B. & Shapir, N. (2002). Biodegradation of atrazine and related s-triazine compounds: from enzymes to field studies. *Applied Microbiology and Biotechnology*, Vol. 58, No. 1, (January 2002), pp. 39–45, ISSN 0175-7598

Wendelaar Bonga, S.E. (1997). The stress response in fish. *Physiology Reviews*, Vol. 77, No. 3, (July 1997), pp. 591–625, ISSN 0031-9333

Yadav, R.S.; Srivastava, H.C.; Adak, T.; Nanda, N.; Thapar, B.R.; Pant, C.S.; Zaim, M. & Subbarao, S.K. (2003). House-scale evaluation of bifenthrin indoor residual spraying for malaria vector control in India. *Journal of Medical Entomology*, Vol. 40, No. 1, (January 2003), pp. 58–63, ISSN 0022-2585

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Genotoxicity Testing in Pesticide Safety Evaluation

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1. Introduction

Genotoxicity is a recently developed branch of toxicology, which deals with the study of deleterious effects of toxic agents present in the environment on the structure and function of DNA. Direct damage to DNA is an increasingly more essential focus on ecotoxicology research for two reasons; firstly, because of the far reaching effects of genotoxins on the health of an organism and the possible future implications if the germline is affected, and secondly, because extremely sensitive methods of detecting DNA damage have been developed, which allowed the improvement of early biomarkers for xenobiotic exposure.

Genotoxicity can result in three types of genetic lesions. Firstly, single-gene mutations, also called point mutations, which include alterations in the nucleotide sequence of DNA, and may involve either the base substitution or frame – shift mutations. Second are the structural chromosomal mutations or genomic mutations which include changes in chromosomal structure, such as breaking of chromosome, or translocation of an arm, commonly called clastogenensis. Third are numerical changes in the genome; aneuploidy and/or polyploidy (Cajaraville et al., 2003).

Induction of DNA damage is one of the primary events in the initiation of carcinogenesis by chemicals. Several chemical pollutants can produce carcinogenic effects through the induction of genetic lesions.

Carcinogens can be divided into two categories; genotoxic and epigenetic. Compounds that react directly or indirectly with DNA are, in most cases genotoxic carcinogens for example; polycyclic aromatic compounds, hydrocarbons, heavy metals, alkylating agents etc. are called as genotoxic compounds.

Epigenetic carcinogens such as pesticides, asbestos, silica, immunosupressors etc. cause carcinogenesis by inducing a multitude of mechanisms that ultimately bring changes in the DNA of the organism (Cajaraville et al., 2003).

Tests for genotoxicity include systems which give an indication of damage to DNA. End points determined are unscheduled DNA synthesis (UDS), sister-chromatid exchange (SCE), single cell gel electrophoresis (SCGE), formation of DNA adducts and mitotic

recombination, chromosomal aberrations studies, micronuclei tests and many modern molecular tools and techniques which will be discussed in the subsequent sections.

1.1 Pesticides

Pesticides are substances used to control pests, including insects and plant diseases. Naturally-occurring pesticides have been in use since centuries, but widespread production and use of modern synthetic pesticides did not begin until 1940s. On a global scale approximately over five billion pounds of conventional pesticides are being in use in different areas like agricultural lands, forests, rangelands management, disease control, domestic use and many more areas annually (EPA,2001). Use of pesticides in India began in 1948 when dichloro diphenyl trichloroethane (DDT) was imported for malaria control and benzene hexachloride (BHC) for locust control (Gupta, 2004). India started pesticide production with manufacturing plant for DDT and BHC in the year 1952. In 1958, India was producing over 5000 metric tonnes of pesticides. Currently, there are approximately 145 pesticides registered for use, and production has increased to approximately 85,000 metric tonnes (Gupta, 2004). However, it is estimated that often less than 0.1 percent of an applied pesticide reaches the target pest, leaving 99.9 percent as an unintended pollutant in the environment, including in soil, air, and water, or on nearby vegetation. Pesticides can also move from the site of application via drift, volatilization, leaching and runoff. In addition to killing insects or weeds, pesticides can be toxic to a host of other non-target organisms including birds, fish, beneficial insects, plants and humans.

Pesticides have the potential to enter aquatic habitats from direct application, terrestrial runoff or wind borne drift. Aquatic toxicology is the study of the effects of environmental contaminants on aquatic organisms, such as the effect of pesticides on the health of fish or other aquatic organisms. A pesticide's capacity to harm fish and aquatic animals is largely a function of its toxicity, exposure time, dose, and persistence in the environment. Exposure of fish and other aquatic animals to a pesticide depends on its biological availability (bioavailability), bioconcentration, biomagnification, and persistence in the environment (Van der Werf, 1996; Louis *et al.*, 1996).

Fish appear to possess the same biochemical pathways to deal with the toxic effects of endogenous and exogenous agents as do mammalian species. Fish species are sensitive to enzymic and hormone disruptors (Grabuski *et al*., 2004). Chronic exposure to low levels of pesticides may have a more significant effect on fish populations than acute poisoning. Doses of pesticides that are not high enough to kill fish are associated with subtle changes in behavior and physiology that impair both survival and reproduction. Biochemical changes induced by pesticidal stress lead to metabolic disturbances, inhibition of important enzymes, retardation of growth and reduction in the fecundity and longevity of the organism (Murty, 1986; Khan & Law, 2005). Liver, kidney, brain and gills are the most vulnerable organs of a fish exposed to the medium containing any type of toxicant. Fish show restlessness, rapid body movement, convulsions, difficulty in respiration, excess mucous secretion, change in color, and loss of balance when exposed to pesticides. Some agrochemicals can indirectly affect fish by interfering with their food supply or altering the aquatic habitat, even when the concentrations are too low to affect fish directly. It is important to examine the toxic effects of pesticides on fish since they constitute an important link in food chain and their contamination by pesticides imbalances the aquatic system.

Organophosphate (OPs) and the carbamate group of pesticides are the recently developed pesticides used against many pests. The chemicals in these classes kill insects by disrupting

the brain and the nervous system. Some of the commonly used OPs in use are phorate, disulfoton, dimethoate, dichlorvos, diazinon, sevin, chlorpyrifos, etc. These pesticides are esters, amides, or simple derivatives of phosphoric and thiophosphoric acids. Chemically carbamate is a salt of or an ester of carbamic acid. The carbamate compounds include furadan, carbaryl, carbofuran, methomyl, and oxamyl.

Phorate and furadan are two most widely used pesticides in agriculture in India. Phorate is an organophosphate whereas furadan is a carbamate. According to World Health Organisation (WHO), phorate is a Class IA (Extremely Hazardous) pesticide. Phorate 10% CG falls under Class IB (Highly Hazardous). The Food and Agriculture Organisation (FAO) recommends that products that fall under Class IA and Class IB should not be used in developing countries because of safety concerns related to these products. Furadan 3G has been classed as highly toxic in relation to other pesticides (Palmer & Schlinke, 1973). Together with organophosphorus compounds it is held responsible for most of the accidental poisonings in animal agriculture.

Aquaculture has emerged as one of the most promising and fastest growing food producing sectors in the world. It has grown at an annual rate of 10% from 1984 to 1995. India occupies second position in the global aquaculture production. Carp accounts for half of the world inland aquaculture production. India is also regarded as 'Carp country' as carps contributes maximum to the fisheries industry of the country (FAO, 2005) and the major species which contributes to the production are Indian major carps (IMCs) *viz*., *Labeo rohita, Catla catla, Cirrihinus mrigala.* Together these carp species contribute about 23, 00, 000 tonnes per year of total aquaculture production (FAO, 2005). Among all the IMCs rohu is the most preferred species and contribute about 41% of the total carp production (FAO, 2005).

In such a scenario it is important to monitor the activities of the fish in the changing environmental conditions. It is important to focus on the changes in the genetic system as some of the changes very often show lethal effects in the individual organism.

2. Genotoxicity

Genotoxicity is a recently developed branch of toxicology and is a general term that refers to alterations to the gross structure or content of chromosomes (clastogenicity) or base pair sequence (mutagenicity) by exposure to toxic agents. Genotoxicants are very important components to be monitored as they cause mutations that often lead to cancers. Further, understanding the changes at the DNA level of an organism exposed to pollutants is essential to demonstrate an impact at the ecological relevant population or community level (Shugart &Theodorakis, 1996).

An understanding of the processes and mechanisms operating at the genetic level would help to identify the more complex changes at higher levels of organization. DNA molecules bear highly reactive groups and are thus targeted and modified by a range of genotoxic compounds, including reactive oxygen species (ROS), metabolites and organic and inorganic electrophiles, such as heavy metals (Adams, 2001). Contaminants may impact genetic material either directly through interaction with nucleotides or indirectly through impacting natural cellular function such as impeding DNA replication, transcription etc. Direct acting genotoxins include chemical compounds that are electrophilic and hence can potentially react directly with the nucleophilic sites within DNA molecules, and such compounds include carbonium ions, episulfonium ions, free radicals, diazonium ions, strained lactones, sulfonates, halo ethers etc. (Williams & Weisburger, 1991).

In addition to such direct binding chemicals, a large number of chemically inert compounds may be transformed into metabolites with electrophilic and/or nucleophilic properties and so become able to form different changes in the structure and function of DNA (Hodgson & Levi, 1996). Substances such as polycyclic aromatic hydrocarbons, aromatic amines, azo compounds, nitroaryl compounds and nitrosamines are non-polar lipophilic components, which would build up in the organism if they were not actively transformed into water soluble derivatives and excreted out (Sipes and Gandolfi, 1991). This cellular detoxification mechanism produces intermediates, which are more reactive than the parent compound and/or their metabolites, and may therefore act as genotoxins forming DNA adducts. A direct relationship between exposure to polycyclic aromatic compounds and the level of DNA adducts has been shown in several fish species, including English sole (*Pleuronectes vetulus*), winter flounder (*Pseudopleuronectes americanus*), and oyster toadfish (*Opsanis tau*) (Collier *et al.,* 1993).

Apart from forming DNA adducts, genotoxins bring major forms of damage to DNA which include damage to the phosphodiester backbone, changes in ribose sugars and in the purine and pyrimidine bases.

2.1 Genotoxic effects of pesticides in living organisms

Pesticides form an important group of environmental pollutants and the genotoxic effects of several chemical groups of pesticides have been shown by *in vivo* and *in vitro* experiments (Bolognesi, 2003; Abdollahi *et al.,* 2004; Kaushik & Kaushik 2007). However, genotoxicity data for a great majority of pesticides are scanty (Gandhi *et al*., 1995), and where ever exist; the findings from different laboratories are contradictory for many formulations.

Among pesticides, organophosphates and organochlorines are constantly a matter of worry because of their wide use. Both groups of chemicals bear the potentiality to cause genotoxicity and carcinogenicity (Kaushik & Kaushik, 2007). Apart from the OPs and OCs, a new generation of pesticides, the synthetic pyrethroids, once claimed to possess a great safety factor (Kaushik & Kaushik, 2007), is reported to be genotoxic (Bhunya & Pati, 1990). In a survey including halogenated hydrocarbons, organophosphates, carbamates and other classes of pesticides, Borzsonyi *et al*. (1984) found 29 pesticides to be definite or suspected genotoxic agents.

Several studies on the effect of pesticides in different fish species have been carried out recently using different genotoxicological tools (Hai *et al.,* 1997; Das & John, 1999; Pena-Llopis *et al.,* 2003). However, genotoxicity studies of pesticides on various indigenous fish species of India are very limited. Banu *et al.* (2001) studied the genotoxic effects of monocrotophos, one of the popular organophosphate pesticides on the fish *Tilapia mossambica* using comet assay and found a dose-related increase and time-related decrease of comet tail length. Pandey *et al*. (2006) evaluated the genotoxic potential of Endosulphan in *Channa punctatus*. They exposed the fish to different doses of pesticides and assessed the DNA damage in gill and kidney tissues by comet assay. The authors found a dose-dependent response in both the tissues.

Oxidative damage is thought to be an important mechanism in the DNA damage caused by organophosphate pesticides (Hodgson & Levi, 1996). More than 100 different oxidative modifications to DNA by OPs have been described (Loft & Poulsen, 2000), and several DNA base oxidation products are known to be mutagenic, including 8-oxo-7, 8-dihydro-2-deoxyguanosine and thymine glycol (Halliwell, 2002). The adverse effects caused due to the generation of ROS and reactive oxygen intermediates (ROI) are lifted off in the organism as

soon as the antioxidant system present in the organisms gets activated in the organism. Wild (1975) focused attention on the electrophilic activity as the fundamental cause of the toxicity of these compounds and considered DNA alkylation as one of the reasons for the production of genotoxicity (Hodgson & Levi, 1996; Yadav & Kaushik, 2002). Oxidative stresses due to the OP pesticides are also well evidenced in fishes like *Cyprinus carpio,* catfish *Ictalurus nebulosus* (Hai *et al.,* 1997) and in the European eel *Anguilla anguilla* (Pena-Llopis *et al.,* 2003).

Carbamates constitute another major group of pesticides and many of them have been reported to show mutagenic properties in various test systems. Zineb, a carbamate fungicide, has been reported to be mutagenic in both somatic and germ-line cells in Drosophila (Tripathy *et al*., 1988). In another report, the same research group has reported that the fungicide ziram is mutagenic in the wing, eye and female germ-line mosaic assays, and in sex linked recessive lethal test in *Drosophila melanogaster* (Tripathy *et al*., 1989). In a more recent study, Franekic *et al*. (1994) reported that ziram, zineb and thiaram are mutagenic in a battery of bacterial test systems. The thiocarbamate pesticide malinate and vernolate have been reported to cause chromosomal changes like SCE and chromosomal aberrations *in vitro* and increased frequency of polychromatic erythrocytes in mouse bone marrow cells (Pinter *et al*., 1989). Studying on the genotoxicity of aldicarb, aldicarbosulfone, aldicarb oxide, carbofuran and propoxur, Canna-Michaelidou & Nicolaou (1996) reported that all the pesticides were 'suspect genotoxic' directly and after S9-actvation in mutatox test. Genotoxicity of carbofuran, carbosulfan and methyl isothiocyanate, a component of the pesticide carbaryl, has also been reported (Chauhan *et al*., 2000; Rencuzogullari and Topaktas, 2000; Kassie *et al*., 2001).

3. Tools in the study of Genotoxicity

In recent years a number of assays have been framed to evaluate the genotoxic effects of chemicals and other potent environmental toxicants in microbes, plants and animals. Emphasis has been given on the effective detection of mutations as it not only provides the basis for biomonitoring, but also serves to identify vulnerable stages in the life history of a species, the nature and dynamics of causal agents and associated phenotypic and population-level effects (Shugart and Theodorakis, 1994).

Several genotoxic effects like, DNA adducts, DNA breakage, chromosomal aberrations and sister chromatid exchanges can be observed in organisms exposed to various pollutants (Venier *et al.,* 1997; Das & John, 1999). Several assays have been successfully employed in the detection of the various genotoxic effects in the living organisms.

Sister chromatid exchange has successfully been used in many fish to assess the toxicity of the test chemical on the DNA. It was used by Kligerman (1979) in *Umbra limi* in the assessment of the affect of some mutagenic agents.

Chromosomal aberration is another tool, which is applied in the field of genotoxicological studies. Different chromosomal aberrations, such as breaks, ring chromosomes and dicentric chromosomes, have been detected in kidney cells after the injection of three fish species (common carp*, Cyprinus carpio*, tench, *Tinca tinca*; grass carp, *Ctenopharyngodon idella*) with aflatoxins B, aroclor 1254, benzidine, benzo [a] pyrene and 20-methylcholanthrene (Al-Sabiti, 1985; Cajaraville *et al.,* 2003). Das & John (1999) evaluated the effect of two organophosphorus pesticides, methyl parathion and phosphamidon on *Etroplus suratensis* using chromosomal aberration as the genetoxicological test tool.

Another less time consuming and authentic way of monitoring the genotoxic effects of pollutants and mutagens is micronuclei formation. There has been an increasing interest in the use of micronucleus test (MNT) as an index of cytogenetic damage in fish and other marine vertebrates and invertebrates (Al-Sabiti, 1994; Venier *et al.,* 1997). Various studies have been shown that the peripheral erythrocytes of fish have a high incidence of micronuclei under laboratory conditions. Among the recent assays, single cell gel electrophoresis or called comet assay has immense use in the detection and evaluation of genotoxic compounds in several test systems (Singh *et al*., 1988; Collins, 2004; Pandey *et al*., 2006). Several reviews have been published on the acceptance of comet assay in monitoring the effects of several potent genotoxic agents on the DNA of different animals (Moller *et al.,* 2000; Bolognesi, 2003; Collins, 2004).

Recently, polymerase chain reaction (PCR) has been used in the studies of genotoxicity (Atienzar & Jha, 2006). The detection of unknown mutations involves the identification of heteroduplexes or mismatches between mutated and wild type sequences, based either upon the electrophoretic properties of the sequences or upon the selective chemical modifications of such sequences. The two main types of electrophoretic methods are denaturing gradient gel electrophoresis (DGGE) assay, and the single stranded conformational polymorphisms (SSCP) assay (Cajaraville *et al.,* 2003). The DGGE separates the wild type and mutant DNA heteroduplexes, whereas the SSCP separates single stranded wild type and mutant DNA sequences due to differences in secondary structure (Cajaraville *et al.,* 2003). Although such procedures detect a variety of base substitutions, frame shifts and deletions, the methods fail to detect all mutations present (Cajaraville *et al.,* 2003). Detection of known mutations involves mismatched primer techniques such as the allele-specific oligonucleotide technique, or the allele-specific amplification method. Both of these involve the amplification of mutant and wild type sequences. These approaches are based on the successful amplification of mutant sequences with primers specific to the suspected mutation and therefore require sequence information of the targeted areas (Cajaraville *et al.,* 2003).

Among different types of PCRs, RAPD-PCR and AP-PCR offers a great scope in the detection and comparison of changes between the normal and genotoxicants exposed groups of animals in genotoxicity studies. Despite the problems concerning with the reproducibility and complexity of patterns (Atienzar *et al.,* 1998; Singh & Roy, 1999), these techniques have shown several advantages for the detection of genomic mutations, such as ease, speed and low cost of experiments and the ability to clone aberrant fragments (Navarro & Jorcano, 1999). While these techniques have so far been mainly used for investigation of human cancer tissues, its potential has been shown in the study on Japanese medaka (*Oryzias latipes*) where a correlation between gamma-rays induced genomic damage and embryo malformations was demonstrated (Kubota *et al.,* 1992).

Advances in transgenic approaches that include knockout gene technology and gene silencing have been proven to be powerful assays towards the observation of mutational changes, whereby transgenes are introduced at the zygotic stage of development act as target genes, capable of a phenotypic response to mutational events (Gossen & Vijig, 1993; Bailey *et al.,* 1994). The greatest potential for new biomarkers of early effect lies in toxicogenomics, a field of study that examines how the entire genome responds to toxicants or other hazards (Toraason *et al.,* 2004).

3.1 Comet assays

Comet assay otherwise called single cell gel electrophoresis was first described by Ostling & Johanson (1984) and numerous modifications have been reported to date to allow

detection of various types of DNA damage. The principle of the test is remarkably simple. DNA damage is quantified by the proportion of DNA which migrates out of the nuclei toward the anode when individual cells or isolated nuclei, embedded in a thin agarose layer, are subjected to electrophoresis that results in a "comet-like" shape of nuclei. This enables quantification of DNA in comet tails after staining with an appropriate fluorochrome (e.g. ethidium bromide) or with silver staining protocol (Garcia *et al.,* 2007). The comets can be either classified by visual examination (visual scoring) (or measured from morphological parameters obtained by image analysis and integration of intensity profiles (Collins, 2004).

According to visual scoring, the comets are classified into five different classes, from 0 (no tail) to 4 (almost all DNA in tail), which give sufficient resolution to make distinction among all the five comet classes. The comets are given different class on the basis of the length of the tail, amount of DNA present in the tail. If 100 comets are scored, and each comet assigned a value of 0 to 4 according to its class, the total score for the sample gel will be between 0 and 400 "arbitrary units or damage index"(Collins, 2004, Heaton *et al.,* 2007).

Different types of comet assay for different purposes have been described by Collins (2004). The different modified versions of comet assays were standardized by the employment of various combinations of neutral and alkali pH solutions immediately prior and during electrophoresis. Exposure of DNA to high alkali prior to electrophoresis allows for the preferential detection of DNA single-strand breaks (SSB). This procedure also detects some alkali labile sites depending on the pH of the alkali unwinding solution and the duration of exposure. The majority of alkali labile sites become detectable when electrophoresis is performed in alkaline solution.

Major advantage of comet assay over other techniques is the highly sensitive detection ability for both double and single-strand breaks. Levels of detection have been reported to be as low as one break per chromosome (Mitchelmore & Chipman, 1998) or as few as 200 breaks per cell (Rojas *et al.,* 1999). Double-strand breaks (DSB) cause comet formation even under completely neutral conditions. Treatment of DNA after lysis with specific DNA repair enzymes can be used for the selective detection of abasic sites or pyrimidine dimers (Angelis *et al.,* 2000).

The comet assay being a short - term genotoxicity test has been widely used to reveal a broad spectrum of DNA-damaging agents capable of inducing strand breakage, cross-links and alkali - labile sites (Singh *et al.,* 1988; Fairbairn *et al.,* 1995; Pandey *et al.,* 2006). This technique has been applied in several genotoxicity studies (Pandey *et al.,* 2006), ecotoxicology (Cotelle & Ferard, 1999), biomonitoring (Collins *et al.,* 1997) and clinical radiobiology (Olive, 1999). Its versatility has allowed the investigation of repair mechanisms (Alapetite *et al.,* 1997), in the detection of apoptosis, alkylating, oxidizing and cross-linking agents.

Many reviews details the employment of comet assay in the assessment of the genotoxic potential of many compounds, which notably include metals, pesticides, opiates, nitrosamines and anticancer drugs (Collins, 2004). A significant advantage of the comet assay is its applicability to any eukaryotic organism and nucleated cell type (Mohanty *et al.,* 2009a, 2009b, 2011). This assay can be applied both *in vitro* and *in vivo* conditions after an exposure to different potent genotoxic and mutagenic agents (. This assay is also very useful, as it requires very small cell samples (<10 000 cells) (Shugart, 2000).

In last two decades comet assay has been adapted by many workers to evaluate the genotoxicity potentialities of pesticides in human populations (Garaj-Vrhovac & Zeljezic,

2001). During a study on workers involved in the production of a variety of pesticides, an increase in DNA damage in peripheral blood lymphocytes was found (Garaj-Vrhovac & Zeljezic, 2000). In another experiment on Croatian workers occupationally exposed to a complex mixture of pesticides showed an increase in the values of the comet assay parameters (Garaj-Vrhovac & Zeljezic, 2001) indicating that the pesticides to be the potent genotoxic substances. Grover *et al.* (2003) evaluated the DNA damage in Indian pesticide production workers. Blood leukocytes of a group of 54 pesticide workers and an equal number of control subjects were examined for genotoxicity in this study. The two groups had similar mean ages and smoking prevalence. The mean comet tail length was set as the parameter to measure the extent of DNA damage. The exposed workers had significantly greater mean comet tail lengths than those of control group. The authors put forwarded the possible reasons of comet formation with greater tail length in exposed groups could have happened due to the single-strand breaks in DNA and/or during the repair of DNA strand breaks, DNA adduct formation or DNA-DNA and DNA-protein cross links. Occupational exposure to xenobiotics might have resulted in their covalent binding to DNA, which might lead to chromosome alterations and could be an initial event in the process of chemical carcinogenesis (Fairbairn *et al.,* 1995; Shah *et al.,* 1997).

Comparably fewer studies have been conducted with aquatic invertebrates and these have been restricted to bivalve species such as the marine mussel (*Mytilus edulis*) (Accomando *et al.,* 1999), the zebra mussel (*Dreissena polymorpha*) (Pavlica *et al.,* 2001), Mediterranean mussel (*Mytilus galloprovincialis*) (Frenzilli *et al.,* 2001) and oyster (*Crassostrea americanus*) (Nacci *et al.,* 1996).

This assay has been carried out in fishes such as bullhead (*Ameriurus nebulosus*) Common carp (*Cyprinus carpio*) (Pandrangi *et al.,* 1995), brown trout (*Salmo trutta*) (Belpaeme *et al.,* 1996), flounder (*Pleuronectes americanus*) (Nacci *et al.,* 1996), rainbow trout (*Oncorhynchus mykiss*) (Devaux *et al.,* 1999), butterfish (*Pholis gunnellus*) (Bombail *et al.,* 2001), zebra fish (*Danio rerio*) (Schnurstein and Braunbeak, 2001) tilapia (*Tilapia mossambica*) (Banu *et al.* 2001) and *Channa punctatus* (Pandey *et al.,* 2006) etc.

3.2 RAPD - PCR

The 1993 Nobel Prize for chemistry was awarded to Dr. Kary Mullis, for having invented the polymerase chain reaction (PCR). This remarkable technology has revolutionized the field of molecular biology and has been used in diverse areas of research such as evolution clinical medicine, forensic science, pathogen detection, genotoxicant detection etc. Subsequently, new PCR based methods have been developed. In particular, Williams *et al.* (1990) and Welsh & McClelland (1990) developed the random amplified polymorphic DNA (RAPD) and arbitrarily primed PCR (AP-PCR), respectively. The random amplified polymorphic DNA technique uses single primer of arbitrary sequence to amplify the segment of DNA between two inverted priming sites on complementary strands, separated by 150-5000 base pairs. This technique has many advantages over other molecular techniques engaged in the similar type of works. The advantages include the need of minute quantity of template DNA. The technique has the potential to access many loci, thus lead to the identification of loci those were not previously known to be under contaminate-selective pressure within the DNA. Finally, RAPD primers amplify from inverted repeat sites within the DNA. This is important because certain sequences that contain inverted repeats (e.g. transposons) have shown to be responsive to DNA damaging agents (Theodorakis & Shugart, 1997).

Moreover, RAPD is more cost effective and less labour intensive than the similar kinds of molecular techniques like arbitrarily primed-PCR (AP-PCR). This technique does not require any previous knowledge of the species genome sequence. It also avoids the use of radioisotopes. In the field of ecotoxicology, most RAPD studies describe the RAPD changes such as differences in band intensity as well as gain/loss of stable RAPD bands (Atienzer & Jha, 2006).

RAPD-PCR has been used to detect not only DNA damage and mutations but also changes in genetic diversity and gene frequencies. The first study measuring genotoxic effects using the RAPD assay was performed by Savva *et al.* (1994). In the study, the RAPD profiles generated from rats exposed to benzo [a] pyrene (B [a] P) revealed the appearance and disappearance of bands in comparison to control patterns. These changes observed in the fingerprints of exposed animals were supposed to be produced due to the presence of DNA adducts; mutations or DNA strand breaks. In genotoxicity studies, the RAPD approach adopts comparison of RAPD profiles obtained from control and treated population at a defined time. Krane *et al.* (1999) suggested that RAPD based measures of genetic diversity may be suitable for development as a sensitive means of directly assessing the impact of environmental contaminants upon ecosystems. Theodorakis *et al.* (1999) indicated that the probability of survival and degree of DNA strand breakage in radionuclide-exposed mosquitofish were dependent on RAPD genotype, and were consistent with the hypothesis that the contaminant-indicative RAPD bands were markers of loci which imparted a selective advantage in radionuclide-contaminated environments.

RAPD-PCR has also been successfully utilized in *in vitro* genotoxicity test. Becerril *et al.* (1999) studied the effect of the well known carcinogen, mitomycin C in RTG-2 fish cell line by RAPD-PCR. The bands obtained were analyzed to show a difference in the banding pattern of control with that of the exposed groups.

4. Application of comet assay and RAPD – PCR in the assessment of genotoxic effects of two pesticides on *Labeo rohita* fingerlings

4.1 Comet assay

A study was carried out by Mohanty *et al.,* (2009a, 2009b and 2011) with an objective to study the genotoxic effects of two pesticides, phorate, an organophosphate and furadan, a carbamate on rohu (*Labeo rohita*) fingerlings.

To evaluate the DNA damage, rohu fingerlings were exposed to control, 0.001, 0.002 and 0.01ppm of phorate control, 0.002, 0.004 and 0.02ppm of furadan for a total time period of 96h. Samplings were carried out at 24, 48, 72 and 96h, and six numbers of fishes were sampled at each sampling hour for each dose of pesticide. Three tissue samples such as blood, liver and gill were selected for the study. DNA damage in these tissue samples of pesticide treated fish was carried out by alkaline comet assay and the comet slides were stained with silver stain for visualization. Two slides per fish were prepared and 100 randomly selected non-overlapping cells were scored for comets. The comets were visually assigned a score on an arbitrary scale of 0–4 (i.e., ranging from 0 – undamaged to 4 - maximum DNA damage) based on perceived comet tail length migration and relative proportion of DNA in the comet tail. The mean percent of overall DNA damaged cells was calculated by adding the number of cells scored under comet classes 1, 2, 3 and 4, and was termed as total percentage of damaged cells. The extent of DNA damage score in terms of arbitrary units (AU) for each slide was derived by multiplying the number of cells assigned

to each class of damage by the numeric value of that class and summing the overall values {may vary within a range of 0 (all cells undamaged - 0×100) to 400 (all cells damaged at class 4 – 4 X 100)} (Fig.1). The statistical analyses were carried out with Microsoft excel 2007 and SPSS statistical package version 10. The test of significance was determined by nonparametric Mann-Whitney U test at 5% level.

Results obtained from alkaline comet assay in the blood, liver and gill cells of rohu fingerlings exposed phorate and furadan showed that the baseline damage was minimal in blood cells compared to the liver and gill cells, whereas the DNA damage was maximum in liver cells followed by the gill and blood cells. The fishes treated with 0.001ppm phorate dose did not show much difference in DNA damage compared to the control groups and hence was concluded that the dose was low to cause genotoxic effects in rohu fingerlings. However, the significant differences in DNA damage observed at 0.002 and 0.01ppm dose levels proved phorate to possess potent genotoxic effects on rohu DNA (Fig 2 and Table 1). Similarly, results obtained from furadan treated fishes revealed that furadan at 0.002ppm dose or more was capable to produce sufficient DNA damage and hence, was also highly genotoxic to rohu (Fig 3; Table 4).

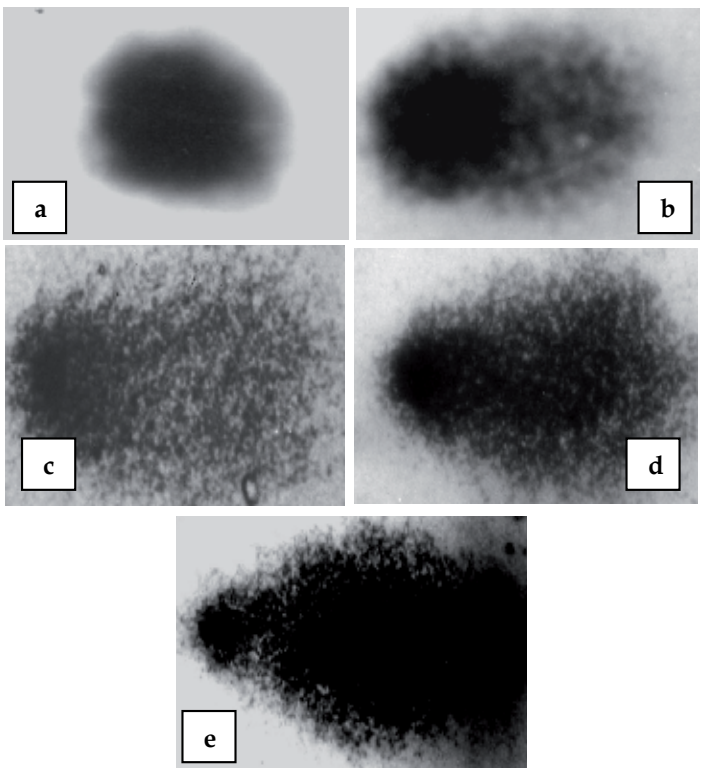
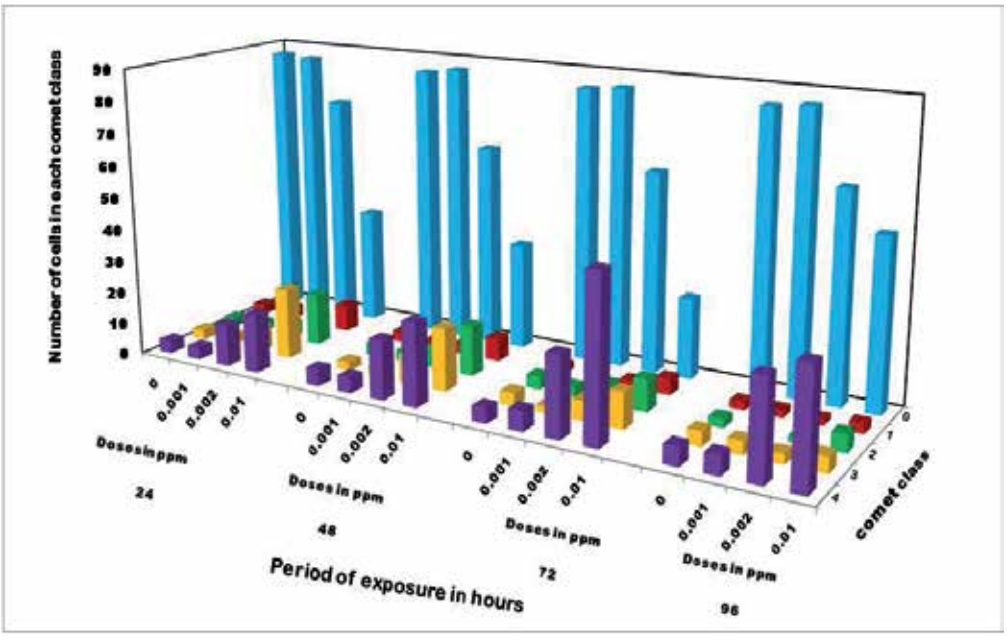
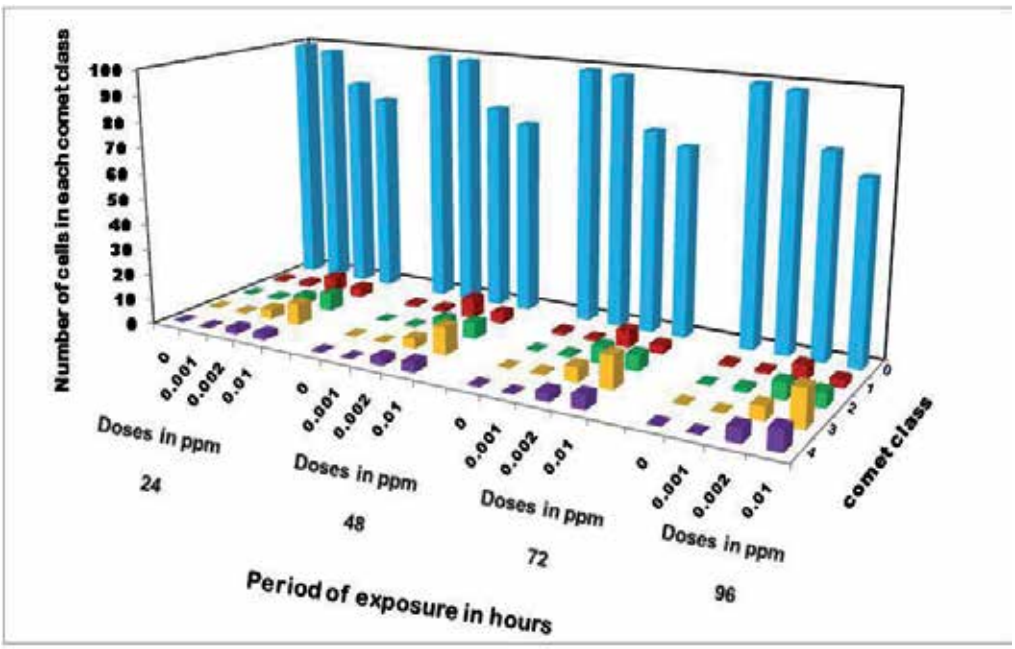


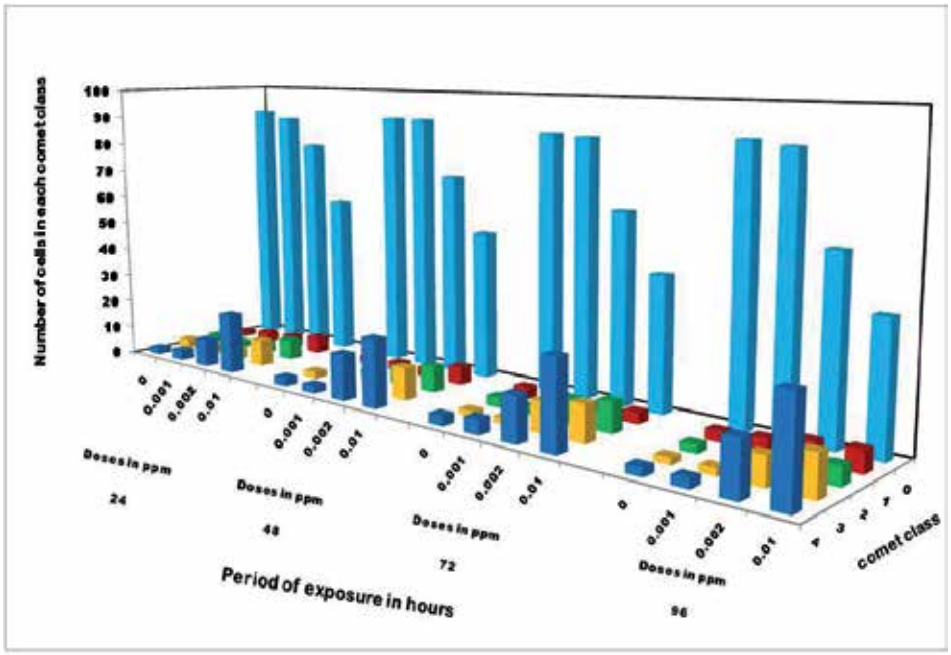
Fig. 1. Representative comet images of nuclei from cells of *Labeo rohita*. a. class 0 (undamaged), b. class 1, c. class 2, d. class 3, e. class 4 (maximum damage).


Number of cells in each comet class
Doses in ppm
24
48
72
96
Period of exposure in hours
comet class

(a)

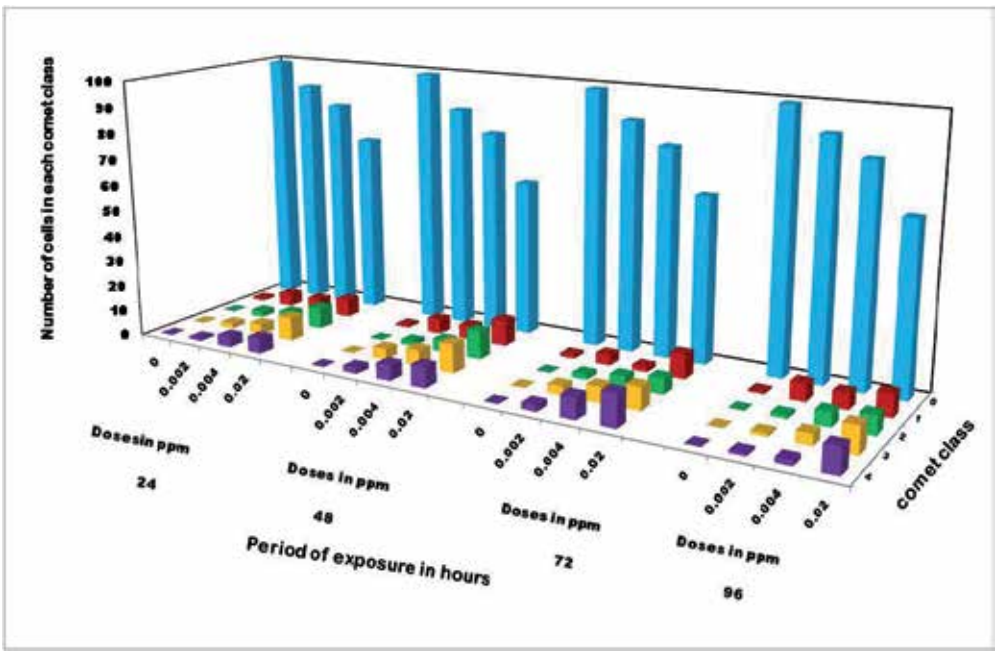

Number of cells in each comet class
Doses in ppm
24
48
72
96
Period of exposure in hours
comet class

(b)

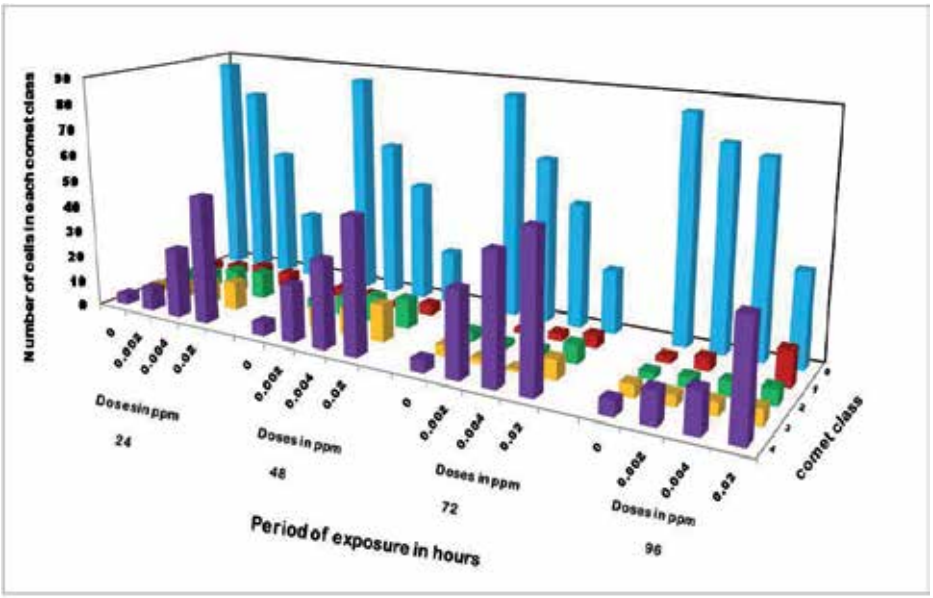


(c)

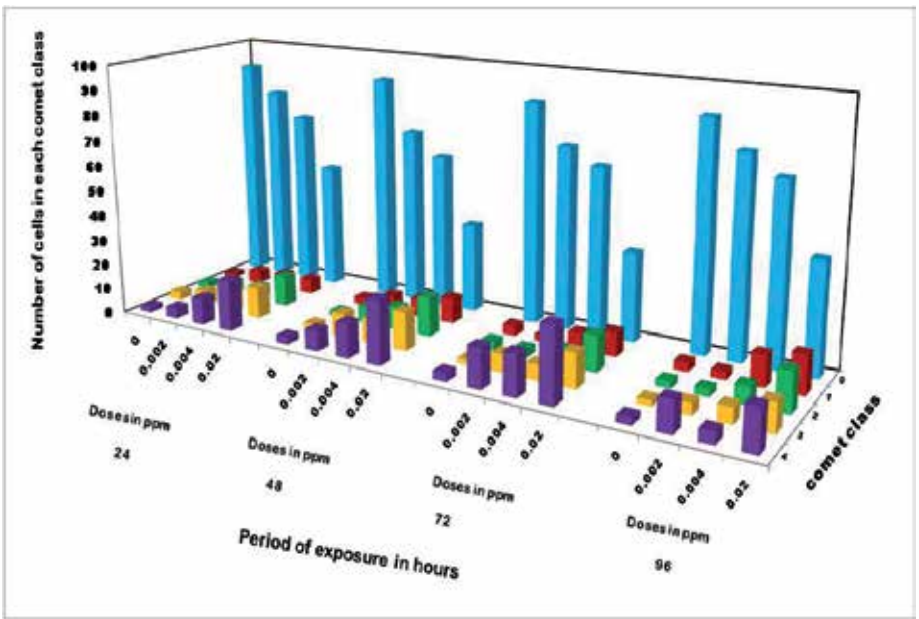
Fig. 2. Frequency of cells in each comet class (%) measured by alkaline comet assay in (a) blood (b) liver (c) gill cells of *Labeo rohita* following exposure to different doses of phorate for various time periods.



(a)



(b)



(c)

Fig. 3. Frequency of cells in each comet class (%) measured by alkaline comet assay in (a) blood (b) liver (c) gill cells of *Labeo rohita* following exposure to different doses of furadan for various time periods.

Hours of exposure	Phorate doses (ppm)	DNA damaged cells (%) (mean ± SE)	AU (mean ± SE)	DNA damaged cells (%) (mean ± SE)	AU (mean ± SE)	DNA damaged cells (%) (mean ± SE)	AU (mean ± SE)
		Blood Cells		Liver Cells		Gill Cells	
24	0.00	0.83± 0.30[a]	0.83 ±0.30[a]	13.17 ± 0.79[a]	34.33 ± 3.21[a]	9.67 ± 0 .71[a]	25.02 ± 1.80[a]
	0.001	2.67± 0.42[b]	4.51± 1.05[a]	13.83 ± 0.60[a]	34.33 ± 2.40[a]	12.00 ± 0.36[a]	30.33 ± 1.47[b]
	0.002	15.5± 0.56[c]	32.66±1 .56[b]	27.83 ± 0.47[b]	85.83 ± 1.92[a]	22.00 ±1.06[b]	63.84 ± 3.28[c]
	0.01	21.01± 0.57[d]	51.52±1 .45[c]	64,00 ± 0.57[c]	177.5 ± 1.95[a]	43.00 ± 0.57[c]	131.34 ± 1.83[d]
48	0.00	1.00± 0.36[a]	1.00±0. 36[a]	14.84 ± 0.60[a]	39.36 ± 2.76[a]	10.00 ± 0.57[a]	25.32 ± 2.33[a]
	0.001	1.67± 0.49[a]	1.84±0. 60[a]	13.16 ± 0.60[a]	36.64 ± 2.29[b]	10.00 ± 0.51[a]	24.84 ± 1.01[a]
	0.002	19.66± 0.61[b]	41.16±1 .47[b]	37.34 ± 0.33[b]	116.36 ± 1.52[c]	29.84 ± 0.94[b]	92.68 ± 2.55[b]
	0.01	24.68± 0.42[c]	63.16±1 .77[c]	67,00 ± 0.36[c]	196.66 ± 1.89[d]	48.83 ± 0.30[c]	148.66 ± 2.88[c]
72	0.00	1.33± 0.21[a]	1.33±0. 21[a]	15.17 ± 0.60[a]	41.18 ± 1.57[a]	12.49 ± 0.61[a]	29.47 ± 1.20[a]
	0.001	2.32± 0.42[a]	4.47±1. 11[a]	13.99 ± 0.57[a]	39.49 ± 1.91[b]	13.00 ± 0.68[a]	35.17 ± 2.18[b]
	0.002	22,00± 0.44[b]	49.83±1 .49[b]	38.16 ± 0.47[b]	128.98 ± 2.51[c]	36.00 ± 0.36[b]	109.00 ±1.61[c]
	0.01	26.67± 0.66[c]	72.67±1 .66[c]	75.17 ± 0.60[c]	255.34 ± 2.51[d]	55.00 ± 0.36[c]	176.34 ± 1.25[d]
96	0.00	0.83± 0.30[a]	0.83±0. 30[a]	15.17 ± 0.65[a]	43.85 ± 1.70[a]	11.17 ± 0.65[a]	26.67 ± 1.87[a]
	0.001	2.16± 0.30[b]	3.49±0. 42[a]	14.00 ± 0.25[a]	41.67 ± 1.22[b]	12.49 ± 0.56[a]	28.98 ± 2.11[a]
	0.002	22.34± 0.42[c]	54.52±1 .38[b]	36.00 ± 0.68[b]	131.83 ± 2.74[c]	41.17 ± 0.47[b]	119.35 ± 3.19[b]
	0.01	30.34± 0.76[d]	87.19±2 .98[c]	47.83 ± 0.30[c]	167.3 ± 2.27[d]	58.17 ± 0.65[c]	186.68 ± 1.58[c]

* indicates significant difference from respective control (0.00ppm phorate) at $p < 0.05$ by Mann-Whitney U nonparametric test.
Different alphabets in damage cell percentage within each time period indicate significant difference at $p < 0.05$ by Mann-Whitney U nonparametric test.

Table 1. DNA damage measured by alkaline comet assay {DNA damaged cells (%) and DNA damage scores in arbitrary units (AU)} in different cells of *Labeo rohita* following exposure to different doses of phorate for various time periods.

Hours of exposure	Furadan doses (ppm)	DNA damaged cells (%) (mean ± SE)	AU (mean ± SE)	DNA damaged cells (%) (mean ± SE)	AU (mean ± SE)	DNA damaged cells (%) (mean ± SE)	AU (mean ± SE)
		Blood Cells		Liver Cells		Gill Cells	
24	0.00	0.83 ± 0.30[a]	0.83 ± 0.30[a]	13.17 ± 0.79[a]	34.33 ± 3.21[a]	9.67 ± 0.71[a]	25.02 ± 1.80[a]
	0.001	10.17 ± 0.30[b]	19.34 ± 0.76[b]	25.17 ± 0.30[b]	70.17 ± 1.30[b]	20.17 ± 0.30[b]	49.68 ± 0.98[b]
	0.002	17.17 ± 0.30[c]	41.68 ± 1.62[c]	49.83 ± 0.40[c]	154.83 ± 1.42[c]	29.82 ± 0.47[c]	82.80 ± 0.49[c]
	0.01	30.34 ± 0.42[d]	74.85 ± 1.53[d]	74.16 ± 0.30[d]	250.98 ± 1.63[d]	50.00 ± 0.36[d]	146.00 ± 1.21[d]
48	0.00	1.00 ± 0.36[a]	1.00 ± 0.36[a]	14.84 ± 0.60[a]	39.36 ± 2.76[a]	10.00 ± 0.57[a]	25.32 ± 2.33[a]
	0.001	14.00 ± 0.57[b]	31.16 ± 1.51[b]	40.00 ± 0.25[b]	128.01 ± 1.46[b]	30.17 ± 0.30[b]	79.34 ± 0.66[b]
	0.002	22.17 ± 0.30[c]	57.18 ± 1.85[c]	54.83 ± 0.47[c]	183.66 ± 1.62[c]	39.00 ± 0.51[c]	107.33 ± 2.12[c]
	0.01	39.83 ± 0.40[d]	96.66 ± 1.28[d]	79.83 ± 0.16[d]	271.32 ± 1.33[d]	64.99 ± 0.51[d]	184.81 ± 2.86[d]
72	0.00	1.33 ± 0.21[a]	1.33 ± 0.21[a]	15.17 ± 0.60[a]	41.18 ± 1.52[a]	12.66 ± 0.61[a]	29.64 ± 1.30[a]
	0.001	11.83 ± 0.47[b]	27.99 ± 1.75[b]	38.00 ± 0.25[b]	140.34 ± 1.94[b]	28.17 ± 0.30[b]	91.33 ± 1.89[b]
	0.002	20.00 ± 0.57[c]	60.17 ± 2.12[c]	53.33 ± 0.42[c]	199.17 ± 1.42[c]	34.16 ± 0.30[c]	99.65 ± 1.66[c]
	0.01	36.33 ± 0.33[d]	96.66 ± 1.35[d]	76.33 ± 0.42[d]	269.16 ± 2.76[d]	65.32 ± 0.33[d]	192.30 ± 1.49[d]
96	0.00	0.83 ± 0.30[a]	0.83 ± 0.30[a]	15.17 ± 0.65[a]	43.85 ± 1.70[a]	11.17 ± 0.65[a]	26.67 ± 1.87[a]
	0.001	10.17 ± 0.30[b]	16.84 ± 0.30[b]	24.67 ± 0.56[b]	72.17 ± 2.67[b]	22.17 ± 0.40[b]	69.51 ± 1.30[b]
	0.002	17.33 ± 0.33[c]	36.49 ± 0.61[c]	28.00 ± 0.25[c]	87.83 ± 1.19[c]	30.00 ± 0.16[c]	63.83 ± 0.98[c]
	0.01	35.32 ± 0.42[d]	90.98 ± 1.77[d]	65.32 ± 0.42[d]	204.3 ± 1.81[d]	56.83 ± 0.30[d]	141.49 ± 3.15[d]

*indicates significant difference from respective control (0.00ppm furadan) at $p < 0.05$ by Mann-Whitney U nonparametric test.

Different alphabets in damage cell percentage within each time period indicate significant difference at $p < 0.05$ by Mann-Whitney U nonparametric test.

Table 2. DNA damage measured by alkaline comet assay {DNA damaged cells (%) and DNA damage scores in arbitrary units (AU)} in different cells of *Labeo rohita* following exposure to different doses of furadan for various time periods.

4.2 RAPD-PCR

RAPD-PCR was carried out with DNA samples of blood from 0.01ppm phorate-treated and 0.02ppm furadan-treated fishes. The fishes were exposed to pesticides for a period of 96h and samplings were carried out at 24, 48, 72 and 96h. A total of six 10 mer oligonucleotide primers were selected for the study on the basis of their capability to amplify rohu DNA to generate multiple bands. Three individual samples of each of the four sampling hours were run in 1.8% agarose gel along with the controls for each primer. Three control samples out of 12 samples collected at four different time periods were run in individual gel as all 12 samples for each primer showed similar banding pattern. Each change observed in RAPD profiles i.e. disappearances and appearance of bands in comparison to the control RAPD profiles, was given the arbitrary score of +1. The average was then calculated for each experimental group exposed to the pesticides for varying time periods. The template genomic stability (%) was calculated as '100 - (100a/n)' where 'a' is the average number of changes in DNA profiles and 'n' the number of bands selected in control DNA profiles. The

Name of the Primer	Change in the RAPD profile	24h		48h		72h		96h	
		control	treated	control	treated	control	treated	control	treated
OPC 08	No of bands disappeared	0	6	0	4	0	2	0	3
	No of bands appeared	0	0	0	0	0	0	0	0
OPC 11	No of bands disappeared	0	0	0	6	0	0	0	0
	No of bands appeared	0	0	0	0	0	0	0	0
OPC 19	No of bands disappeared	0	1	0	1	0	5	0	4
	No of bands appeared	0	0	0	0	0	0	0	0
OPY 04	No of bands disappeared	0	2	0	2	0	2	0	0
	No of bands appeared	0	0	0	0	0	0	0	0
OPY 13	No of bands disappeared	0	1	0	1	0	5	0	3
	No of bands appeared	0	0	0	0	0	0	0	0
OPY 19	No of bands disappeared	0	5	0	5	0	6	0	7
	No of bands appeared	0	0	0	0	0	0	0	0

Table 3. Frequency of appearance and disappearance of bands in the RAPD profiles of genomic DNA from blood samples of *Labeo rohita* following exposure to 0.01ppm of phorate for various time periods.

template genomic stability for blood DNA samples of phorate treated fishes showed significant changes compared to the control group only at 72h (Fig. 4, Table 3). In furadan treated fishes the blood DNA samples failed to show any significant change in the template stability at any time period (Fig. 5, Table 4).

Name of the Primer	Change in the RAPD profile	24h		48h		72h		96h	
		control	treated	control	treated	control	treated	control	treated
OPC 08	No of bands disappeared	0	8	0	2	0	9	0	5
	No of bands appeared	0	0	0	0	0	0	0	0
OPC 11	No of bands disappeared	0	7	0	1	0	3	0	2
	No of bands appeared	0	0	0	0	0	0	0	0
OPC 19	No of bands disappeared	0	0	0	0	0	2	0	3
	No of bands appeared	0	0	0	0	0	0	0	0
OPY 04	No of bands disappeared	0	2	0	1	0	1	0	1
	No of bands appeared	0	0	0	0	0	0	0	0
OPY 13	No of bands disappeared	0	0	0	0	0	0	0	0
	No of bands appeared	0	0	0	0	0	0	0	0
OPY 19	No of bands disappeared	0	4	0	1	0	2	0	4
	No of bands appeared	0	0	0	0	0	2	0	2

Table 4. Frequency of appearance and disappearance of bands in the RAPD profiles of genomic DNA from blood samples of *Labeo rohita* following exposure to 0.02ppm of furadan for various time periods.

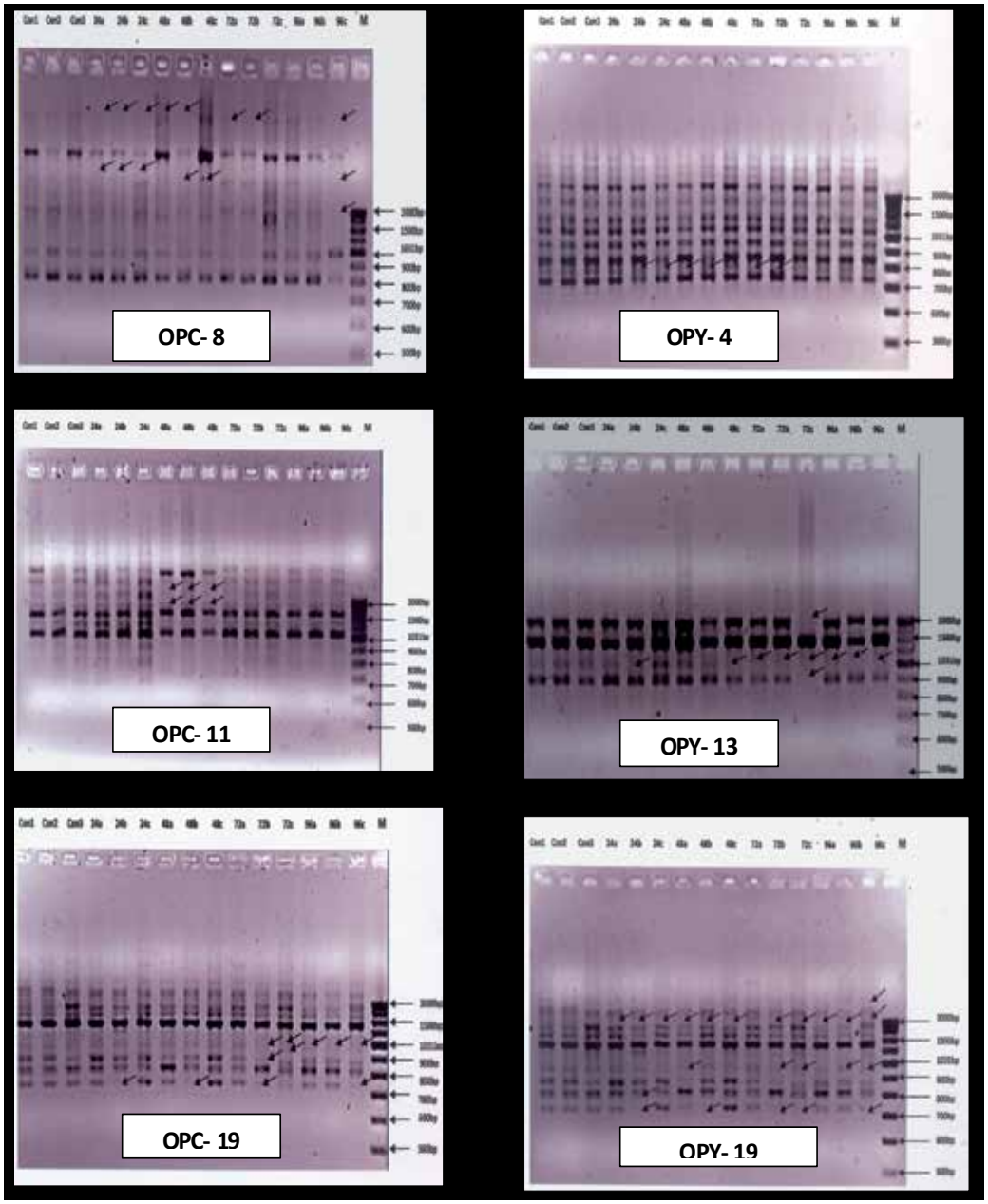


Fig. 4. RAPD profiles of genomic DNA from blood samples of *Labeo rohita* following exposure to 0.01ppm phorate for various time periods. The primers used to amplify are indicated below each photograph. White arrows show appearance of bands and black arrows show disappearance of bands in comparison to the controls. Lanes: con 1. con 2 and con 3 – three control fishes; 24 a. 24 b. 24 c – three fishes with 24 h exposure to phorate; 48 a. 48 b. 48 c – three fishes with 48 h exposure to phorate; 72 a. 72 b. 72 c – three fishes with 72 h exposure to phorate; 96 a. 96 b. 96 c – three fishes with 96 h exposure to phorate; M – molecular size marker.

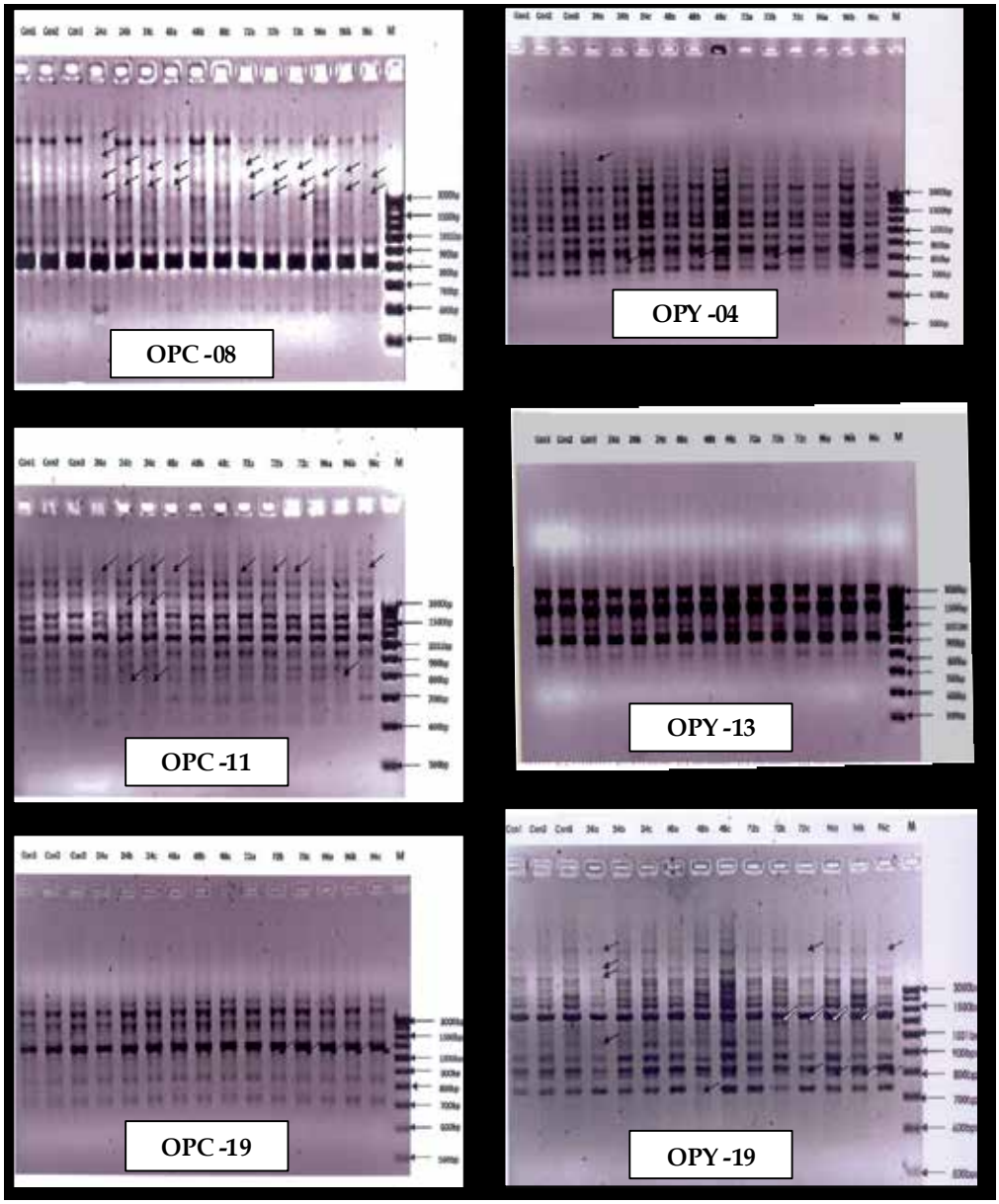


Fig. 5. RAPD profiles of genomic DNA from blood samples of *Labeo rohita* following exposure to 0.02ppm furadan for various time periods. The primers used to amplify are indicated below each photograph. White arrows show appearance of bands and black arrows show disappearance of bands in comparison to the controls. Lanes: con 1. con 2 and con 3 – three control fishes; 24 a. 24 b. 24 c – three fishes with 24 h exposure to phorate; 48 a. 48 b. 48 c – three fishes with 48 h exposure to phorate; 72 a. 72 b. 72 c – three fishes with 72 h exposure to phorate; 96 a. 96 b. 96 c – three fishes with 96 h exposure to phorate; M – molecular size marker.

5. Conclusion

From the entire study it could be generated that rohu (*Labeo rohita*) may serve as a model organism in genotoxicity studies of potential genotoxins. Blood, liver and gill cells were found to be suitable tissues system in aquatic organisms for the genotoxic studies. Alkaline Comet assay and RAPD - PCR were found to be appropriate tools in measurement of DNA damage. The two pesticides phorate and furadan used in the study were found to possess genotoxic potentials to *Labeo rohita.* RAPD-PCR employed in our study showed as a promising tool in the analysis of DNA damage due to pesticide effects.

6. References

Abdollahi, M.; Ranjbar, A.; Shadnia, S.; Nikfar, S. & Rezaiee, A. (2004). Pesticides and oxidative stress: A review. *Med. Sci. Monit.,* Vol. 10, pp. RA144 - RA147.

Accomando, R.; Viarengo, A.; Moore, M. N. & Marchi, B. (1999). Effects of ageing on nuclear DNA integrity and metabolism in mussel cells (*Mytilus edulis* L.). *Int. J. Biochem. Cell.,* Vol. 31, pp. 443 - 450.

Adams, S. M. (2001). Biomarker/bioindicator response profiles of organisms can help differentiate between sources of anthropogenic stressors in aquatic ecosystems. *Biomarkers*., Vol. 6, pp.33-44.

Alapetite, C.; Benoit, A.; Moustacchi, E. & Sarasin, A. (1997). The comet assay as a repair test for prenatal diagnosis of xeroderma pigmentosum and trichothiodystrophy. *J. Invest. Dermatol.,* Vol. 108, pp.154 - 159.

Al - Sabiti, K. (1985). Carcinogenic - mutagenic chemicals induced chromosomal aberrations in the kidney cells of three cyprinids. *Comp. Biochem. Physiol. Part C*., Vol. 82, pp.489 - 493.

Al - Sabiti, K. (1994). Micronuclei induced by selenium, mercury, merthyl mercury and their mixtures in binucleated blocked hepatic cell techniques fish erythrocytes cells. *Mutat. Res.,*Vol. 320, pp.157 - 163.

Angelis, K. J.; McGuffie, M.; Menke, M. & Schubert, I. (2000). Adaptation to alkylation damage in DNA measured by the comet assay. *Environ. Mol. Mutagen.,*Vol. 36, pp. 146 - 150.

Atienzar, F. A. & Jha, A. N. (2006). The random amplified polymorphic DNA (RAPD) assay and related techniques alied to genotoxicity and carcinogenesis studies: A critical review. *Mutat. Res.,* Vol. 613, pp. 76 - 102.

Atienzar, F.; Child, A.; Evenden, A.; Jha, A.; Savva, D.; Walker, C. & Depledge, M. (1998). Alication of arbitrarily primed polymerase chain reaction foe the detection of DNA damage. *Mar. Environ. Res.,* Vol 46, pp. 331 - 335.

Bailey, G. S.; Hendricks, J. D. & Williams, D. E. (1994). Improved food safety through discovery and control of natural and induced toxicants and antitoxicants. United States Department of agriculture/cooperative State, Fedrip Database, National Technical Information Service (NTIS).

Banu, B. S.; Danadevi, K.; Rahman, M. F.; Ahuja, Y. R. & Kaiser, J. (2001). Genotoxic effect of monocrotophos to sentinel species using comet assay. *Food Chem. Toxicol.,*Vol. 39, pp. 361 - 366.

Becerril, C.; Ferrero, M.; Sanz, F. & Castano, A. (1999). Detection of mitomycin C-induced genetic damage in fish cells by use of RAPD. *Mutagenesis,* Vol.14, pp.449 - 456.

Belpaeme, K.; Delbeke, K.; Zhu, L. & Kirsch-Volders, M. (1996). Cytogenetic studies of PCB77 on brown trout (*Salmo trutto*) using the micronucleus test and the alkaline comet assay. *Mutagenesis*, Vol. 11, pp. 485 - 492.

Bhunya, S. P. & Pati, P. C. (1990). Effect of deltamethrin, a synthetic pyrethroid, on the induction of chromosome aberrations, micronuclei and sperm abnormalities in mice. *Mutagenesis*, Vol.5, pp. 229 - 232.

Bolognesi, C. (2003). Genotoxicity of pesticides: a review of human biomonitoring studies. *Mutat. Res*., Vol. 543, pp. 251 - 272.

Bombail, V.; Aw, D.; Gordon, E. & Batty, J. (2001). Application of the comet assay and micronucleus assays to butterfish (*Pholis gunnellus*) erythrocytes from the Firth of Forth, Scotland. *Chemosphere,* Vol. 44, pp. 383 - 392.

Borzsonyi, M.; Torok, G.; Paintèr, A. & Surán, A. (1984). Agriculturally - related carcinogenic risk. *IARC Sci. Publ.,* Vol. 56, pp.465 - 486.

Cajaraville, M. P., Hauser, L., Carvalho, G., Hylland, K., Olabarrieta, I., Lawrence, A. J., Lowe, D. and Goksoyr, A. (2003). Genetic damage and the molecular/ cellular response to pollution. In. *Effects of pollution on fish: molecular effects and Population responses.* (Eds). Lawrence, A. J. and Hemingway, K. L. pp 14 – 62, Blackwell Publishing Company, ISBN 0-632-06406-4, USA.

Canna-Michaelidou, S. & Nicolaou, A. (1996). Evaluation of the genotoxicity potential (by Mutatox TM test) of ten pesticides found as pollutants in Cyprus. *Sci. Tot. Environ.* Vol. 193, pp. 27 - 35.

Chauhan, L. K. S.; Pant, N.; Gupta, S. K. & Srivastava, S. P. (2000). Induction of chromosome aberrations, micronucleus formation and sperm abnormalities in mouse following carbofuran exposure. *Mutat. Res.,* Vol. 465, pp.123 - 129.

Collier, T. K.; Stein, J. E.; Goksoyr, A.; Myers, M. S.; Gooch, J. W.; Huggett, R. J. & Varanasi, U. (1993). Biomarkers of PAH exposure in oyster toadfish (*Opsanis tau*) from the Elizabeth River. Virginia. *Environ. Sci.,*Vol 2, pp.161 - 177.

Collins, A. R.; Dusinská, M.; Franklin, M.; Somorovská, M.; Petrovská, H.; Duthie, S.; Fillion, L.; Panayiotidis, M.; Raslová, K. & Vaughan, N. (1997). Comet assay in human biomonitoring studies: reliability, validation, and applications. *Environ. Mol. Mutagen.,*Vol. 30, pp. 139 - 146.

Collins, A. R. (2004). The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol. Biotech*., Vol. 26, pp. 249 - 261.

Cotelle, S, and Ferard, J. F. (1999). Comet assay in genetic ecotoxicology: a review. *Environ. Mol. Mutagen.*,Vol. 34, pp. 246 - 255.

Das, P. & John, G. (1999). Induction of sister chromatid exchanges and chromosome aberrations *in vivo* in *Etroplus suratensis* (Bloch) following exposure to organophosphorus pesticides. *Toxicol Lett*., Vol.104, pp. 111 - 116.

Devaux, A.; Pesonen, M. & Monod, G. (1999). Alkaline comet assay in rainbow trout hepatocytes. *Toxicol. In vitro*., Vol. 11, pp. 771 - 779.

EPA, (2001). Pesticides industry sales and usages: 1996 and 1997 market estimates. www.epa.gov/obeas1/pestsales/97pestsales/table1.html

Fairbairn, D. W.; OliveJ'. L. & O'Neill, K. L. (1995). The comet assay: a comprehensive review. *Mutat. Res.,*Vol. 339, pp. 37 - 59.

FAO. (2005). Fisheries Statistics Aquaculture Production, *FAO year book*, Vol. 100 (2), pp. 54 - 55.

Franekic, J.; Bratulic, N.; Pavlica, M. & Papes, D. (1994). Genotoxicity of dithiocarbamates and their metabolites. *Mutat. Res.,* Vol. 325 (2 - 3), pp. 65 - 74.

Frenzilli, G.; Nigro, M.; Scarcelli, V.; Gorbi, S. & Regoli, F. (2001). DNA integrity and total oxyradical scavenging capacity in the Mediterranean mussel, *Mytilus galloprovincialis*: a field study in a highly eutrophicated coastal lagoon. *Aquat. Toxicol.,* Vol. 53, pp. 19 - 32.

Garaj-Vrhovac, V. & Zeljezic, D. (2000). Evaluation of DNA damage in workers occupationally exposed to pesticides using single-cell gel electrophoresis (SCGE) assay. Pesticide genotoxicity revealed by comet assay. *Mutat. Res.,* Vol.469, pp. 279 - 285.

Garaj-Vrhovac,V. & Zeljezic, D. (2001) Cytogenetic monitoring of a Croatian population occupationally exposed to a complex mixture of pesticides. *Toxicology,* Vol.165, pp.153 - 162.

Garcia, O.; Romero, I.; Gonz´alez, J. E. & Mandina, T. (2007). Measurements of DNA damage on silver stained comets using free internet software. *Mutat. Res.,* Vol. 627, pp. 186 - 190.

Gossen, J. A. & Vijg, J. (1993). Transgenic mice as model systems for studying gene mutation *in vivo*. *Trends Genet.*, Vol. 9, pp. 27 - 31.

Grabuski, J.; Martin, P. A. & Struger, J. (2004). Pesticides in Ontario: A critical assessment of potential toxicity of urban use products to wildlife, with consideration for endocrine disruption. Phenoxy herbicides, chlorothalonil and chlorpyrifos. *Technical Report Series No. 410.* Vol. 3, Canadian Wildlife Service, Ontario Region, Burlington, Ontario, Canada.

Grover, P.; Danadevi, K.; Mahboob, M.; Rozati, R.; Banu, B. S. & Rahman, M. F. (2003). Evaluation of genetic damage in workers employed in pesticide production utilizing the Comet assay. *Mutagenesis,* Vol. 18, pp. 201 - 205.

Gupta, P. K. (2004). Pesticide exposure - Indian scene, *Toxicology,* Vol. 98, pp. 83 - 90.

Hai, D. Q.; Varga, S. I. & Matkovics, B. (1997). Organophosphate effects of antioxidant system of carp (*Cyprinus carpio*) and catfish (*Ictalurus nebulosus*). *Comp. Biochem. Physiol. Part C.*, Vol. 117, pp. 83 - 88.

Halliwell, B. (2002). Effect of diet on cancer development: is oxidative DNA damage a biomarker? *Free Radic. Biol. Med.*, Vol. 32, pp. 968 - 974.

Heaton, P. R.; Ransley, R.; Charlton, C. H.; Mann, S. J.; Stevenson, J.; Smith, B. H. E., Rawlings, J. M. & Harper, E. J. (2007). Application of single-cell gel electrophoresis (Comet) assay for assessing levels of DNA damage in canine and feline leukocytes. *The J. Nutrit.*, Vol. pp. 1598 - 1603.

Hodgson, E. & Levi, P. E. (1996). Pesticides: an important but underused model for the environmental health sciences. *Environ. Health Perspect.,*Vol. 104, pp. 97 - 106.

Kassie, F.; Laky, B.; Nobis, E.; Kundi, M. & Knasmüller, S. (2001). Genotoxic effects of methyl isothiocyanate. *Mutat. Res.,* Vol. 490, pp.1 - 9.

Kaushik, P. & Kaushik, G. (2007). An assessment of structure and toxicity correlation in organochlorine pesticides, *J. Hazard. Materials*, Vol.143, pp.102 -111.

Khan, M. Z. & Law, F. C. P. (2005). Adverse effects of pesticides and related chemicals on enzyme and hormone systems of fish, amphibians and reptiles: a review. Proc. *Pakistan Acad. Sci.,*Vol. 42, pp. 315 - 323.

Kligerman, A. D. (1979). Induction of sister chromatid exchanges in the central mudminnow following *in vivo* exposure to mutagenic agents. *Mutat. Res.,* Vol. 64, pp. 205 - 217.

Krane, D. E.; Sternberg, D. C. & Burton, G. A. (1999). Randomly amplified polymorphic DNA profile based measures of genetic diversity in crayfish correlated with environmental impacts. *Environ. Toxicol. Chem.*, Vol. 18, pp. 504 - 508.

Kubota, Y.; Shimada, A. & Shima, A. (1992). Detection of Gamma ray-induced DNA damages in malformed dominant lethal embryos of the Japanese medaka (*Oryzias latipes*) using AP - PCR fingerprinting. *Mutat. Res.,*Vol. 283, pp. 263 - 270.

Loft, S. & Poulsen, H. E. (2000). Antioxidant intervention studies related to DNA damage, DNA repair and gene expression. *Free Radical. Res.,* Vol. 33, pp. S67 - S83.

Louis, A. H.; Weighmann, D. L.; Hipkins. P. & Elizabeth, R. S. (1996). Pesticides and Aquatic Animals: A Guide to Reducing Impacts on Aquatic System. *Virginia Cooperative Extention,* Publication number 420-013.

Mitchelmore, C. L. & Chipman J. K. (1998). Detection of DNA strand breaks in brown trout (*Salmo trutta*) hepatocytes and blood cells using the single cell gel electrophoresis (comet) assay. *Aquat. Toxicol.*, Vol. 41, pp. 161 - 182.

Mohanty, G.; Mohanty, J.; Garnayak, S. K. & Dutta, S. K. (2009a) PCR based detection of furadan genotoxicity effects in rohu (*Labeo rohita*) fingerlings. *Vet. Res. Commun.*, Vol. 33, pp. 771-780.

Mohanty, G.; Mohanty, J.; Garnayak, S. K. & Dutta, S. K. (2009b) PCR based detection of Phorate genotoxicity effects in Rohu (*Labeo rohita*) fingerlings. *Res J Biotech,* pp. 23-30

Mohanty, G.; Mohanty, J.; Nayak, A. K.; Mohanty, S. & Dutta, S. K. (2011) Application of comet assay in the study of DNA damage and recovery in rohu (*Labeo rohita)* fingerlings after an exposure to phorate, an organophosphate pesticide. *Ecotoxicol.,*Vol. 20, pp. 283-292.

Moller, P.; Knudsen, L. E.; Loft, S. & Wallin, H. (2000). The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors. *Cancer. Epidemiol. Biomar.*, Vol. 9, pp.1005 - 1015.

Murty, A. S. (1986). Toxicity of Pesticides to fish. C.R.C Press Inc, I and II. pp. 483 - 355.

Nacci, D. E.; Cayulab, S. & Jackim, E. (1996). Detection of DNA damage in individual cells from marine organisms using the single cell gel assay. *Aquat. Toxicol.,* Vol. 35, pp.197 - 210.

Navarro, J. M. & Jorcano, J. L. (1999). The uses of arbitrarily primed polymerase chain reaction in cancer research. *Electrophoresis,*Vol. 20, pp. 283 - 290.

Ostling, O. & Johanson, K. J. (1984). Microelectrophoretic study of radiation - induced DNA damage in individual mammalian cells. *Biochem. Biophys. Res. Commun.,* Vol. 123, pp. 291 - 298.

Palmer, J. S. & Schlinke. J. C. (1973). Toxic effects of carbofuran in cattle and sheep. *J. Am. Vet. Med. Assoc.,* Vol. 162, pp. 561 - 563.

Pandey, S.; Nagpure, N. S.; Kumar, R.; Sharma, S.; Srivastava, S. K. & Verma, M. S. (2006). Genotoxicity evaluation of acute doses of endosulfan to freshwater teleost *Channa punctatus* (Bloch) by alkaline single cell gel electrophoresis. *Ecotoxicol. Environ. Saf.*, Vol. 65, pp. 56 - 61.

Pandrangi, R.; Petras, M. ; Ralph, S. & Vrzoc, M. (1995). Alkaline single cell gel (comet) assay and genotoxicity monitoring using bullheads and carp. *Environ. Mol. Mutagen.*, Vol. 26, pp. 345 - 356.

Pavlica, M.; Klobucar, G. I. V.; Mojas, N.; Erben, R. & Papes, D. (2001). Detection of DNA damage in haemocytes of zebra mussel using comet assay. *Mutat. Res.,* Vol. 490, pp. 209 - 214.

Pena-Llopis, S.; Ferrando, M. D. & Pena, J. B. (2003). Increased recovery of brain acetylcholinesterase activity in Dichlorvos-intoxicated European eels *Anguilla anguilla* by bath treatment with N-acetylcysteine. Dis. Aquat. Org. 55: 237 - 245.

Pinter, A.; Torok, G.; Sutjan, A.; Csik, M.; Bonsonyi, M. & Kelescsenyi, Z. (1989) Genotoxicity of selected herbicides. *Ann. 1st Super Sanita.,* Vol. 25, pp. 577 - 582.

Rencuzogullari, E. & Topaktas, M. (2000). Chromosomal aberrations in cultured human lymphocytes treated with the mixtures of carbosulfan, ethyl carbamate and ethyl methanosulfonate. *Cytologia,* Vol. 65, pp. 83 - 92.

Rojas, E.; Lopez, M. C. & Valverde, M. (1999). Single cell gel electrophoresis assay: methodology and applications. *J. Chromat. Part B.* Vol. 722, pp. 225 - 254.

Savva, D.; Castellani, S.; Mattei, N.; Renzoni, A. & Walker, C. H. (1994). The use of PCR and DNA fingerprints to detect the genotoxic effects of environmental chemicals. In.

Environmental Contamination, *Proceedings of the 6th International Conference,* (Eds. Varnavas, S. P.), Delphi, Greece, CEP Consultants, Edinburgh. pp. 105 - 110.

Schnurstein, A. & Braunbeak, T. (2001). Tail moment versus tail length—application of an *in vitro* version of the comet assay in biomonitoring for genotoxicity in native surface waters using primary hepatocytes and gill cells from zebrafish (*Danio rerio*). *Ecotoxicol. Environ. Saf.*,Vol. 49, pp.187 - 196.

Shah, R. G.; Lagueux, J.; Kapur, S.; Levallois, P.; Ayotte, P.; Tremblay, M.; Zee, J. & Poirier, G. G. (1997). Determination of genotoxicity of the metabolites of pesticides guthion, sencor, lorox, reglone, daconil and admire by 32P postlabeling. *Mol. Cell. Biochem.*, Vol. 169, pp. 177 - 184.

Shugart, L. & Theodorakis, C. (1994). Environmental genotoxicity: probing the underlying mechanisms. *Environ. Health Perspect.,* Vol.102 (12), pp. 13 - 17.

Shugart, L. R. & Theodorakis, C. (1996). Genetic ecotoxicology: the genotypic diversity aroach. *Comp. Biochem. Physiol.* Vol. 113C (2), pp. 273- 276.

Shugart, L. R. (2000). DNA damage as a biomarker of exposure. *Ecotoxicology*, Vol. 9, pp. 329 - 340.

Singh, K. P. & Roy, D. (1999). Detection of mutation(s) or polymorphic loci in the genome of experimental animal and human cancer tissues by RAPD/AP-PCR depend on DNA polymerase. *Internat. J. Oncol.*, Vol. 14, pp. 753 - 758.

Singh, N. P.; McCoy, M. T.; Tice. R. R. & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* Vol. 175, pp. 184 – 191.

Theodorakis, C. W. & Shugart, L. R. (1997). Genetic ecotoxicology II: population genetic structure in mosquitofish exposed in situ to radionuclides. *Ecotoxicol.,* Vol. 6, pp. 335 - 354.

Theodorakis, C. W.; Elbl, T. & Shugart, L. R. (1999). Genetic ecotoxicology IV: survival and DNA strand breakage is dependent on genotype in radionuclide exposed mosquitofish. *Aquat. Toxicol.,* Vol. 45, pp. 279 - 291.

Toraason, M.; Albertini, R.; Bayard, S.; Bigbee, W.; Blair, A. & Boffetta, P. (2004). Alying new biotechnologies to the study of occupational cancer—a workshop summary. *Environ. Health Perspect.,* Vo1. 12, pp. 413 - 416.

Tripathy, N. K.; Dey, L.; Majhi, B. & Das, C. C. (1988). Genotoxicity of zineb detected through the somatic and germ - line mosaic assays and the sex - linked recessive - lethal test in *Drosophila melanogaster*. *Mutat. Res.,* Vol. 206, pp. 351-360.

Tripathy, N. K.; Majhi, B.; Dey, L. & Das, C. C. (1989). Genotoxicity of ziram established through wing, eye and female germ - line mosaic assays and the sex - linked recessive lethal test in *Drosophila melanogaster*. *Mutat. Res.,* Vol. 224, pp.161-169.

Van der Werf, H. M. G. (1996). Assessing the impact of pesticides on the environment. *Agric. Ecosyst. Environ.,* Vol. 60, pp. 81 - 96.

Welsh, J. and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers, *Nucleic Acids Res.,* Vol. 18, pp. 7213 - 7218.

Williams, G. M. & Weisburger, J. H. (1991). Chemical carcinogenesis. In. *Casarett and Doull's Toxicology*. *The Basic Science Poisons* (Eds. Amdur, M. O., Doull, J. and Klaassen, C. D.), 4th ed. Pergamon Press, New York. pp. 127 - 200.

Williams, J. G. K.; Kubelik, A. R.; Livak, K. J.; Rafalski, J. A. & Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.,* Vol. 18, pp. 6531 - 6535.

Yadav, J. S. & Kaushik, V. K. (2002). Studies on the Genotoxicity of an Organophosphorous Pesticide Baytex-1000. *IJHG.,* Vol. 2, pp. 19-25.

19

Gene Expressions of the *Dhb*, *Vtg*, *Arnt*, *CYP4*, *CYP314* in *Daphnia magna* Induced by Toxicity of Glyphosate and Methidathion Pesticides

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1. Introduction

Pesticides, the chemicals used commonly in agriculture to control pests, pathogens and weeds, have been contributing to the serious contamination of the aquatic environment through spray drift, volatilization, drainage and leaching (Cerejeira et al., 2003; Pereira et al., 2009). Among various pesticides, methidathion is a highly toxic insecticide used to control a wide spectrum of agricultural insect and mite pests. In the other hand, glyphosate, the active ingredient in many commercial weed-killing formulation (e.g., Roundup), is widely used in agricultural, silvicultural and urban environment (Borggaard, K.O., and Gimsing, L.A., 2008). The results are an increasing detection of these pesticides in the environment, especially in aquatic system may have some ecotoxicological impacts on non-target aquatic organisms (Tsui et al., 2003; Vorkamp et al., 2002). *D. magna*, a freshwater crustacean, has been used extensively to evaluate the toxic effects of chemical on aquatic system (EPA, 2002) because of their high sensitivity to a wide range of chemicals, a short lifecycle, and ease of manipulation in the laboratory. In addition, the daphnia are ubiquitous and play a key role in aquatic food web (Soetaert et al., 2006). Conventionally, the toxicity assays (e.g., acute or chronic toxicity tests) have widely been used to evaluate the aquatic toxicity as well as the adverse impacts of the toxic chemicals on aquatic organisms based on the phynotic endpoints such as the survival, growth, and reproduction (Colleen et al., 2005; Heckmanna et al., 2007). These body responses result from some molecular responses (e.g., gene expression) in organisms that expose to a toxic environment. Therefore, changing in gene expression in the organisms should happen first, and be a more sensitive indicator to the toxic chemicals than the body responses (Jo et al., 2008; Le et al., 2010; Le et al., 2011). Notably, when an organism is exposed to a toxic environment, the metabolic activity in the organism will change to overcome the adverse effects (Ankley and Villeneuve, 2006). Certain genes, possessing the particular functions, express differently in organisms that are exposed to toxicants. For instance, hemoglobins (*Dhb*), the protein are distributed widely in all organisms, are composed of multiple two-domain chains with a relatively normal oxygen binding activity when found in the hemolymph of *D. magna* (Tokishita et al., 1997; Anderson et al., 2008). Vitellogenin (*Vtg*) is a major lipoprotein in many oviparous animals and has

been used as a useful biomarker to examine the hazardous effects of endocrine disrupting compounds (EDCs) (Jones et al., 2000; Kato et al., 2004). Aryl hydrocarbon receptor nuclear translocator (*Arnt*) activates the transcription of the genes that encode the enzymes involved in metabolizing aryl hydrocarbons, such as dioxin and endocrine disruptors in mammalian cells and marine, freshwater and avian species (Tokishita et al, 2006). Hence, the *Arnt* gene probably responds to the altered metabolic effect of exposure to aryl hydrocarbons. Cytochrome P450s (CYPs) are a large and ubiquitous super-family of heme proteins that are encoded by receptor-dependent transcriptional activation genes, and are a class of proteins that respond to the hazardous effect of toxic chemicals (Snyder, 2000). CYPs are categorized into 4 different families, i.e., mitochondrial, CYP2, CYP3-like, and *CYP4* family (Bradfield et al., 1991; Baldwin et al., 2009). In the present study, the hazardous effects of two selected pesticides (i.e., glyphosate, and methidathion) on *D. magna* were examined by studying the changes in the gene expressions of five stress responsive genes, including *Dhb, Arnt*, *Vtg*, *CYP4*, and *CYP314* using the method of reverse transcription polymerase chain reaction (RT-PCR). This technique is a simple and effective tool to study the gene expression (Stephen et al., 2008). The expression level of a gene is measured by determining the mRNA amount generated by that gene via the transcription. Through the gene expression analysis, the molecular responses of *D. magna* exposed to pesticides can be determined to provide an insight into the action mode of chemicals.

The aim of this study was to evaluate toxicity of the two pesticides and to analyze their adverse effects on the expression patterns of different genes in *D. magna*. Thereby, the mechanisms controlling the gene expressions were indirectly studied in response to the addition of pesticides, i.e., glyphosate and methidathion. Five different genes including hemoglobin (*Dhb*) (Ha and Choi, 2009), vitellogenin (*Vtg*) (Jones et al., 2000), aryl hydrocarbon receptor nuclear translocator (*Arnt*) (Tokishita et al., 2006), cytochrome P450 *CYP4* family (*CYP4*) (Scharf et al., 2001), and cytochrome P450 mitochondrial family 314 (*CYP314*) (Shen et al., 2003) were selected for this study to quantify the expression level and analyze the physiological changes and different expression mechanisms for each of the genes.

2. Experimental methods

2.1 Daphnia magna culture

D. magna (the Korea Institute of Toxicology, Daejeon, Korea) were cultured and handled according to the USEPA manual (US Environmental Protection Agency, 2002). The organisms were cultured at 20 ± 1°C in 2 L beakers containing 1.5 L of hard reconstituted water (HRW) prepared by adding 0.12 g/L $MgSO_4$, 0.192 g/L $NaHCO_3$, 0.008 g/L KCl, 0.12 g/L $CaCO_3$ into deionized water distilled using a Minipore Milli-Q apparatus. This HRW was controlled at a pH of 8.2 ± 0.2 and aerated for at least 24 h prior to use. Three times per week, the medium for the *D. magna* culture was renewed with fresh HRW and fed with algae (*Chlorella vulgaris*) and YTC (a mixture of yeast, cerophyll, and Trout chow) purchased from Aquatic Biosystem Inc. (Colorado, US). The number of *D. magna* was adjusted to about 30 to 50 organisms per 2 L culture vessel. A photoperiod of 16 h light : 8 h darkness was applied.

2.2 Acute toxicity test

Acute toxicity test was performed following the standard US EPA manual (2002) to determine the lethal endpoint caused by glyphosate and methidathion (Fluka, US). In all of

the tests, the less-than-24h-old *D. magna* neonates collected from the less-than-30 d-old female organisms were used for the experiment. Ten neonates per test vessel were exposed to several concentrations of glyphosate and methidathion. Each test concentration was prepared using a volume of 50ml with three replicates and maintained at 20 ± 1 ^{o}C during a 24 h photoperiod of 16 h light : 8 h darkness without any feeding.

2.3 Chronic toxicity test

The chronic effects on the physiological responses of *D. magna* were studied in this experiment. All of the test conditions were performed according to the US EPA protocol (2002). One daphnia neonate (less than 24-h old) was individually cultured in a 50ml vessel and continuously exposed to three sublethal concentrations (1/100LC50, 1/50LC50, 1/10LC50) of the pharmaceuticals for 21 days. Ten replicates were conducted for each chemical concentration. The experimental conditions and feeding regime were similar to the conditions that were described for the daphnia culture. The test solutions were renewed every two days. The oxygen concentration and the pH of the HRW were checked weekly to ensure that they were not affected by the biological responses. The growth, survival, and reproduction were the three endpoints for evaluating the chronic effects of the pharmaceuticals on the physiological responses of *D. magna*. The growth was determined by measuring the length of the daphnia after the 21 day test using a microscope (Olympus SZX9, Japan). Mortality was observed everyday at the same time. The reproduction was evaluated using two different parameters, the number of offspring per tested organism and the time of first reproduction.

2.4 Exposure experiments to analyze the gene expression

To study the effect of acute toxicity of chemical on the gene expression, the tested concentrations of glyphosate and methidathion were LC5, LC10, LC20, and LC50, which were defined as the concentrations where the percentages of dead testing daphnia were 5, 10, 20, and 50%, respectively. These sublethal concentrations were determined previously through the acute toxicity test. This exposure test was performed according to the USEPA manual 2002. The 20d-old daphnia collected from less than 30-d old mother daphnia were used in this experiment. Before being exposed to the chemicals, the organisms were starved for 24 h under the culturing conditions described above. After that, the exposure tests were conducted using ten organisms per 200ml test solution volume in 500ml vessel with three replicates. Test conditions were controlled at 20 ± 1 ^{o}C for 24 h with a photoperiod of 16-h light : 8-h darkness. In the other hand, effect of chronic toxicity of glyphosate and methidathion on the gene expression was studied on all daphnia used for the 21d chronic toxicity assay. Particularly, at the day 21 of the chronic assay, all alive tested organisms were collected into the 1.5ml eppendorf tube and then isolated the total RNA using Trizol method. Next, the amount of messenger RNA for each specific gene, which is the result of the gene expression, was measured using the semi-quantitative reverse transcriptase polymerase chain reaction (RTPCR).

2.5 Isolation of RNA samples and Semi-quantitative reverse transcriptase-polymerase chain reaction

A semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used to analyze the gene expression in *D. magna* that were exposed to pesticides. The pesticide-

treated daphnia were harvested, and the total RNA was isolated using Trizol method. After isolation, the concentrations of the total RNA in the samples were measured using a Qubit kit (Invitrogen, US). The mRNA levels were determined using the semi-quantitative RT-PCR with a TaKaRa RNA PCR™ Kit (TaKaRa Inc., Japan). In the case of *D. magna*, the PCR primers were designed for six genes, where the actin gene was used as an internal standard (Table 1). On the other hand, five specific genes were used to analyze the expression patterns in *D. magna* after exposure to the pesticides. The PCR experiments were carried out using 30 cycles with a T Gradient Thermocycler (Biometra, Germany) to amplify the specific genes. All of the RNA samples were also used without a RT step for PCR to detect the presence of the genomic DNA contamination. No DNA was detected at any time. The PCR products were visualized on an EtBr-stained agarose gel. The intensities of DNA band observed on the UV light are quantified for the gene expression level using GelScope 1.5 software (Imageline Inc., USA). The PCR was carried out three different times to confirm the reproducibility of the results. All of the results were reported as mean values. The relative expression level of a gene is determined by dividing the expression level of that gene to that of an internal standard (i.e., actin gene).

Genes	Description	Gene bank accession No.	Primer (5′-3′)	Amplicon length (bp)
Dhb	Hemoglobin	AAC47544	Forward: CACCACTGTGACTACCACTG Reverse: CAGCTTTCTTGAGGTTTTTG	506
Vtg	Vitellogenin	AB252738	Forward: ATGCTGAGAACACCGTCTAC Reverse: GTGGGTCTTGTAGTCGTCAT	530
Arnt	Aryl hydrocarbon Receptor Nuclear Translocator	AB242866	Forward: CAGTTTCTGGAGAGGTTACG Reverse: GGTGGAACTACAGGTGATTG	508
CYP4	Cytochrome P450 4 family	AB257772	Forward: ATGGATTTACCAACAAGGTG Reverse: AGGTAATACGAGCAGATGGA	502
CYP314	Cytochrome P450 314 family	AB257771	Forward: ACGCGTAGTGAAAGTGATTT Reverse: TTACAGTATGATCCCCAAGG	203
Act	Actin	AJ292554	Forward: GAGACCGTCTACAACTCGAT Reverse: GTGTCGACAGAGACAATGAG	491

Table 1. Primer sequences of the genes used for the RT-PCR

2.6 Statistical analysis

All of the data were obtained from three independent samples carried out simultaneously for error analysis, and the results were shown along with the standard deviations and

correlations between the cell mortality and the experimental conditions. The data were analyzed using a Sigma Plot (SPS Chicago, IL. USA), and a p value < 0.05 was considered significant. The probit method, which is a parametric statistical method for estimating with 95% confidence limits, was used to calculate the sub-lethal concentrations of pesticides such as LC5, LC10, LC20, and LC50.

3. Results and discussion

3.1 Acute toxicity of glyphosate and methdiathion

The cellular toxicities of glyphosate and methidathion as pesticides were investigated using an acute 24-h toxicity test with 10 neonates of *D. magna* (less than 24-h old). In Figure 1, the mortality percentages of the test organisms increased in a sigmoid-curve relationship with increasing concentrations of glyphosate and methidathion. In particular, all of the neonates died when exposed to 350 mg/L glyphosate, as well as 0.10 mg/L methidathion. Based on the regression curves of the acute toxicity data, LC5, LC10, LC20, and LC50 were determined as the lethal concentrations of a chemical when the percentage of *D. magna* mortality was 5%, 10%, 20% and 50%, respectively. These values related to the lethal concentrations showed a 95% confidence interval. In this study, four lethal concentrations were used to show the specific response patterns of *D. magna* to glyphosate and methidathion through the analysis of the gene expression patterns using a semi-quantitative RT-PCR.

3.2 Effects of acute toxicity of glyphosate and methidathion on gene expression

To examine the effects of glyphosate on the gene expression in *D. magna*, ten 20-d old organisms were exposed to four different LC values (LC5, LC10, LC20 and LC50) 190, 202, 214, and 234 mg/L for 24 hours. In this study, the *Act* gene was selected as an internal standard gene. The expression level of the *Act* was constant in all experimental conditions (data not shown). The relative expression levels of the five selected genes were determined by normalizing to the *Act* expression level (Fig. 2). Three of the five genes selected in this study, excluding *Vtg* and *CYP314*, showed sensitive responses to glyphosate. Particularly, *Arnt* and *CYP4* were down-regulated after glyphosate exposure, and although *Dhb* transiently increased at 190 mg/L of glyphosate, the expression patterns of *Dhb* decreased with increasing concentrations of glyphosate.

On the other hand, four different LC values (LC5, LC10, LC20 and LC50) of methidathion, i.e., 0.024, 0.029, 0.034 and 0.044 mg/L were also used to study the expression patterns of the five selected genes in this study (Fig. 3). The effect of methidathion on the expression of *Dhb* and *Arnt* was the same as glyphosate, indicating the reduced gene expression after methidathion exposure. Interestingly, *CYP4* and *CYP314*, which are categorized into the CYP family, had significantly different expression patterns for glyphosate and methidathion. These results showed that exposure of *D. magna* to glyphosate led to a down regulated expression of the *CYP4* gene but no significant responses in the expression of the *CYP314* gene. However, in exposure to the increasing concentrations of methidathion, the *CYP4* gene expression did not differ, but the expression of *CYP314* gradually decreased compared to control.

Cytochrome P450s (*CYPs*), a large and ubiquitous super-family of heme proteins encoded by receptor-dependent transcriptional activation genes, are a class of proteins that respond to the hazardous effects of toxic chemicals (Snyder, 2000). Although both *CYP4* and *CYP314* belong to the same group of functional proteins, their different families elicited different susceptibilities to glyphosate. *CYP4*, which metabolizes endogenous compounds such as

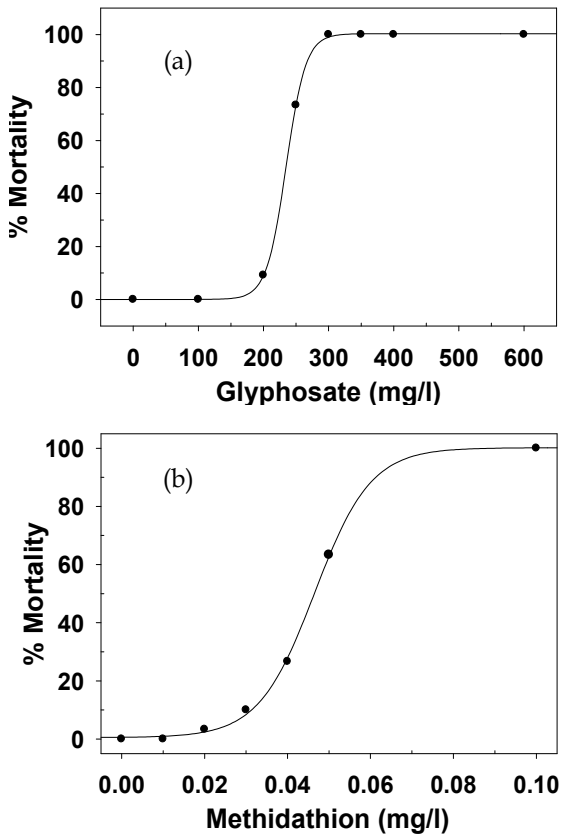

Fig. 1. Acute toxicity assay of *D. magna* for glyphosate (a) and methidathion (b).

fatty acids and steroids (Brandfield et al., 1991), is used for insecticide resistance in insects (Pittendrigh et al., 1997; Scharf et al., 2001). On the other hand, *CYP314* was subcategorized into mitochondrial CYPs and is required for the conversion of ecdysone to its active form (Shen et al., 2003). Therefore, based on these results, glyphosate probably creates more serious effects in fatty acids and steroids metabolisms and the ecdysis of *D. magna* would be influenced by methidathion.

Hemoglobin (*Dhb*), an important protein in many advanced organisms, is required for the formation of the constitutive part of their oxygen transport system (Aderson et al., 2008). Its expression is usually increased by most of the tested chemicals that reduce the oxygen level in aquatic systems (Ha et al., 2009). However, the increasing oxygen consumption is not always associated with an up-regulated expression of the hemoglobin gene (Anderson et al., 2008). For instance, the exposure of Chironomus midge to atrazine herbicide resulted in the elevated oxygen consumption in atrazine-treated midge that associated with a decreased expression level of the *Hb* genes (Anderson et al., 2008). In this study, the expression level of *Hb* in *D. magna* that was exposed to glyphosate slightly increased at a low concentration (LC5) and decreased gradually in response to the higher concentrations (LC20, LC50) in Figure 2a. Similarly, the expression of the *Hb* gene was significantly down regulated in *D. magna* after methidathion exposure (Fig. 3a).

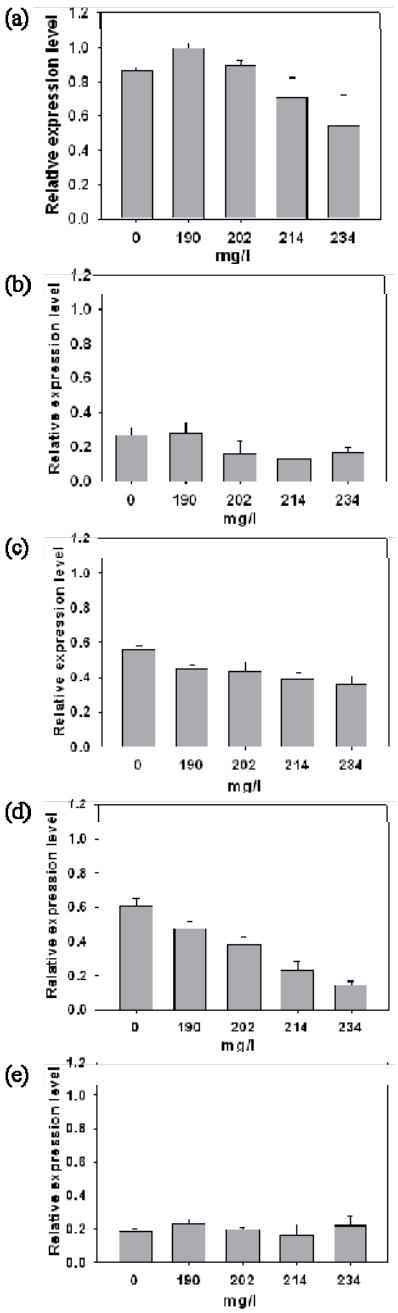

Fig. 2. Relative expression levels of 5 selected genes after 24h exposures of 0, 190, 202, 214, and 234 mg/L glyphosate. (a) *Dhb*, (b) *Vtg*, (c) *Arnt*, (d) *CYP4* and (e) *CYP314*. All of the data correspond to the expression level relative to the *Act* gene.

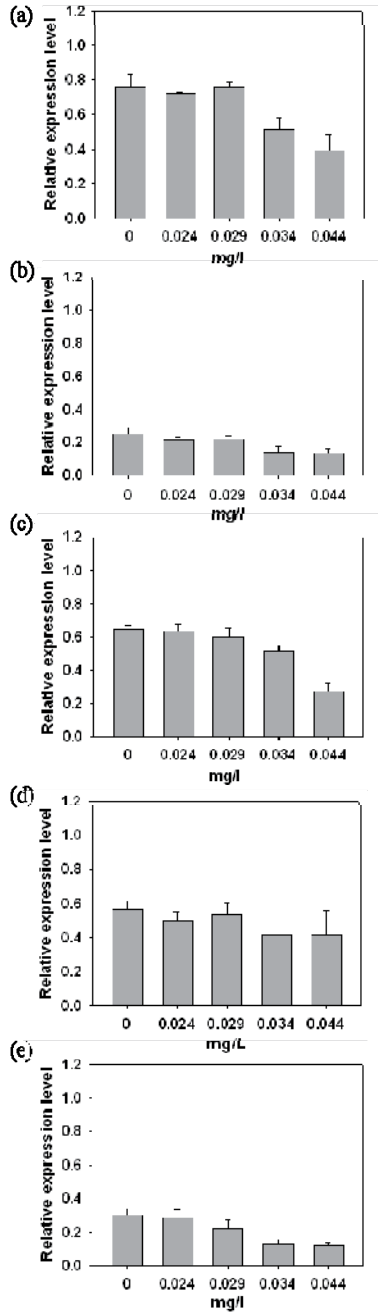

Fig. 3. Relative expression levels of 5 selected genes after 24h exposures of 0, 0.024, 0.029, 0.034, and 0.044 mg/L methidathion. (a) *Dhb*, (b) *Vtg*, (c) *Arnt*, (d) *CYP4* and (e) *CYP314*. All of the data correspond to the expression level relative to the *Act* gene.

The aryl hydrocarbon receptor nuclear translocator (*Arnt*) activates the transcription of the genes that encode the enzymes involved in metabolizing aryl hydrocarbons, such as dioxin and endocrine disruptors in mammalian cells and marine, freshwater and avian species (Tokishita et al., 2006). In this study, the expression of *Arnt* in *D. magna* gradually decreased in the dose-dependent response to both glyphosate (Fig. 2c) and methidathion, leading to the metabolism of the toxic compounds tested in this study (Fig. 3c).
Finally, vitellogenin is the precursor of the egg-yolk protein, vitellin, which provides an energy supply for embryo development in oviparous animals. Vitellogenin is considered as a biomarker for estrogenic compound because the production of vitellogenin is controlled by some estrogen hormones (Jones et al., 2000; Matozzo et al, 2008). In this study, no significant differences were discovered for the vitellogenin expression in *D. magna* in response to the sublethal concentrations of glyphosate (Fig. 2b) and methidathion (Fig. 3b) for the 24h duration. These results indicated that neither the acute toxicity of glyphosate nor methidathion induced estrogenically.

3.3 Chronic toxicity of glyphosate and methdiathion

The chronic toxicity of pesticides (i.e., glyphosate and methidathion) was examined based on their adverse impacts on the survival, growth and reproduction of *D. magna* after 21d exposing to sublethal concentrations. Three sublethal concentrations were selected for the chronic test included 1/100, 1/50, and 1/10 of LC50 glyphosate and methidathion in Table 2, and 3, respectively.

Glyphosate (ppm)	Suvival (%)	Body length (mm)	Time of first reproduction (day)	Number of offsprings per female (organism)
Control	100	3.87±0.18	13.2±1.03	28.8±8.26
2.34	100	3.86±0.16	13.0±1.05	28.2±11.3
4.68	100	3.90±0.24	13.6±1.33	25.3±7.65
23.4	100	3.45±0.15	15.4±0.97	11.3±2.54

Table 2. Survival and reproduction of *D. magna* after a 21d exposure to glyphosate

Methidathion (ppb)	Suvival (%)	Body length (mm)	Time of first reproduction (day)	Number of offsprings per female (organism)
Control	100	3.89±0.08	12.6±1.2	49.5±14.1
0.02% ethanol	100	3.77±0.16	12.8±0.8	48.8±9.70
0.44	100	3.88±0.06	13.9±1.9	46.6±16.6
0.88	90	3.74±0.11	14.3±2.8	38.0±25.1
4.4	0	na	na	0

Table 3. Survival and reproduction of *D. magna* after a 21d exposure to methidathion

The addition of 0.02% ethanol into culture media, the highest content of ethanol among all experiments, was also tested in comparison with control because ethanol was used to

prepare stock solution of methidathion. There were no significant differences of *D. magna* behaviors between addition of 0.02% ethanol and control (Table 3).

Except the highest of these tested concentrations (1/10 LC50), which gave an obvious effect on the survival and reproduction of organisms, the other concentrations including 1/100 and 1/50 LC50 showed a little difference compared to control. After 21d chronic toxicity experiment, the survival of daphnia exposed to 1/100, 1/50, 1/10 LC50 glyphosate were all equal to control (100%) while those exposed to methidathion were 100%, 90%, and 0%, respectively. The body length of daphnia organisms exposed to the low concentrations (1/100 and 1/50 LC50) of both pesticides were not significantly different from those of control. However, the size of daphnia exposed to 1/10 LC50 glyphosate was only 3.45±0.15mm, and about 10.85% smaller than that of control daphnia, which was 3.87±0.18mm. In case of exposing to 1/10LC50 methidathion, the size of daphnia could not be measured because all of the tested organisms died before the day 21.

Additionally, the reproduction ability of daphnia, which was represented by two tesed parameters including time-of-first-reproduction and number-of-offsprings, was also examined by the 21d chronic toxicity experiment. As shown in the table 2, no significant differences in these parameters were observed for the daphnia exposed to both 1/100 and 1/50 LC50 glyphosate compared to the control organisms. But, the time-of-first-reproduction was delayed from 13.2±1.03d to 15.4±0.97d and the number-of-offspring was dramatically reduced from 28.8±8.26 offsprings to 11.3±2.54 offsprings when daphnia was exposed to the 1/10LC50 glyphosate during 21d experiment. In case of methidathion, while the 1/10LC50 was high enough to kill all organisms before their reproduction, the 1/100LC50 was too weak to make any obvious effects on the reproduction ability (Table 3). In case of the exposure to 1/50LC50 methidathion, there was slightly adverse influence on the reproduction of organisms. Particularly, the time-of-first-reproduction was delayed from 12.6±1.2d to 14.3±2.8d and the number-of-offspring was reduced from 49.5±14.1 offsprings to 38.0±25.1 offsprings. The results indicated that only the 1/10LC50 concentration, which is highest among the tested concentrations, significantly affected on the physiological behaviors of organisms while the 1/100 and 1/50LC50 gave almost no obvious influences on the survival, and reproduction of *D. magna*.

3.4 Effects of chronic toxicity of glyphosate and methidathion on gene expression

The gene expressions of the five genes in *D. magna* were investigated by RTPCR technique using the total RNA isolated from organisms exposed to glyphosate and methidathion for 21 days. Among these examined genes, *Dhb* was the only gene of which expression level was not changed in daphnia following the 21d exposures to all tested doses including 2.34, 4.68, and 23.4 mg/L glyphosate compared to control (Fig. 5a) while the expression level of *CYP4* gene was slightly increased in response to those glyphosate concentrations (Fig. 5d). However, the other genes such as the *Vtg*, *Arnt*, and *CYP314* genes were significantly up-regulated after 21d exposing to glyphosate (Fig. 5b, 5c, 5e). The expression level of these genes substantially rose when the exposing concentration increased from 0 to 4.68 mg/L, and kept maintaining when it increases from 4.68 mg/L to 23.4 mg/L. Ethanol is the solvent used to dissolve methidathion and prepare some stock solutions for the chronic toxicity experiment. The 0.02% ethanol, a maximum percentage of ethanol exposed to tested *D. magna* in all experiments, was examined to confirm its effects on the gene expression. As shown in the Figure 4, there were no significant differences of the expression level of all five selected genes in *D. magna* after a 21d exposure to 0.02% ethanol compared to the control.

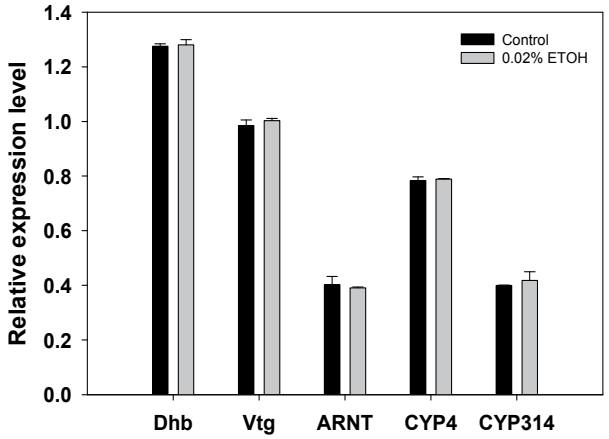

Fig. 4. Effects of 20% ethanol on the relative expression levels of 5 selected genes.

In case of methidathion, the gene expression of the five genes can be examined for only daphnia exposed to 0.44 and 0.88 μg/L because all daphnia exposed to 4.4 μg/L methidathion were died before the day 21 (Fig. 6). Unlike glyphosate, which was tolerantly influenced on the gene expression, methidathion significantly induced the expression level of the five genes when daphnia exposed to low concentration (0.44 μg/L), but reduces the expression level of those genes when daphnia exposed to the higher concentration (0.88 μg/L). It can be explained that methidathion may damage the normal functions of DNA in *D. magna* when the exposure concentration reaches to the 0.88 μg/L. In the other hand, the exposure to the 0.44 μg/L methidathion up-regulated the expression of the *Dhb*, *Vtg*, and *CYP314* genes stronger than that of the *Arnt* and *CYP4* genes. Notably, while the chronic toxicity assay based on the conventional endpoints such as growth, survival, and reproduction gave out some general information about the impacts of glyphosate and methidathion on the physiological behaviors of *D. magna*, the study on expression pattern of the five selected genes including *Dhb*, *Vtg*, *Arnt*, *CYP4*, and *CYP314* may provide a better insight into the action mode of these pesticides.

Hemoglobin is an important protein for the oxygen transport system of animals. Its expression is usually upregulated by the chemicals that reduce the oxygen level in aquatic environment (Ha et al. 2009). In this study, the expression level of *Dhb* was not changed after 21d exposure to the three concentrations of glyphosate (Fig. 5a), but slightly upregulated in response to 0.44 μg/L methidathion (Fig. 6a). This indicates that methidathion may have more possibility than glyphosate to inhibit the regular interaction between hemoglobin and free oxygen molecule in the aquatic environment. Vitellogenin, which its production is controlled by some estrogen hormones (Jones et al., 2000; Matozzo et al, 2008), is considered as a biomarker for estrogenic compound. In this study, while expression level of *Vtg* gene reduced in response to acute toxicity of glyphosate (Fig. 2b) and methidathion (Fig. 3b), that increased when exposing organism to the low concentration of these pesticides for 21days (Fig. 5b and 6b). This indicates that chronic toxicity of both glyphosate and methidathion may stimulate the synthesis of some estrogenic compounds in *D. magna*. The aryl hydrocarbon receptor nuclear translocator (*Arnt*), which plays a role in the synthesis of the enzymes involved in metabolizing aryl hydrocarbons (Tokishita et al.,

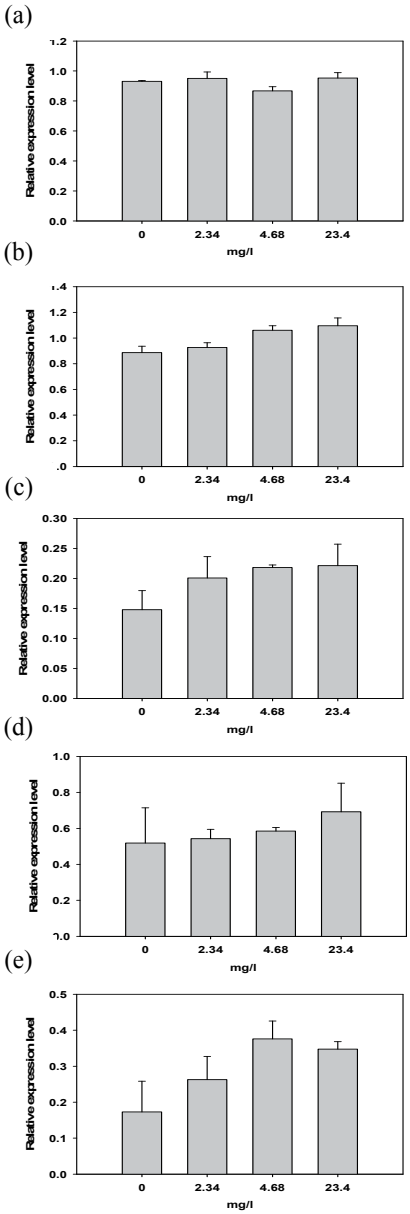

Fig. 5. Relative expression levels of 5 selected genes after 21d exposures of 0, 2.34, 4.68 and 23.4 mg/L glyphosate. (a) Dhb, (b) Vtg, (c) Arnt, (d) CYP4 and (e) CYP314. All of the data correspond to the expression level relative to the Act gene.

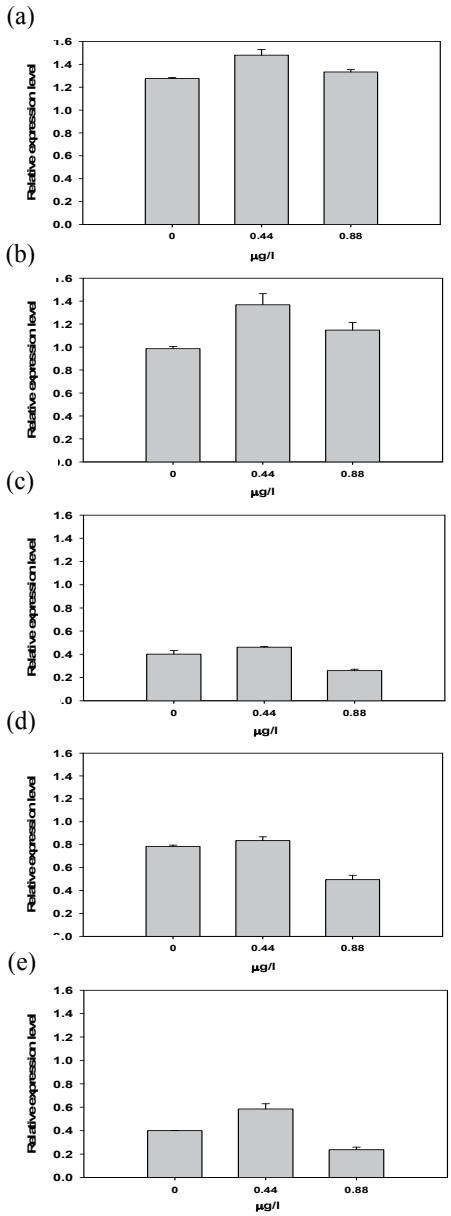

Fig. 6. Relative expression levels of 5 selected genes after 21d exposures of 0, 0.44, and 0.88 µg/L methidathion. (a) Dhb, (b) Vtg, (c) Arnt, (d) CYP4 and (e) CYP314. All of the data correspond to the expression level relative to the Act gene

2006), was differently regulated by the short-time and long-time exposure to both glyphosate and methidathion (Fig. 2c, 3c, 5c, and 6c). Particularly, the expression level of *Arnt* gradually decreased in *D. magna* when these organisms were exposed to the high doses of pesticides for 24h (Fig. 2c, and 3c), but it slightly increased when exposing to the low concentration of glyphosate (2.34μg/L) and methidathion (0.44μg/L) for 21d (Fig. 5c, and 6c). These results suggested that the low concentrations of glyphosate and methidathion could activate the expression of the *Arnt* gene in *D. magna* while the exposure to high dose of these pesticides even just for the short time can post some adverse effects on the molecular responses in organism. Although both proteins encoded by *CYP4* and *CYP314* genes belong to a same class of proteins that respond to the hazardous effects of toxic chemicals (Snyder, 2000), their different families caused different susceptibilities to pesticides. Particularly, in the 21d exposure to the low concentrations of glyphosate (Fig. 5d) and methidation (Fig. 6d), expression level of *CYP4* gene was slightly raised while that of *CYP314* was substantially increased compared to control. Therefore, *CYP314*, which is required for the conversion of ecdysone to active form (Shen et al., 2003), could be a more sensitive indicator to glyphosate and methidathion than *CYP4*, which metabolizes endogenous compounds such as fatty acids and steroids (Brandfield et al., 1991).

4. Conclusions

Glyphosate and methidathion, which have widely been used in agriculture to protect the crop productivity, are contaminating the aquatic environment through some main routes such as spray drift, volatilization, drainage, and leaching (Cerejeira et al., 2003; Pereira et al., 2009). Commonly, the toxicity tests based on the physiological responses (e.g., survival, growth rate, and reproduction) have been used to examine the biological effects of chemicals (Sakai, 2002; De Schamphelaere, 2004). In this study, the results indicated that both glyphosate and methidathion posted some adverse effects on the non-target aquatic organisms (e.g., *D. magna*) by both the acute and chronic toxicity. However, these physiological responses primarily result from molecular responses such as changes in gene expressions that happen in organisms in exposure to toxicants. Therefore, the changes in gene expressions should be more sensitive indicators than conventional endpoints to assess toxicity of chemicals. While *D. magna* showed specific response patterns according to the toxic effects caused by glyphosate and methidathion, the manners of expression for *Dhb*, *Vtg*, *Arnt*, *CYP4* and *CYP314* were quite different in *D. magna*. In the 24h exposure, the high concentrations of glyphosate and methidation may damage the regular functions of DNA in *D. magna*, and that result in the significant down-regulation of expression of the selected genes. *CYP4* and *CYP314* most sensitively responded as biomarkers to identify glyphosate and methidathion among the various pesticides. However, the results of the 21d exposure indicated that the low concentration of these pesticides induced the gene expression in daphnia. Among the five selected genes, *Vtg* and *CYP314* genes are substantially upregulated by both glyphosate and methidathion in long time exposure. Probably, the daphnia has a specific mechanism to adapt to the toxic influences of both glyphosate and methidathion. Therefore, the results of this study suggested that the differences between the gene expression patterns caused by glyphosate and methidathion must be considered to further understand the action mode of these chemicals and the specific molecular responses in *D. magna* as well. In further study, the effects of these toxic chemicals on the expression level of some other genes (not only the five genes used in this study) as well as their protein

expression level should be investigated to understand molecular mechanisms underlying the ability of *D. magna* to adapt to the toxic environments.

5. Acknowledgements

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6. References

Ankley, G.T., Villeneuve, D.L., 2006. The fathead minnow in aquatic toxicology: Past, present and future. Aquat. Toxicol. 78, 91–102.

Anderson, T.D., Ying, J.C., Begum, K., Starkey, S.R., Zhu, K.Y., 2008. Gene expression profiling reveals decreased expression of two hemoglobin genes associated with increased consumption of oxygen in *Chironomus tentans* exposed to atrazine: A possible mechanism for adapting to oxygen deficiency. Aquat. Toxicol. 86, 148–156.

Baldwin, W.S., Marko, P.B., Nelson, D.R., 2009. The cytochrome P450 (CYP) gene superfamily in Daphnia pulex. BMC Genomics 10, 169.

Bradfield, J.Y., Lee, Y.H., Keeley, L.L., 1991. Cytochrome P450 family 4 in a cockroach: molecular cloning and regulation by hypertrehalosemic hormone. P. Natl. Acad. Sci. USA 88, 4558–4562.

Borggaard, K.O., Gimsing, L.A., 2008. Fate of Glyphosate in soil and the possibility of leaching to ground and surface water: a review. Pest. Manag.Sci. 64, 441-456.

Cerejeira, M.J., Viana, P., Batista, S., Pereira, T., Silva, E., Valerio, M.J., Silva, A., Ferreira, M., Silva-Fernandes, A.M., 2003. Pesticides in Portuguese surface and ground waters. Water Res. 37, 1055-1063.

Colleen M.F., Stanley I.D., 2005. Effects of pharmaceuticals on Daphnia survival, growth, and reproduction. Chemosphere 61, 200–207.

De Schamphelaere, K.A.C., Canli, M., Van Lierde, V., Forrez, I., Vanhaecke, F., Janssen, C.R., 2004. Reproductive toxicity of dietary zinc to Daphnia magna. Aquat. Toxicol. 70, 233-244.

Ha, M.H., Choi, J., 2009. Effects of Environmental Contaminants on Hemoglobin Gene Expression in Daphnia magna: a Potential Biomarker for Freshwater Quality Mornitoring. Arch. Environ. Con. Tox. 57, 330-337.

Heckmanna, L.H., Callaghan, A., Hooper, L.H., Connona, R., Hutchinson, T.H., Maundc, J. S., Sibly, M.R., 2007. Chronic toxicity of ibuprofen to Daphnia magna: Effects on life history traits and population dynamics. Toxicology Letters 172, 137–145

Jo, H.J., Jung, J., 2008. Quantification of differentially expressed genes in Daphnia magna exposed to rubber wastewater Chemosphere 73, 261–26.

Jones, P.D., De Coen, W.M., Tremblay, L., Giesy, J.P., 2000. Vitellgenin as a biomarker for environmental estrogens. Water Research and Technology 42, 1-14.

Kato, Y., Tokishita, S., Ohta, T., Yamagata, H., 2004. A vitellogenin chain containing a superoxide dismutase-like domain is the major component of yolk proteins in cladoceran crustacean Daphnia magna. Gene 334, 157-165.

Le, T.H., Lim, E.S., Lee, S.K., Choi, Y.W., Kim, Y.H., Min, J., 2010. Effects of glyphosate and methidathion on the expression of the Dhb, Vtg, Arnt, CYP4 and CYP314 in Daphnia magna. Chemosphere 79, 67–71.

Le, T.H., Lim, E.S., Lee, S.K., Park, J.S., Kim, Y.H., Min, J., 2011. Toxicity Evaluation of Verapamil and Tramadol Based on Toxicity Assay and Expression Patterns of Dhb, Vtg, Arnt, CYP4, and CYP314 in Daphnia magna. Environmental Toxicology. DOI 10.1002/tox.

Matozzo, V., Gagné, F., Marin, G.M., Ricciardi, F., Blaise, C., 2008.Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review article. Environment International 34, 531–545

Pereira, J.L., Antunes, S.C., Castro, B.B., Marques, C.R., Goncalves, A.M.M., Goncalves, F., Pereira, R., 2009. Toxicity evaluation of three pesticides on non-target aquatic and soil organism: commercial formulation versus active ingredient. Ecotoxicology 18, 455-463.

Scharf, M.E., Parimi, S., Meinke, L.J., Chandler, L.D., Siegfried, B.D., 2001. Expression and induction of three family 4 cytochrome P450 (CYP4) genes identiWed from insecticide-resistant and susceptible western corn rootworms, Diabrotica virgifera virgifera, Insect Mol. Biol. 10, 39–46.

Sakai M., 2002. Use of chronic tests with Daphnia magna for examination of diluted river water. Ecotox. Environ. Safe. 53,376-381

Shen, B., Dong, H.Q., Tian, H.S., Ma, L., Li, X.L., Wu, G.L., Zhu, C.L., 2003. Cytochrome P450 genes expressed in the deltamethrin-susceptible and -resistant strains of Culex pipiens pallens, Pestic. Biochem. Phys. 75, 19–26.

Snyder, M.J., 2000. Cytochrome P450 enzymes in aquatic invertebrates: recent advances and future directions. Aquat. Toxicol. 48, 529-547.

Soetaert, A., Moens L.N., Van der Ven, K., Van Leemput, K., Naudts, B., Blust, R., De Coen, M. W., 2006. Molecular impact of propiconazole on Daphnia magna using a reproduction-related cDNA array. Comp. Biochem. Phys. C. 142, 66-76.

Stephen, J. W., Travis, J. W., Kent, E. V., 2008 Semiquantitative Real-Time PCR for Analysis of mRNA Levels. Methods in Molecular Medicine. 79, 1940-6037.

Tokishita, S., Shiga, Y., Kimura, S., Ohta, T., Kobayashi, M., Hanazato, T., Yamagata, H., 1997. Cloning and analysis of a cDNA encoding a two-domain hemoglobin chain from the water flea *Daphnia magna*. Gene 189,73-78.

Tokishita, S.I., Kimura, S., Mandokoro, Y., Kato, K., Shiga, Y., Takahashi, Y., Ohta, T., Yamagata, H., 2006. Tissue-specific expression of a bHLH-PAS protein homologous to ARNT during the development of crustacean Daphnia magna. Gene 376, 231–239.

Tsui, M.T.K., Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations:
comparison between different organisms and the effects of environmental factors Chemosphere 52, 1189–1197.

US EPA 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. 5th ed. EPA-821-R-02-012, Office of Research and Development, Washington, DC.

Vorkamp, K., Kellner, E., Taube, J., M€oller, K.D., Herrmann, R., 2002. Fate of methidathion residues in biological waste during anaerobic digestion. Chemosphere 48, 287–297.

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Challenges of Anticoagulant Rodenticides: Resistance and Ecotoxicology

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1. Introduction

Control of rodent pests worldwide relies heavily on the use of rodenticides. In the 40s', O'Connor (1948) isolated and first suggested the use of dicoumarol (a naturally occurring substance responsible for the "sweet clover disease" in cattle) as a rodenticide. In the early 50s', anticoagulant rodenticides (warfarin and later indane-dione derivatives) replaced the acute poisons with great success. It is well known that Norway rats (*Rattus norvegicus*) are very suspicious to new, unfamiliar items in their environment (neophobia) and may eat only a small, non-lethal dose of a new bait. If they survive, they learn to avoid the bait. This phenomenon is also known as food aversion (Lund, 1972). Neophobia may also extend to bait boxes and traps when first introduced in the rat environment. Unlike rats, house mice do not exhibit neophobia (Bonnefoy et al, 2008). As Anticoagulant (or anti-vitamine K AVK) compounds are cumulative, small amounts ingested daily will eventually lead to the consumption of a lethal dose. In the early 60s', massive use of these first generation AVK was considered a great opportunity to reduce or even eradicate rat populations from many areas at that time, despite their behavioral traits. First-generation AVK include warfarin, coumafuryl, coumachlor, coumatetralyl, diphacinone, pindone and chlorophacinone (Bentley, 1972).

Unfortunately, a first detection of a resistant strain of rats was reported in Scotland in 1958, followed by similar reports in other areas in Europe: Wales, Denmark, the Netherlands, and Germany (Lund, 1972). At the same time, Brooks and Bowerman (1973) tested several strains of Norway rats from New York City and confirmed that warfarin resistance was also common among rat populations heavily treated with warfarin in the US. The World Health Organization (WHO) rapidly recognized this event and suggested some guidelines for the rapid detection of resistant rodent, based on feeding tests (Drummond and Rennison, 1973). Pelz et al. (2005) published a survey of countries reporting resistance in commensal rodents (2005). The major findings are described in Table 1 below.

Recently, warfarin resistance has also been reported in another rat species: the Lesser Rice-field rat (Rattus losea) (Wang et al., 2008).

Rodent species can develop complex behavioral patterns and one of this is considered to be "behavioral resistance". There is evidence, for instance in the roof rat, that animals can learn to avoid toxic baits (Leung and Clark, 2005). This complex approach has not been given full attention and will not be developed in this review.

Country	*Rattus norvegicus*	*Rattus rattus*	*Mus musculus*	Reference
Belgium	+	?	+	Lund 1984; Baert, 2003
Danemark	+	+	+	Myllymaki, 1995, Lodal, 2001
Finland	?	?	+	Myllymaki, 1995
France	+	+	+	Myllymaki, 1995, Pelz et al., 2005, Lasseur et al., 2005
Germany	+	+	+	Myllymaki, 1995, Pelz, 2001, Pelz et al., 2005
Italy	+	?	?	Alessandroni et al., 1980
Sweden	?	?	+	Lund, 1984
Switzerland	?	?	+	Muhr, 1981
United Kingdom	+	+	+	Myllymaki, 1995, Kerins et al., 2001
Argentina	?	?	+	Guidobono et al., 2009
Canada	+	?	+	Siddiqi and Blaine, 1982
USA	+	+	+	Jackson and Ashton, 1981
Australia	?	+	?	Saunders, 1978
New-Zealand	+	?	+	De Jonge, 1994
Japan	?	+	?	Naganuma et al, 1981
Korea	+	?	?	Rost et al., 2009

Table 1. Warfarin resistance in the Norway rat (*Rattus norvegicus*), the roof rat (*Rattus rattus*) and the house mouse (*Mus musculus*) around the world (*completed from Pelz et al., 2005*)

As a consequence and in order to overcome this resistance phenomenon, other AVK were developed. These compounds are active after a single feeding, which makes them even more effective against neophobic rodents. They are usually more toxic and more persistent in animal tissues. These second-generation AVK include bromadiolone, difenacoum, brodifacoum and flocoumafen (Redfern and Gill, 1980 ; Petterino and Paolo, 2001).

Table 2 reports the name, rat LD50s for AVKs currently used in most countries around the world.

All the anticoagulants developed so far are based on three moieties:

- the coumarin derivatives (7)
- the indanedione derivatives (3)
- the benzothiopyranone derivatives (1)

The structures of all these compounds are described in Figure 1 below.

Compound	Rat Oral LD50 (mg/kg)	Source
Warfarin	1.6 - 50	Toxnet
Coumachlor	187	Toxnet
Coumatetralyl	30	Toxnet
Chlorophacinone	2.1 – 50	Toxnet
Diphacinone	1.5	Toxnet
Pindone	280	Toxnet
Bromadiolone	0.49	Toxnet
Brodifacoum	0.16	Toxnet
Difenacoum	0.68	Toxnet
Flocoumafen	0.25	Toxnet
Difethialone	0.55	Toxnet

Table 2. Rat oral LD50 of anticoagulant rodenticides

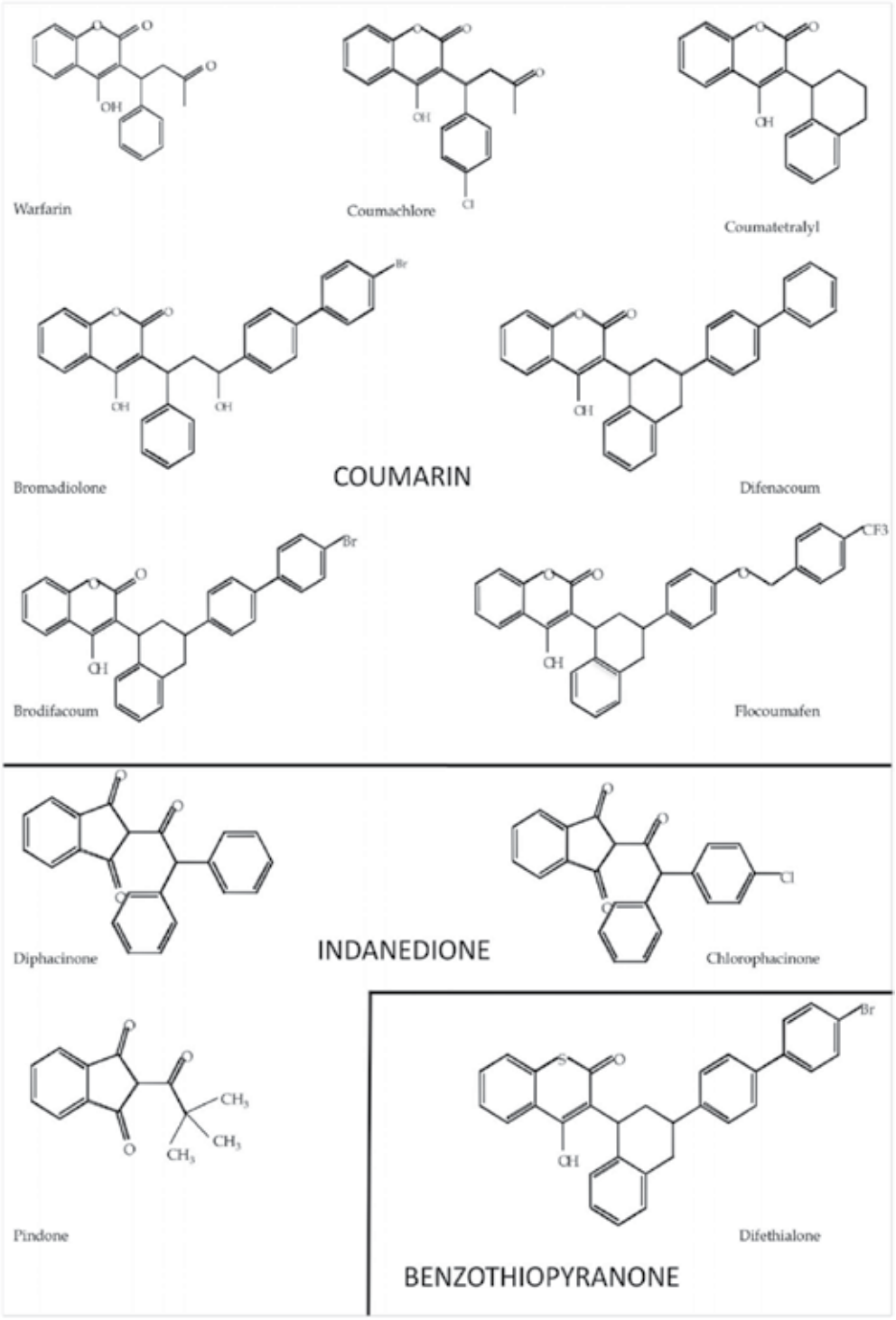

Fig. 1. Chemical structure of anticoagulant rodenticides grouped by moiety

These anticoagulant rodenticides share their mode of action: they all are anti-vitamine K compounds and their effects on mammals and birds are very similar. The general overview of this mechanism of action is described in figure 2.

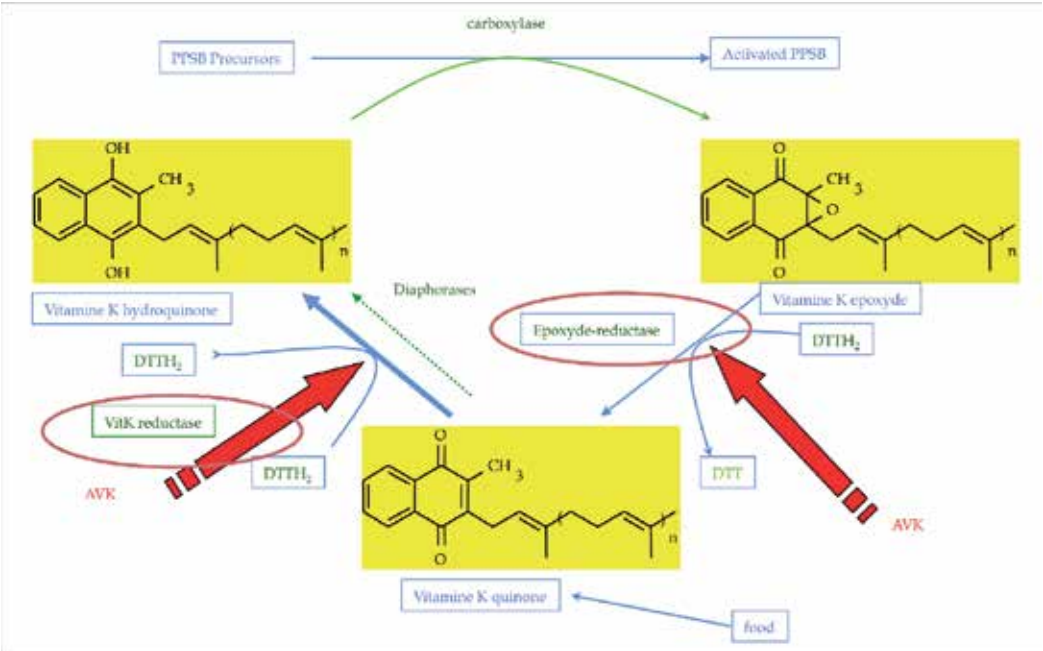

Fig. 2. Mechanism of action of anticoagulant rodenticides on the Vitamin K cycle

AVKs can inhibit two different enzymes of the vitamine K cycle: the epoxyde reductase and the vitamine K reductase (although some scientists consider these two enzymes are, in fact a single protein as suggested by Fasco et al., 1982) (Petterino and Paolo, 2001). Clearly, as indicated by the arrow size, the epoxyde reductase is the rate-limiting step and inhibition by AVK will result in the accumulation of Vitamine K epoxide, which is not active. The second step is not as critical, since other pathways may lead to the activation of vitamine K, such as the diaphorases. Inhibition of this vitamin K cycle results in a decreased production of active coagulation factors which, in turn, will result in coagulation disorders and hemorrhages after 48/72h.

2. The genetic basis of resistance

2.1 Background

Evidence of resistance to AVK was clearly reported at the end of the 50s' (Boyle, 1960) and observed in several European countries shortly thereafter as reported by Lund (1972). For years, the only evidence of resistance was based on feeding tests, as recommended by the WHO (Drummond and Rennison, 1973). Feeding tests performed with warfarin over 4 days are still considered a good indicator of phenotypic resistance and recommended by the WHO in order to evaluate the resistance of a given population in live-trapped animals (WHO, 1982). Basically, rodents are fed a diet made of wheat+250 mg/kg warfarin for 4 days and mortality is evaluated until day 21. Phenotypic evaluation of resistance to AVK

also relied on evaluation of coagulation (WHO, 1982). The biochemical basis of resistance was eventually established in the 1990s' when VKOR enzymatic activity could be assessed in liver microsomes. Misenheimer and Suttie (1990) established the enzymatic characteristics of a Chicago-resistant strain of rats, and showed that VKOR was inhibited by warfarin, but that this inhibition was partially reversible. Later, Misenheimer et al. (1993) showed that, in a Danish resistant strain of mice, VKOR enzymatic constants were altered: reduced Vmax, reduced Km, and also this enzyme was highly insensitive to in vitro inhibition by bromadiolone or warfarin.

It was rapidly established that resistance was an inheritable trait associated to a single autosomal gene Rw (Resistance to warfarin) located on chromosome 1 (Greaves and Ayres, 1969). This gene was later found to be linked with the microsatellite D1Rat219 (Lasseur et al, 2005). The first reports of resistance were mostly concerned with first generation AVKs. It soon became obvious, however, that some strains of rats had also developed resistance to the newer second generation AVK, such as difenacoum, bromadiolone or even brodifacoum. Greaves et al (1982), for instance, report a field trial in a wild population showing evidence of difenacoum resistance in Southern UK. Although bromadiolone and brodifacoum had proven effective in laboratory tests, field trials were surprisingly disappointing with 51% lethality over 14 days and 83% after 35 days. As a consequence, in order to overcome this resistance, the authors suggested at that time to increase the rodenticide concentration in baits (up to 500 mg/kg bromadiolone), since they did not experience any loss of palatability in laboratory tests. Eventually, Greaves (1994) defined resistance as a major lack of efficacy of AVKs used correctly and under normal circumstances. This lack of efficacy is due to the presence of a strain of rodents less susceptible to AVKs, and this reduced susceptibility is genetically transmitted.

In the house mouse, resistance was also rapidly described (Wallace and McSwiney, 1976) and closely linked with the Frizzy (fr) gene on chromosome 7, in a linkage group similar to the one carrying Rw in the rat. Already in these early works on resistance, females appeared more resistant than males. The results also suggested that resistance was a dominant trait influenced by sex.

These historical data and early works on resistance suffered from one major problem: the genetic basis of resistance was not known and could not be further investigated. Some studies are in favor of a metabolic resistance (i.e. increased degradation by cytochrom P450), especially in the roof rat or the house mouse (Sutcliffe et al., 1990, Sugano et al., 2001). Very few data are available on this metabolic hypothesis in the Norway rat. Metabolic resistance will be discussed for each species.

Recent advances in genetics in the Norway rat constituted a real breakthrough.

2.2 The Norway rat

Although the biochemical tools to study VKOR have long been available, it was not until recently that biochemical investigation and biochemical characterization of VKOR activity was given full attention in rats. This approach has also been associated with recent genetic advances and the identification of the first gene involved in the synthesis of the VKOR enzyme (often presented as a complex, since many authors considered that VKOR activity was supported by several proteins) (Li et al., 2004, Rost et al., 2004).

This first gene (Vkorc1) is clearly located on the chromosome 1 of the rat, associated with the D1Rat219 microsatellite. Mutated forms are associated with severe changes in VKOR

activity (Rost et al, 2004). The gene is rather small (1800 bp), with 3 exons and encodes a small trans-membrane protein (163 AA). This small protein (18kDa) has been computed and a suggested structure has been published (Tie et al, 2005) as depicted in Figure 3. This protein has three trans-membrane domains. It is embedded in the endoplasmic reticulum. The catalytic center is considered to be the redox center C-X-X-C (C132 and C135), essential to the activity of the enzyme (Wajih et al., 2004). Back-crossing of resistant rats carrying the Y139F mutation into Spraque-Dawley susceptible rats over 7 generations was used to demonstrate the implication of this mutation in the phenotypic resistance observed via coagulation tests. Indeed, homozygous resistant rats (with less than 1.5% genetic material from the original resistant male, around the Vkorc1 gene) developed a phenotypic resistance similar to their wild counterparts. At the same time, it was demonstrated that Vkorc1 resistance is a co-dominant resistance, since heterozygous individuals are less intermediate between resistant and susceptible rodents (Grandemange et al., 2009).

In Europe, several strains of rats, known to be genetically resistant display some mutations at various loci, with some "hotspots". For instance, Pelz et al (2005) analyzed resistant strains of rats from several countries in Europe and described several mutations associated with the phenotypic resistance, as well as the VKOR activity in recombinant cells transfected by VKORC1 as described in Table 3.

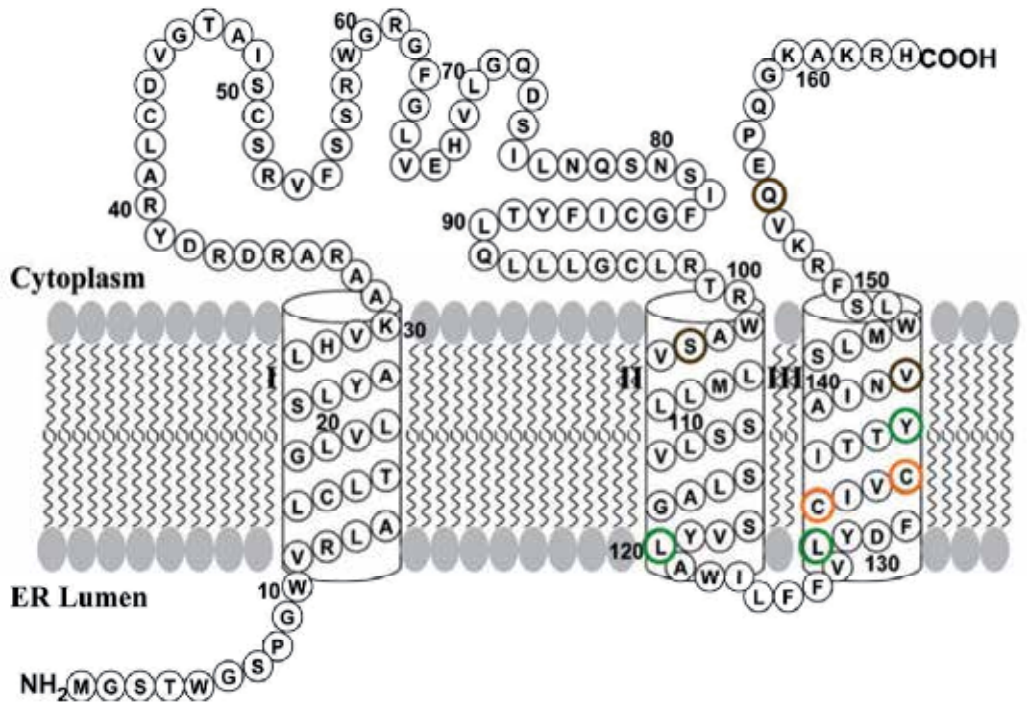

Fig. 3. Theoretical topology of VKORC1 (163 Amino Acids), with some of the known mutation points (green and brown circles : L120, L128, Y139, V142 for instance)- *adapted from Tie, JK et al. J. Biol. Chem. 2005; 280: 16410-16416*

Species / strain	Area	Codon and mutation
Rattus norvegicus	UK, Hampshire	L120Q
Rattus norvegicus	UK, Berkshire	L120Q
Rattus norvegicus	UK, Scotland	L128Q
Rattus norvegicus	UK, Wales	Y139S
Rattus norvegicus	DK	Y139C
Rattus norvegicus	D	Y139C
Rattus norvegicus	B	Y139F
Rattus norvegicus	F	Y139F
Rattus norvegicus	HU	Y139C

Table 3. Some common mutations described in the Norway rat in Europe (*adapted from Pelz et al., 2005*).

Recent studies have identified numerous mutations in Vkorc1 around Europe and especially in France. Indeed, a survey conducted with Pest Control Operators (PCOs') on more than 260 rat samples revealed several interesting data :

- over 92 sampling sites, at least one mutations was identified in 40 sites
- 37% of the rat samples were mutated
- homozygous mutated individuals were very common. The geographical distribution of these mutations is described in figure 4.

This figure illustrates the widespread distribution of the Y139F mutation in France (almost 28% of all samples). Some mutations (E155K, L128S, S103Y) had never been described before. Haplotypes C and D are silent mutations observed on amino acids 107, 137 and 143 and on amino acids 97, 137 and 143 respectively (Grandemange et al., 2010). In their work, Pelz et al (2005) showed that recombinant cells including the Y139 mutations still displayed a high VKOR activity in the presence of warfarin, as compared with other mutations or the wild type. Investigation of the catalytic activity of the mutated enzyme was carried on by Lasseur et al. (2007) and showed that the mutated enzyme is very poorly susceptible to first generation AVKs ' as reported in Table 4 below.

Apart from these two studies, the actual impact of the various mutations detected has not been elucidated and our group is currently working on recombinant cell system dot express these mutants and evaluate the catalytic consequences of the various mutations identified so far, both on the basic activity level of Vkor, but also in response to AVK exposure, since this information can be of critical importance when deciding which rodenticide to use in the field.

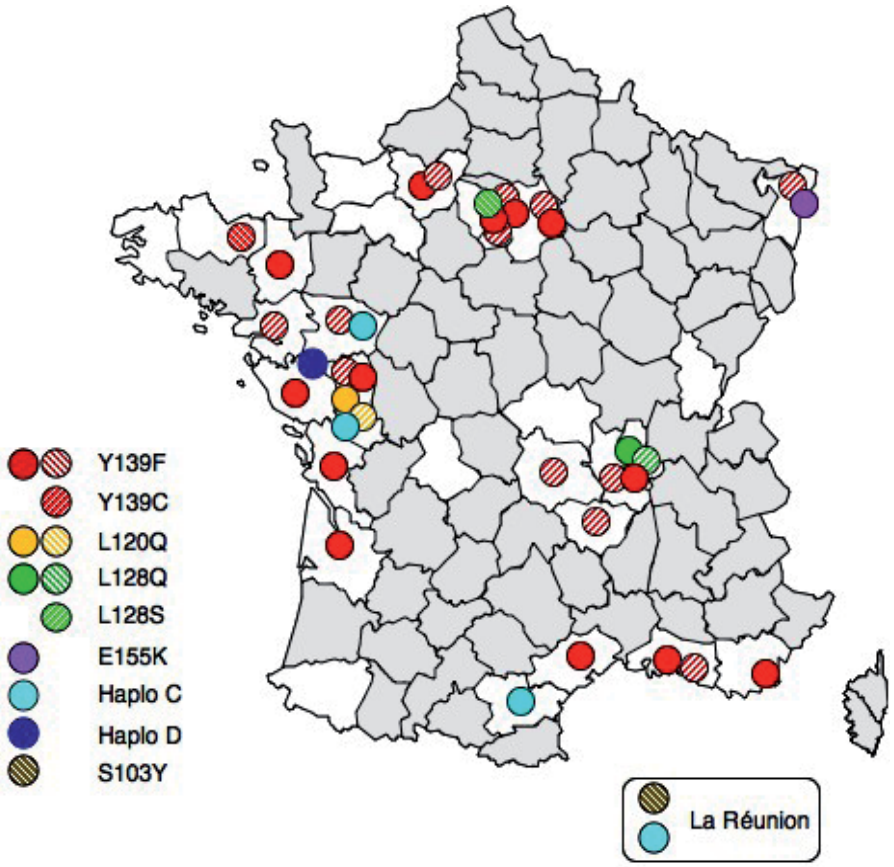

Fig. 4. Sampling area and mutations detected in Norway rats caught in France (full circle: presence of homozygous individuals, dashed circles: only heterozygous individuals) (*adapted from Grandemange et al. 2010*)

AVK	Ki susceptible microsomes (µM)	Ki resistant microsomes (µM)
Warfarin	0.72	29.0
Coumachlor	0.12	1.9
Coumatetraly	0.13	2.7
Diphacinone	0.07	5.0
Chlorophacinone	0.08	1.6
Bromadiolone	0.13	0.91
Difenacoum	0.07	0.26
Brodifacoum	0.04	0.09

Table 4. Inhibition constant (Ki, µM) for wild-type or resistant rat microsomes. adapted from Lasseur et al., 2007

There is some evidence, at least in the Chicago-resistant strain, that some other proteins may play a role in the resistance phenomenon. Indeed, in this strain, Vkorc1 is not modified but a chaperone protein (calumenine) is over-expressed in resistant rats and not expressed in susceptible rats (Wajih et al., 2004). This pathway has not been described anywhere and, for instance, in the French strain carrying the Y139 mutation, calumenin is not overexpressed (Lasseur et al., 2005).

The metabolic hypothesis has been suggested for several years. Several isoforms of Cytochrom P450 (CYP450) are involved in the biotransformation of warfarin. Hydroxylation of warfarin by CYP450 is mainly due to CYP2C, CYP2B, CYP1A and CYP3A subfamilies. Several hydroxides are identified in the rat: 4'-, 6-, 7-, 8-, and 10-OH warfarin. These metabolites are more water soluble and may also undergo glucuronidation and urinary excretion (Ishizuka et al., 2006). There is however, to date, limited evidence of any metabolic resistance in the Norway rat published. Markussen et al. (2007) reported higher constitutive expression of various CYP isoforms in a resistant strain from Denmark. The over-expressed CYP were CYP2C13, CYP3A2. Upon bromadiolone exposure, several isoforms were induced: CYP1A2, CYP2C13, CYP2E1, CYP3A2 and CYP3A3. It is noteworthy that some of these isoforms are involved in the metabolism of warfarin. There is no evidence, however, that this overexpression or induction results in increased metabolism and reduced half-life of bromadiolone, which would be expected as a resistance phenomenon. Similarly, Heiberg (2009) confirmed the presence of phenotypic resistance to bromadiolone in wild rats from Denmark, without any mutation detected in the Vkorc1 gene.

One of the hypothesized consequences of Vkorc1 mutations is a potential vitamin K deficiency. Some work has been conducted on that matter and there is conflicting evidence on vitamin K requirements of resistant rats. For instance, Hermodson et al (1969), Greaves and Ayres (1973) clearly showed that the Welsh resistant strain was highly dependent on high daily vitamine K (about 13 times the standard requirements) in order to maintain normal coagulation. More recently Markussen et al. (2003) showed, on a Danish resistant strain, that the animals were moderately but significantly deprived of vitamin K and had higher daily requirements than susceptible individuals. Comparatively, based on enzymatic evaluation, the French resistant strain described by Lasseur et al., does not present any vitamin K deficiency (Km/Vm ratio constant) (Lasseur et al., 2005). This vitamin K deficiency could be a biological cost associated with resistance to AVK. In the Danish strain, Heiberg et al. (2006) showed that homozygous resistant rats had a lower reproductive success than expected and that heterozygous males or females had a better reproductive success. They suggested that the vitamin K deficiency may play a role in this phenomenon, especially in pregnant females, for which vitamine K is primarily directed to developing fetuses, thereby reducing their vitamin K status.

As a conclusion, Vkorc1 mutations appear to play a major role in the development of resistance in the Norway rat, with evidence from all over the world that these mutations are common and vary diverse in nature. A lot of work remains to be done in order to evaluate the individual consequences of each of these mutations on the catalytic properties of the enzyme, in order to adapt the AVK use to the resistance level observed in a given area, and some work is still needed to investigate the biological costs potentially associated with vkorc1 resistance.

2.3 The roof rat

Comparatively few information is available for the roof rat. There is no evidence of Vkorc1-dependent resistance, but the gene has been sequenced and is highly conserved (Ishizuka et

al., 2006). Species-spcific microsatellitaes have also been identified to help determine Vkorc1 sequence and potentially identify SNPs' in the gene sequence (Diaz et al., 2010). In this study, none of the roof rat tested had any mutation known to confer resistance. Sugano et al (2001) described a resistant strain in Tokyo with evidence of metabolic resistance. . In a wild resistant *Rattus rattus* population in Tokyo, Ishizuka et al. (2006) failed to detect any mutation in Vkorc1. The authors investigated the potential involvement of CYP isoforms in the metabolism of warfarin. Based on a one-month feeding trial, they selected surviving rodents as resistant. A first evidence of metabolic differences was detected with higher plasma concentrations of warfarin in susceptible rats vs resistant rats. The CYP profile exhibited an increased expression of CYP3A subfamily, known to be involved in the metabolism of warfarin, and especially in the production of 10-OH warfarin, which was clearly more produced in resistant rats (Ishizuka et al., 2006). These hydroxylated metabolites are known to be less or even not active on the coagulation process, thereby confirming the lack of susceptibility of rodents. Ishizuka et al. (2007) also demonstrated that NADPH cytochrome c reductase activity (dependent on NADPH cytochrome P450 reductase) wasmarkedly higher in resistant rats, with increased general metabolic activity in all degradation pathways of warfarin in resistant animals. As a confirmation, use of a P450 inhibitor (SKF-525A) resulted in a higher mortality rate in animals exposed to warfarin. These results tend to show that CYP-dependent metabolism of warfarin is a resistance pathway for the roof rat (Ishizuka et al., 2008). Unfortunately, there is no other evidence or work related to resistance in the roof rat and it is difficult to compare the resistance level achieved with metabolic resistance as compared with genetic resistance conferred by vkorc1 mutations. Obviously, much still needs to be done on the roof rat, one of the most abundant rat species around the world.

2.4 The House mouse

Few studies have considered the House mouse and its resistance status towards AVKs. Indeed, at least from a European perspective, there are still other rodenticides marketed to control mice populations, as for instance alpha-chloralose. Nevertheless, resistance has been rapidly identified in mice after AVKs were introduced on the market in the 50s'. For instance, Redfern and Gill (1980) described warfarin resistance in mice from the UK and the effective use of bromadiolone to overcome this resistance and Lund (1984) also reported resistance in mice. A recent investigation in farm mice in Argentina concluded, however, that resistance was present in South America as well as in other areas of the world (Guidobono et al., 2009). Countries reporting house mouse resistant to AVKs are listed in Table 1.

Despite the limited number of studies available, the results show that the two major resistance pathways exist in the house mouse. Indeed, genetic alterations of vkorc1 have been described and altered VKOR activity or lack of susceptibility to AVKs has been reported (Lasseur et al, 2006) together with a mutation in vkorc1 (W59G). Rost et al. (2009) also described and identified several strains of resistant mice with mutated vkorc1 (R12W, R58G, R61L for instance) and these amino acid substitutions resulted in reduced VKOR activity (33, 39 and 49% respectively). They also reported a R58G with no evidence of VKOR activity modification and the more common Y139C mutation, known to confer resistance in rats, in mice from Germany and the Azores. VKOR resistance has been known in mice for at least 15 years and some strains have been described as resistant to both warfarin and bromadiolone (Misenheimer et al., 1993).

In the paper by Lasseur et al. (2006) the catalytic properties of VKOR in the house mouse have been investigated and the results are quite surprising (Figure 5). These data complete the first work by Misenheimer et al. (1993), who only described reduced Km and Vm in resistant Danish mice.

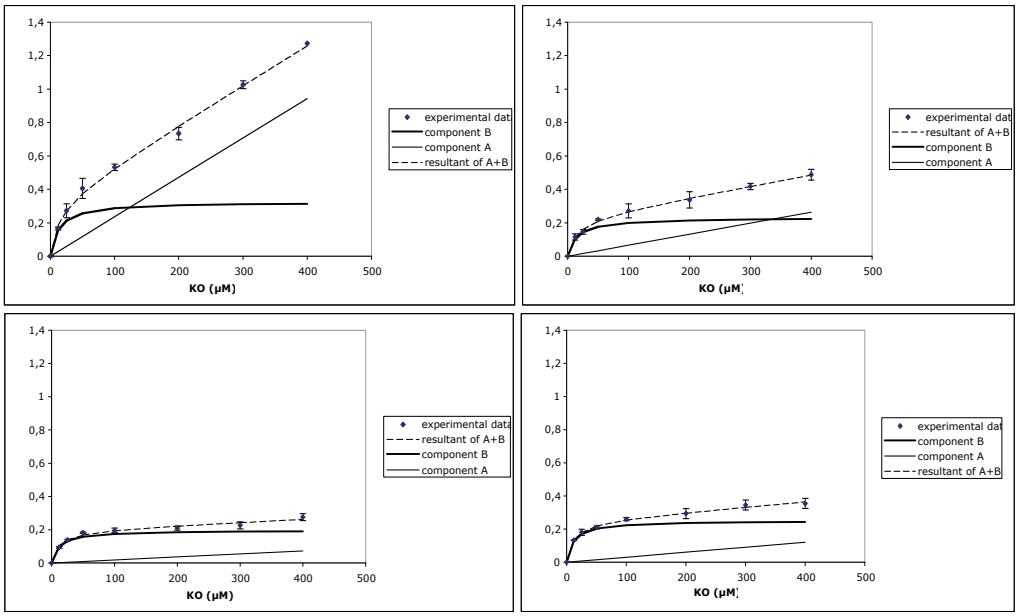

Fig. 5. VKOR activity in the susceptible (left) and resistant (right) mouse without (upper) or with (lower) 1 µM warfarin (*adapted from Lasseur et al., 2006*)

The interpretation of the enzyme activity could only be possible with a bi-component model in which component A is highly active in the susceptible mouse and highly susceptible to AVK, while the resistant mouse seems to lack this susceptible component A. This complex model advocates for more biochemical studies of the VKOR complex in the House mouse.

Investigation of the metabolic pathways of AVKs also showed evidence of increased metabolic degradation of AVKs. Indeed, Sutclife et al. (1990) treated mice with various P450 inducers and showed that the metabolic profile for Warfarin 4'-, 6-, 7-, and 8-OH metabolites were different between susceptible and resistant strains. Based on this very different metabolic profile and CYP induction pattern, they concluded that CYP450 was highly involved in the resistance of the house mouse to AVKs. The metabolic resistance has not been fully evaluated, though and is only reported for warfarin metabolism. There is still much work to be done in order to evaluate the potential for CYP450 to metabolize other AVKs, especially second generation products such as bromadiolone or difenacoum for instance.

2.4 Other rodent species

Apart from the three feral species, very limited information is available on wild rodent resistance status. A Chinese study described resistance in Lesser Rice-field rat (*Rattus losea*)

(Wang 2008). This resistance concerns warfarin and has been determined by means of the traditional feeding test.
We have recently published some enzymatic evidence of resistance to warfarin in water voles (*Arvicola terrestris*) trapped in areas where bromadiolone is repeatedly used to control vole populations, at least several times per year (Vein et al., in press). This resistance does not appear to be linked to a modification of the vkorc1 gene but rather to alterations of the VKOR enzyme function as could be determined by promoter alteration for instance.

2.5 Evaluating and managing resistance in rodents

Since resistance is a well-established problem in commensal rodent species, testing for its existence is a necessary preliminary step in order to adapt the AVK control treatment. As of today, there are few relevant techniques : in vivo assays (feeding test, Blood Clotting Resistance test) and in vitro assays (VKOR activity, CYP450 metabolism and vkorc1 testing) (Grandemange et al., 2010).

2.5.1 Feeding test

This strategy has been the first developed by the WHO (Drummond and Rennison, 1973). After several revisions, it has been established in its present form in 1982 (WHO, 1982), with various doses of the AVK to test. It is basically a no-choice feeding test over 6 days with a 50 ppm warfarin, bromadiolone or difenacoum bait for instance. Rodents surviving the 5-day test period (+14 days observation) are classified as resistant to the AVK tested. This test needs to be conducted on a sufficient number of animals in order to be significant. It also involves intense trapping and maintaining rat colonies in order to evaluate the resistance status. Some authors consider that this test has several limitations, especially with regards to local variations in the resistance of the strain, which would need adaptation of the exposure period to cover a wider range of susceptibility. Unfortunately, this approach requires a large number of animals and is ethically critical (Gill and McNicoll, 1991).

2.5.2 BCR testing

The Blood Clotting Response test (BCR) was first developed by the EPPO (European Plant Protection Organization) (Martin et al., 1979). In its present form, the BCR can be conducted in two ways:

- the first approach is to determine the rat capacity to use the vitamin K epoxide substrate (1 mg/kg) as a vitamin K source in the presence of an AVK such as warfarin (5 mg/kg). Determination of the clotting response (Prothrombin time) 24hours later is a good indicator of the resistance status. A revised version of this test only relies on the administration of a low dose (1 mg/kg) warfarin, with no vitamine K epoxyde and investigation of the clotting capacity 24hours later. The recent developments of this approach are based on the works by Gill and McNicoll (1993) and Prescott and Buckle (2000), who tested several protocols with various AVKs. These works led to the production of a technical Monograph by the Rodenticide Resistance Action Committee (http://www.rrac.info/) in 2003. The idea is to administer normalized and increasing doses of AVK to rodents (as multiples of a known effective dose 50 for which 50% of the animals treated will have an INR>5, in which the INR is a standardized ratio for the measurement of the prothrombin time). Table 5 below gives a list of ED50s of various AVKs in the Norway rat and the House mouse for resistance investigation.

Species / strain	AVK	Gender	ED50 (mg/kg)
R. norvegicus (CD)	Warfarin	M	1.51
		F	2.13
	Diphacinone	M	0.86
		F	1.12
	Chlorophacinone	M	0.54
		F	0.67
	Coumatétralyl	M	0.36
		F	0.44
	Bromadiolone	M	0.47
		F	0.61
	Difenacoum	M	0.65
		F	0.79
	Difethialone	M	0.43
		F	0.49
	Flocoumafen	M	0.29
		F	0.34
	Brodifacoum	M	0.22
		F	0.23
M. musculus (CD-1)	Bromadiolone	M	1.96
		F	1.68
	Difenacoum	M	0.85
		F	0.56
	Difethialone	M	0.83
		F	0.83
	Flocoumafen	M	0.51
		F	0.44
	Brodifacoum	M	0.39
		F	0.35

Table 5. ED50's of various AVKs in susceptible strains of Norway rat (*Rattus norvegicus*) and House Mouse (*Mus musculus*) (*adapted from RRAC, 2003*).

This approach gives phenotypic results and a good idea of the general resistance status of a given strain, both in terms of molecules and level of resistance (a "resistance factor" can be computed for each AVK tested and is a reliable estimate of the potential efficacy of an AVK), in 24hours. Its limitations are still similar to the feeding test, i.e. manipulation of live-trapped animals, sanitary and ethical problems with the manipulation of wild mammals. It is very commonly used nowadays.

- the second approach has been less developed and investigates the rat vitamin K deficiency status (Martin et al., 1979).

2.5.3 VKOR activity

Numerous studies have reported the determination of kinetic constants and/or enzyme activity for VKOR in susceptible and resistant rats. Several protocols may be used (Lasseur et al., 2005 ; Lasseur et al., 2007) on liver microsomes or any other enzyme system (recombinant cells for instance as in Rost et al., 2009). This assay can be conducted on a limited number of animals and does not require extensive rodent trapping. Also, animals do not need to be maintained in laboratory facilities. This approach provides a very good estimate of the enzyme activity and the resistance status of a population. It is also rapid and cost-effective, and all AVKs can be tested in a very short time period (Lasseur et al., 2006). This determination requires analytical material (HPLC or LC-MS) for routine determination. Only a few strains will not respond to this assay : metabolic resistance will not be detected, for instance.

2.5.4 CYP metabolism

Although CYP metabolism of warfarin in the Norway rat (Ishizuka et al., 2006), in the roof rat (Sugano et al., 2001; Ishizuka et al., 2006) and in the House Mouse (Sutclife, 1990), it is not a standard tool for the monitoring of resistance so far. More work needs to be done to determine the CYP450 isoforms involved, as well as the AVK concerned, in order to develop this approach as a routine monitoring tool for metabolic resistance in rodent species. Undoubtedly, this resistance pathway needs to be more deeply investigated at that point. Nevertheless, it is an in vitro approach, like the VKOR activity assay, and requires microsoms and analytical devices to look at warfarin metabolites produced.

2.5.5 Vkorc1 sequencing or genotyping

This last in vitro approach has been suggested in our work (Grandemange et al., 2010). Basically, sequencing of Vkorc1 only requires a piece of animal tissue (tail, ear, fur may be used) and does not necessitate live-trapping of rodents. In the Norway rat, considering the major importance of the SNPs' identified so far, sequencing of Vkorc1 appears as one of the most interesting and cost effective tools to date. As compared with other resistance detection assays, it can be applied rapidly on large scale samples, even across a country (Grandemange et al., 2010). Coupled with VKOR activity measurement in recombinant cells, it can also provide a good indication of the resistance level conveyed by a given mutation. The following primers have been used in the rat : rat VKORC1 exon 1 (VKORC1 GenBank accession no. NM-203 335) are exon1-forward 5′ -GTGGCGGGTTCTTCCCTC-3′) and exon1-reverse primer (5′-GACTCCAAAATCATCTGGCAACC-3′).

In very specific situations, especially when only one mutation is expected or known to occur, this approach may be simplified even further with the use of qPCR and specific primers. In this case, the different genotypes (homozygous, heterozygous, resistant and susceptible) are tested and their characteristic cycle threshold values (ΔCt, i.e. the difference between the matched and the mismatched primer extension for homozygous rats and the absence of such a difference in Ct values for heterozygous animals) gives significantly different results (see figure 6).

This last approach is the most cost-effective one when the resistance status of a population is known. It is used extensively in our group to investigate wild populations of rats, since the Y139F mutation is the only SNP detected so far in our study sites. Nevertheless, a similar approach can be conducted with specific primers for each SNP and the results combined.

Only very small pieces of tissue are required and we have evidence that this technique can be applied to fecal samples, which are extremely common and easy to collect when rat populations are installed.

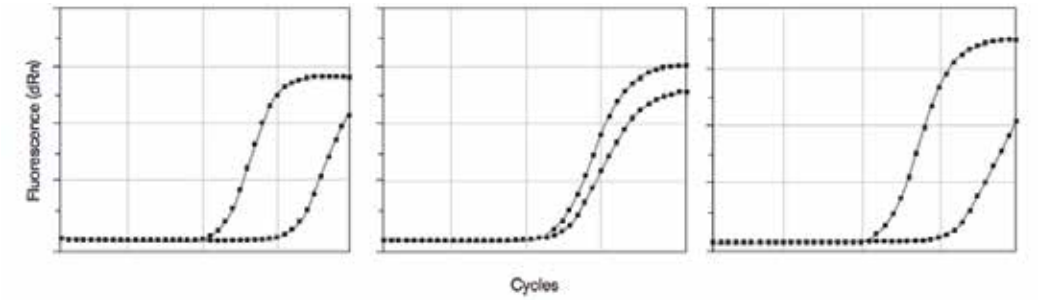

Fig. 6. Fluorescence and qPCR determination of the genotype of Norway rats (for the Y139F mutation). The first graph represents the SYBER green fluorescence curves for a SD/SD animal, with a cycle threshold (DCt) of – 9.28 cycles, the second graph represents the SYBER green fluorescence curves for a SD/Y139F animal, with a DCt of 0.91 cycles and the third graph represents the SYBER green fluorescence curves for a Y139F/Y139F animal, with a DCt of 9.83 cycles. dRn, baseline corrected normalized fluorescence (*adapted from Grandemange et al., 2009)*

2.5.6 Management of resistance

When resistance is confirmed in a given population, a proper strategy needs to be implemented, in order to avoid spreading of this resistance and over selection of hyper resistant individuals.

A first step (if we exclude construction and rodent-proofing techniques) is to place baits judiciously to facilitate regular and massive uptake by rodents. Endepols et al. (2003) clearly showed that each site needs to be visited and baiting must be carefully planned in order to achieve excellent baiting results and to overcome resistance. Similarly, Whisson and Salmon (2002) showed that, in another rodent species, bait uptake does not need to be continuous (even one feeding every 48h is sufficient to result in death) but well planned and with adapted baits (good palatability).

In the EU, AVKs are the only chemical option against rats. Therefore, it is impossible to switch to another family of rodenticide as can be done with insecticides for instance (http://www.rrac.info/). In mice, this is still an option to consider.

The RRAC suggests to avoid permanent baiting with AVKs and to use them only when rodent infestation is observed. Bait station and boxes should be replenished as needed (every day or every other day) until consumption is no longer detected. Then all baits should be removed.

One important issue still unresolved is the choice of active AVK to use. Indeed, the Danish situation, in which warfarin, then coumatetralyl, followed by difenacoum have been used successively, shows that this progressive strategy has resulted in extremely high levels of resistance of rats, even to difenacoum, in most of the country (Lodal, 2001). The opposite strategy would then appear to be a major shift towards newer second generation AVKs (brodifacoum, difethialone for instance), hoping that moving from chlorophacinone or warfarin to one of these AVKs represents a change that cannot result in an adaptation of the

rat population. We have tested (unpublished results) such a situation, and there seems to be a dramatic increase in the proportion of homozygous resistant animals in the resulting population. However, lowering AVK selection pressure results in a rapid return of susceptible individuals, thereby suggesting that there is a real biological cost associated with the resistance status and therefore resistant individuals remain only as long as AVK selection pressure is high. Lodal (2001) and Quy et al. (1995) suggest that second generation AVKs should be used first as a treatment against well-established rodent populations. Then, as prevention, first generation AVKS, especially if no resistance is suspected.
In any case, several strategies need to be evaluated more completely in order to be considered for general application. One major disadvantage of this many strategies is certainly the use of second generation AVKs, which are obviously more toxic and may have more severe detrimental effects on non-target species.
As already advocated by Pelz et al. (2005) and ourselves (Grandemange et al., 2010), testing for resistance in the Norway rat should be considered as much as possible before starting a baiting campaign, since genetic tools are available, easy to use in lab situations and provide rapid answers to customers, in order to decide which AVK should be used.

3. Ecotoxicity of rodenticides

When we consider the AVK family, one striking characteristic is that development has always led, so far, to newer, more potent and more persistent compounds, which is by no means acceptable for ecological reasons (Lasseur et al., 2006). AVKs are quite unique because they have a common mode of action and, as a consequence, all AVKs share the clinical features of poisoning with severe bleeding and coagulopathy (Kolf-Clauw et al, 1995). . The only differences of interest are the toxic doses and potential for secondary poisoning.

3.1 Primary toxicity in non-target species

Primary poisoning is the result of direct exposure to the toxic bait. Several reviews are available on that topic. Petterino and Paolo (2001) published a paper providing many toxic doses in domestic or laboratory animal species. Watt et al. (2005) also provided a general toxicology paper on human toxicity of AVKs. Recently, we published a survey of AVK poisoning in human beings, domestic and wild animals based on poison control center data (Berny et al., 2010). Human exposure is fairly limited, thanks to the use of bittering agents and the vast majority of AVK exposure in humans result in no clinical signs. Only suicidal attempts may result in severe poisoning cases, but, generally speaking, AVK exposure in humans does not result in prolonged surveillance of patients (Caravatti et al., 2007) The high frequency of rodenticide primary poisoning in companion animals is fairly widespread across Europe and the US (Roben et al., 1998, Guitart et al., 2010a, Guitart et al., 2010b). Most cases involve dogs, although cats may sometimes be affected as well (Kohn et al., 2003). A summary of the major toxicity data is provided in Table 6 below.
The values reported show the wide variability of toxicity for each AVK across species. Nevertheless, some surprising results need to be considered. For instance, chlorophacinone appears to be moderately toxic. Indeed, the lowest reported LD50 In the dog is 50 mg/kg. Considering the amount available in most baits in the EU (50 to 75 mg/kg bait), a 10-kg dog would need to eat 6.6 kg bait to reach this LD50. Even considering $1/10^{th}$ of this dose still potentially lethal, the same dog would still need to eat about 700g bait to be poisoned.

Clinical data reported in our survey are quite opposite to that, since chlorophacinone is one of the most commonly involved AVK in companion animal poisoning (Berny et al., 2010). It is our hypothesis that AVK toxicity may be more pronounced in real case situations, mainly because animals are more active and may suffer from hemorrhages more rapidly than laboratory animals, confined and quiet. A second possibility can be deduced from Table 5. Warfarin and coumatetralyl, for instance, have very low LD50s' when administered over several days, as compared with acute oral LD50s'. This is probably due to the accumulation of AVKs in the liver. None of the other AVK has been specifically tested to validate this hypothesis but we suggest that repeated exposure (even 2 or 3 times) could significantly reduce the toxic dose necessary to poison a dog and certainly be more consistent with the results of the survey.

AVK	Rat	Mouse	G. pig	Rabbit	Dog	Cat	Quail	Chicken	Cattle
Warfarin	186 (1-5d)	374	-	-	3 (5d)	3 (5d)	>2150°	-	200 (5d)
Coumatetralyl	16.5 (0.3-5d)	-	>250	>500	-	50	2000	-	-
Bromadiolone	1.12	1.75	-	1.0	8.1	>25	1600	-	-
Difenacoum	1.8	0.8	50	2.0	>50	100	-	>50	100**
Flocoumafen	0.25	0.8	>50*	0.2	0.075-0.25	>10	>300	>100	
Brodifacoum	0.27	0.4	2.8	0.3	0.25-1	25	-	4.5	>3**
Difethialone	0.56	1.29	-	-	4	16	-	-	-
Diphacinone	2.3	340	-	35	3-7.5	14.7	>400°	-	-
Chlorophacinone	6.26	1.06	-	50	50-100	-	258°	-	-

*hamster, **goat and sheep, °Northern Bobwhite quail

Table 6. Toxicity data (acute oral LD50s in mg/kg/day) for AVKs in animals (adapted from Petterino and Paolo, 2001, Toxnet® : values in bold+italics and USEPA : values in blue)

Among domestic species, herbivores appear to be absent. A survey at the Animal Poison Control Center (Lyon, France) confirmed that cattle, sheep and goat are rarely affected by AVK poisoning, as compared with other species (Berny et al, 2005). These cases, however, usually involve several animals (mean = 6 for cattle, 33 for sheep/goat) and raise questions regarding residues of AVK in milk, for which there is no published data. Affected animals develop hemorrhagic disorders (digestive and respiratory mostly). Pre-ruminant animals are usually considered to be more susceptible and, indeed, they are usually more affected (Berny et al., 2005) and sometimes also more exposed (Braselton et al., 1992, Del Piero and Poppenga, 2003). Some cases of AVK poisoning are reported in horses (Guitart et al, 2010a),

although rarely. Lethal cases are also described with hemorrhages in horses. This seems to occur only with potent, second generation AVKs such as Brodifacoum (Ayala et al., 2007): a pony ingested *ca* 2 kg of a commercial bait, reaching almost 0.8 mg/kg, which is a lethal dose for many species as can be seen in Table 5.
Questions may also be raised with respect to wildlife. Direct poisoning may occur when baits are applied to extensive areas. This is frequently observed in rodent eradication campaigns, for instance, when aerial application or wide application of AVKs is considered. Thorsen et al. (2000) have evaluated the consequences of brodifacoum application in the Seychelles archipelago and discuss the cost/benefit ratio. In a more generalized view, Howald et al. (2007) reviewed the invasive rodent eradication campaigns in islands and showed that the use of tampered bait stations could successfully reduce primary non-target poisoning. Many species can be affected, depending on the bait nature. One can cite hares, rabbits (Erickson and Urban, 2002), roe deers, wild boars (Berny et al., 2005). In wildlife, however, primary poisoning is not a major issue and most concern is raised by secondary poisoning.

3.2 Secondary toxicity

Secondary poisoning is quite unique to AVKs and can be defined as clinical poisoning occurring in predators and scavengers feeding on contaminated preys. Fairly soon after AVKs started to be used, secondary poisoning was considered as a potential adverse effect of these compounds. Cases of warfarin secondary poisoning are reported by Bentley (1972) in dogs fed poisoned Coypu. At that time, the author concluded that warfarin should not be used to control coypu invasive populations in Florida for this reason. It was not until second-generation AVKs were marketed that secondary poisoning was given full attention. Evidence was published by Gray et al. (1994a) that some of the most recent AVKs (namely : brodifacoum, difenacoum, flocoumafen) were experimentally responsible for secondary poisoning in Barn owls (*Tyto alba*). This first trial, however, only showed that owls fed contaminated mice over 15 days would accumulate AVK residues in the liver, considered as the primary target organ for accumulation. This information was used in surveys that started soon afterwards to be published. For instance, evidence of secondary poisoning was confirmed in red foxes (*Vulpes vulpes*) and buzzards (*Buteo buteo*) (Berny et al., 1997), but also in non-target native species in New-Zealand (Eason et al., 2002). Liver samples were used as an indicator of AVK exposure in animals found dead. AVKs are very specific in that their clinical and pathological features are fairly similar across species and animals always die of hemorrhages. Gross necropsy is usually indicative of AVK poisoning with evidence of massive hemorrhages and lack of coagulation (Berny et al., 2007). There is evidence that vitamine K may play a role in bone metabolism and humans exposed to prolonged AVK therapy may experience increased frequency of bone fractures and osteoporosis. In wildlife species exposed to AVKs, there is, as of to day, no evidence that this is the case, but only one study has been dealing with this issue so far (Knopper et al., 2007). In the UK, small mustelids also were detected with secondary AVK poisoning (Shore et al., 1999). These first reports clearly confirmed the high susceptibility of canids and birds of prey to AVK rodenticides. In some instances, critically endangered sand protected species may be concerned and unexpected death from AVK exposure may be dramatic, such as shown for the Red kite (*Milvus milvus*) (Berny and Gaillet, 2008). Some unusual species such as the Otter (Lutra lutra) and the European mink (Mustela vison) have also been found to be

exposed to AVKs (Fournier-Chambrillon et al., 2004), with reference to the use of AVK against coypu in wetlands in France.

More generalized surveys have also been conducted on animals found dead and submitted to a diagnostic laboratory. Several publications and countries now report a high prevalence of AVK exposure (i.e. detection of AVK, with or without evidence of poisoning). This is the case in birds of prey from New York (Stone et al., 2003), who showed that 49% of the 265 animals analyzed contained detectable residues of AVKs in the liver. Similarly, in a survey conducted on 58 birds received dead at a rehabilitation center in France (Lambert et al., 2007), 73% of the animals contained detectable residues. In Great Britain, a survey on Tawny owls (Walker et al., 2008) also indicated a high prevalence of AVK exposure (>20%), very stable over time. In Canada, there is also evidence that owl species are highly exposed to AVK rodenticides (Albert et al., 2010). More recently, even insectivores such as the Hedgehog have been shown to accumulate AVK to significant extent (Dowding et al., 2010). Probably the most severe contamination appears to be in Californian Mountain Lions (*Puma concolor*) and bobcats (*Lynx rufus*), since almost 90% of them have been found with residues (Riley et al., 2007). The authors even consider the interaction of AVK exposure with the development of other diseases such as a parasitic infestation with notoedric mange in this case.

Secondary poisoning is certainly a result of the prolonged half-life of most AVKs in biological fluids and tissues, as can be seen in Table 6.

AVK	Rat-blood	Dog-blood	Sheep-blood	Rabbit-blood	Rat-liver	Dog-liver	Sheep-liver	Rabbit-liver
Warfarin	0.7-1.2 14.9°°		9.5	0.2	66.8°°	-	-	-
Coumatetralyl	0.5°°	-	-	-	15.8°°	-	-	-
Bromadiolone	1-2.4 33.3°°	12.7-72.2†	49.5	-	170-318 28.1°°	-	256*	-
Difenacoum	20.4°°	-	-	-	61.8°°	-	-	-
Flocoumafen	26.6°°	-	-	-	93.8°°	-	-	-
Brodifacoum	6.5 91.7°°	0.9-4.7	-	2.5	128-350 307°°	-	>128*	-
Difethialone	2.3 38.9°°	2.2-3.2	-	-	74-126 28.5°°	-	-	-
Diphacinone		-	-	-	-	-	>90*°	-
Chlorophacinone	0.4 11.7°°	-	30.1	-	35.4°°	-	-	-

*estimated liver retention in days, ° cattle, °°mouse, in days, †in the red fox
from Erickson and Urban, 2002, Robben et al., 1998, Berny et al., 2006, Vandenbroucke et al., 2008a, Sage et al, 2010

Table 6. Half-lives of selected AVKs in plasma (h) and liver (days) of various species.

Despite some discrepancies, probably due to different analytical techniques and sampling times, the general trend points out the prolonged liver retention of second generation AVKs.

Species	N	COUMAT	CHLORO	BRODIF	BROMA	DIFEN	FLOCOUM
Hedgehog (*Erinaceus europaeus*)	120	-	-	3,3	10,8	13,3	0
Polecat (*Mustela putorius*)	100	-	-	3,0	12,0	22,0	0
Stoat (*Mustela erminea*)	40	15,0	-	2,5	6,7	-	-
Weasel (*Mustela nivalis*)	10	30,0	-	-	10,0	-	-
Red fox (*Vulpes vulpes*)	92	7,6	-	5,4	26,1	16,3	-
Ref Fox (F)	62	0	4,8	0	12,9	1,6	0
Barn owl (*Tyto alba*)	717	-	-	3,9	11,0	16,7	1,1
Buzzard (*Buteo buteo*)	40	-	-	2,5	5	32,5	2,5
Buzzard (F)	98	0	5,1	0	14,2	0	0
Red kite (*Milvus milvus*)	20	-	-	-	1,2	-	0
Red kite (F)	62	0	4,8	0	38,7	0	0

Coumat=coumatetraly, Chloro = chlorophacinone, Brodif = Brodifacoum, Broma = Bromadiolone, Difen = Difenacoum, Flocoum = Flocoumafen

* completed from Dowding et al., 2010

Table 7. Proportion of animals with detectable residues in France (F) and in the UK (in%)*

These data clearly confirm that secondary poisoning with AVKs is not a theoretical problem. In a review paper on island preservation and rodent eradication, Howald et al. (2007) obviously confirm that secondary poisoning occurs regularly after aerial or general bait application, but they also show that, generally speaking, the local population recovers shortly after bait removal. As a general overview, Table 7 describes the proportion of animals detected with AVK residues in France and the UK, based on published data (Dowding et al., 2009) and unpublished data (France). These results clearly point out the high frequency of detection of residues of AVKs despite limited outdoor use in the UK and restricted used in France.

Studies on secondary poisoning have been possible because of the rapid evolution of analytical techniques. Today, many LC-MS-MS techniques are available to detect minute amounts of AVKs in various biological samples such as the liver, plasma or fecal samples (Jin et al., 2008, Vandenbroucke et al., 2008b, Fourel et al., 2010 ; Sage et al., 2010, Vudathala et al., 2010). We have even shown that non-invasive monitoring, as already suggested by Gray et al (1994) on pellets of birds of prey could easily use fecal samples of foxes for instance: it is possible to confirm exposure even one month after the last ingestion of a

contaminated prey (Sage et al., 2010). Using this approach could certainly improve our knowledge of AVK exposure in non-target predators and be used as monitoring tools for wide-scale surveys. This would certainly be of help, in order to monitor potential exposure, since there is evidence that AVK user behavior could still improve to reduce unnecessary exposure of non-target species (Tosh et al., 2011).
The question of concern remains of the long-term impact of such a bottleneck in genetics. Instead of facing such an adverse effect in non-target species, some authors consider alternatives to the use of AVKs (Donlan et al., 2003).

4. Conclusions - perspectives

AVKs have considerably changed our practice and perspectives for rodent control. Unfortunately, the intensive use of these compounds has been rapidly followed by the development of resistant strains in Norway rats, roof rats and house mice. These resistant strains have developed specific genetic traits either via a modification of the VKOR enzyme involved in the catalytic cycle of vitamin K or via metabolic profile modification via induction and overexpression of selected CYP450 isoforms. The most widely spread resistance mechanism appears to be related to VKOR alteration and specifically SNPs in vkorc1 gene. As of today, however, very little is known of the effects of these mutations on the enzyme activity and, similarly, the biological cost of these mutations and resistance is not known.
In order to overcome AVK resistance, second generation compounds have been developed. These compounds are more toxic and more persistent and, therefore, can be successfully used when resistance to first generation AVK is suspected. As a consequence, non-target poisoning is more common, especially secondary poisoning of predators and scavengers. In some instances, endangered species may even be critically affected, although eradication programs in islands have usually shown that most species promptly recover if the eradication campaign is short. This secondary poisoning issue has been raised to a new level by recent surveys showing that many different species (mammals and birds) contain detectable residues of AVKs in various organs, at low levels but still of concern, raising the question of when and where animals have been exposed.
There is some debate as to whether these compounds should be banned. This issue has no easy answer, considering the two problems developed above, but also there are no other chemicals of help, with the advantages of AVKs (specific antidote, delayed poisoning) and many countries, especially in Europe, do not consider any other option for the time being. A valid possibility would be, based on enzymatic and ecotoxicology evaluation, to develop AVK compounds with a strong affinity for the enzyme and a short residence time, in order to avoid secondary poisoning, but no publication has developed that topic recently.

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6. References

Albert, C., Wilson, L., Mineau, P., Trudeau, S., & Elliott, J. (2010). Anticoagulant Rodenticides in Three Owl Species from Western Canada, 1988–2003. *Archives of Environmental Contamination and Toxicology , 58* (2), 451-459.

Alessandroni, P., Marchini, S., Bernardo, A., Terranova, F., Turillazi, P. (1980). Valutazione della presenza di ratti resistenti al warfarin nella cita di reggio Calabria. *Ann. 1st Super Sanita*, 16: 271-286.

Ayala, I., Rodríguez, M., Martos, N., & Zilberschtein, J. (2007). Fatal brodifacoum poisoning in a pony. *The Canadian Veterinary Journal* . 48 : 627-629

Baert, K. (2003). Bestrijding van de Bruine rat. Resistentie tegen anticoagulantia bij de Bruine rat. In: IBW (ed.). Jaarrapport-Onderzoeksgroep Rattenbestijding. AMINAL afdeling Water, IBW, Geraardsbergen, 18-23.

Bentley, E. (1972). A review of anticoagulant rodenticides in current use. *Bulletin of the World Health Organization , 47* (3), 275-80.

Berny, P., Buronfosse, T., Buronfosse, F., & Lamarque, F. (1997). Field evidence of secondary poisoning of foxes (Vulpes vulpes) and buzzards (Buteo buteo) by bromadiolone : a 4-year survey. *Chemosphere* 35 (8) : 1817-1829

Berny, P, Alves, L., Simon V., & Rossi, S. (2005) Intoxication des ruminants par les raticides anti-coagulants: quelle réalité? *Revue de médecine vétérinaire* 156, (8-9): 449-454.

Berny, P, Alves de Oliveira L., Videmann, B., & Rossi S. (2006) Assessment of ruminal degradation, oral bioavailability, and toxic effects of anticoagulant rodenticides in sheep. *American Journal of Veterinary Research* 67 (2) : 363-371

Berny, P. (2007). Pesticides and the intoxication of wild animals. *Journal of Veterinary Pharmacology and Therapeutics*. 30 : 93-100

Berny, P., & Gaillet, J.-R. (2008). Acute poisoning of Red Kites (*Milvus milvus*) in France: Data from the SAGIR network. *Journal Of Wildlife Diseases , 44* (2), 417-426.

Berny, P., Velardo, J., Pulce, C., D'Amico, A., Kammerer, M., & Lasseur, M (2010a) Prevalence of anticoagulant rodenticide poisonings in humans and animals in France and substances involved. *Clinical Toxicology*, 48 : 935-941

Bonnefoy, X., Kampen, H., & Sweeney, K. (2008). Public Health Significance of Urban Pests. 1-292.

Boyle, C. (1960). Case of apparent resistance of *Rattus norvegicus* Berkenhout to anticoagulant poisons. Nature 188: 517-525

Braselton, W., Regg, D., & Poppenga, R. (1992). Confirmation of indanedione rodenticide toxicoses by mass spectrometry/mass spectrometry. *J. vet. Diagn. Invest. , 4*, 441-446.

Brooks, J., & Bowerman, A. (1973). Anticoagulant resistance in wild Norway rats in New York. *The Journal of hygiene , 71* (2), 217-22.

Caravatti, E.M., Erdman, A.R., Scharman, E.J., Woolf, A.D., Chyka, P.A., Cobaugh, D.J., Wax, P.M., Manoguerra, AS., Christianson, G., Nelson, L.S., Olson, K.R., Booze, L.L., Troutman, W.G. (2007) Long-acting anticoagulant rodenticide poisoning: an evidence-based consensus guideline for out-of-hospital management. *Clin Toxicol* 42: 1-22

De Jonge, J. (1994). Resistentieproblemen bij de bestrijding van knaagdieren in Nederland. *Dierplagen en Milieu* 42: 99-101

Del Piero, F., & Poppenga, R. (2006). Chlorophacinone exposure causing an epizootic of acute fatal hemorrhage in lambs. *Journal of Veterinary Diagnostic Investigation* 18 : 483-485

Díaz, J., Song, Y., Moore, A., Borchert, J., & Kohn, M. (2010). Analysis of vkorc1 polymorphisms in Norway rats using the roof rat as outgroup. *BMC genetics , 11*, 43.

Donlan, C., Howald, G., Tershy, B., & Croll, D. (2003). Evaluating alternative rodenticides for island conservation: roof rat eradication from the San Jorge Islands, Mexico *Biological Conservation* 114 : 29-34

Dowding, C., Shore, R., Worgan, A., Baker, P., & Harris, S. (2010). Accumulation of anticoagulant rodenticides in a non-target insectivore, the European hedgehog (Erinaceus europaeus). *Environmental pollution (Barking, Essex : 1987) , 158* (1), 161-6.

Drummond, D., & Rennison, B. (1973). The detection of rodent resistance to anticoagulants. *Bulletin of the World Health Organization , 48* (2), 239-42.

Eason, C. T., Murphy, E.C., Wright, G. R. & SPURR, E.B. (2002). Assessment of risks of brodifacoum to non-target birds and mammals in New- Zealand. *Ecotoxicology* 11: 35–48

Endepols, S., Klemann, N., Pelz, H., & Ziebell, K. (2003). A scheme for the placement of rodenticide baits for rat eradication on confinement livestock farms. *Preventive Veterinary Medicine* 58 : 115-123

Erickson, W., & Urban, D. (2002). Comparative Rodenticide Risk Assessment pp1-202. United States Environmental Protection Agency, Washington DC

Fasco, M., Principe, L. (1982). R- and S-Warfarin inhibition of vitamin K and vitamin K 2,3-epoxide reductase activities in the rat. *J. biol. Chem.* 257, (9): 4894-4901

Fourel, I., Hugnet, C., Goy-Thollot, I., & Berny, P. (2010). Validation of a new liquid chromatography- tandem mass spectrometry ion-trap technique for the simultaneous determination of thirteen anticoagulant rodenticides, drugs, or natural products. *Journal of Analytical Toxicology , 34* (2), 95-102.

Fournier-Chambrillon, C., Berny, P., Coiffier, O., Barbedienne, P., Dassé, B., Delas, G., Galineau, H., Mazet, A., Pouzenc, P., Rosoux, R., & Fournier, P. (2004). Evidence of secondary poisoning of free-ranging riparian mustelids by anticoagulant rodenticides in France: implications for conservation of European mink (*Mustela lutreola*). *Journal of Wildlife Diseases , 40* (4), 688-695.

Gill, J., MacNicoll, A. (1991). Determination of the susceptibility of wild populations of Norway rat (*Rattus norvegicus*) to the anticoagulant rodenticide brodifacoum. *Z. Fuer Angewandte Zool*. 78: 101-117.

Grandemange, A., Kohn, M., Lasseur, R., Longin-Sauvageon, C., Berny, P., & Benoit, E. (2009). Consequences of the Y139F Vkorc1 mutation on resistance to AVKs: in-vivo investigation in a 7th generation of congenic Y139F strain of rats. *Pharmacogenetics and Genomics , 19* (10), 742.

Grandemange, A., Lasseur, R., & Longin- …, C. (2010). Distribution of VKORC1 single nucleotide polymorphism in wild Rattus norvegicus in France *Pest management* 66 (3) : 270-276

Gray, A., Eadsforth, C., Dutton, A., & Vaughan, J. (1994). The toxicity of three second-generation rodenticides to barn owls. *Pesticide Science , 42* (3).

Greaves, J., & Ayres P. (1967). Heritable resistance to warfarin in rats. *Nature* 215: 877-878

Greaves, J., & Ayres, P. (1973). Warfarin resistance and vitamin K requirement in the rat. *Laboratory animals , 7* (2), 141-8.

Greaves, J., Sheperd, D., Gill, J. (1982). An investigation of difenacoum resistance in the norway rat populations in Hampshire. *Ann. appl. Biol.* 100: 581-587

Guidobono, J., León, V., Gómez Villafañe, I., & Busch, M. (2010). Bromadiolone susceptibility in wild and laboratory *Mus musc*ulusL.(house mice) in Buenos Aires, Argentina. *Pest Management Science* , 66 : 162-167.

Guitart, R., Croubels, S., Caloni, F., Sachana, M., Davanzo, F., Vandenbroucke, V., &Berny, P (2010a). Animal poisoning in Europe. Part 1: Farm livestock and poultry. *The Veterinary Journal* , 183, (3) : 249-254

Guitart, R., Sachana, M., Caloni, F., Croubels, S., Vandenbroucke, V., & Berny, P. (2010b). Animal poisoning in Europe. Part 3: Wildlife. *Veterinary journa , 183* (3) : 260-265.

Heiberg, A. (2009). Anticoagulant resistance: a relevant issue in sewer rat (Rattus norvegicus) control? *Pest. Management Scie.* 65 : 444-449

Heiberg, A.-C., Leirs, H., & Siegismund, H. (2006). Reproductive success of bromadiolone-resistant rats in absence of anticoagulant pressure. *Pest Management Science , 62* (9), 862-871.

Hermodson, M. A., Suttie, J. W. & Link, K. P. (1969). Warfarin metabolism and vitamin K requirement in the warfarin-resistant rat. *American Journal of Physiology* 217 : 1316-1319

Howald, G., Donlan, C., Galván, J., Russell, J., Parkes, J., Samaniego, A., Wang, Y., Veitch, D., Genovesi, P., Pascal, M., Sanuders, A., Tershy, B. (2007). Invasive rodent eradication on islands. *Conservation Biology , 21* (5), 1258-68.

Ishizuka, M., Okajima, F., Tanikawa, T., Min, H., Tanaka, K., Sakamoto, K., et al. (2006). Elevated Warfarin Metabolism in Warfarin-Resistant Roof Rats (Rattus rattus) in Tokyo. *Drug Metabolism and Disposition , 35* (1), 62-66.

Ishizuka, M., Tanikawa, T., Tanaka, K., & Heewon, M. (2008). Pesticide resistance in wild mammals-Mechanisms of anticoagulant resistance in wild rodents. *The Journal of Toxicological Sciences* 33 (3) : 283-291

Jackson, W., Ashton, A. (1986). Case histories of anticoagulant resistance. In: Committee on strategies for the management of pesticide resistant pest populations, National Research Council (eds). Pesticide resistance: strategies and tactics for management, National Academy Press, Washington pp: 335-369.

Jin, M., Chen, X., Ye, M., & Zhu, Y. (2008). Analysis of indandione anticoagulant rodenticides in animal liver by eluent generator reagent free ion chromatography coupled with electrospray mass spectrometry. *Journal of Chromatography A , 1213* (1), 77-82.

Knopper, L., Mineau, P., Walker, L., & Shore, R. (2007). Bone Density and Breaking Strength in UK Raptors Exposed to Second Generation Anticoagulant Rodenticides *Bulletin of Environmental Contamination and Toxicology* 78 : 249-251

Kohn, B., Weingart, C., & Giger, U. (2003). Haemorrhage in seven cats with suspected anticoagulant rodenticide intoxication. *Journal of Feline Medicine and Surgery* 5 : 295-304

Kolf-Clauw, M., Alvarez, E., Maytray, O. (1995). Anticoagulants rodenticides: étiologie, diagnostic et traitement des intoxications. *Rec. Méd. vét.* 171, 2/3: 127-134

Lambert, O., Pouliquen, H., Larhantec, M., Thorin, C., & L'Hostis, M. (2007). Exposure of raptors and waterbirds to Anticoagulant Rodenticides (Difenacoum, Bromadiolone, Coumatetralyl, Coumafen, Brodifacoum): epidemiological survey in Loire Atlantique (France) *Bulletin of Environmental Contamination and Toxicology* 79 : 91-94

Lasseur, R., Grandemange, A., & Longin-Sauvageon, C. (2007). Comparison of the inhibition effect of different anticoagulants on vitamin K epoxide reductase activity from warfarin-susceptible and resistant rat. *Pesticide Biochemistry and Physiology* 88 : 203-208

Lasseur, R., Grandemange, A., Longin-Sauvageon, C., Berny, P., & Benoît, E. (2006). Heterogeneity of the coumarin anticoagulant targeted vitamin K epoxide reduction

system. Study of kinetic parameters in susceptible and resistant mice (*Mus musculus domesticus*J. Biochem. Mol . Toxicol. 20, (5) : 221-229 .

Lasseur, R., Longin-Sauvageon, C., & Videmann, B. (2005). Warfarin resistance in a French strain of rats. *J. Biochem. Mol. Toxicol* .19 (6) : 379-385

Li, T., Chang, C., Jin, D., Lin, P., Khvorova, A., Stafford, D. (2004). Identification of the gene for vitamin K epoxide reductase. *Nature*, 427, 541-544.

Leung, L., & Clark, N. (2005). Bait avoidance and habitat use by the roof rat, Rattus rattus, in a piggery. *International Biodeterioration & Biodegradation* 55 : 77-84

Lodal, J. (2001). Distribution and levels of anticoagulant resistance in rat (Rattus norvegicus) in Denmark. In: Peltz, H.J., Cowan, D., Feare, C. (eds). Advances in Vertebrate Pest Management II, Finlander Verlag, Fürth, Germany, 139-148.

Lund, M. (1972). Rodent resistance to the anticoagulant rodenticides, with particular reference to Denmark. *Bulletin of the World Health Organization , 47* (5), 611-8.

Lund, M. (1984). Resistance to the second-generation anticoagulant rodenticides. In: Clark, D.O. (ed.). Proceedings of the 11th Vertebrate Pest Conference, Rodent Biology, Davis, California, USA, 23-26 May 1983, Academic Press, New York, p. 89-94.

Markussen, M., Heiberg, A., Alsbo, C., Nielsen, P., Kauppinen, S., & Kristensen, M. (2007). Involvement of hepatic xenobiotic related genes in bromadiolone resistance in wild Norway rats, Rattus norvegicus (Berk.). *Pesticide Biochemistry and Physiology , 88* (3), 284-295.

Markussen, M., Heiberg, A.-C., Nielsen, R., & Leirs, H. (2003). Vitamin K requirement in Danish anticoagulant-resistant Norway rats (Rattus norvegicus). *Pest Management Science , 59* (8), 913-920.

Martin, A., Steed, L., Redfern, R., Gill, J., Huson, L. (1979). Warfarin-resistance genotype determination in the Norway rat, *Rattus norvegicus*. *Lab. Anim*. 13 : 209-214

Misenheimer, T., Suttie, J. (1990). Warfarin resistance in a Chicago strain of rats. *Biochem. Pharmacol.* 40: 2079-2084.

Misenheimer, T., Lund, M., Baker, E.M., Suttie, J. (1993). Biochemical basis of warfarin and bromadiolone resistance in the house mouse, *Mus musculus domesticus. Biochem. Pharmacol.* 47 (4): 673-678.

Muhr, A. (1981). Zur problematik der Hausmausbekämpfung, resistenzprobleme und Möglichkeiten ihrer Uberwindung. In: Iglisch, I. (ed.). Aktuelle Probleme der Bekämpfung und Abwehr von Ratten und Hausmäusen, Pentagon Publishing GmbH, Frankfurt, 93-109

Myllymaki, A. (1995). Anticoagulant resistance in Europe: appraisal of the data from the 1992 EPPO questionnaire. *Pestic. Sci*. 43: 69-72.

Naganuma, K., Fukita, A., Taniguchi, N., Takada, S. (1981). Warfarin susceptibility in the roof rat, *Rattus rattus*, in some locations of Tokyo. *Jap. J. sanit. Zool*. 32: 243-245

O'Connor, J. (1948). The use of blood anticoagulants for rodent control. *Research* 1: 334-336.

Pelz, H.J., Hanisch, D., Lauenstein, G. (1995) Resistance to anticoagulant rodenticides in Germany and future strategies to control Rattus norvegicus. *Pestic. Sci.* 43: 61-67.

Pelz, H.J. (2001). Extensive distribution and high frequency of resistance to anticoagulants rodenticides in rat populations from north-western Germany. In: Pelz, H.J., Cowan, D., Feare, C. (eds). Advances in Vertebrate Pest Management volume II, Finlander Verlag, Fürth, Germany, 161-170

Pelz, J., Rost, S., Hünerberg, M., Fregin, A., Heiberg, A.C., Baert, K., MacNicoll, A., Prescott, C., Walker, A.S., Oldenburg, J., and Müller, C. (2005) The genetic basis of resistance to anticoagulants in rodents. *Genetics* 170: 1839-1847.

Petterino, C., & Paolo, B. (2001). Toxicology of Various Anticoagulant Rodenticides in Animals. *Veterinary and Human Toxicology* 43 (6) : 353-360

Prescott, C., & Buckle, A. (2000). Blood-clotting response tests for resistance to diphacinone and chlorophacinone in the Norway Rat (*Rattus norvegicus*, Berk) *Crop Protection* 19 : 291-296

Quy, R., Cowan, D., Prescott, C., Gill, J., Kerins, G., Dunsford, G., Jones, A., MacNicoll, A. (1995). Control of a population of Norway rats resistant to anticoagulant rodenticides. *Pest. Sci.* 45: 247-256.

Redfern, R., & Gill, J. (1980). Laboratory evaluation of bromadiolone as a rodenticide for use against warfarin-resistant and non-resistant rats and mice. *The Journal of hygiene , 84* (2), 263-8.

Robben, J., Kuijpers, E., & Mout, H. (1998). Plasma superwarfarin levels and vitamin K1 treatment in dogs with anticoagulant rodenticide poisoning *Vet Quarterly* 20 : 24-27

Rost, S., Fregin, A., Ivaskevicius, V., Conzelmann, E., Hörtnagel, K., Pelz, H-J., Lappegard, K., Seifried, E., Scharrer, I.,Tuddenham, E., Müller, C., Strom, T., Oldenburg, J. (2004). Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* 427: 537-541.

Rost, S., Pelz, H.-J., Menzel, S., Macnicoll, A., Leon, V., Song, K.-J., et al. (2009). Novel mutations in the VKORC1 gene of wild rats and mice - a response to 50 years of selection pressure by warfarin? *BMC Genetics , 10* (1), 4.

Sage, M., Fourel, I., Cœurdassier, M., Barrat, J., Berny, P., & Giraudoux, P. (2010). Determination of bromadiolone residues in fox faeces by LC/ESI-MS in relationship with toxicological data and clinical signs after repeated exposure. *Environmental Research* 110 (7) : 664-674

Saunders, G. (1978). Resistance to warfarin in the roof rat in Sydney. *New South Wales Search,* 9: 39-40

Shore, R., Birks, J., & Freestone, P. (1999). Exposure of non-target vertebrates to second-generation rodenticides in Britain, with particular reference to the polecat Mustela putorius. *New Zealand Journal of Ecology* 23 (2) : 199-206

Siddiqi, Z., Blaine WD. (1982). Anticoagulant resistance in house mouse in Toronto, Canada. *Environ. Health Rev.*, 26: 49-51.

Stone, W., Okoniewski, J., & Stedelin, J. (2003). Anticoagulant rodenticides and raptors: Recent findings from New York, 1998-2001. *Bulletin of Environmental Contamination and Toxicology* 70 : 34-40

Sugano, S., Kobayashi, T., Tanikawa, T., Kawakami, Y., Kojima, H., Nakamura, K., Uchida, A., Morishima, N., Tamai, Y. (2001) Suppression of CYP3A2 mRNA expression in the warfarin-resistant roof rat, *Rattus rattus* : possible involvement of cytochrome P450 in the warfarin resistance mechanism. *Xenobiotica* 31: 399-407

Sutcliffe, F., MacNicoll, A., Gibson, G. (1990) Hepatic microsomal warfarin metabolism in warfarin-resistant and susceptible mouse strains: influence of pretreatment with cytochrome P-450 inducers. *Chem. biol. Interact* 75: 171-184.

Thorsen, M., Shorten, R., Lucking, R., & Lucking, V. (2000, Jan 1). Norway rats (Rattus norvegicus) on Frégate Island, Seychelles: the invasion; subsequent eradication

attempts and implications for the Island's fauna *Biological Conservation* 96 : 133-138

Tie, J., Nicchitta, C., von Heijne, G., Stafford, D. (2005). Membrane topology mapping of vitamin K epoxide reductase by in vitro translation/cotranslocation. *J. biol. Chem.* 280: 16410-16416.

Tosh, D., Shore, R., Jess, S., Withers, A., Bearhop, S., Montgomery, W., et al. (2011, Jan 25). User behaviour, best practice and the risks of non-target exposure associated with anticoagulant rodenticide use. *Journal of Environmental Management* , 92 (6) : 1503-1508

Vandenbroucke, V., Bousquet-Melou, A., De Backer, P., & Croubels, S. (2008). Pharmacokinetics of eight anticoagulant rodenticides in mice after single oral administration. *Journal of Veterinary Pharmacology and Therapeutics , 31* (5), 437-445.

Vandenbroucke, V., Desmet, N., Debacker, P., & Croubels, S. (2008). Multi-residue analysis of eight anticoagulant rodenticides in animal plasma and liver using liquid chromatography combined with heated electrospray ionization tandem mass spectrometry. *Journal of Chromatography B , 869* (1-2), 101-110.

Vein J., Grandemange A., Cosson, J.F., Benoît, E ., & Berny P. (in press) Are water voles (Arvicola terrestris) resistant to anticoagulant rodenticides, following field treatments ? *Ecotoxicology*

Vudathala, D., Cummings, M., & Murphy, L. (2010). Analysis of multiple anticoagulant rodenticides in animal blood and liver tissue using principles of QuEChERS method. *Journal of analytical toxicology , 34* (5), 273-9.

Wajih, N., Sane, D., Hutson, S., Wallin, R. (2004) The inhibitory effect of calumenin on the vitamin K-dependent –carboxylation system. Characterization of the system in normal and warfarin-resistant rats. *Biochem. Pharmacol*., 279: 25276-25283.

Walker, L., Turk, A., Long, S., Wienburg, C., Best, J., & Shore, R. (2008). Second generation anticoagulant rodenticides in tawny owls (Strix aluco) from Great Britain. *Science of the Total Environment , 392* (1), 93-98.

Wallace, M., & MacSwiney, F. (1976). A major gene controlling warfarin-resistance in the house mouse. *The Journal of hygiene , 76* (2), 173-81.

Wang, J., Feng, Z., Yao, D., Sui, J., Zhong, W., Li, M., & Dai, J. (2008). Warfarin resistance in Rattus losea in Guangdong Province, China. *Pesticide Biochemistry and Physiology , 91* (2), 90-95.

Watt, B., Proudfoot, A., Bradberry, S., & Vale, J. (2005). Anticoagulant rodenticides. *Toxicological reviews , 24* (4): 259-269.

Whisson, D., & Salmon, T. (2002). Effect of the timing of applications and amount of 0.01% diphacinone consumed on mortality of California ground squirrels (*Spermophilus beecheyi*) *Crop Protection* 21 : 885-889

World Health Organization (1982). Instructions for determining the susceptibility or resistance of rodents to anticoagulants rodenticids. *WHO Vector Biol. Control. Ser.* 8: 843-849.

21

Morphogenetic Activities of Bendiocarb as Cholinesterase Inhibitor on Development of the Chick Embryo

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1. Introduction

1.1 Xenobiotics and pesticides

Xenobiotics are exogenous foreign substances with different effects on living organism. One group of xenobiotics present agrochemicals including pesticides that can be related to many health disorders. Besides acute intoxication these can also cause motor and sensory disorders (e. g. Parkinson´s disease), carcinogenic diseases, reproduction disorders, immunosupression and allergic states, etc. From a medical view these exogenous foreign environmental compounds commend great respect. The animal eukaryotic multicellular organism is a complicated multistage process at the molecular, physiological and morphological levels. Various negative regulating factors can pass into their processes. Teratogenesis and consequently malformation, eventually death of embryo or foetus can be a result. Some pesticide action of cholinesterase inhibitors, which prevent of inhibit cholinesterase from cleaning up unused acetylcholine also belong to this group. Many of these xenobiotics are certified or assumed to be carcinogenic, embryotoxic or foetotoxic and teratogenic chemical compounds.

Agrochemicals, as chemicals used in agriculture, include a broad range of products used for the nutrition of plants, protection of fruits, protection and nutrition of animals. There are pesticides, substances used as repellents or destroyers of all sorts of plant and animal pests. At present, among more than 700 registered pesticides around the world, there are herbicides, insecticides, fungicides, rodenticides, nematocides, bactericides, algicides, molluscocides, ascaricides, etc. They are exogenous foreign substances, xenobiotics, with various consequences for living organisms. Their effect depends mainly from substances activity, from their metabolizing, respectively (Wylie et al., 2005).

It is known that pesticides can cause acute intoxication or carcinomas. Many of these suppress the immune system which increases its susceptibility to virus, bacteria and parasitic infections and to tumours. It is known that farmers are population with a high risk of Hodgkin´s disease, melanomas, multiply myeloma and leukaemia.

Many agrochemicals (xenobiotics) including pesticides, in the liver through detoxication can be transformed to reactive compounds with mutagenic, carcinogenic or teratogenic metabolites (so-called biotransformation).
Many pesticides, mainly insecticides are introduced among cholinesterase inhibitors, which commonly have an intimate relation to animal morphogenesis.Pesticides are a group of chemicals with high biological activity that are worldwide introduced into the environment and expose large populations of living organisms. Pesticides possess properties that make them different from other chemicals mainly because they are introduced to environment. They are important for their stability in the environment, exposure of population and high biological activity. Though, toxic effect of the pesticides is specialized to specific species. They may endanger also human health, and both domestic and wildlife animals. The annual application of synthetic pesticides to food crops in the EU exceeds 140,000 tones, an amount that corresponds to 280 grams per EU citizen per year. Many pesticides, mainly insecticides are introduced among cholinesterase inhibitors, which commonly have an intimate relation to animal morphogenesis.

1.2 Morphogenetic roles of acetylcholine and cholinesterase

The biological process of morphogenesis is a process in which living systems produce forms and structures through mechanical and biological factors including morphogens. These are developmental signals that exert specific effects on receptive cells depending on their concentration. In developing tissues, neurotransmitters subserve growth regulatory and morphogenetic functions. The stimulation of cholinergic receptors in target cells during a critical developmental period provides signals that influence cell replication and differentiation. Accordingly, environmental agents that promote cholinergic activity evoke neurodevelopmental damage because of the inappropriate timing or intensity of stimulation.
Morphogens are developmental signals that exert specific effects on receptive cells, depending on concentration. Morphogens are present in gradients created by the presence of a “source” and “sink”. Developing cells are affected in specific ways along this concentration gradient. This concept has traditionally been applied to substances involved in pattern formation and morphogenesis, such as retinoic acid. However, it may also be appropriate to consider neurotransmitters as morphogens when they act as dose-dependent morphogenetic signals in neural and non-neural tissues (Lauder, 1988 as cited in Lauder & Schambra, 1999).
Neurotransmitters participate in various forms of intra- and intercellular signaling throughout all stages of ontogenesis, (Buznikov et al., 1996) and they exert their effects using receptors and signal transduction mechanisms similar to those in the adult nervous system (Lauder, 1985, as cited in Slotkin, 1999). These substances and their specific receptors has been identified during ontogeny of the mammalian nervous system, and it is now certain that transmitters play essential roles in the cellular and architectural development of the brain (Whitaker-Azmitia, 1984, as cited in Slotkin, 1999). During this period, receptor stimulation uniquely communicates with the genes that control cell differentiation, changing the ultimate fate of the cell. The ontogenetic state of the target cell is critical in determining whether the outcome of receptor stimulation is an effect on cell replication, differentiation, growth, death (apoptosis), or "learning," that is, determining the future set-point for responsiveness of the cell. At the same time, these multiple roles create a wide

window of vulnerability in which exposure of the brain to neuroactive chemicals that elicit or block neurotransmitter responses can alter development (Yanai, 1984, as cited in Slotkin, 1999). Thus, unlike classical teratology, in which the first trimester of fetal development is the most sensitive target for adverse effects of drugs or chemicals may make developing neurotransmitter system especially vulnerable to environmental neurotoxins, such as pesticides, designed to target receptors for these neurochemicals in lower organisms (Slotkin, 1999).

Acetylcholine (ACh) is synthesized from acetyl coenzyme A and choline by the enzyme choline acetyltransferase. In addition to its synthesis in the liver, choline employed in acetylcholine production is derived from dietary sources. There is a carrier system in capillary endothelial cells that is responsible for transport of choline, in its free and phospholipid forms, into the brain.

ACh is a neurotransmitter widely diffused in central, peripheral, autonomic and enteric nervous system. Presynaptic choline transport supports ACh production and release, and cholinergic terminals express a unique transporter critical for neurotransmitter release. Neurons cannot synthesize choline, which is ultimately derived from the diet and is delivered through the blood stream (Amenta & Tayebati, 2008).

ACh plays regulatory roles throughout ontogenesis, including stages prior to development of the nervous system (Buznikov et al., 1996). Acetylcholine is a major excitatory neurotransmitter in the nervous system of vertebrates and invertebrates. Accumulated evidence suggests that ACh also plays a key role in regulation of morphogenetic cell movements, cell proliferation, growth, and differentiation in species as diverse as echinoderms, insects, worms, avians, rodents, and humans (Lauder & Schambra, 1999). Evidence that ACh plays a key role in neural development suggests that disruptors of cholinergic function could disturb these actions if present during key critical periods. The cerebral cortex may be especially vulnerable to such insults because of important roles cholinergic afferents play in cerebral morphogenesis and synaptogenesis. Disruptors of cholinergic function that may have significant effects on brain development include alcohol, nicotine, and cholinergic pesticides (Slotkin, 1999).

In sea urchin embryos, cell movements occurring during gastrulation and postgastrulation stages appear to be regulated by ACh and biogenic monoamines (Falugi, 1993). Similar functions of ACh during gastrulation of vertebrate embryos are suggested by the presence of AChE during gastrulation in the chick embryo (Laasberg et al., 1987 as cited in Lauder & Schambra, 1999).

Developing animals are more sensitive than adults to acute cholinergic toxicity from anticholinesterases, including organophosphate and carbamate pesticides, when administered in a laboratory setting. It is also possible that these agents adversely affect the process of neural development itself, leading to permanent deficits in the architecture of the central and peripheral nervous systems. New evidence that AChE may have a direct role in neuronal differentiation provides additional grounds for interest in the developmental toxicity of anticholinesterases. Still, developing rats recover faster from AChE inhibition than adults, largery due to the fact that developing organisms have a rapid synthesis of new AChE molecules. It therefore seems that either developmental toxicity may unrelated to AChE inhibition, or that even a brief period of AChE inhibition is sufficient to disrupt development (Slotkin, 2004). Some selective cholinesterase inhibitors effectively suppress neurite outgrowth in model systems like differentiating neuroblastoma cells and explanted

sensory ganglia. Certain of these "morphogenic" effects may depend on protein-protein interactions rather than catalytic AChE activity. It remains possible that some pesticides interfere with important developmental functions of the cholinesterase enzyme family (Brimijoin & Koenigsberger, 1999). Insecticides which enhance cholinergic effects through inhibition of cholinesterase are the most widespread chemical assaults on the fetus.

AChE is the enzyme that hydrolyzes the neurotransmitter acetylcholine at cholinergic synapses and neuromuscular junctions. The reaction that is catalysed by AChE is: acetylcholine + H_2O → choline + acetate. AChE is present in mammals, birds, fish, reptiles and insects (Fukuto, 1990, as cited in Van Dyk & Pletschke, 2011). AChE predominates in neurons and muscle cells wherever cholinergic synapses are found. But the full picture is more complex, since AChE also occurs in nonneural and embryonic tissues like red blood cells, megakaryocytes, and migrating neural crest cells (Brimijoin & Koenigsberger, 1999). Proposed noncholinergic roles for AChE range from neuromodulation by secreted forms to promotion of cell proliferation in tumor growth and hematopoiesis (Soreq et al., 1994).

Prenatal exposure to organophosphate and carbamate pesticides could have adverse effects on neural development by interfering with the morphogenic function of AChE. Accumulating evidence indicates that AChE has extrasynaptic functions during neural development (Layer & Willbold, 1995, as cited in Bigbee et al., 1999). This idea was initially based on in vivo observations that AChE is transiently expressed by neurons throughout periods of axonal outgrowth prior to synaptogenesis, a period during which the classical cholinolytic role for AChE in terminating nervous transmission is unnecessary. In the chick, transient AChE expression occurs in developing spinal cord neurons, which coincides with axonal outgrowth from these cells (Weikert et al., 1990). In the peripheral nervous system, AChE is transiently expressed by developing dorsal root ganglion neurons and later in their axons and growth cones in the spinal cord. Together, these data strongly suggest that AChE plays a developmental role in the morphogenesis of the nervous system (Bigbee et al., 1999).

Although AChE may affect morphogenesis by noncatalytic mechanisms such as structural recognition, these mechanisms could certainly be vulnerable to pesticides. However, the toxicologic data reviewed earlier indicate that those agents may have additional actions that would be deleterious to a growing nervous system. It seems wise to re-evaluate the developmental risks of anticholinesterases as data become available from ongoing studies of environmentally relevant molecules in neuronal culture and sensitive embryologic models of neural development (Brimijoin & Koenigsberger, 1999).

Today we know three isoforms of AChE:

- AChE - S (synaptic soluble) the major multimeric enzyme of brain and muscles,
- AChE - R ('readthrough') monomeric enzyme of embryonic and tumor cells
- AChE - E (erythocytes) associated with erythrocyte membranes.

Prenatal development of the central cholinergic nervous system coupled with the important developmental roles played by ACh in both neural and non-neural tissues should make the vertebrate embryo especially vulnerable to the gestational effects of environmental neurotoxins that target cholinergic receptors or choline esterase. It seems especially important to study of effects of chronic prenatal exposure to cholinergic pesticides on pre- and postnatal brain development as well as behavioral consequences of these exposures (Lauder & Schambra, 1999). Drugs or chemicals that target cholinergic neurotransmission probably represent the largest source of neurobehavioral teratogenesis. Agricultural and

household pesticides that target AChE could interfere with non-cholinergic role of AChE if exposure occurs during critical periods of nervous system development (Bigbee et al., 1999). Given the widespread use and exposure to pesticides, the general lack of data on developmental neurotoxicity is a serious impediment. For certain pesticides, a requirement exists for neurotoxicity tests in adult animals, but developmental neurotoxicity is usually not considered when determining pesticide safety. Experimental, clinical, and epidemiologic evidence suggests that neurotoxic pesticides can also cause developmental neurotoxicity, and that the effects are more severe and lasting, and that they occur at much lower exposure levels. Given the likely environmental etiology of neurodevelopmental deficits and their importance to families and to society, prevention of exposures to neurotoxic pesticides should be made a public health priority (Bjørling-Poulsen et al., 2008).

1.3 Carbamates and organophosphates like inhibitors of cholinesterase

Decades ago the activity of acetylcholinesterase (AChE) was used as a sensitive indicator of exposure to organophosphate and carbamate pesticides. The inhibition of AChE is associated with a toxic mechanism of pesticide, with the pesticide´s reversible or irreversible binding to the ester-point of enzyme and with increasing of cholinergic effects on the nervous system (Kristoff et al., 2006). The inhibition of AChE by organophosphates takes place as a result of the phosphorylation of the serine residue in the active site of the enzyme. Carbamate pesticides are cholinesterase inhibitors with a similar mechanism of action as organophosphate pesticides. However, carbamates cause only reversible inhibition of AChE. Thus, AChE inhibition by carbamates lasts only minutes or hours, whereas the effects of organophosphates (OPs) with respect to AChE can last for 3–4 months (Van Dyk & Pletschke, 2011). A number of pesticides belong to the group called cholinesterase inhibitors (ChEI), which not only in one case report the relation to morphogenesis. ChEI form a group of chemical compounds that prevent hydrolysis of acetylcholine (ACh; as a classical neurotransmitter of the autonomic nervous system providing communication between cells), thereby allowing accumulation of ACh in the reactive sites of the living organism. Such activity may have some drugs (eg. Alzheimer's disease and other dementia diseases called memory drugs) but also insecticides, respectively some chemical warfare agents that can be fatal for humans and animals (Krall et al., 1999).

Due to the fact that AChE is inhibited, ACh is not hydrolyzed and its accumulation occurs on the receptors of target cells. Cumulation effects are manifested by excessive stimulation of cholinergic synapses in the central and peripheral nervous system (Huff et al., 1989, Ratner et al. 1983). The most common accompanying symptom is decreasing of AChE activity in blood and dysregulation of ions between the external and internal environment causing failure of formation of action potentials in nerve endings (Kassa & Samnaliev, 2004; Mignini et al., 2003).

Changes induced by the action of ChEI can be divided into two effects:

1. muscarinic effect and
2. nicotine effect.

Both have central and peripheral effects.

Peripheral nicotinic effects manifest on the periphery causing fasciculation, muscle contraction, muscle pain, general weakness, tachycardia, hypertension, hyperglycaemia and mydriasis.

Peripheral muscarinic effects in the periphery cause contraction of smooth muscles, stimulation of the glands, increased salivation, lacrimation, rhinorrhoea, bronchial secretion

(bronchorea) bronchial constriction, cyanosis, nausea, vomiting, incontinence (incontinentio urinae), bradycardia and pulmonary edema.
Central effect causes insomnia, sleep disorders, headaches, dizziness, behavioral disorders, tremor, ataxia, respiratory depression, convulsions and coma (Levelridge, 1998).

1.3.1 Organophosphates

0Ps belong to a group of insecticides, which have been discovered in 1938 by German chemists. OPs were used as nerve poisons during the 2nd World War. At present, the OPs are used in agriculture and also used as antiparasitic substances for destroying insects such as fleas, louse and mosquitoes.
OPs replace forbidden organic chlorine compounds and they are a major cause of poisoning in animals. They vary in toxicity, the level of residue and excretion. Many OP were developed to protect plants and animals and generally have an advantage by creating little or no residues in tissues and environment. But it seems that chlorinated OP compounds have greater potential for producing tissue residues. Many OPs used as pesticides are not strong inhibitors of esterases, until they are activated in the liver by microsomal enzymes (Toxicology, 1998).
In severe cases of OP poisoning in adults (AChE inhibition exceeding 70%), a "cholinergic syndrome" is elicited, including various central nervous system effects such as headache, drowsiness, dizziness, confusion, blurred vision, slurred speech, ataxia, coma, convulsions and block of respiratory centre. Some OPs can also induce a delay neuropathy which does not involve inhibition of AChE but rather the neuropathy target esterase (NTE). The physiological functions of NTE are still unknown, and it is obscure how phosphorylation and aging of NTE leads to axonal degeneration. The syndromes described above are observed only following high dose, acute exposures to OPs. Survivors recover from these syndromes, but it is likely that the exposure also produces long-term adverse health effects. In rats, a single high exposure to an OP can cause long lasting behavioural effects and the same has been reported from several human studies (Bjørling-Poulsen et al., 2008). WHO estimates that each year there are 3 million cases of acute pesticide poisoning, and it ends with 220 000 deaths (Jaga & Dharmani, 2003).

1.3.2 Carbamates

Carbamates (CAs) are substances that were originally extracted from the bean called calabar, which is the home plant of Western African States. The obtained extract contained esters of physostigmine and methylcarbamate. CA is considered a derivative of carbamic acid. CAs act as acetyl cholinesterase (AChE) inhibitors that affect lots of organs such as peripheral and central nervous systems, muscles, liver, pancreas, and brain. There are several reports about metabolic disorders, hyperglycemia, and also oxidative stress in acute and chronic exposures to pesticides that are linked with diabetes and other metabolic disorders. Induction of oxidative stress by some carbamates might also cause developmental neurotoxicity. In this respect, there are several in vitro and in vivo but few clinical studies about mechanism underlying these effects (Karami-Mohajeri & Abdollahi, 2010). When comparing the clinical course of carbamate poisoning (by aldicarb or methomyl) in young children (1–8 years old) and adults (17–41 years old), it was found that the predominant symptoms in children were CNS depression and hypotonia, and the most common muscarinic effect was diarrhoea. In adults the main symptoms were miosis and

fasciculations, whereas CNS depression, hypotonia, and diarrhoea were uncommon. As for the OPs, it is likely that poisoning with carbamates may result in long term neurological effects. Two patients showed cognitive deficit in attention, memory, perceptual, and motor domains 12 months after a poisoning incident. With respect to long term, low level exposures to carbamates, reports concerning chronic toxicity are almost non-existent. (Bjørling-Poulsen et al., 2008). Results indicated that CAs impair the enzymatic pathways involved in metabolism of carbohydrates, fats and protein within cytoplasm, mitochondria, and proxisomes (Karami-Mohajeri & Abdollahi, 2010). Also, carbamate insecticides inhibit cellular metabolism including energy, protein, and nucleic acid metabolism, thereby, causing cell regression and death (Mohd.Amanullah & Hari, 2011).

Carbamates represent except AChE inhibitors also inhibitors of brain esterase (NTE neuropathy target esterase) leading to polyneuropathy. This neuropathy arises due to degeneration of long axons of nerve cells. Brain NTE esterase is a protein that is present in neurons as well as in other vertebrate cells and plays a role in the interaction between neurons and glial cells relevant in the evolving nervous system (Glynn, 1999). Recent studies suggest that carbamates cause virtually 100% inhibition of NTE and polyneuropathy in chicken models (Lotti & Moretto, 2006).

No epidemiological studies of developmental neurotoxicity of carbamates in humans could be found, and data from animal experiments are very sparse as well. Assuming that some of the neurotoxic effects observed in association with prenatal exposure to OPs, such as chlorpyrifos, are due to inhibition of AChE, it is possible that carbamates may have similar developmental effects, even though the inhibition of AChE by carbamates is only transient (Bjørling-Poulsen et al., 2008).

1.4 Bendiocarb

Bendiocarb (2,3–isopropyledene–dioxyphenyl methylcarbamate) is a carbamate insecticide, which also belongs among the ChEIs, and is used to control disease vectors such as mosquitoes and flies, as well as household and agricultural pests. Most formulations of bendiocarb are registered for general use, except to Turcam, Turcam 2.5 G and the best-known product Ficam (Flesarova et al., 2007). The blockage of enzyme cholinesterase (ChE) caused by bendiocarb persists for approximately 24 hours and subsequently the situation returns to normal after acute exposure because the insecticide does not accumulate in mammalian tissues (Sirotakova et al., 2005). Bendiocarb is the pesticide acting upon invertebrates by irreversibly blocking the activity of the ChE, which is critical in allowing muscle relaxation by removing the neuromuscular mediator ACh. Acute toxic symptoms of carbamate poisoning, e.g. miosis, urination, diarrhea, diaphoresis, lacrimation, salivation, and excitation of the central nervous system, are generally caused by inhibition of the AChE, which leads to accumulation of ACh. An acute oral toxicity (LD_{50}) was investigated in different adult mammals; rat 34-156 mg kg^{-1}, guinea pig 35 mg kg^{-1}, rabbit 35-40 mg kg^{-1}; and also in non-mammalian species like birds: mallard duck 3.1 mg kg^{-1}, bobwhile quail 16 mg kg^{-1}, hen 137 mg kg^{-1} (World Health Organization [WHO], 2007), fish 0.7 – 1.8 mg kg^{-1} (LC_{50}, Hayes & Lawes, 1990); bee 0.1 μg per bee (Wright et al., 1981).

Studies on chronic exposure to carbamate insecticides and case reports of long-term exposure give equivocal results. Chronic intoxication with bendiocarb was investigated in 2-year study on adult rats that administered bendiocarb orally in a dose of 10 mg/kg/day. The author observed changes in the weight of organs, composition of blood and urine and

also increased occurrence of stomach and eye lesions (Baron, 1991). Adult rabbits that were administered bendiocarb per os for 90 days at the dose 5 mg/kg/day, showed slight toxic effect of bendiocarb. However, no negative effect of bendiocarb was observed on formation of thymus structures (Flesarova et al., 2007). In a three-generation reproductive study in rats, the no-effect levels of bendiocarb administered in the diet were considered to be 0.6 and 3 mg kg^{-1} body weight per day for reproductive effects respectively. No teratogenic effects were seen in the offspring of rats given 4 mg kg^{-1} or in rabbits given 5 mg kg^{-1} per day of bendiocarb during gestation (Kamrin, 1997). Up to now, in the organotoxic (acute and chronic toxicity) and ecotoxic properties, moreover reproductive, teratogenic, mutagenic and carcinogenic effects of this insecticide have been studied.No epidemiological studies of developmental neurotoxicity of carbamates in humans could be found, and data from animal experiments are very sparse as well.
Currently the oral LD_{50} of bendiocarb for hen is 137 mg/kg.b.w (WHO, 2007). Up until now no detailed studies were conducted regarding the embryotoxic effects of bendiocarb on birds which are more sensitive to the action of toxic substance.

1.5 Animal model - chick embryo

Animal models play a crucial role in fundamental and medical research. Progress in the fields of drug study, regenerative medicine and cancer research among others are heavily dependent on *in vivo* models to validate *in vitro* observations, and develop new therapeutic approaches. However, conventional rodent and large animal experiments face ethical, practical and technical issues that limit their usage. The chick embryo represents an accessible and economical *in vivo* model, which has long been used in developmental biology, gene expression analysis and loss/gain of functional experiments.
Chick embryo is a popular model for developmental pharmacological and toxicological studies. It is readily available, cost-efficient, and presents an alternative approach to treatment of pregnant mammals. The concordance of data from CHEST and mammals is excellent, and it avoids potentially confounding effect of different maternal metabolism between species by allowing for separate testing of human-relevant metabolites. Given the absence of maternal metabolism, it requires considerably smaller amounts of administered substances per embryo, which is particularly useful for testing rare or expensive compounds, or when maternal toxicity is of concern.
The nervous system of the chick embryo is formed from neural plate and the neural crest. At 2 ED the neural tube possesses two layers, the *ependyma*, which contains a large number of mitotic cells and the *marginal layer*. By 3 ED the *mantle layer* is also recognizable. Neuroblasts are visible from about 2 ED in the ventro-lateral part of tube. By 3 ED spinal nerves have developed and by 3-4 ED regions of grey and white matter are recognizable. Dorsal and ventral horns can be seen in the grey matter from 7 ED, and glial cells in the white matter. During the following days the spinal cord becomes larger in transverse section and there is a change in shape of the lumen from a longitudinal slit to an almost square or round shape. The presumptive liver areas of the chick embryo are closely associated with those of the heart and together are known as the cardio-hepatic regions. Whereas the heart is an entirely mesodermal structure, the liver is formed from both, mesoderm and endoderm. The liver primordium is visible at the end of 2 ED. As it grows, it comes into contact with the body wall (Bellairs & Osmond, 2005).
In order to assess to the fullest extent the possible embryotoxic potential, we performed a detailed study of bendiocarb effects in the chick embryo.

We observed the toxicity (mortality and weight of survived embryos - LD_{50}) of bendiocarb and the associated occurrence of malformations during various developmental stages (embryonic days 2-5 and 10). Then we observed the organ toxicity as well as the programmed cell death (apoptosis) after bendiocarb administration. Bendiocarb was administered to individual developmental stages in various doses (8-1600 μg/egg).
Agrochemicals, including pesticides, are being used in increasing amounts in agriculture and are therefore potential environmental contaminants which may affect a variety of biological systems. The pesticides residues directly affect the embryos, disturbing their normal development and causing pathophysiological and morphological changes. The aim of our study was to investigate toxicity of cholinesterase inhibitor bendiocarb to organs (liver, CNS) of the chick embryo and also the entire embryotoxicity in the chick embryo.

2. Material and methods

2.1 Eggs

Fertilized white Leghorn chicken eggs were purchased from the animal facility of the Institute of Molecular Genetics (Koleč, Czech Republic) and incubated without storage blunt end up in a forced-draft constant-humidity incubator at 37.5 °C with continuous rocking and relative humidity 60 % until embryonic days (ED) 2-10 of the (21-day) incubation period. Embryos were observed during incubation and dead, growth retarded or dysmorphic individuals at the time of treatment were excluded from further study.

2.2 Bendiocarb

The bendiocarb (2,2-dimethyl-1,3-benzzodiol-4-yl-*N*-methyl carbamate, Bendiocarb tech, 98.9%, Bayer, Germany) was dissolved in acetone and diluted with sterile water intended for tissue culture to obtain the required concentrations.

2.2.1 Application of bendiocarb

At embryonic days 2, 3, 4, 5 and 10, the eggs were opened by the modified „window technique" (Jelinek, 1977). The blunt end of eggs was cleaned with 70 % alcohol and covered by a transparent adhesive tape (Sedmera et al., 2002). Subsequently, using serrated scissors (FST 14071-12), an opening was cut for application of the respective doses of bendiocarb. The tested solution was applied directly over the embryo on the top of inner shell membrane (*membrane papyracea*). Controls received the same volume of solvent alone – 10 μl of acetone in 200 μl of water for injection. The ranges of concentration as well as the total number of embryos and the days of application are listed in Table 1.

2.2.2 Application dose

The application dose per one egg was 200 μL, with acetone concentration equal to 10 μL/200 μL of application dose.

2.3 Processing of the chick embryo

At the time of bendiocarb application on ED 2-5 the chick embryos were removed on ED 9. At the time of bendiocarb application on ED 10 the chick embryos were removed from eggs on ED 17. The chick embryos were removed from the eggs using a crook, weighed and examined under a dissecting microscope for external (eye, beak, palate, body wall, limbs) and internal (gastrointernal system, microdissection of the heart) anomalies.

	ED	dose (μg)	*N*
Embryotoxicity	**2**	control	35
		8	20
		80	22
		200	13
		400	11
		800	21
		1200	22
	3	control	36
		16	24
		160	19
		500	15
		1000	22
		1600	50
	4	control	23
		16	17
		160	18
		500	19
		1000	20
		1300	21
		1600	12
	5	control	40
		80	22
		160	21
		320	21
		500	23
		1000	19
		1600	21
	10	control	12
		800	17
		1600	15
Organ toxicity	3	control	36
		500	15
	10	control	12
		800	17

Table 1. Application doses, embryonic days (ED) of application and number of embryos (*N*)

2.4 Methods

2.4.1 Light microscopy – organ toxicity - general microscopic changes

After the examination under the dissecting microscope the embryos were fixed for 24 hours in Dents´solution (20% dimethyl sulphoxide and 80% methanol) and processed by a standard way for histological examination. Neck and part of the liver were separated from the fixed chicken embryos (exposure at 3 ED and 10 ED, right liver lobe from embryos

exposed at 10 ED). The respective parts of embryos were embedded in paraffin and after 24 hours a microtome (Leica RM 2265) was used to cut sections of thickness 10 μm.
To observe the microscopic changes in the liver and CNS, part of the sections was stained with haematoxylin-eosin. The microscopic examination was carried out under optical microscope Olympus BX 51 using a dry objective with 60 x magnification. Pictures were taken subsequently using a digital camera DP 70 and Cell P (Olympus) software.

2.4.2 Fluorescence microscopy – caspase activity

The remaining part of the sections was stained immunohistochemically for observation of caspases activity. The caspases activity was observed in the liver and CNS by means of primary murine monoclonal antibody IgG 1—Caspase-3/CPP32 (BD Pharmingen) and secondary antibody conjugated with Rhodamine Red dye (Jackson ImmunoResearch). To visualize the nuclei in the liver, the respective sections were stained with Hoechst 33258 dye (Calbiochem). The Rhodamine Red-conjugated antibody was red under a fluorescent microscope when using a suppression filter (465 nm) while Hoechst 33258 stain was blue when using an excitation filter (420 nm). Autofluorescence in the fluorescein channel was used for tissue contrast. Microscopic examination was carried out by means of a fluorescence microscope Leica using a dry objective with 60 x magnification.

2.4.3 Confocal microscopy – embryotoxicity - detection of dead cells

In a separate group of 11 embryos treated with 400 μg of bendiocarb on ED 3 and 5 controls, the sampling was performed at 24 and 48 h intervals for the purpose of whole-mount detection of dead cells using Lysotracker Red (Invitrogen, USA; Schaefer et al., 2004). After staining, the embryos were fixed with 4% paraformaldehyde (Sigma-Aldrich. Germany) in phosphate-buffered solution (PBS; NBS Biologicals, England) for 24 h at 4 °C, rinsed in PBS, dehydrated through graded ethanol (Sigma-Aldrich, Germany) series and cleared in benzyl alcohol (99%)–benzyl benzoate (99%, Sigma-Aldrich, Germany; in mixing rate (1:1) for examination on a confocal microscope (Miller et al., 2005). Validity of using Lysotracker Red for whole-mount detection of cell death was verified using bromodeoxyuridine (BrdU; 99%, Fluka, Switzerland) in the positive control group. An applied dose of 5 μg bromodeoxyuridine is considered to be embryotoxic on ED 3–5, causing alterations of programmed cell death and deviation of limb development (Sedmera & Novotna, 1994). Images acquired in the green and red channels on a Leica SPE confocal microscope were processed using Adobe Photoshop.

2.5 Statistical analysis

Statistical comparison of different groups was performed using the statistical software GraphPad Prism. Value of $P<0.05$ were considered significant.

3. Results and discussion

3.1 Embryotoxicity

Total embryotoxicity of a single dose of bendiocarb after application on ED 2, 3, 4, 5 and 10 was investigated on the sampling days (ED 9 and 17). The embryolethality (expressed as LD50; Table 2) decreased with increasing age (Fig. 1), except on ED 3, when the LD_{50} was the lowest. The embryolethality after bendiocarb application on ED 10 could not be determined

because of solubility limits (200 g l^{-1} of acetone, but lower in mixture with water for injection; too much of concentrated acetone is toxic to the embryo).

ED	Dose (µg)	N	Dead	Mortality (%)	Malformed	Mean weight (g)*	Weight SD	LD_{50} (mg/egg)
2	0	35	5	14	3	1,355	0,272	
	8	20	0	0	0	**1,546**	0,218	
	80	22	1	5	1	1,399	0,378	
	200	13	5	38	0	1,363	0,119	**0,973**
	400	11	5	46	0	1,305	0,081	
	800	21	7	33	0	1,196	0,245	
	1600	22	16	73	0	1,304	0,267	
3	0	36	4	11	0	1,446	0,165	
	16	24	6	25	1	1,421	0,183	
	160	19	2	11	0	1,536	0,111	**0,646**
	500	15	10	67	0	1,312	0,131	
	1000	22	18	82	0	1,331	0,139	
	1600	50	47	94	0	1,544	0,031	
4	0	23	2	9	1	1,139	0,201	
	16	17	1	6	0	1,162	0,151	
	160	18	1	6	0	1,196	0,119	**1,783**
	500	19	0	0	0	1,225	0,134	
	1000	20	10	50	0	1,115	0,113	
	1300	21	6	29	1	1,188	0,163	
	1600	12	6	50	0	1,145	0,129	
5	0	40	4	10	0	1,496	0,126	
	80	22	9	41	0	1,445	0,187	
	160	21	11	52	1	1,635	0,220	**28,571**
	320	21	10	48	1	**1,364**	0,152	
	500	23	1	4	0	**1,306**	0,133	
	1000	19	1	5	0	**1,394**	0,182	
	1600	21	1	5	0	**1,382**	0,151	
10	0	12	0	0	0	17,25	1,324	
	800	17	3	18	3	**14,86**	2,543	**not determined**
	1600	15	5	33	0	**15,30**	1,337	
Total		**651**	**197**		**12**			

* Wet weight of embryos sampled on ED10, except for application at ED10 when sampling was done at ED17.

Table 2. Bendiocarb embryotoxicity at different stage of development

Table 2 lists the wet weight of embryos in different treatment groups. In general, administration of bendiocarb resulted in a small decrease in embryonic weight, with a clear correlation of dose at later developmental stages (ED 5 and 10).
P values (differences in weight) considered statistically significant are in **bold**.

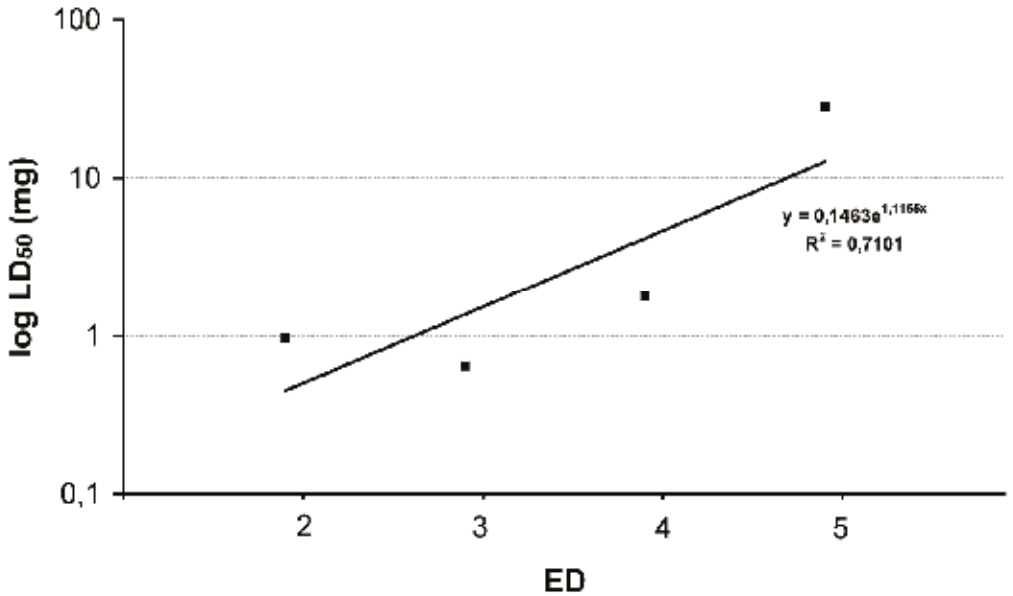

Fig. 1. LD_{50} of bendiocarb increases with development. The graph is based on values from Table 2

Fig. 2. Opening of the body cavity of the chick embryo

The values of mortality are used for construction of Figure 1. The weights of embryos at incubation days 2, 3, 4, 5, and 10 according to Clark et al. (1986) are 5, 18, 80, 149, and 2820 mg, respectively.
The embryolethality (LD_{50}) could not be determined on ED 10 because of solubility limits (200 g/l of acetone, but lower in mixture with water for injection).
The malformations were observed sporadically in both treated and control groups, with overall frequency below 2% against mortality (30%). Examples of malformations included defects of body wall, microphthalmia, anophthalmia, cleft beak and general growth retardation (Fig. 2). No specific pattern of malformations was observed among the treated embryos, irrespective of the dose and embryonic stage at its application.

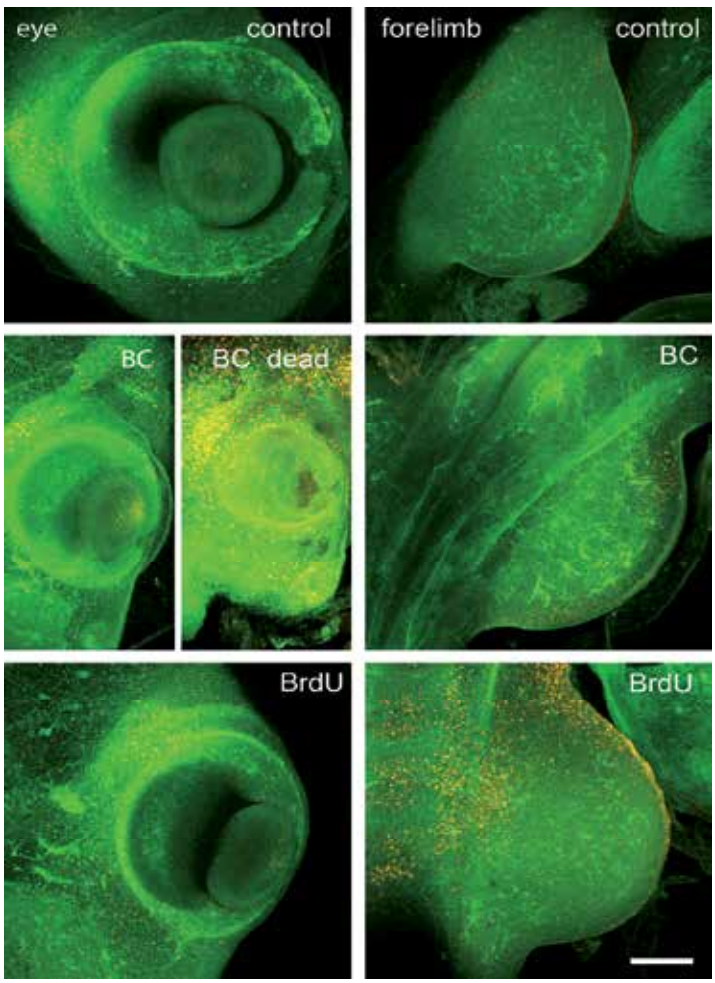

Fig. 3. Lysotracker Red (LTR) staining of ED 4 embryos in control eye and wing bud 24 hours after administration of bendiocarb (BC). A mild increase in the number of dead cells (red or yellow colored, dotted areas) is observed in the treated group, but a much more pronounced effect is visible in those treated with a teratogenic dose of BrdU (positive control). Scale bar 100 μm.

To discern potentially subtle toxic effects that are compensated later on by increased cell proliferation, we performed whole-mount staining with Lysotracker Red to detect dead cells in the whole embryo 24 h (ED 4) and 48 h (ED 5) after application on ED 3 (Fig. 3). The wing and leg buds are similar to one another morphologically, but by about ED 4 they have begun to acquire their individual characteristics. Hirata & Hall (2000) concluded that cell death is a feature of development. The programmed cell death plays an important role in shaping and patterning of organs during morphogenesis and organogenesis on ED 1–9 (Bellairs & Osmond, 2005). Since there was no specific pattern of malformations following bendiocarb administration, we did not expect to find any significant changes in cell death patterns that were shown to be a common mechanism of pathogenesis of congenital anomalies (Sedmera & Novotna, 1994). There were no gross anomalies or overt growth retardation among the survivors. The areas of programmed cell death, revealed by Lysotracker Red staining, were observed in the developing eye, face (branchial region), limbs and tail. There was a mild increase in the intensity of staining between bendiocarb-treated and control embryos at 24 h but no difference at 48 h sampling interval. The extent of cell death was remarkably increased in freshly dead treated embryos at 24 h.

The annual application of the synthetic pesticides to food crops in the European Union exceeds 140,000 tonnes, an amount that corresponds to 280 grams per EU citizen per year. Thus, many pesticides such as organophosphates, carbamates and pyrethroids are widely used in agriculture and households (Bjørling- Poulsen et al., 2008). Carbamate insecticides have different degrees of acute oral toxicity (Costa et al., 2008). No epidemiological studies of developmental neurotoxicity of carbamates in humans could be found, and data from animal experiments are very sparse as well (Bjørling-Poulsen et al., 2008).

This study provides the detailed analysis of bendiocarb toxicity in the chick embryo. Acute oral toxicity of bendiocarb has been investigated in adult mammals such as rat, guinea pig and rabbit, as well as the LD_{50} in non-mammalian species [e.g. mallard duck 3.1 mg kg^{-1}, bobwhile quail 16 mg kg^{-1}, hen 137 mg kg^{-1} (WHO, 2007), and fish 0.7–1.8 mg l^{-1} (LC_{50}, Hayes & Lawes, 1990). Similar to findings in adult mammals and birds, the embryotoxicity of a single dose is rather low in the chick, with the youngest stages being the most sensitive (Table 2; calculated LD_{50} doses based on the embryonic weight are in the range 20–200 g kg^{-1}; considering the whole ~30 g egg as a distribution space, the range would be 24–924 mg kg^{-1} according to stage). It is unlikely that such doses or concentration would be achieved during environmental exposure; however, it does not necessarily mean that even lower concentrations could not cause harm to more sensitive individuals.

There were no specific malformations associated with bendiocarb exposure in our set of experiments. Those encountered were also seen in the controls, and the frequency did not exceed 2%, which is considered background noise in the pre-hatching chicks (Novotna et al., 1994). While embryonic mortality is clearly correlated to the size of the dose, the number of malformed embryos does not change very much as the dose increases and may even decline (Peterka et al., 1986). We thus conclude that bendiocarb does not possess a significant teratogenic potential, at least in the avian embryo. Nevertheless, overriding differences in biotransformation in the fetus is the probable role of maternal metabolism of xenobiotics affecting the level of fetal toxicant exposure (Garry, 2004). This could cause secondary problems to the developing embryo or fetus in mammals.

Cell death detected in the developing embryo could be the most sensitive indicator of toxic effects of a substance, even if they are compensated later on by increased proliferation of the remaining cells and thus fail to translate into overt malformations (Novotna & Jelinek, 1990). We noted a mild increase in the number of dead cells revealed by whole-mount staining with the vital dye, but it did not result in any congenital anomalies and was substantially smaller than the increased of cell death associated with, for example, bromodeoxyuridine embryotoxicity (Sedmera & Novotna, 1994), which does result in limb defects. It is possible that this mild reduction in cell number could underlie the small dose-dependent decrease in embryonic weights observed at the time of autopsy. The validity of using Lysotracker Red for whole-mount detection of cell death was verified using bromodeoxyuridine in a parallel experiment. Embryos treated with 5 μg of bromodeoxyuridine served as internal controls, and showed clearly increased amounts of cell death at 24 h but not 48 h interval (Fig. 3).
BrdU incorporation into DNA induces a dose-dependent cytotoxic effect (Fränz and Kleinebrecht, 1982). The rate of cell death in consequence of BrdU-induced DNA single strand breaks (Novotná et al., 1994) must undoubtedly influence the pattern of programmed cell death in embryonic development as well as the resulting spectrum of malformations (Sedmera & Novotna, 1994).
The lack of excessive cell death in the bendiocarb group could be in consequence of less DNA damage. Subsequently, the number of malformations was low in survivors. The next bendiocarb effect could be an influence on the other cell structures and processes which cause death of the chick embryo.
Toxicity to specific target organs such as liver and central nervous system could be another manifestation of deleterious effects of bendiocarb in the chick embryo. Histological examination of these structures did not show any significant morphological or caspase immunopositivity.
Our analysis of bendiocarb embryotoxic potential in the chick embryo supports the earlier observations in other animal models, testifying to the relative safety of bendiocarb for the embryo or fetus.

3.2 Organ toxicity

At 3 ED the mortality of 51 chicken embryos used was 28% (51/14).An average weight of the control (n=36) was 1446 ± 0.165 mg. The chicken embryos exposed to bendiocarb were lower weight than control within 9% (1312 ± 0.131 mg). At 10 ED the mortality rate of 29 chicken embryos was 10% (29/3). An average weight of the control (n = 12) was 17.25 ± 1.324 g. The chicken embryos exposed to bendiocarb were lower weight than control within 14% (14.86 ± 2.544 mg).

3.2.1 Liver

Comparison with the control showed neither macroscopic nor microscopic changes in chicken embryos exposed to bendiocarb at 3 ED at concentrations of 500 μg/egg. Macroscopic observation revealed no changes in the size or shape of the liver. The organs were yellow, with a shiny surface and the sections showed preservation of characteristic liver structure. Histology of liver tissue was unchanged. We failed to observe any changes in hepatocytes or the intracellular space.
Similarly the examination of chicken embryos exposed to bendiocarb at 10 ED at doses of 800 μg/egg, failed to show any macroscopic or microscopic changes in the liver in

comparison with the control. Macroscopic examination detected no changes in the size or shape of the liver. The organ was yellow, with a shiny surface and respective sections showed that the liver structure was preserved. Histological examination of liver also failed to detect any changes as in the hepatocytes as in the intracellular space (Fig. 4).

3.2.2 Central nervous system

The microscopic findings in CNS in chicken embryos exposed to bendiocarb at 3 ED and 10 ED were negative when compared to the control. Part of the neck was sampled for this examination (including spinal cord cross section) and no histological changes were observed in CNS as far as neurons and intracellular space was concerned (Fig. 5).

Our experiment showed that application of bendiocarb to chicken embryos produced no macroscopic or microscopic changes in the liver and CNS tissues in comparison with the control. There were no changes in the tissues of liver and CNS when bendiocarb was administered (500 µg/egg) at 3 ED, nor were these changes after application of bendiocarb (800 µg/egg) at 10 ED.

A two-year study on dogs which received bendiocarb in food, revealed no changes in the weight of organs or any harmful effect of the pesticide on dog tissues. The daily dose used corresponded to 12.5 mg/kg b.w. and the authors detected increased serum cholesterol and decreased bloodstream level of calcium (Baron, 1991).

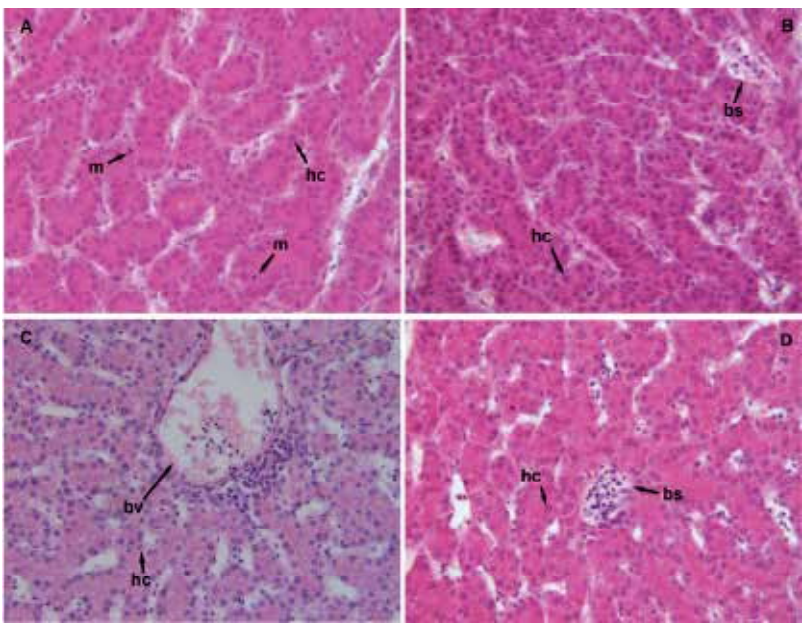

Fig. 4. Toxic action of BC on liver exposed on 3 ED (9 ED – A: control embryo, B: treatment embryo; 500 µg/egg) and 10 ED (17 ED - C: control embryo, D: treatment embryo; 800 µg/egg). mitosis (m); hepatocyte (hc); blood vessel (bv); blood sinusoid (bs) [H-E, 60x]

Toxicity of bendiocarb to organs was investigated in adult rabbits which received bendiocarb per os at a dose of 5 mg/kg/day. In this study, based on long-term (90 days)

application of bendiocarb, the authors observed increased volume of cortex and decreased volume of thymus pulp. In addition to that, the morphometric analysis detected lower number of cells and also smaller diameter of cells in the thymus in comparison with the control (Flesarova et al., 2007).
Male rats showed a significant increase in incidence of nuclear cataract related to bendiocarb dose (20 and 200 mg/kg; Hunter et al., 2008).

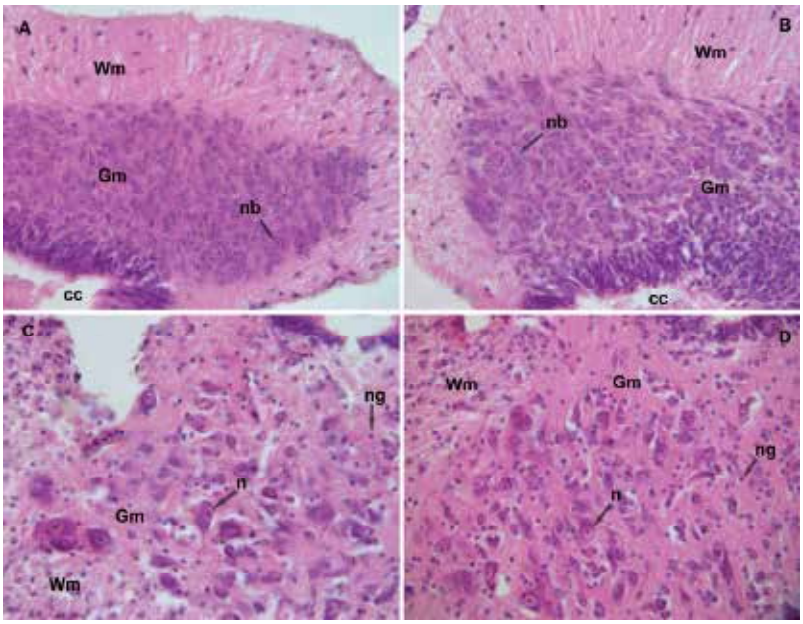

Fig. 5. Toxic action of BC on CNS of chicken embryos exposed on 3 ED (9 ED - A: control embryo, B: treatment embryo; 500 µg/egg) and 10 ED (17 ED - C: control embryo, D: treatment embryo; 800 µg/egg). white matter (Wm); gray matter (Gm); central canal (cc); neuroblast (nb); neuron (n); neuroglia (ng) [H-E, 60x]

3.3 Caspase activity

3.3.1 Liver

The chicken embryos that were exposed to bendiocarb at 3ED at concentrations of 500 µg/egg, showed low caspases activity in comparison with the control. After application of bendiocarb at a dose of 500 µg/egg at 3 ED, we observed that in the viewing field of size 887.5 µm3 were 850 liver cells (with the mean number equal to one cell/μm^3), any liver cells showed caspase activity of treatment embryos in comparison with the control.
In chicken embryos that were exposed to bendiocarb at 10 ED at doses of 800 µg/egg, was detected low caspases activity in comparison with the control. After application of bendiocarb at a dose of 800 µg/egg at 10ED, were found three (0.40%) liver cells with caspase activity contrary to the control with two (0.20%) caspase activity of the red stained cells (Fig. 6).

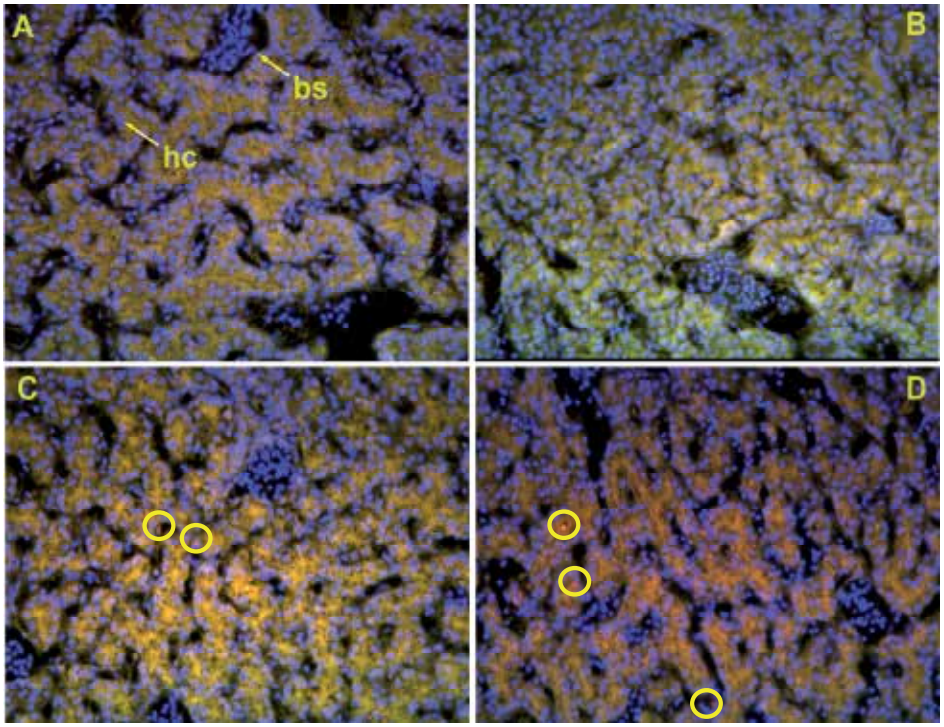

Fig. 6. Caspase activity of liver cells (in yellow circle) after application of BC on 3 ED (9 ED - A: control embryo, B: treatment embryo; 500 µg/egg) and 10 ED (17 ED - C: control embryo, D: treatment embryo; 800 µg/egg). blood sinusoid (bs); hepatocyte (hc) [stained immunohistochemically, 40x]

3.3.2 Central nervous system

Chicken embryos were administered bendiocarb at 3 ED at doses of 500 µg/egg. Among them, we observed 450 nerve cells (with the mean number of one nerve cell/2µm^3, in the viewing field of size 887.5µm^3. One cell (0.20%) showed caspase activity in comparison with the control.

In chicken embryos which were administered bendiocarb at 10 ED at doses of 800 µg/egg, one cell (0.20%) with caspase activity was found in comparison with the control which contained three (0.7%) red-stained nerve cells. In chicken embryos that were exposed to bendiocarb at 3 ED and 10 ED low caspase activity was detected in comparison with the control. The presence of apoptotic cells in CNS after exposure to bendiocarb can be related to physiological elimination of excessive neurons at generation of synapses (Fig. 7).

The chicken embryos exposed to bendiocarb showed low caspase activity of liver cells. The presence of apoptotic cells in the liver after application of bendiocarb may be related to physiological apoptosis occurring during embryogenesis. Apoptosis is also known as "programmed cell death" because in many cases the patches of cells die in a particular location of the embryo at a specific time in development and play an important role in morphogenesis (Bellairs, 1961). Caspase-3 is a member of the family of cysteine proteases. An apoptotic signal such as granzyme B of cytotoxic T-cells induces the intracellular clevage of Caspase-3 from the inactive proform to the active form. The active form of Caspase-3

cleaves several other apoptotic proteins (Fernandes-Alnemri et al., 1994). The experiment based on application of bendiocarb to chicken embryos at 3 ED and 10 ED showed no increase in the number of cells with caspase activity in comparison with the control. This applies to both the liver and CNS of chicken embryos.

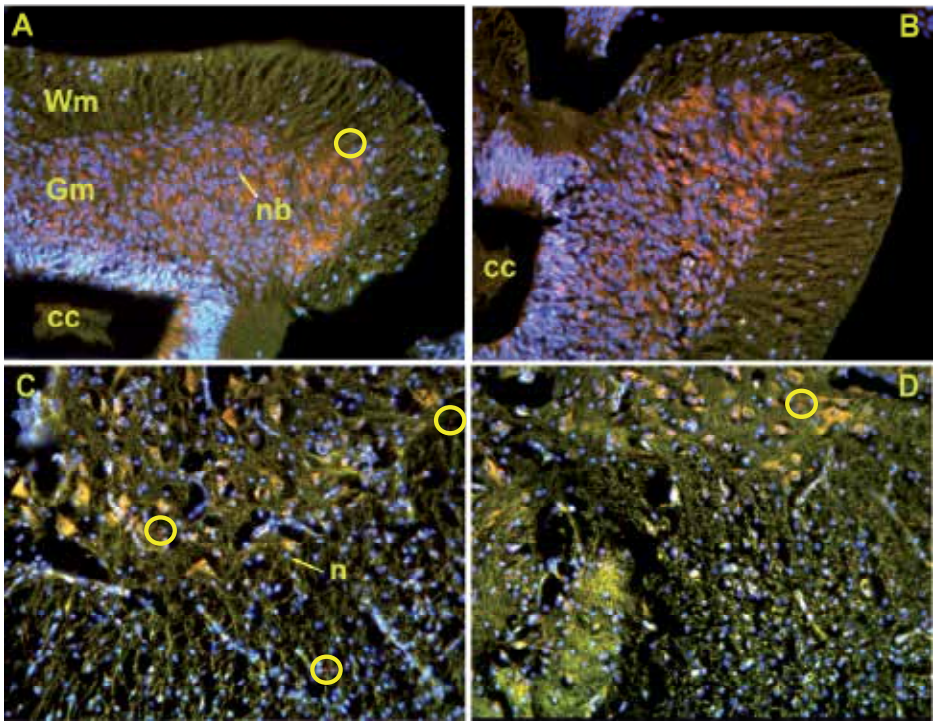

Fig. 7. Caspase activity of nervous cells (in white circle) after application of BC on 3 ED (9 ED - A: control, B: treatment embryo; 500 μg/egg) and 10 ED (17 ED - C: control, D: treatment embryo; 800 μg/egg). white matter (Wm); gray matter (Gm); central canal (cc); neuroblast (nb); nervous cell (n) [stained immunohistochemically, 40x]

Cell death with its well-known role in morphogenesis is an important characteristic of developing legs in chicken embryos (Dawd & Hinchliffe, 1971). During the development of limbs the cell death results in removal of interdigital tissue and in birds also to vanishing of the 1st and 5th toe. In this way the cell death participates in formation and development of toes of bird legs. Cell apoptosis is species-specific not only from temporal but also from the spatial point of view (Zakeri & Lockshin, 2002).

Cell apoptosis occurs in chicken embryos for the first time at 2 ED (somites and neural tube). The interdigital regions of mesenchyma are subject to regression an in this way they likely participate in formation of toes in amniotic embryos (chicken embryo, murine embryo and others) and also in humans (Dawd & Hinchliffe, 1971). Cell apoptosis has an important role also in the nervous system. In the course of development of vertebrates the nerve cells are produced in excessive numbers and therefore cellular apoptosis involving 20–80% of neurons is physiological. Fetal neurons thus compete for nerve growth factor (NGF) which ensures their survival and is produced not only by neurons but also by other cells. However,

not all cells obtain the required quantity of NGF for their survival. Therefore apoptosis adjusts the total number of produced neurons to such quantity which is supported physiologically (Zakeri & Lockshin, 2002).

4. Conclusion

Pesticides represent the significant environmental contaminants, but often we cannot avoid their use in agriculture for plant protection and health of people and animals from diseases spread by vectors. Their extensive use raises the question about the toxicity of pesticides to non-target organisms, persistence, accumulation and combined effect with other agrochemicals. A great attention is paid to the study of impact of extraneous substances from external environment on humans and animal lately. It is mainly due to the high contamination of environment by chemical substances used in industry and agriculture. The most pesticides are not highly selective, and are generally toxic to many nontarget species, including humans. Adverse health effects of pesticides in humans cover a variety of domains; some compounds may only exert some mild irritant in the skin, while others may affect liver or lung functions. Some are carcinogenic, other may cause reproductive toxicity or have endocrine disrupting properties. Many pesticides target the nervous system of insect pests. Because of the similarity of neurochemicals processes, these compounds are also likely to be neurotoxic to human. Prenatal development of the central cholinergic nervous system coupled with the important developmental roles played by ACh in both neural and non-neural tissues should make the vertebrate embryo especially vulnerable to the gestational effects of environmental neurotoxins that target cholinergic receptors or choline esterase. In vitro systems such as neural cell lines or embryo cultures can play key roles in elaborating of the effects of prenatal exposure to cholinergic pesticides and in establishing new safety thresholds for insecticide exposure during development. Carbamate insecticides have different degrees of acute oral toxicity, ranging from moderate to low toxicity (carbaryl - 250 mg/kg), to extremly high toxicity (aldicarb – 0,8 mg/kg;). Given that the purity of environment and the negative impact of contamination on human and animal health is currently highly topical problem, we consider it necessary to pay attention to the action of pesticides on living organisms and thus to expand and acquire new knowledge about their potentially harmful effects. We thus conclude that bendiocarbamate does not possess a significant toxic potential, at least in the avian embryo. Nevertheless, large doses that would impair maternal metabolism could cause secondary problems to the developing embryo or fetus in mammals.

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6. References

Amenta, F. & Tayebati, S.K. (2008). Pathways of acetylcholine synthesis, transport and release as targets for treatment of adult-onset cognitive dysfunction. *Current Medicinal Chemistry*, Vol.15, No.5, pp. 488-489, ISSN 0929-8673

Baron, R.L. (1991). Carbamate insecticides, In: *Handbook of Pesticide Toxicology*, W.J.,Jr. Hayes, E.R.,Jr. Laws, (Eds.), 3-6, Academic Press, ISBN 978-0123341600 New York, USA

Bellairs, B. (1961). Cell death in chick embryos as studied by electron microscopy. *Journal of anatomy,* Vol. 95, No.1, (January 1961), ISSN 0021-8782

Bellairs, R. & Osmond, M. (2005). Apoptosis, In: *The Atlas of chick development*. R. Bellairs, M. Osmond, (Eds.), 42-43, Academic Press, ISBN 978-0-12-084791-4, San Diego, USA

Bigbee, J.W.; Sharma, K.V.; Gupta, J.J. & Dupree, J.L. (1999). Morphogenic Role for Acetylcholinesterase in Axonal Outgrowth during Neural Development, *Environmental Health Perspectives,* Vol.107, Suppl. 1, (February 1999), pp. 81-87, ISSN 0091-6765

Bjørling-Poulsen, M.; Andersen, H.R. & Grandjean, P. (2008). Potential developmental neurotoxicity of pesticides used in Europe. *Environmental Health*, Vol.7, No.1, (October 2008), pp. 1-22, ISSN 1476-069X

Brimijoin, S., Koenigsberger, C. (1999). Cholinesterases in Neural Development: New Findings and Toxicologic Implications. *Environmental Health Perspectives,* Vol.107, Suppl 1, (February 1999), pp. 59-64, ISSN 0091-6765

Buznikov, G.A.; Shmukler, Y.B. & Lauder, J.M. (1996). From oocyte to neuron: do neurotransmitters function in the same way throughout development? *Cellular and molecular neurobiology*, Vol.16, No.5, (October 1996), pp. 537-539, ISSN 0272-4340

Clark, E.B; Hu, N.; Dummett, .JL.; Vandekieft G.K.; Olson, C. & Tomanek, R. (1986). Ventricular function and morphology in chick embryo from stages 18 to 29. *American journal of physiology. Heart and circulatory physiology, Vol*. 250, No. 3, (March 1986), pp. 407-413, ISSN 0363-6135

Costa, L.G.; Giordano, G.; Guizzetti, M. & Vitalone, A. (2008). Neurotoxicity of pesticides: a brief review, *Frontiers in Bioscience*, Vol. 13, (January 2008), pp. 407- 413, ISSN 1093-9946

Dawd, D.S. & Hinchliffe, J.R. (1971). Cell death in the "opaque patch" in the central mesenchyme of the developing chick limb: a cytological, cytochemical and electron microscopic analysis. *Journal of Embryology and Experimental Morphology*, Vol.26, No.3, pp. 401-424, ISSN 0022-0752

Falugi, C. (1993). Localization and possible role of molecules associated with the cholinergic system during „non-nervous" developmental events. *European journal of histochemistry*, Vol.37, No.4, pp. 287-294, ISSN 1121-760X

Fernandes-Alnemri, T.; Litwack, G. & Alnemri, E.S. (1994). CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. *The Journal of biological chemistry*, Vol. 269, No.49, (December 1994), pp. 30761-30764, ISSN 0021-9258

Flesarova, S.; Lukac, N.; Danko, J. & Massanyi, P. (2007). Bendiocarbamate induced structural alterations in rabbit thymus after experimental peroral administration. *Journal of environmental science and health. Part. B, Pesticides, food*

contaminants, and agricultural wastes, Vol.42, No.3, (March-April 2007), pp. 329- 34, ISSN 0360-1234

Franz,J. & Kleinebrecht, J. (1982). Teratogenic and clastogenic effects of BUdR in mice. *Teratology*, Vol.26, No.2, (October 1982), pp. 195-202, ISSN 0040-3709

Garry, V.F. (2004). Pesticides and children. *Toxicology and applied pharmacology*, Vol. 198, No. 2, (July 2004), pp. 152-163, ISSN 0041-008X

Glynn, P. (1999). Neuropathy target esterase, *The Biochemical journal*, Vol.344, No.3, (December 1999), pp.625-631, ISSN 0264-6021
1970), pp.102-117, ISSN 0014-4827

Hayes, W.J. & Lawes, E.R. (1990).*Handbook of pesticide toxicology. Classes of pesticides*, Academic Press, ISBN 978-0123341600, New York, USA

Hirata, M. & Hall, B.K. (2000). Temporospatial patterns of apoptosis in chick embryos during the morphogenetic period of development, *The International journal of developmental biology,* Vol.44, No.7, (October 2000), pp. 757-768, ISSN 0214-6282

Huff, F.J.; Reiter, C.T. & Rand, J.B. (1989). The ratio of acetylcholinesterase to butyrylcholinesterase influences the specificity of assays for each enzyme in human brain. *Journal of Neural Transmission*, Vol.75, No.2, 129-134, ISSN 0300-9564

Hunter, B.; Watson, R.; Street, A.E.; Prentice, D.E.; Gopinath, C. & Gibson, W.A. (2008). NC 6897 toxicity and tumorigenicity to rats in long-term dietary administration. Unpublished report, Hantingdon Research Centre: England. Submitted to the World Health Org. by FBC, Limited, 1981. Available from http://www.hc- sc.gc.ca/ewh-semt/pubs/water-eau/bendiocarb-bendiocarbe/index-eng.php (accessed February 2008).

Jaga, K. & Dharmani, Ch. (2003). Sources of exposure to and public health implications of organophosphate pesticides, *Revista panamericana de salud pública*, Vol.14, No.3, (September 2003), pp. 171-185, ISSN 1020-4989

Jelinek, R. (1977). The chick embryotoxicity screening test (CHEST), In: Methods in prenatal toxicology, D. Neubert, J. Merker, T.E. Kwasigroch (Eds.), 381-386, George Thieme Publishers, ISBN 0884162397, Sttutgart, Germany

Karami-Mohajeri, S.; Abdollahi, M. (2010). Toxic effects of organophosphate, carbamate, and organochlorine pesticides on cellular metabolism of lipids, proteins, and carbohydrates: A comprehensive review, *Human & experimental toxicology*, November 11, [Epub ahead of print], ISSN 0960-3271

Kamrin, M.A. (1997). *Pesticide profiles: Toxicity, Environmental Impact and Fate*, CRC Press: Boca Raton, ISBN 978-1566701907, Michigan, USA

Kassa, J. & Samnaliev, I. (2004). Assessment of the therapeutic and anticonvulsive efficacy of a drug combination consisting of trihexyphenidyl and HI-6 in soman – poisoned rats, *Acta Medica (Hradec Kralove),* Vol.47, No.3, pp. 171-175, ISSN 1211-4286

Krall, W.J.; Sramek, J.J. & Catler, N.R. (1999). Cholinesterase inhibitors: a therapeutic strategy for Alzheimer disease. *The Annals of Pharmacotherapy*, Vol.33, No.4, (April 1999), pp. 441-450. ISSN 1060-0280

Kristoff, G.; Guerrero, N.V.; Pechén de D´Angelo, A.M. & Cochón, A.C. (2006): Inhibition of cholinesterase aktivity by azinphos-methyl in two freshwater invertebrates: Biomphalaria glabrata and Lumbriculus variegatus. *Toxicology*, Vol.222, No.3, (May 2006), pp. 185-194, ISSN 0300-483X

Lauder, J. M. & Schambra, U.B. (1999). Morphogenetic roles of acetylcholine, *Environmental Health Perspectives,* Vol.107, Suppl. 1, (February 1999), pp. 65-69, ISSN 0091-6765

Leveridge, Y.R. (1998). Pesticide poisoning in Costa Rica during 1996, *Veterinary and human toxicology*, Vol.40, No.1, (February 1998), pp. 42-44, ISSN 0145-6296

Littleton, J.T.; Bhat, M.A. & Bellen, H.J. (1997). Deciphering the function of neurexins at cellular junctions, *Journal of Cell Biology,* Vol.137, No.4, (May 1997), pp. 793-796, ISSN 0021-9525

Lotti, M. & Moretto, A. (2006). Do carbamates cause polyneuropathy? *Muscle & Nerve*, Vol.34, No.4, (October 2006), pp. 499-502, ISSN 0148-639X

Mignini, F.; Streccioni, V. & Amenta, F. (2003). Autonomic innervation of immune organs and neuroimmune modulation, *Autonomic & autacoid pharmacology*, Vol.23, No.1, (February 2003), pp. 1-25, ISSN 1474-8665

Miller, C.E.; Thompson, R.P.; Bigelow, M.R.; Gittinger, G.; Trusk, T.C. & Sedmera, D. (2005). Confocal imaging of the embryonic heart: How deep? *Microscopy and Microanalysis*, Vol. 11, No.3, pp.216-223, ISSN 1431-9276

Mohd. Amanullah, Hari B.Y. (2011). Evaluation of carbamate insecticides as chemotherapeutic agents for cancer, *Indian journal of cancer*, Vol. 48, No. 1, (January 2011), pp. 74-79, ISSN 0019-509X

Novotna, B.; Hubalek, F. & Bednar, V. (1994). Genotoxic and embryotoxic effects of 5-bromodeoxyuridine in the chick embryo, *Teratogenesis, carcinogenesis, and mutagenesis*, Vol.14, No.3, pp. 123-134, ISSN 0270-3211

Novotna, B. & Jelinek, R. (1990). Mutagenic and teratogenic effects of cyclophosphamide on the chick embryo: chromosomal aberrations and cell proliferation in affected and unaffected tissues. *Teratogenesis, carcinogenesis, and mutagenesis*, Vol.10, No.4, pp. 341-250, ISSN 0270-3211

Peterka, M; Havranek, T. & Jelinek, R. (1986). Dose-response relationships in chick embryos exposed to embryotoxic agents, *Folia morphologica*, Vol.34, No.1, pp. 69-77, ISSN 0015-5640

Ratner, D.; Oren, B. & Vidger, K., 1983: Chronic dietary anticholinesterase poisoning. Israel journal of medical sciences, Vol.19, No.9, (September 1983), pp. 810-814, ISSN 0021-2180

Schaefer, K.S.; Doughman, Y.Q.; Fisher, S.A. & Watanabe, M. (2004). Dynamic patterns of apoptosis in the developing chicken heart, *Developmental dynamics: an official publication of the American Association of Anatomists*, Vol. 229, No.3, (March 2004), pp. 489-499, ISSN 1058-8388

Sedmera, D.; Hu, N.; Weiss, K.M.; Keller, B.B.; Denslow, S. & Thompson, R.P. (2002). Cellular changes in experimental left heart hypoplasia, *The anatomical record,* Vol. 267, No.2, (June 2002), pp. 137-145, ISSN 1932-8486

Sedmera, D & Novotna, B. (1994). Bromodeoxyuridine-induced shift of malformation spectrum in the chick embryo: stage effect, dose effect and role of cell death, *European archives of biology*, Vol. 105, No.1-2, pp. 41-49, ISSN 0777-0553

Sherman, J.D. (1996). Chlorpyrifos (Dursban)-associated birth defects: report of four cases. *Archives of Environmental Health,* Vol.51, No.1, (January-February, 1996), pp. 5-8, ISSN 0003-9896

Sirotakova, M.; Schmidtova, K. & Ucekaj, N. (2005). Microscopic images of adrenergic and BuChE - positive innervation of the spleen in rabbits after application of bendiocarb, (in Slovak), In: *Xenobiotics in relation to health*, J. Danko & F. Lesnik, (Eds.), pp. 75-81, Seminar sv. Karola Boromejskeho, Kosice, Slovakia

Slotkin, T.A. (1999). Developmental Cholinotoxicants: Nicotine and Chlorpyrifos. *Environmental Health Perspectives*, 107, Suppl. 1, (February 1999), pp. 71-80, ISSN 0091-6765

Slotkin, T.A. (2004). Cholinergic system in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. *Toxicology and applied pharmacology*, Vol. 198, No. 2, (June 2004), pp. 132-151, ISSN 0041-008X

Soreq, H.; Patinkin, D.; Lev-Lehman, E.; Grifman, M.; Ginzberg, D.; Eckstein, F. & Zakut, H. (1994). Antisensense oligonucleotide inhibition of acetylcholinesterase gene expression induces progenitor cell expansion and suppresses hematopoietic apoptosis *ex vivo*. *Proceedings of the National Academy of Sciences of the United States of America,* Vol.91, No.17, (August 1994), pp. 7907-7911, ISSN 1091-6490

Toxicology (1998). In: *The Merck Veterinary Manual (Eight Edition).* S.E. Aiello (Ed.), pp. 2015-2161, Merck and Co., INC. Whitehouse Station, ISBN 0-911910-29-8. N. J., USA

Van Dyk, J.S. & Pletschke, B. (2011). Review on the use of enzymes for the detection of organochlorine, organophosphate and carbamate pesticides in the environment. *Chemosphere,* Vol.82, No.3, (January 2011), pp. 291-307, ISSN 0045-6535

Weikert, T.; Rathjen, F.G. & Layer, P.G. (1990). Developmental maps of acetylcholinesterase and G4-antigen of the early chicken brain: long distance tracts originate from AChE-producing cell bodies. *Journal of Neurobiology,* Vol.21, No.3, (April 1990), pp. 482-498, ISSN 0022-3034

World Health Organization [WHO]. (June 2007). Data Sheets on pesticides, No.52, Bendiocarb, Bureau of Chemical Standards, Environmental Health Directorate, Health & welfare: Canada, 14. 06. 2007, Available from http://www.intox.org/databank/documents/chemical/bendiocb/pest52_e.ht m

Wright, C.G.; Leidy, R.B. & Dupree, H.E.Jr.(1981). Insecticides in the ambient air of rooms following their application for control of pests, *Bulletin of environmental contamination and toxicology*, Vol. 26, No. 4, (April 1981), pp. 548-553, ISSN 0007-4861

Wylie, P. L.; Szelewski, M.J. & Meng, Chin-Kai. (2005). Pesticide screening by GC/MSD using spectral deconvolution, *Chemistry in Australia*, Vol. 72, No. 1, pp. 4-8. ISSN 0314-4240

Zakeri, Z. & Lockshin, R.A. (2002). Cell death during development. *Journal of Immunological Methods*, Vol.265, No.1-2, (July 2002), pp. 3-20, ISSN 0022-1759

22

Age-Related Differences in Acetylcholinesterase Inhibition Produced by Organophosphorus and *N*-Methyl Carbamate Pesticides

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1. Introduction

The concern that infants and children may be more susceptible to the toxic effects of chemicals, including pesticides, has received much attention in the scientific literature and the public media. Greater toxicity may be evident as long-term adverse outcomes, *e.g.*, neurological and IQ deficits from early exposure to lead, or else as increased toxicological effects of acute or short-term exposures. A National Academy of Science panel reported in 1993 on the scientific and regulatory issues regarding relative sensitivity of the young (National Research Council 1993). This report stressed how little is understood regarding the magnitude and mechanisms of these differences, and called for systematic research on pesticide toxicity in developing organisms. The concern that regulatory practices may not adequately protect these subpopulations further led to the passage of the Food Quality Protection Act in 1996 (FQPA, Public Law 104-170, August 1996), which required the US Environmental Protection Agency (EPA) to take extra steps to protect infants and children in the regulation of pesticides. Specifically, the FQPA instructed that "an additional tenfold margin of safety" be applied for non-cancer effects of pesticides "to take into account potential pre-and postnatal toxicity and completeness of data with respect to exposure and toxicity to infants and children". Currently, the EPA Office of Pesticide Programs addresses this additional margin of safety during the risk characterization process (Lowit, 2006; US EPA, 2002). With pesticides for which direct or acute effects drive the assessment, one approach for determining this factor has often been an evaluation of relative sensitivity of young compared to adult animals (US EPA, 2006).

There are several factors impacting greater pesticide toxicity in children (Faustman *et al.* 2000). Exposures from intake of water contaminants and food residues are higher, because children take in considerably more food and water than adults on a per body weight basis (NRC, 1993). Behaviors of infants and toddlers (*e.g.*, crawling, hand-to-mouth) also increase the likelihood of coming into contact with pesticides through dust and soil. Greater exposure levels in children have been documented in children of agricultural workers, comparisons of organic and standard diets, and in numerous housing surveys (*e.g.*, Curl *et al.* 2003; Fenske *et al.* 1990; Loewenherz *et al.* 1997; Lu *et al.* 2001; Simcox *et al.* 1995). Higher

exposures, combined with immature and developing biological systems, underscore the potential for children's susceptibility to pesticides as well as other environmental factors.

Organophosphorus (OP) and *N*-methyl carbamate compounds inhibit acetylcholinesterase, the enzyme that preferentially hydrolyzes acetylcholine at cholinergic nerve terminals. This prolonged half-life of acetylcholine may cause an overstimulation of the cholinergic pathways and produce central and peripheral toxicities. Signs of acetylcholinesterase inhibition include salivation, lacrimation, gastrointestinal stimulation, muscular tremors to convulsions, ataxia: respiratory paralysis is the ultimate cause of death (reviewed in Fukuto, 1990; Pope, 2006). Many of these inhibitors are used as pesticides. The majority of OP pesticides have a long duration of inhibition due to the very slow regeneration of the enzyme (inhibition that lasts days to weeks), whereas the *N*-methyl carbamates are reactivated more quickly (minutes to hours) and thus have a much shorter time-course of toxicity (Aldridge and Reiner, 1975). While these chemicals are highly successful insecticides, their agricultural and especially household usage in the US have been somewhat curtailed due to concerns of toxicity, particularly to infants and children. They remain, however, to be widely used for agriculture in the US and throughout much of the rest of the world (US EPA, 2004).

The earliest studies describing greater toxicity in the young compared lethal doses of a number of pesticides. In many cases, but not all, the young were more sensitive than adults (Brodeur and DuBois 1963; Gaines and Linder 1986; Harbison 1975; Lu *et al.,* 1965). Using sublethal doses, many studies have also documented greater sensitivity in the young when comparing maximum-tolerated doses or else equi-effective doses producing cholinesterase (ChE) inhibition and/or behavioral changes (*e.g.*, Atterberry *et al.* 1997; Benke and Murphy 1975; Moser 1999, 2000; Moser and Padilla 1998; Pope *et al.* 1991; Pope and Chakraborti 1992; Zheng *et al.* 2000). Over the years researchers have examined potential kinetic or dynamic factors that may account for these differences (*e.g.*, Atterberry *et al.* 1997; Benke and Murphy 1975; Brodeur and DuBois 1967; Karanth and Pope 2000; Mortensen *et al.* 1998; Moser *et al.* 1998; Sterri *et al.* 1985). For most of the literature, there has been considerable variability in the ages tested, as well as different species, strain, gender, routes of administration, and vehicles. These different experimental details have not allowed direct quantitative comparisons across studies. In addition, much of this literature has focused on a relatively few pesticides that clearly demonstrate greater effects in the young compared to adults. Chlorpyrifos especially has been studied in considerable detail, perhaps more than any other ChE inhibitor. Chlorpyrifos, parathion, methyl parathion, and malathion have been repeatedly shown to produce greater toxicity in the young (*e.g.*, Benke and Murphy, 1975; Brodeur and DuBois 1963, 1967; Lu *et al.* 1965; Karanth and Pope 2000; Moser, 2000; Moser and Padilla, 1998; Pope *et al.* 1991; Pope and Chakraborti 1992; Zheng *et al.* 2000). Because of the extensive literature on these few chemicals, there is a tendency to assume that they are representative of the entire chemical class. Beyond these few pesticides, however, there is a paucity of data with which to determine the overall occurrence or the magnitude of such age-related sensitivity differences. The purpose of this review is to summarize and evaluate a number of studies of age-related differences in response to OP and carbamate pesticides.

2. Methods

The overall aim of this chapter is to provide a retrospective analysis of ChE-inhibiting pesticides and their potential to be more toxic in the young. This laboratory is in a unique

position to provide this review, since a total of 18 pesticides have been systematically evaluated in both adult and young rats. Consistency in execution of the studies as well as the ChE assay provides confidence in the comparisons.

2.1 Study description

Over the years, this laboratory has conducted acute dose-response studies in adult and preweanling, 17-day old (postnatal day 17, PND17) Long-Evans hooded rats for a total of 10 OPs and 8 carbamates. In a few cases, PND11 rats were also included. ChE activity was measured at the time of peak acute effect, often derived from range-finding or time-course studies. Across studies, there was consistency in the general experimental design. For almost all studies, the assay for ChE activity used a radiometric procedure that is modified to minimize potential reactivation of carbamylated tissues (Johnson and Russell 1975), and modified for use in this laboratory (Moser *et al.* 2010). This aspect of the assay is critical for studies of carbamates, since reactivation of tissues during the assay process could underestimate the degree of *in vivo* inhibition. The exception was methamidophos-treated tissues, which were analyzed using an automated Hitachi 911 analyzer as previously described (Hunter *et al*., 1997). ChE activity was measured in brain and either whole blood or red blood cells (RBCs). For 7 carbamates, the ChE assays for adult rats were physically conducted in another EPA laboratory. While the brain ChE inhibition data were subsequently confirmed in this laboratory, the RBC data were not and therefore are not included here.
In almost all of our studies, behavioral evaluations were included to correlate with the biochemical changes; however, since the focus of this review is to compare ChE inhibition, that aspect of the studies will not be further described.

2.2 Data analysis

In order to make direct comparisons across dose-response curves, all data were fit to a logistic equation (Hill plot; Barlow and Blake, 1989) using SAS (version 9, Cary, NC) for estimation of doses producing 50% ChE inhibition (ED50). The ratios of ED50 values in adults compared to young rats indicate the magnitude of sensitivity differences.

3. Results

3.1 ED50s

Doses which produce 50% inhibition were derived and compared by taking the ratio of the adult ED50 to that for the younger rat; values >1 indicate higher ED50s and therefore less sensitivity in the adults, *i.e.*, greater sensitivity in the young. For the purposes of this review, ratios ≥5-fold are considered "large", and <2-fold suggest little or no differences. These calculated ED50 values and ratios for each pesticide are listed in Table 1. In all cases, whole brain was used, whereas RBC was tested for some chemicals, and whole blood for others. As described above, adult RBC data for five carbamates are not available from this laboratory. In addition, the lowest doses used for aldicarb produced almost 70% blood ChE inhibition, and the lowest dose of methamidophos produced considerable blood ChE inhibition (40-60%), and thus dose-response curves could not be fit reasonably well. Dose-response data for adult brain ChE inhibition produced by carbofuran and carbaryl were conducted twice (McDaniel *et al.* 2007; Moser *et al.* 2010). The calculated ED50 values were essentially the same (carbaryl, 29.1 and 29.8; carbofuran, 1.06 in both), and therefore are

averaged for the purposes here. The highest dose of malathion used in adults was 500 mg/kg, and range-finding studies went as high as 750 mg/kg (unpublished); these doses produced no inhibition of brain ChE. Therefore an ED50 could not be calculated, but it is evident that the ratio would be at least 3-fold given that the ED50 value in PND17 rat pups was less than half of the doses that were ineffective in adults.

Tissue	Brain			Blood		
Pesticide/Age	Adult	PND17	Ratio	Adult	PND17	Ratio
Acephate[a,b]	14.7	14.2	1.0	20.1[1]	13.2[1]	1.5
Aldicarb[c]	0.27	0.096	2.8	-- [3]	-- [3]	
Carbaryl[d,e]	29.5[4]	32.8	0.9	6.64[2]	11.5[2]	0.6
Carbofuran[d,e]	1.06[4]	0.40	2.7	0.39[2]	0.096[2]	4.1
Chlorpyrifos[a,b]	22.6	5.29	4.3	1.72[1]	2.40[1]	0.7
Diazinon[a,b]	121	22.8	5.3	5.24[1]	8.96[1]	0.6
Dicrotophos[f]	0.75	0.31	2.4	0.45[2]	0.36[2]	1.3
Dimethoate[a,b]	21.5	17.9	1.2	15.8[1]	8.72[1]	1.8
Formetanate[d,e]	1.35	0.49	2.8	-- [3]	0.28[2]	
Malathion[a,b]	>750[3]	241	>3	494[1]	146[1]	3.4
Methamidophos[c]	1.47	1.94	0.8	-- [3]	-- [3]	
Methiocarb[d,e]	13.8	8.44	1.6	-- [3]	2.94[2]	
Methomyl[d,e]	3.86	2.74	1.4	-- [3]	1.44[2]	
Mevinphos[f]	1.04	0.29	3.6	0.27[2]	0.21[2]	1.3
Monocrotophos[f]	0.49	0.35	1.4	0.28[2]	0.35[2]	0.8
Oxamyl[d,e]	1.13	0.59	1.9	-- [3]	0.19[2]	
Phosphamidon[f]	2.55	1.40	1.8	1.34[2]	1.12[2]	1.2
Propoxur[d,e]	21.5	7.05	3.0	-- [3]	3.09[2]	

[1] whole blood
[2] RBC
[3] could not calculate
[4] Average value
[a] Moser *et al.*, 2005
[b] Moser *et al.*, 2006
[c] Moser, 1999
[d] McDaniel *et al.*, 2007
[e] Moser *et al.*, 2010
[f] Moser, 2011

Table 1. ED50 values (mg/kg) for brain and blood ChE inhibition for all pesticides in adult and PND17 rats, with the ratio calculated as adult:PND17. Blood assays involved RBC for some, and whole blood for other chemicals.

ED50 values obtained in PND11 rat pups are presented in Table 2; adult values in this table are taken from Table 1. For carbaryl and carbofuran, but not dicrotophos, the ratios of brain ED50 values were greater, indicating more sensitivity, in the PND11 rat compared to PND17.

Overall, it is clear that while the young are much more sensitive to some of these pesticides, there are no such differences with others. This is illustrated in Figure 1, showing brain ChE dose-response data for both ages for diazinon (brain ratio >5) and acephate (brain ratio=1).

Tissue	Brain			RBC		
Pesticide/Age	Adult	PND11	Ratio	Adult	PND11	Ratio
Carbaryl[a,b]	29.5[1]	18.1	1.6	6.64	9.36	0.7
Carbofuran[a,b]	1.06[1]	0.18	5.9	0.39	0.090	4.3
Dicrotophos[c]	0.75	0.43	1.7	0.45	0.40	1.1

[1] Average value
[a] McDaniel *et al.*, 2007
[b] Moser *et al.*, 2010
[c] Moser, 2011

Table 2. ED50 values (mg/kg) for brain and RBC ChE inhibition for three pesticides in adult and PND11 rats, with the ratio calculated as adult:PND11.

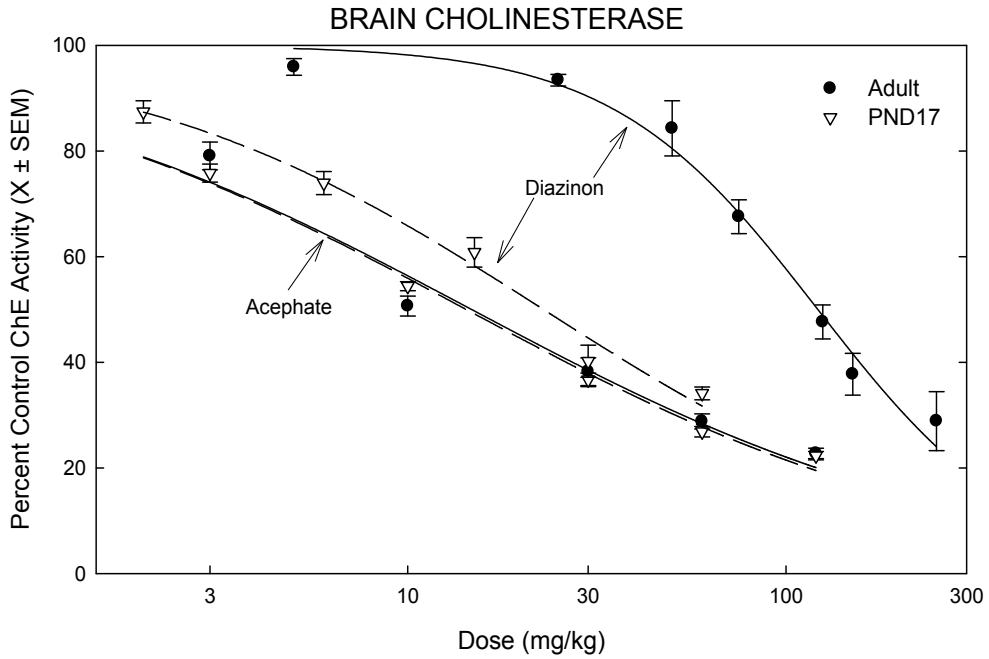

Fig. 1. Brain ChE inhibition in adult and PND17 rats treated with either acephate or diazinon. Data presented as percent control (X±SEM)

For most pesticides where data are available, there were less obvious age differences in blood ChE inhibition, with ratios <2 on this measure for all except carbofuran and malathion: this was true for both PND17 and PND11 ages. Finally, even where brain ChE was more sensitive, in many cases the blood ChE inhibition was similar.

3.2 Summary

Table 3 summarizes and bins the adult:PND17 ratios from Table 1 by chemical class. A comparison of ED50s for brain ChE inhibition revealed that slightly more than a quarter of the pesticides showed increased sensitivity in the young of 3-fold or greater, slightly less showed about 2- to 3-fold greater sensitivity, and fully half of the pesticides showed

essentially no marked differences (<2-fold) in sensitivity. With both carbamates and OPs, half of the tested chemicals had low ratios (<2-fold), but there were more OPs producing relatively large age differences than carbamates. OPs with the largest ratios were diazinon, chlorpyrifos, mevinphos, chlorpyrifos, and malathion, and carbamates were propoxur and carbofuran. In contrast to brain, many ED50 values for blood ChE inhibition showed little or no differences (<2-fold) between ages. For the two pesticides that showed greater age differences in blood values, there was one from each chemical class.

Tissue	Brain ChE ratio			Blood ChE ratio	
Chemical/Bin	<2	2.0-2.9	≥3	<2	>3
Combined	9	4	5	9	2
OPs	5	1	4	8	1
Carbamates	4	3	1	1	1

Table 3. Grouping of adult:PND17 ratios from Table 1. There were 10 OPs (brain for all, whole blood or RBC for 9) and 8 carbamates (brain for all, RBC for 2).

Similar grouping for the adult:PND11 ratios was not reasonable due to the low number of pesticides tested. However, of these three, the highest ratio for both brain and blood ChE inhibition was observed with carbofuran, a carbamate.

4. Discussion

4.1 Organophosphates

The majority of the literature in this area has addressed chlorpyrifos, parathion, methyl parathion, and malathion. The data presented here for chlorpyrifos and malathion further support previous findings of juvenile sensitivity, and in general show similar magnitudes of differences. In this study, the ratio of brain ChE ED50s for chlorpyrifos was 4.3, similar to the 5-fold difference in maximally tolerated dose (MTD) at PND17 (Moser and Padilla 1998). While others have reported somewhat greater MTDs and LD10s in younger rats (PND7, MTD ratio 6.2-fold, LD10 ratio 9.1-fold; Pope *et al.* 1991; Zheng *et al.* 2000), they found the ratio of brain ED50 values at PND7 to be less (2.2-fold; Pope and Liu 1997) than reported here for older rat pups. In our data, RBC ChE inhibition was not greater in the pups, in contrast to other reports of juvenile sensitivity of around 4-fold (Pope *et al.* 1991; Zheng *et al.* 2000). The reason for these tissue-dependent differences in ChE inhibition ratios is unclear.

The only dose-response studies for malathion that could be found in the literature measured lethality. The greatest difference in LD50 values, 27.5-fold, was measured in newborn rats (Lu *et al.* 1965). As the pups matured, the LD50 ratios decreased, being measured at 7.2-fold in PND12, 4-4.5-fold in PND14-18, and 2.2-fold in weanling rats (Brodeur and DuBois 1963; Lu *et al.* 1965). Our difference of 3-fold or greater for ChE inhibition agrees well with these values, despite the different endpoints.

The ratios presented here are generally in agreement with the few available studies for a few other OPs. Acephate showed little to no differences in sensitivity, as was reported for lethality (Gaines and Linder 1986). Likewise, methamidophos showed no differences in terms of lethality or MTDs (Gaines and Lindner, 1986; Moser, 1999), agreeing with the similar brain ChE inhibition obtained here. While our data report increased sensitivity of 3.6-fold in PND17 rats with mevinphos, an earlier study showed only a 1.5-fold difference in

LD50s in PND23 rats (Brodeur and DuBois, 1963). No other dose-response data with which to compare point estimates could be found for the remaining OPs.

It is important to note that while we have not studied parathion or methyl parathion, ratios of sensitivity differences from the literature range from around 8-fold in newborn to PND7 rats, to less than 2-fold in weanling pups (Benke and Murphy, 1975; Brodeur and DuBois, 1963; Harbison, 1975; Pope and Chakraborti, 1992). This information could add two more OP pesticides to the group that show sensitivity ratios >3-fold at approximately PND17; however, they are not added to Table 1 or 3 since we did not test those pesticides in this laboratory.

4.2 Carbamates

There are many fewer studies of juvenile sensitivity in carbamate toxicity. Besides our studies on ChE inhibition, the literature has only provided lethality data. Methomyl and carbaryl were not more sensitive to lethality in post-weaning (3-6 weeks of age) rats (Brodeur and DuBois, 1963; Gaines and Lindner, 1986). We observed essentially no differences in PND17 rats with either pesticide, but younger rats (PND11) were 1.6-fold more sensitive with carbaryl. We had previously reported about 2-fold more sensitivity with aldicarb for lethality and MTDs, similar to the 2.4-fold difference in ChE brain inhibition (Moser, 1999).

As part of an ongoing pesticide registration process by the EPA Office of Pesticide Programs, manufacturers have submitted comparative ChE studies in which inhibition in adult and PND11 rats is measured following acute and/or short-term repeated exposures. These data were modeled to calculate values that inhibit 10% brain ChE. While most of the studies are not available in the peer-reviewed literature, summaries are reported in the carbamate cumulative risk assessment document (US EPA, 2007). Sensitivity ratios based on these values for formetanate and carbofuran were similar to those obtained here for ED50 values, but the >3-fold ratios for methomyl and oxamyl were greater than those reported here. Some of these discordant results may be due to differences in levels of effect (10% vs 50%), age (PND11 vs PND17), as well as other experimental factors (*e.g.*, rat strain, ChE assay, etc.). Values for carbaryl and aldicarb were calculated using the same data presented here, so it is not surprising that the sensitivity ratios are similar for those carbamates.

4.3 Kinetics

Considerable evidence suggests that immature detoxification mechanisms in the young account for much of the reported age-related differences in sensitivity (*e.g.*, Atterberry *et al.* 1997; Benke and Murphy 1975; Chanda *et al.* 1997; Mendoza 1976; Mortensen *et al.* 1996; Sterri *et al.* 1985). All of these chemicals are detoxified through a combination of P450 microsomal enzymes, carboxylesterases, and/or A-esterases, but the metabolic patterns differ greatly (Chambers *et al.*, 2010). For some chemicals such as chlorpyrifos, sensitivity in young rats has been directly correlated with maturing carboxylesterase and A-esterase systems (Chanda *et al.* 1997,. 2002; Karanth and Pope 2000; Mortensen *et al.* 1996; Moser *et al.* 1998). In addition to chlorpyrifos, esterase detoxification, determined *in vivo* and/or *in vitro*, is known to be important for diazinon, mevinphos, malathion, and propoxur (*e.g.*, Cashman *et al.* 1996; Cohen and Murphy 1971, 1974; Gupta and Dettbarn 1993; Gupta and Kadel 1990; Main and Braid 1962; Moser and Padilla, 2011; Padilla *et al.* 2000, 2004; Poet *et al.* 2003; Walker and Mackness 1987). These chemicals all showed ≥3-fold increased sensitivity in the

young. Using *in vitro* tests, measurements of esterase (carbarylesterase, A-esterase) detoxification have also revealed good concordance between juvenile sensitivity and degree of esterase detoxification (Moser and Padilla, 2011; Padilla *et al.* 2000, 2004). Extrapolation of these findings suggest that the chemicals most dependent on esterases for detoxification will be more toxic to the young, and that screening for this can be predictive of juvenile sensitivity.

In these studies, the magnitude of age-related differences in sensitivity did not correlate with potency. Juvenile sensitivity was notable for malathion, the least potent (highest ED50) of the chemicals tested, as well as for aldicarb, the most potent. While a highly potent pesticide may produce more environmental risk, it may not necessarily be more toxic to the young.

4.4 Considerations

The ratios presented here may not be quantitatively exact or fixed. For example, point estimates depend on the curve-fitting model used. The logistic function was used here for all pesticides instead of chemical-specific models, even though the latter may fit better specific shapes of the dose-response curves. These different models may produce different estimates, and similar but different ratios. In a previous report (Moser *et al*., 2010), we used a four-parameter logistic model to fit the adult, PND17, and PND11 data for carbaryl and carbofuran. Most of the ratios were essentially the same as what is reported here, the largest discrepancy being the carbofuran brain, where the PND11 comparison is 5.9 here, and reported as 5.3 previously (Moser *et al*., 2010). Here we have also only compared 50% inhibition values, but the choice of this level could also impact the ratios, especially where the curves may not be parallel.

The age of the pups is an important factor. Progressively decreasing sensitivity from birth to weaning has been demonstrated for several pesticides, and may correlate with maturing esterase detoxification as described in section 4.3. Similar evidence is presented here, since for carbaryl and carbofuran, the brain ChE ratios were greater in PND11 pups compared to PND17. On the other hand, dicrotophos ED50 values for PND11 pups were slightly higher than for PND17, resulting in somewhat lower ratios in the youngest rats.

The interpretation of the magnitude of age differences in terms of "large" or "small" is relative. For example, even a 5-fold difference, which is considered here as a "large" difference, is less than a 10-fold uncertainty factor for intraspecies variability. On the other hand, a 5-fold difference is clearly larger than 2-fold, allowing the pesticides to be directly compared. Furthermore, as mentioned above, these ratios depend on the age at testing. Finally, it is important to note that these ED50 values are based on administered dose in mg/kg. Considering the large differences in body weight, on a total dose level, the differences are greater. For example, for chlorpyrifos the ED50 values for adult and PND17 brain ChE inhibition are 22.6 and 5.3 mg/kg, respectively (Table 1). Given an average weight of 330g for adults, and 28 g for PND17 pups, the total doses administered average about 7.5 mg for adults and 0.15 mg for pups, which is a 50-fold difference in intake. Thus, these differences in sensitivity can be considered several different ways.

5. Conclusions

Generalizing these data along with other literature reports leads to a conclusion that relatively large age-related differences are evident more often with OP pesticides, whereas

carbamates showed more moderate differences. However, fully half of both classes of chemicals showed essentially no age differences. These outcomes are mostly chemical-specific, and therefore assumptions that the young are always more sensitive to ChE inhibition are incorrect. For children's health, logic would dictate the use of pesticides showing less juvenile sensitivity. This retrospective analysis informs estimation of the likelihood for age-related differences in sensitivity for acute cholinesterase inhibition.

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7. References

Aldridge, W.N., and Reiner, E. (1975). Enzyme Inhibitors as Substrates: Interaction of Esterases with Esters of Organophosphorus and Carbamic Acids, Elsevier, New York.

Atterberry, T. T., Burnett, W. T., and Chambers, J. E. (1997). Age-related differences in parathion and chlorpyrifos toxicity in male rats: target and nontarget esterase sensitivity and cytochrome P450-mediated metabolism. Toxicol. Appl. Pharmacol. 147:411-418.

Barlow, R., and Blake, J.F. (1989). Hill coefficients and the logistic equation. Trends Pharmacol. Sci. 10:440-441.

Benke, G. M., and Murphy, S. D. (1975). The influence of age on the toxicity and metabolism of methyl parathion and parathion in male and female rats. Toxicol. Appl. Pharmacol. 31:254-269.

Brodeur, J., and DuBois, K. P. (1963). Comparison of acute toxicity of anticholinesterase insecticides to weanling and adult male rats. Proc. Soc. Exp. Biol. Med. 114:509-11.

Brodeur, J., and DuBois, K. P. (1967). Studies on factors influencing the acute toxicity of malathion and malaoxon in rats. Can. J. Physiol Pharmacol. 45:621-631.

Cashman, J. R., Perotti, B. Y., Berkman, C. E., and Lin, J. (1996). Pharmacokinetics and molecular detoxication. Environ. Health Perspect. 104 Suppl 1:23-40.

Chambers, J.E., Meek, E.C., Chambers, H.W. (2010). The metabolism of organophosphorus insecticides. In: Krieger, R. (ed), Hayes' Handbook of Pesticide Toxicology, Academic Press, NY, pp. 1399-1407.

Chanda, S. M., Lassiter, T. L., Moser, V. C., Barone, S. J., and Padilla, S. (2002). Tissue carboxylesterases and chlorpyrifos toxicity in the developing rat. Human Ecol.Risk Assess. 8:75-90.

Chanda, S. M., Mortensen, S. R., Moser, V. C., and Padilla, S. (1997). Tissue-specific effects of chlorpyrifos on carboxylesterase and cholinesterase activity in adult rats: an in vitro and in vivo comparison. Fundam. Appl. Toxicol. 38:148-157.

Cohen, S. D., and Murphy, S. D. (1971). Carboxylesterase inhibition as an indicator of malathion potentiation in mice. J. Pharmacol. Exp. Ther. 176:733-742.

Cohen, S. D., and Murphy, S. D. (1974). A simplifed bioassay for organophosphate detoxification and interactions. Toxicol.Appl.Pharmacol. 27:537-550.

Curl, C. L., Fenske, R. A., and Elgethun, K. (2003). Organophosphorus pesticide exposure of urban and suburban preschool children with organic and conventional diets. Environ. Health Perspect. 111:377-382.

Faustman, E. M., Silbernagel, S. M., Fenske, R. A., Burbacher, T. M., and Ponce, R. A. (2000). Mechanisms underlying children's susceptibility to environmental toxicants. Environ. Health Perspect. 108 Suppl 1:13-21.

Fenske, R. A., Black, K. G., Elkner, K. P., Lee, C. L., Methner, M. M., and Soto, R. (1990). Potential exposure and health risks of infants following indoor residential pesticide applications. Am. J. Public Health 80:689-693.

Fukuto, T.R. (1990). Mechanism of action of organophosphorus and carbamate insecticides. Environ. Health Perspec. 87:245-254.

Gaines, T. B., and Linder, R. E. (1986). Acute toxicity of pesticides in adult and weanling rats. Fundam. Appl. Toxicol. 7:299-308.

Gupta, R. C., and Dettbarn, W. D. (1993). Role of carboxylesterases in the prevention and potentiation of N-methylcarbamate toxicity. Chem. Biol. Interact. 87:295-303.

Gupta, R. C., and Kadel, W. L. (1990). Toxic interaction of tetraisopropylpyrophosphoramide and propoxur: some insights into the mechanisms. Arch. Environ. Contam Toxicol. 19:917-920.

Harbison, R. D. (1975). Comparative toxicity of some selected pesticides in neonatal and adult rats. Toxicol. Appl. Pharmacol. 32:443-446.

Hunter, D. L., Marshall, R. S., and Padilla, S. (1997). Automated instrument analysis of cholinesterase activity in tissues from carbamate-treated animals: a cautionary note. Toxicol. Methods 7:43-53.

Johnson, C. D., and Russell, R. L. (1975). A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations. Anal. Biochem. 64:229-238.

Karanth, S., and Pope, C. (2000). Carboxylesterase and A-esterase activities during maturation and aging: relationship to the toxicity of chlorpyrifos and parathion in rats. Toxicol. Sci. 58:282-289.

Loewenherz, C., Fenske, R. A., Simcox, N. J., Bellamy, G., and Kalman, D. (1997). Biological monitoring of organophosphorus pesticide exposure among children of agricultural workers in central Washington State. Environ. Health Perspect. 105:1344-1353.

Lowit, A.B. (2006). Federal regulations and risk assessment of organophosphate and carbamate pesticides. In: Toxicology of Organophosphate and Carbamate Compounds. R.C. Gupta, ed. Elsevier Academic Press, New York, pp 617-632.

Lu, C., Knutson, D. E., Fisker-Andersen, J., and Fenske, R. A. (2001). Biological monitoring survey of organophosphorus pesticide exposure among pre-school children in the Seattle metropolitan area. Environ. Health Perspect. 109:299-303.

Lu, F. C., Jessup, D. C., and Lavallee, A. (1965). Toxicity of pesticides in young versus adult rats. Food Cosmet. Toxicol. 3:591-596.

Main, A. R., and Braid, P. E. (1962). Hydrolysis of malathion by aliesterases in vitro. Biochem. J. 84:255-263.

McDaniel, K. L., Padilla, S., Marshall, R. S., Phillips, P. M., Podhorniak, L., Qian, Y., and Moser, V. C. (2007). Comparison of acute neurobehavioral and cholinesterase inhibitory effects of N-methylcarbamates in rat. Toxicol. Sci. 98:552-560.

Mendoza, C. E. (1976). Toxicity and effects of malathion on esterases of suckling albino rats. Toxicol. Appl. Pharmacol. 35:229-238.

Mortensen, S. R., Chanda, S. M., Hooper, M. J., and Padilla, S. (1996). Maturational differences in chlorpyrifos-oxonase activity may contribute to age-related sensitivity to chlorpyrifos. J. Biochem. Toxicol. 11:279-287.

Mortensen, S. R., Hooper, M. J., and Padilla, S. (1998). Rat brain acetylcholinesterase activity: developmental profile and maturational sensitivity to carbamate and organophosphorus inhibitors. Toxicology. 125:13-19.

Moser, V. C. (1999). Comparison of aldicarb and methamidophos neurotoxicity at different ages in the rat: behavioral and biochemical parameters. Toxicol. Appl. Pharmacol. 157:94-106.

Moser, V. C. (2000). Dose-response and time-course of neurobehavioral changes following oral chlorpyrifos in rats of different ages. Neurotoxicol. Teratol. 22:713-723.

Moser, V.C. (2011). Age-related differences in acute neurotoxicity produced by mevinphos, monocrotophos, dicrotophos, and phosphamidon. Neurotoxicol. Teratol. in press.

Moser, V.C., Casey, M., Hamm, A., Carter, Jr., W.H., Simmons, J.E., and Gennings, C. (2005). Neurotoxicological and statistical analyses of a mixture of five organophosphorus pesticides using a ray design. Tox. Sci. 86:101-115.

Moser, V. C., Chanda, S. M., Mortensen, S. R., and Padilla, S. (1998). Age- and gender-related differences in sensitivity to chlorpyrifos in the rat reflect developmental profiles of esterase activities. Toxicol. Sci. 46:211-222.

Moser, V. C., McDaniel, K. L., Phillips, P. M., and Lowit, A. B. (2010). Time-course, dose-response, and age comparative sensitivity of N-methyl carbamates in rats. Toxicol. Sci. 114:113-123.

Moser, V. C., and Padilla, S. (1998). Age- and gender-related differences in the time course of behavioral and biochemical effects produced by oral chlorpyrifos in rats. Toxicol. Appl. Pharmacol. 149:107-119.

Moser, V.C., and Padilla, S. (2011) Esterase metabolism of cholinesterase inhibitors using rat liver in vitro. Toxicol. 281, 56-62.

Moser, V.C., Simmons, J.E., and Gennings, C. (2006). Neurotoxicological interactions of a five-pesticide mixture in preweanling rats. Tox. Sci. 92:235-245.

National Research Council (1993). Pesticides in the Diets of Infants and Children, National Academy Press, Washington, DC.

Padilla, S., Buzzard, J., and Moser, V. C. (2000). Comparison of the role of esterases in the differential age-related sensitivity to chlorpyrifos and methamidophos. Neurotoxicol. 21:49-56.

Padilla, S., Sung, H. J., and Moser, V. C. (2004). Further assessment of an in vitro screen that may help identify organophosphorus pesticides that are more acutely toxic to the young. J. Toxicol. Environ. Health A 67:1477-1489.

Poet, T. S., Wu, H., Kousba, A. A., and Timchalk, C. (2003). In vitro rat hepatic and intestinal metabolism of the organophosphate pesticides chlorpyrifos and diazinon. Toxicol. Sci. 72:193-200.

Pope, C.N. (2006) Central nervous system effects and neurotoxicity. In: Toxicology of Organophosphate and Carbamate Compounds. R.C. Gupta, ed. Elsevier Academic Press, New York, pp 271-291

Pope, C. N., and Chakraborti, T. K. (1992). Dose-related inhibition of brain and plasma cholinesterase in neonatal and adult rats following sublethal organophosphate exposures. Toxicology. 73:35-43.

Pope, C. N., Chakraborti, T. K., Chapman, M. L., Farrar, J. D., and Arthun, D. (1991). Comparison of in vivo cholinesterase inhibition in neonatal and adult rats by three organophosphorothioate insecticides. Toxicology 68:51-61.

Pope, C. N., and Liu, J. (1997). Age-related differences in sensitivity to organophosphorus pesticides. Environ. Toxicol. Pharmacol. 4:309-314.

Simcox, N. J., Fenske, R. A., Wolz, S. A., Lee, I. C., and Kalman, D. A. (1995). Pesticides in household dust and soil: exposure pathways for children of agricultural families. Environ. Health Perspect. 103:1126-1134.

Sterri, S. H., Berge, G., and Fonnum, F. (1985). Esterase activities and soman toxicity in developing rat. Acta Pharmacol. Toxicol. (Copenh). 57:136-140.

US Environmental Protection Agency (2002). Determination of the Appropriate FQPA Safety Factor(s) in Tolerance Assessment. Office of Pesticide Programs, Washington DC. Available at: www.epa.gov/oppfead1/trac/science/determ.pdf

US Environmental Protection Agency (2004). Pesticides Industry Sales and Usage, 2000 and 20001 Market Estimates. Office of Prevention, Pesticides, and Toxic Substances, Washington, DC. Available at: http://www.epa.gov/oppbead1/pestsales/01pestsales/market_estimates2001.pdf

US Environmental Protection Agency (2006). Organophosphorus Cumulative Risk Assessment – 2006 Update. Office of Pesticide Programs, Washington DC. Available at: www.epa.gov/pesticides/cumulative/2006-op/index.htm

US Environmental Protection Agency (2007). Revised N-Methyl Carbamate Cumulative Risk Assessment. Office of Pesticide Programs, Washington DC. Available at: www.epa.gov/oppsrrd1/REDS/nmc_revised_cra.pdf

Walker, C. H., and Mackness, M. I. (1987). "A" esterases and their role in regulating the toxicity of organophosphates. Arch. Toxicol. 60:30-33.

Zheng, Q., Olivier, K., Won, Y. K., and Pope, C. N. (2000). Comparative cholinergic neurotoxicity of oral chlorpyrifos exposures in preweanling and adult rats. Toxicol. Sci. 55:124-132.

23

Side-Effects of Pesticides on the Pollinator *Bombus*: An Overview

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1. Introduction

For the pollination of crops, agriculture relies largely on managed colonies of the honeybee *Apis mellifera* (Gallai et al., 2009). Unfortunately, recent crashes of colonies have been reported worldwide, also better known as "Colony Collapse Disorder" (CCD) (Mullin et al., 2010). In this context several authors pointed out that factors such as parasites and pesticides or a combination of these factors might be responsible for a decline in honeybee health (Van Engelsdorp et al., 2009). As a response multiple studies were conducted to assess pesticide residues in the field. The results were dramatic. For example, a study of apiaries in North American orchards recovered 121 agrochemicals in honeybees, pollen and the wax (Mullin et al., 2010). However the impact of our agricultural landscape is not limited to honeybee colonies. Indeed, also other pollinators suffer. Since 40 years non-*Apis* species such as bumblebees are decreasing in abundance (Goulson et al., 2008). Bumblebees, important for the pollination of many wild flowers, are crucial for the terrestrial ecosystem (Goulson, 2010). In addition, these pollinators as *Bombus terrestris*, *Bombus impatiens* and *Bombus ignitus* are also commercially reared for the pollination of agricultural and horticultural crops (Velthuis & van Doorn, 2006). Therefore, side-effects of pesticides need to be assessed for conservation and economic reasons. However, our current knowledge of pesticide toxicity on pollinating insects is fragmented for bumblebees since it is still mostly restricted to *A. mellifera*. One explanation to this can be found in bumblebees belonging to a less familiar group in the area of environmental protection. To date only a few pesticides have been tested on their compatibility with bumblebees prior to their commercial release, while for honeybees oral and acute toxicity tests are required for pesticide registration. Newer generation pesticides, which are thought to be less harmful to humans and the environment than the older pesticides such as synthetic organophosphate, carbamate and pyrethroid insecticides, are on the current marketplace. Nonetheless, even sublethal effects of pesticides may have significant impact on bees and pollination in addition to the more easily observable mortality.

This chapter provides for the first time an extensive overview of the side-effects of pesticides also called as "Plant Protection Products" (PPPs) on bumblebees. In a first and second part we will discuss the testing strategies so far employed to evaluate pesticide compatibility on bumblebees. Here attention will be given to the different "tier" levels, the various biological endpoints of effect, and the impact of the route of exposure. Then in a third part, an

overview will be given on the compatibility data that are currently available for the different groups of chemical and biological pesticides such as insecticides, acaricides, fungicides. A fourth part will compare the pesticide sensitivity between both pollinators for the different groups of PPPs. Finally, based on our increasing knowledge on the insect body we will make suggestions to improve some existing tests in order to work more standardized which would allow comparison between different PPPs in future.

2. Risk assessment at different "tier" levels with individual workers and micro-colonies in the laboratory to full colonies in the field

When assessing the toxicity of pesticides the first question one should address is: Can exposure to the pesticide occur? In the field, possible routes of exposure for bumblebees are by direct contact after a spray or orally via the consumption of contaminated food. However, evaluating the effect of a single pesticide or residue on an organism under field conditions is complex. However, in the case potential side-effects cannot be excluded, the risks need to be assessed in a stepwise approach with different "tier" levels (Figure 1).

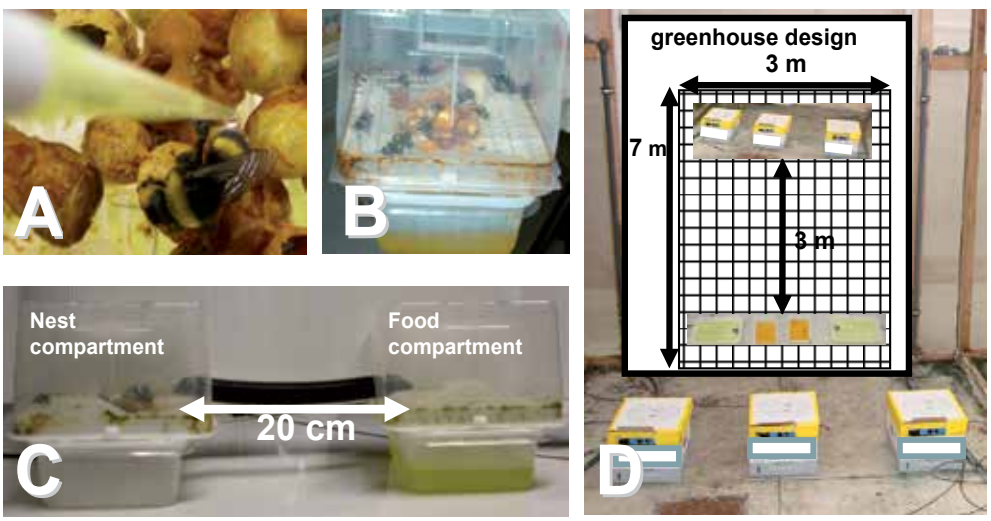

Fig. 1. Schematic overview of the different "tier" levels with (A) individual workers in the laboratory ("tier 1"), (B) micro-colonies in the laboratory without foraging ("tier 2"), (C) micro-colonies in the laboratory including foraging behavior ("tier 2"), and (D) full colonies in small greenhouse compartments ("tier 3"). The inset of D gives the greenhouse design (3 x 7 m) with the bumblebee colonies placed at 3 m from the food (own photographs).

At "tier 1" level, individual bumblebee workers are exposed to a worst case scenario in a laboratory insect toxicity test. To assess direct contact toxicity due to a spray application several experimental setups have been used (see for review Thompson, 2001; van der Steen, 2001). Currently, pesticides are dissolved in acetone and worker bumblebees are anesthetized with carbon dioxide up to 7 s prior to application of specific doses/concentrations to the bumblebee workers. For pesticide application dishes containing individual bumblebee workers are placed under a Potter spray tower (Scott-Dupree et al., 2009; Gradish et al., 2010). After treatment workers are transferred to cups with wax paper

where they are then provided with fresh sugar water. Then 48-72 h post-treatment acute toxicity is evaluated and the median lethal dose/concentration (LC_{50} or LC_{50}) is calculated. For this test at least 30 individual bumblebees need to be exposed. Also for the assessment of the acute toxicity via oral exposure, several protocols have been developed over the years (see for review Thompson, 2001). Bumblebees were first starved for 2-3 h and then fed with a 10 µl mixture of the pesticide dissolved in 50% sucrose which they had to consume within 2 h (see for review van der Steen, 2001). Hereafter the bumblebees were provided with regular sugar water and the LD_{50} was determined after 24-72 h. In the controls, an acceptable mortality level of ≤10% was set. The same method has also been recently used by Wu et al. (2010). These researchers assessed the oral toxicity in the laboratory with individual workers of the three bumblebee species *B. ignitus, Bombus hypocrita* and *Bombus patagiatus* and the diverse pesticides that are frequently used in Chinese greenhouses. In general, acute toxicity studies in the laboratory are easy to perform, but here attention should be given to the age of the individual workers used as susceptibility might change with the worker age. Some studies conduct their risk assessment with callow workers (<24 h), while others use bumblebees between 9-10 days or do not give any information on how the workers were selected.

A criticism on the aforementioned laboratory risk assessment tests with individual bumblebee workers over 72 h, is that side-effects of pesticides might take a longer time (>72 h) before becoming visible under practical conditions and that bumblebee workers show a social organization with the building of a nest (brood) and with foraging behavior to gather food from outside to inside the nest. It is therefore recommended to conduct an extended laboratory test as a second step of the risk assessment ("tier 2"). In order to cover all potential side-effects, bumblebees are exposed as in the insect laboratory test with individual workers ("tier 1") to PPPs concentrations recovered in the field, to concentrations as recommended for use or to the maximum field recommended concentration (MFRC). To date several studies evaluated potential postponed effects up to 11 weeks following exposure to insecticides, acaricides and fungicides by use of micro-colonies (Besard et al., 2010, 2011; Gradish et al., 2010; Mommaerts et al., 2006a,b, 2008, 2009, 2010a,b). Micro-colonies are artificial nests made of 3 to 5 workers of the same age, however a number of 5 workers is to be recommended for long chronic exposure assessments (Figure 1B). The wide application of this method in risk assessment studies with bumblebees can be explained by the low cost, the easy in use, the possibility to work standardized and with multiple replicates resulting in statistical power and thus in reproducible data. For the direct contact toxicity all the workers of the nest are treated by contact with a 50 µl drop of an aqueous solution made of the pesticide and tap water, on the dorsal thorax. These data give already strong indications on the compatibility of the pesticide with bumblebees, but other routes of exposure also occur. In the past, systemic compounds like neonicotinoids have been recovered in pollen. Also more recently, large studies in Europe and North-America showed the presence of PPP residues in pollen collected by honeybees (Skerl et al., 2009; Mullin et al., 2010; Wu et al., 2011). To simulate an oral chronic exposure via contaminated food, the bumblebee workers in the micro-colonies can be fed continuously with treated food (sugar water and/or pollen) over a period up to 11 weeks, or they can be fed for a period of 30 days after which they are then provided for 30 days with untreated food. For the sugar water treatment a solution is made of commercial sugar water (50%) or artificial home-made sugar water and the pesticide. Contaminated pollen paste is prepared by spraying pollen until saturation with an aqueous solution of the pesticide, prepared in tap water (Besard et al.,

2010; Mommaerts et al., 2006a,b, 2008, 2009, 2010a). However, the pesticide can also be dosed at exact amounts to pollen grains, which are then mixed with sugar syrup, and finally offered as a homogenous food source to the bumblebee workers. A final route of exposure is via residues left on plant surfaces. To simulate this situation, Wu et al. (2010) sprayed solutions of the pesticide (as prepared in water) on paper which was then air-dried before exposure to the bumblebees. To assess such effect upon exposure to biological insecticides, Hokkanen et al. (2004) developed two different methods. First, by treatment of the flowers until drip-off, and secondly via a "maximum challenge test". In the latter test bumblebee workers walk through a Petri Dish containing the growing and sporulating fungus. Considering the worker mortality, the aim of the extended laboratory tests is to classify PPPs. Unfortunately, criteria for a classification of substances are up until today not available for bumblebees. However, the side-effects' classification for arthropods and beneficial organisms by the "International Organization for Biological Control of Noxious Animals and Plants" (IOBC) is useful: "class 1": <25% effect, non-toxic; "class 2": 25-50% effect, weakly toxic; "class 3": 50-75% effect, moderately toxic; and "class 4": >75% effect, highly toxic. There is still no validation of this classification at present. For example when a product causes a loss of <25%, it is considered as not toxic. However, Goulson (2010) argued that the effect of a loss on the colony is directly depending on the colony size. We therefore suggest that in future these classification classes should be defined in relation to the range of the colony size.

Besides worker mortality (i.e. lethal side-effects), risk assessment studies also need to cover potential sublethal side-effects on bumblebee reproduction, larval development and the foraging ability of adults. These parameters are of crucial importance to guarantee the crop pollination. At first colonies containing adult workers and brood were fed on a treated 50% sugar solution during 24 h. Then, the brood (consisting of egg cups, open cups containing larval and pupal stages) was evaluated by observations at 3 times per week and this over a period of 3 weeks (see van der Steen, 2001). However, collecting data on effects on brood is difficult and thus de Wael et al. (1995) developed a method where the brood was daily checked and by photographing the brood from a fixed point. Although this was already an improvement a better protocol was developed by Gretenkord & Dresscher (1996). Here a more detailed evaluation was possible as eggs were removed from the colony and incubated until hatching where after the number of larvae was standardized to 10. For exposure, the larvae were placed in small boxes containing 3 workers that fed treated pollen during 24 h. Then, the amounts of pollen consumed by the larvae and the numbers of larvae developing into an adult were determined. Also these sublethal endpoints can be assessed with micro-colonies (Mommaerts et al., 2006a,b, 2010a; Gradish et al., 2010), but this will be discussed in more detail under 2.1. Moreover, in "tier 2" also laboratory trials including side-effects on the foraging behavior can be included. For example, Mommaerts et al. (2010b) recently reported on a "foraging bioassay" which made use of micro-colonies. As depicted in figure 1C, a box containing a micro-colony was connected by a tube of 20 cm in length with an empty nest containing the food (pollen and sugar water). This experimental setup allows the evaluation of interferences with the orientation capacity of the adult bumblebee workers. However, also other endpoints important for the foraging process can become affected after pesticide exposure. Hereto flight cages are a good tool. Morandin et al. (2005) connected colonies to flight cages (1.2 m x 1.2 m x 1 m) wherein artificial flowers were placed to evaluate the impact of an insecticide on the flower handling time and on the foraging speed.

Finally, in a last step, the PPPs are to be tested under semi-field and field conditions ("tier

3"). The aim of such complex studies is to get more insight in the risks for bumblebee colonies under more practical, field-related conditions. However, up until today the numbers of such studies are limited (see for review van der Steen, 2001). Gretenkord & Drescher (1996) was the first to describe a protocol for semi-field testing. According to his method a colony of at least 100 workers was placed in a cool box in the ground. Then this box was connected to a gauze tent (3 m x 2 m x 4 m) containing flowering *Phacelia tanacetifolia* plants. At a foraging intensity of 10 workers the connection tube is closed, the colony is standardized (containing one queen, 10 foragers, 5 nurses, 4-6 egg cups, and brood that is consisting of one cup with larval stages of 1-2 days, 3-4 days, and 5-6 days old and with 10-15 pupae), and the plants are sprayed. Bumblebees are exposed during 2-3 weeks and thereafter lethal and sublethal side-effects are assessed during 2 weeks in the laboratory. Similarly, Sechser & Reber (1996) placed free flying colonies in a tent (5-9 m^2) that was sprayed with the recommended concentration of the pesticides, and in addition colonies were fed with sugar water supplemented with the pesticide. Here effects were evaluated on all stages after 6 weeks. Moreover, next to tents, semi-field tests have also been conducted in small greenhouse compartments (3 m x 2 m) with a crop area of 2 m^2 (Tasei et al., 1993). However, the main problem with the use of crops in small compartments is that the size of the colony is not proportionate to the crop size, resulting in not enough pollen and nectar for the colony. To circumvent the use of plants, as depicted in figure 1D, Mommaerts et al. (2010b) provided bumblebee colonies with commercial pollen and treated sugar water at a distance of 3 m from their nest in greenhouse risk assessment experiments.
For field testing, a first protocol was described by Schaefer & Mühlen (1996). They placed six bumblebee colonies in a 2400 m^2 field with flowering *Phacelia* plants. Here worker mortality, colony activity and colony development were evaluated by collecting dead workers, activity observations on 5 x 1 m^2 for 1 min and by counting adults, dead larvae and photographing the brood. Also here the IOBC classification for side-effects in arthropods and beneficial organisms has been used to classify substances, but again it should be remarked that no validation has been done so far. According to this classification system for (semi-)field testing the following three classes can be distinguished: "class N": harmless or slightly harmful, 0-50%; "class M": moderately harmful, 51-75%; and "class T": harmful, >75%. Besides a lack of a proper classification system, it is still unclear how long bumblebees should be exposed. Some studies provide bumblebees during 5 weeks with treated food followed by a period of 5 weeks of uncontaminated food, while in other studies bumblebees were exposed during their entire life-span. Consequently, comparison between the determined risks resulting from the different assessment tests with the same pesticides is difficult.

2.1 Different biological endpoints for the assessment of side-effects

At present, risk assessments for PPPs follow regulatory guidelines which are for Europe defined by the European Council Directive 91/414. The aim of these guidelines is to protect honeybees and other pollinators. Here only side-effects on adult and larvae of honeybees are considered, while exposure in the field to other pollinators cannot be excluded. For example bumblebees might be exposed to pesticides in greenhouses through spraying via residues left on plants or by consuming contaminated nectar and pollen. Following exposure, the most obvious effect is worker mortality, but pesticides may also cause sublethal effects. Moreover, due to the increasing development of chemicals with different modes of action there is a demand to define valuable endpoints of effects. At present the increasing

economic importance of bumblebees in agriculture results in a growing body of literature on side-effects of pesticides of which on overview is given below.

2.1.1 Lethal effects

For a long time risk assessment studies with bees only considered the LD_{50} or LC_{50} of pesticides. Most likely this approach is probably based on honeybee risk assessments where at first the risk was calculated by the hazard quotient which is the application rate divided by the LD_{50} as calculated after 72 h of exposure (i.e. "tier 1"). To date for acute worker mortality, insect death (i.e. lethal endpoint) which is easy to observe, is not adequate enough. Indeed, the lethal dose is only a partial assessment of the risk for loss of survival as the test runs only for 3 days. Therefore Gradish et al. (2010) scored workers as dead when they did not move upon touching. This criterion considers also the effect of slower acting pesticides such as for the pesticides abamectine and metaflumizone that causes paralysis of the insect, resulting in feeding cessation and death. In conclusion, to date acute toxicity (via oral and contact exposure) is evaluated on the level of individual insects, whereas studies evaluating the long term side-effects (i.e. chronic exposure) make use of micro-colonies of bumblebees. It is to be noticed that the latter experimental setup has the wide advantage to consider potential pesticide transfer between bumblebees which might occur upon contact.

2.1.2 Sublethal effects

Considering the growing interest to determine potential sublethal effects following pesticide exposure, several methods have been reported to identify and characterize these for beneficial arthropods. A first comprehensive review on this research topic was published by Desneux et al. (2007). Here the authors mainly focused on effects on honeybees and natural enemies. However, the use of bumblebees in agriculture demands for examinations of sublethal side-effects as pollination must be guaranteed. For bumblebees, the reported sublethal effects of pesticides include effects on adults and on brood with fecundity and abnormal larval development resulting in reduced offspring. More details are discussed hereunder.

2.2 Exposure to different developmental stages

2.2.1 Exposure to adult workers

Following pesticide exposure, adults can directly be affected. At first adult longevity was shown affected after exposure to lethal and/or sublethal concentrations. For example Gradish et al. (2010) observed a shortened life-span when adult workers were fed on imidacloprid-treated pollen by scoring the number of dead workers.

So far pesticide exposure occurs in long-term studies by feeding the bees with contaminated food. For bumblebees food consumption is crucial as workers need sugar water for energy and pollen for ovary development (Heinrich, 1979). Based on this often also a second endpoint has been evaluated, namely, worker biomass. To determine worker biomass, some studies determined the weight of collected dead workers, while others used newly emerged workers which were cooled before weighed (Gradish et al., 2010; Wu et al., 2010).

Moreover, considering the importance of food for ovary development the moment of first oviposition has been used as a third endpoint. Care is needed as a reduction of the fecundity (oviposition) can be the result of a reduced food uptake or of a physiological effect of the pesticide. For example for diflubenzuron (IGR), Mommaerts et al. (2006a) showed

transovarial transport and accumulation in the eggs after pollen consumption by adults resulting in egg mortality. Next to a reduction, pesticides can also induce a stimulatory effect on the oviposition. Topical contact of adult workers with a sublethal concentration of kinoprene (IGR) resulted in a significant increase of both ovarian length and the numbers of eggs present in the ovaries (Mommaerts et al., 2006b).
Finally, pesticides are known to induce behavioral changes on adults (Thompson, 2003). To date several studies demonstrated that ingestion of small amounts of pesticides (e.g. imidacloprid, deltamethrin) by adult honeybees (Colin et al., 2001; Decourtye et al., 2003) interferes with their learning and orientation capacity. Similarly, sublethal concentrations of imidacloprid affected bumblebee behavior as Mommaerts et al. (2010b) demonstrated with use of the "foraging bioassay", thus when adult bumblebees (*B. terrestris*) needed to gather their food, that adult bumblebees had difficulties to find back the way to their nest resulting in a severe reduction of the offspring. For foragers orientation and memory are essential to find food. Assessment of these side-effects occurs in honeybees by use of the proboscis extension response (PER) (Decourtye & Pham-Delègue, 2002; Decourtye et al., 2004a,b; El Hassani et al., 2008). However, for bumblebees PER has been conducted with *Bombus occidentalis* but not in the context of risk assessments (Riveros & Gronenberg, 2009). Therefore future studies might include this method to broaden the endpoints which might become affected when adult bumblebees are exposed to pesticides.

2.2.2 Exposure to eggs, larvae and pupa

Bumblebee foragers gather pollen and nectar which is transported to the hive. Pesticides can also be brought to the hive via this route. Thus, in the field bumblebee brood can become indirectly exposed to pesticides sprayed on crops when the brood (larvae) is fed with contaminated pollen/nectar. For the assessment of these side-effects micro-colonies have been used successfully. In micro-colonies, comprising of 3 to 5 callow workers, one worker becomes dominant and starts to lay eggs after one week, while the other workers assist her in rearing the brood. Eggs laid in micro-colonies are not fertilized and will develop over 4 larval stages and 1 pupal stage into male adults (drones) after 4 weeks. Effects on brood are scored as the numbers of larvae that are removed from the brood. This criterion is based on the typical behavior of bumblebee workers to remove larval stages with abnormalities or dead larvae from the respective brood clump (Mommaerts et al., 2006a,b, 2009, 2010a; Gradish et al., 2010). Moreover, this endpoint was further refined in accordance with the mechanism of action of pesticides under investigation. For example, in case of the IGRs as developed to interfere with the developmental processes in insects, the different stages of the removed larvae were determined based on their head width (Mommaerts et al., 2006a,b). For effects on the reproduction, the number of offspring (drones) produced was already used by multiple studies as endpoint (Mommaerts et al., 2006a,b, 2008, 2009, 2010a,b; Besard et al., 2010, 2011). Here drones were measured on a weekly basis and this during a period up to 11 weeks; the drones were removed from the micro-colonies after scoring.
Considering behavioral effects, Morandin et al. (2005) showed that spinosad (insecticide) when administered during the entire larval stage affected other parameters crucial for the foraging capacity of adult workers. Hereto the authors connected a bumblebee (*B. impatiens*) colony with a flight cage, containing two different types of artificial flowers. A "simple flower" consisted of an Eppendorf tube without caps, while a "complex flower" an Eppendorf tube with the caps attached leaving an opening of 7 mm. With this experimental setup data were collected on the time period needed to access the first artificial flower, the

handling time, and the foraging rate. However, there exists a debate to date whether sublethal effects must be investigated, particularly at lower tier level, because potential side-effects are expected to become visible in experiments at higher tier level. It should be noted that there is not enough information to make a firm conclusion in this matter.
Next to indirect exposure via food, only a few studies examined the effect of a direct contamination of the brood by contact. For example Mommaerts et al. (2008) treated third- and fourth-instar larvae by dermal contact with a suspension of a biological insecticide in water to assess the larval toxicity of the compound. Also van der Steen (2005) evaluated side-effects on bumblebee brood. Hereto all adult workers were removed from the colony prior to spraying.

3. Different classes of pesticides

3.1 Chemical pesticides

3.1.1 Insecticides

To date risks assessment studies conducting the side-effects of conventional insecticides are mostly limited to acute toxicity studies. A summary of all available data on effects of the older insecticides including the pyrethroids, the carbamates and the organophosphates is given in table 1. Interestingly, van der Steen (1994) found that the acute toxicity (oral and contact) for dimethoate was correlated with the size of the bumblebee. In addition, for the pyrethroid deltamethrin also sublethal effects have been described. At double the recommended rate, Gretenkord & Drescher (1993) reported a repellent effect. Similarly, Tasei et al. (1994) showed an increase of 40-100% in sucrose uptake when *B. terrestris* were treated by dermal contact with 0.08-0.16 mg/kg, whereas a higher dose of 0.1-0.2 mg/kg caused a 47-59% decrease of sucrose uptake. Overall, when considering conventional insecticides it is remarkable that none of all compounds included (n=59) was considered as non-toxic (Figure 2).
Neonicotinoids are systemic insecticides which interfere with the insect nervous system by binding on the nicotinic acetylcholine receptor. The most studied compound within this group is imidacloprid. Exposure to bumblebees (*B. terrestris* or *B. impatiens*) caused acute worker mortality after contact/oral exposure (Incerti et al., 2003; Marletto et al., 2003; Scott-Dupree et al., 2009; Gradish et al., 2010; Mommaerts et al., 2010b). Also effects such as bumblebee trembling, reduced brood production, pollen consumption, vitality, and impaired foraging behavior have been observed after exposure to imidacloprid (Tasei et al., 2000; Gels et al., 2002; Incerti et al., 2003; Morandin & Winston, 2003; Gradish et al., 2010). However Tasei et al. (2001) concluded by use of a greenhouse test that imidacloprid when applied as a seed coating at the registered dose did not affect *B. terrestris* foraging and homing behavior. Although imidacloprid received much attention in risk assessments, this group of neonicotinoids also contains other compounds. Recently, Mommaerts et al. (2010b) reported that the neonicotinoids with a nitro group (imidacloprid and thiamethoxam) caused the greatest side-effects. Here it should also be remarked that not only the mother product but also metabolites were shown to affect bee survival. For example clothianidin, derived from thiamethoxam, was highly toxic after contact on *B. impatiens* workers. In contrast, acetamiprid and thiacloprid both belonging to the group of the cyano-neonicotinoids, were less toxic. In total only 17% of the 6 compounds considered were safe (Figure 2).
IGRs are classified as more selective due to their interference with insect-specific targets however only 47% of the compounds tested has been found non-toxic (Figure 2). Within the IGRs, three different groups can be distinguished: chitin synthesis inhibitors (CSIs), juvenile

hormone analogs (JHAs), and ecdysteroid agonists or also called molting-accelerating compounds (MACs).

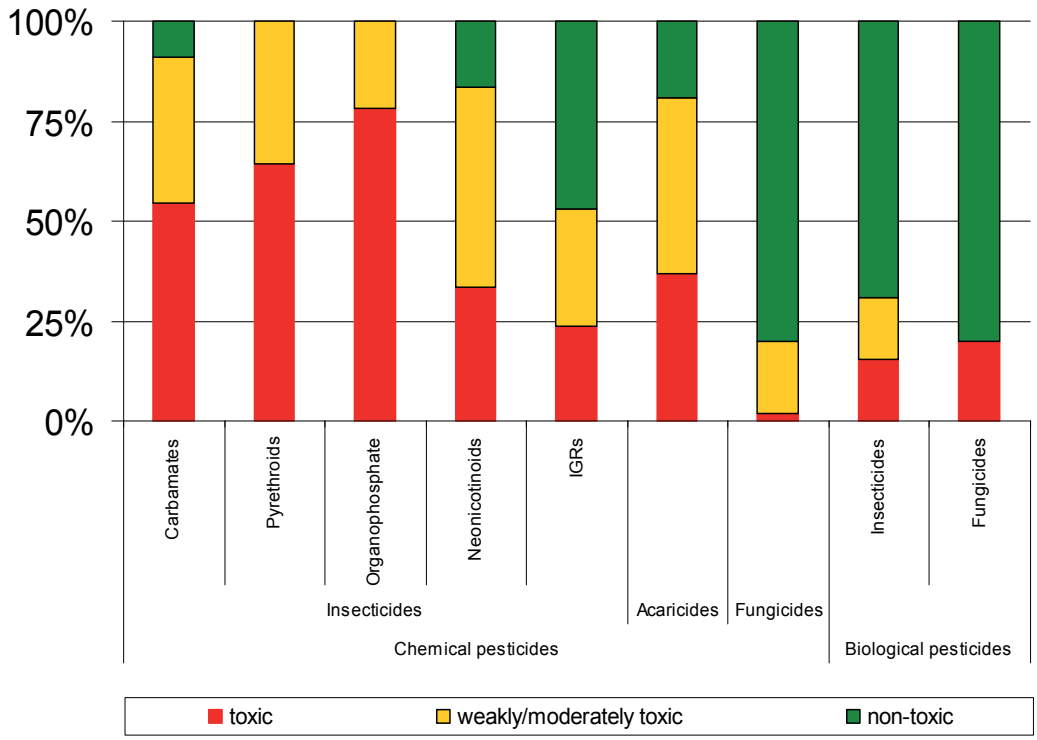

Fig. 2. Overview of the toxicity of chemical and biological pesticides towards bumblebees (*Bombus terrestris*). For each pesticide group the bars represent the percentage of compounds which are non-toxic (green), weakly/moderately toxic (yellow-orange) and toxic (red). The division in toxicity levels is based on the recommendations made by the side-effect list when available, or on the obtained toxicity with micro-colonies ("tier 2"). The numbers of compounds considered per group are n=11 for carbamates, n=14 for pyrethroids, n=32 for organophosphates, n=6 for neonicotinoids, n=17 for IGRs, n=13 for biological insecticides, n=27 for acaricides, n=66 for chemical fungicides, and n=5 for biological fungicides. For details with references, see table 1, 2 and 3.

CSIs are mainly larvicides and act through the inhibition of chitin formation. So far no mortality was reported by CSIs against adult bumblebee workers (de Wael et al., 1995; Tasei, 2001; Mommaerts et al., 2006a; Scott-Dupree et al., 2009). However, severe effects have been observed on reproduction. Dermal contact exposure to the MFRC of diflubenzuron (288 mg/l) and teflubenzuron (150 mg/l) caused a total inhibition of adult formation (Mommaerts et al., 2006a). Also for diflubenzuron transovarial transport was confirmed. The NOEC for this compound was 100-10,000 times lower than the MFRC. Consequently, it is not recommended to use these compounds in combination with bumblebees. Other CSIs tested comprise novaluron, flucycloxuron, flufenoxuron, lufenuron, buprofezin and cyromazine (Mommaerts et al., 2006a; Scott-Dupree et al., 2009). Here the route of exposure will determine the effect with the strongest effects seen when CSIs were administrated via

the pollen. Overall, the MFRC of all CSIs were also detrimental to larval growth as significantly more larvae of the first and second instar were removed due to an abnormally formed cuticle.

The JHAs with a function resembling the juvenile hormone (JH), are contact and stomach poisons. In insects, JH is responsible for the regulation of the metamorphosis and the synthesis of vitellogenin. For *B. terrestris* toxicity tests by use of micro-colonies showed that JHAs (pyriproxyfen, fenoxycarb and kinoprene) did not cause acute/chronic worker mortality by oral/contact exposure (Mommaerts et al., 2006b). Similarly, no effect on the reproduction was reported when *B. terrestris* workers were exposed during 11 weeks to the MFRC of these compounds. In contrast, pollen exposure to pyriproxyfen (25 mg/l) and kinoprene (650 mg/l) resulted in a significantly higher numbers of removed third- and fourth-instar larvae, implying a lethal blockage of the development before metamorphosis (Mommaerts et al., 2006b). Interestingly, for the latter compound a low concentration of 0.0650 mg/l had a stimulatory effect on brood production, resulting in longer ovaries that contained more eggs than in control dominant workers.

The MACs are active after contact and ingestion when they bind on the receptor site of the insect molting hormone 20-hydroxyecdysone, the ecdysone receptor. For the bumblebee *B. terrestris* the MFRC of tebufenozide and methoxyfenozide did not affect worker survival, worker reproduction and larval development (Mommaerts et al., 2006b). In conclusion, the extended laboratory tests with micro-colonies indicated that these MCAs are compatible with the use of bumblebees.

Finally, within the class of the chemical insecticides metaflumizone, chlorantraniliprole and a natural plant derivate Matrine (Kingbo) have also been tested. These insecticides are currently used in the greenhouse vegetable production. For metaflumizone 0.1-1 g/l caused direct contact toxicity, whereas chorantraniliprole was harmless (Gradish et al., 2010). Also both insecticides at the recommended rate did not affect reproduction in *B. impatiens* micro-colonies (Gradish et al., 2010). The natural plant derivate Matrine was only evaluated for its impact on worker survival. After contact exposure to dry residues Wu et al. (2010) observed a significant effect on worker mortality when application doses used in the greenhouse were tested (1/5000, v/v). For oral toxicity it was interesting that the LD_{50} for *B. hypocrita* (0.0019 µg per bee) was significantly higher than for the other bumblebee species (*B. ignitus* and *B. patagiatus*) (Wu et al., 2010).

3.1.2 Acaricides

Studies evaluating the impact of acaricides are limited. Recently Besard et al. (2010) published a first extensive evaluation of 23 acaricides (traditional and novel ones) on *B. terrestris* by using the laboratory micro-colony design. Also here effects are different according to the route of exposure with the strongest effects observed after oral exposure via the drinking of treated sugar water. According to Besard et al. (2010) abamectin, bifenazate, bifenthrin and etoxazole were not compatible with *B. terrestris*. At a concentration of 18 mg/l (i.e. MFRC) abamectine caused 100% worker loss. Similarly, Gradish et al. (2010) reported for *B. impatiens* 80-100% worker mortality after contact to 0.1-1.0 g/l while oral exposure via pollen caused several sublethal effects such as reduced colony lifespan and delay of oviposition. Overall, of the 27 compounds tested only 19% was non-toxic (see figure 2). For more detailed information concerning the different acaricides so far tested see table 2.

3.1.3 Fungicides

Risk assessments including fungicides are limited resulting in only fragmented data (see table 3). Overall, it can be concluded that at the recommended rates the fungicides tested (myclobutanil, potassium bicarbonate, difenoconazole and copper abietate) did not cause a negative effect on *B. impatiens* worker survival and reproduction. Also the side-effect list (see Biobest side-effect list: http://www.biobest.be, and Koppert side-effect list: http://neveneffecten.koppert.nl/), comprising data of more than 50 active ingredients of applied fungicides, recommends that bumblebee hives do not need to be removed before product application, however except for carbendazim, cyprodinil+fludioxonil, dimethomorph, fosetyl-aluminium, penconazole, pyrazofos and tebuconazole. Here it is recommended to remove the hives prior to application and this until 24 h after. On this list only one active ingredient, namely zineb (Zerlate), is indicated as not compatible. Consequently, of the 66 compounds included 66% is classified as non-toxic (Figure 2).

3.1.4 Weed crop control products and plant growth/health regulators

To our knowledge no data is available at present on the compatibility with bumblebees of herbicides, plant growth regulatory hormones (e.g. straw shorteners) and plant health stimulating compounds, such as chemicals that induce systemically acquired resistance (SAR) in the treated crops.

3.2 Biological pesticides

3.2.1 Bio-insecticides

The group of the biological insecticides includes 13 different compounds of which 69% is considered as safe (Figure 1).

Beauveria bassiana GHA and *Metarhizium anisopliae* caused side-effects on *B. terrestris* (Hokkanen et al., 2004; Mommaerts et al., 2009). In the laboratory contact exposure to 2.5 x 10^{10} CFU/l (i.e. MFRC) of *B. bassiana* GHA resulted in 92% worker mortality after 11 weeks, while oral administration did not affect worker survival. In addition, also sublethal effects on the reproduction and changes in the foraging behavior have been observed with *B. bassiana* GHA (Mommaerts et al., 2008, 2009).

For the MFRC of *Cydia pomonella* granulovirus no detrimental effects have been observed after contact and oral exposure (Mommaerts et al., 2009).

In the laboratory with the micro-colony design no worker mortality was seen after contact and oral exposure via eating pollen to the MFRC of *Bacillus thuringiensis* kurstaki and *B. thuringiensis* aizawai (Mommaerts et al., 2010a). In contrast, oral exposure via sugar water treated with *B. thuringiensis* aizawai caused a 100% loss, but this effect disappeared when the concentration was 10 times diluted. Similar effects were also reported on *B. occidentalis* and *B. terrestris* by Morandin & Winston (2003) and Babendreier et al. (2008) when pure Cry proteins (Cry1Ab and Cry1Ac) were taken up via pollen and sugar water. Concerning the sublethal effects on reproduction var. kurstaki was harmless, while var. aizawai administered at 0.01% via the pollen reduced reproduction by 31%. Both strains did not induce behavioral changes.

For the naturalyte spinosad, consisting of spinosyn A and D derived from the fermentation of the bacterium *Saccharopolyspora spinosa*, acute oral and contact toxicity tests demonstrated its toxicity for bumblebees (Mayes et al., 2003). However, according to Morandin et al. (2005) colony losses only occurred when bumblebees (*B. impatiens*) were exposed to an unrealistically high dose of 8.0 mg/kg. Nonetheless, at realistic field concentrations (0.2-0.8

mg/kg) sublethal effects were observed. For example larval exposure to 0.8 mg/kg via the diet (pollen) resulted in adults foraging slower on artificial complex flowers, whereas such effects were not visible at lower concentrations. Similarly, Besard et al. (2011) demonstrated for *B. terrestris* that oral feeding with the MFRC (400 mg/l) of spinosad caused 75% worker mortality after 72 h. Here bumblebee workers showed tremors causing paralysis and finally insect death. Moreover, at 0.4 mg/l spinosad was harmless. In contrast, the novel spinosyn spinetoram was less toxic as the MFRC (25 mg/l) resulted only in 55% worker mortality. No sublethal effects were scored at 0.025 mg/l. In addition, a wet and dry residue test also confirmed the higher toxicity of spinosad over spinetoram.

3.2.2 Bio-fungicides

In total the MFRC of 5 different microbiological fungicides have been tested with the micro-colony design (see table 3). All were classified as harmless via the different routes of exposure, except *Bacillus subtilis* QST713 (Figure 2). Here the MFRC (7.5×10^9 CFU/l) resulted in a severe total loss of adult *B. terrestris* workers ("class 4" for extended laboratory testing) after contact and oral exposure to treated sugar water (Mommaerts et al., 2009).

4. Sensitivity for pesticide side-effects: does there exist a correlation between honeybees and bumblebees?

To determine the sensitivity for pesticide side-effects between closely related pollinators as *B. terrestris* and *A. mellifera* we will first compare the overall toxicity of the different classes of PPPs. Then, for the chemical insecticides we will investigate if a correlation exists on product level between bumblebee and honeybee toxicity by use of a regression analysis with available LD_{50}-24h. Finally, for the insecticides such as the IGRs whereof no LD_{50} could be found the side-effects on bumblebees were compared with those on honeybees.

As mentioned above the toxicity of different PPPs for bumblebees is given in figure 2. At present toxicity data of all PPPs are available for honeybees. An overview of the relative toxicity based on the LD_{50}-48h after contact and oral exposure on honeybees (*A. mellifera*) for the different classes of PPPs (the same selection of PPPs as for figure 2) is given in figure 3. Comparison of both figures 2 and 3 clearly demonstrates a similar trend in sensitivity between bumblebee and honeybee toxicity. For example the overall toxicity of older chemical insecticides (including the carbamates, pyrethroids and organophosphates) is comparable and ranges between high to moderate except for one product (oxamyl) which was safe for bumblebees and highly toxic for honeybees. A similar trend can also be seen for the newer chemical insecticides (IGRs and neonicotinoids), the biological insecticides and the chemical fungicides, although it should be remarked that honeybees were more sensitive than bumblebees. Furthermore, an equal toxicity was observed for the different products belonging to the class of the acaricides and biological fungicides. Based on these results we argue that bumblebee toxicity, can be used as a first indication for honeybee toxicity but care is needed when different endpoints can be affected because honeybees and bumblebees are very distinct (colony live, behavior,…). Nonetheless, this would imply that a first toxicity screening can be done by using bumblebees which are easier to work with as compared to honeybees.

As mentioned above, the toxicity of the chemical insecticide class is comparable between bumblebees and honeybees. However, figure 4 shows a regression analysis with the available LD_{50}s for the different PPPs belonging to the carbamates, pyrethroids,

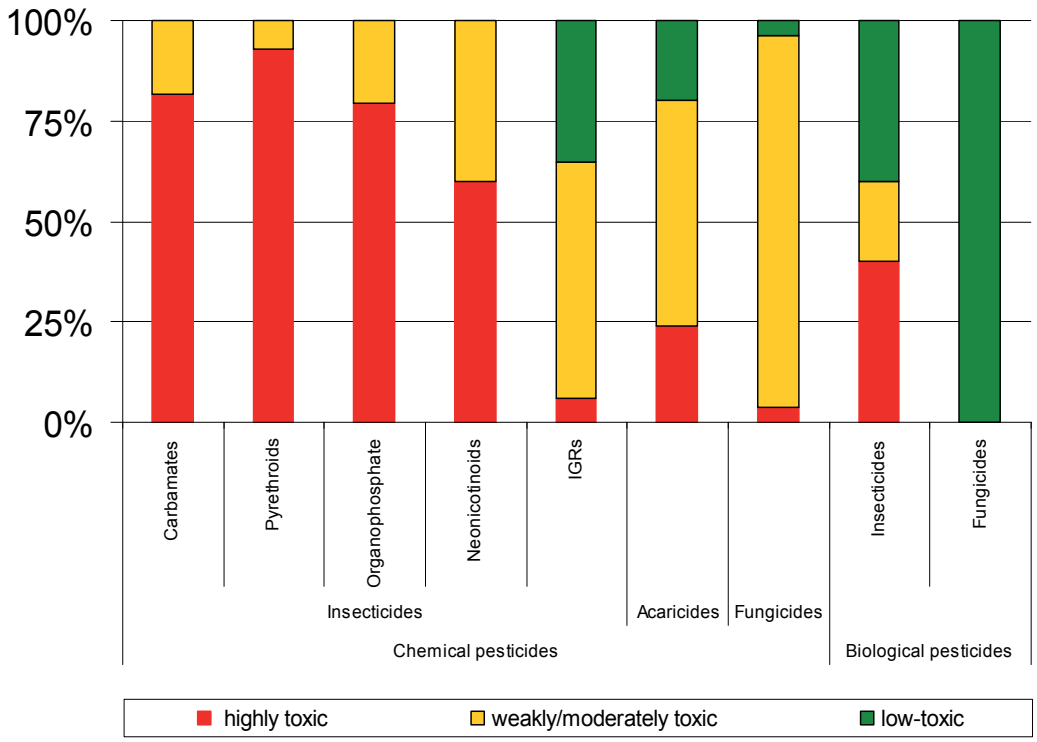

Fig. 3. Overview of the honeybee (*Apis mellifera*) toxicity of chemical pesticides and biological pesticides as available for bumblebees (*Bombus terrestris*). For each pesticide group the bars represent the percentage of compounds which are non-toxic (green), weakly/moderately toxic (yellow-orange) and toxic (red). The toxicity levels are based on the LD_{50}-48h obtained after contact and oral exposure (see http://sitem.herts.ac.uk/aeru/footprint/en/index.htm). The numbers of compounds considered per group are n=11 for carbamates, n=14 for pyrethroids, n=29 for organophosphates, n=5 for neonicotinoids, n=17 for IGRs, n=5 for biological insecticides, n=25 for acaricides, n=62 for chemical fungicides, and n=3 for biological fungicides.

organophosphates, and neonicotinoids. Here the LD_{50}s obtained after 24 h exposure were used and this for 17 insecticides. When the values were expressed as µg/g, then these were recalculated to µg/bee based on the weights as published by Thompson (2001) (with 0.10 g for *A. mellifera* and 0.21 g for *B. terrestris*). The poor linear regression (R=0.36) between the toxicities of the different compounds confirms that extrapolation of toxicity data between these two pollinators is not possible. In case of the carbamates, the LD_{50}s of 4 compounds were obtained. Here it was shown that for 75% of the products (carbaryl, methomyl and propoxur) *B. terrestris* was up to 10 times less sensitive than honeybees. Only the LD_{50} for ethiofencarb was lower (more sensitive) for *B. terrestris* (0.205 µg/bee) than for *A. mellifera* (6.85 µg/bee). For the group of pyrethroids, *A. mellifera* was more sensitive for all 5 products. For the organophosphates, the *B. terrestris* sensitivity was variable. Out of the 7 organophosphates, there were 4 products (acephate, chlorpyrifos, demeton-S-methyl and dimethoate) for which *A. mellifera* showed a higher sensitivity than *B. terrestris*. Equal sensitivity for both pollinators was seen for oxy-demeton-methyl and paraxon while *B.*

terrestris was 10 times more sensitive for chlorpyrifos-methyl. For the neonicotinoids *A. mellifera* was most sensitive to imidacloprid. This is in agreement with Hardstone & Scott (2010) who concluded that *A. mellifera* was among the most sensitive for imidacloprid. In contrast, the sensitivity for acetamiprid was equal between both pollinators.

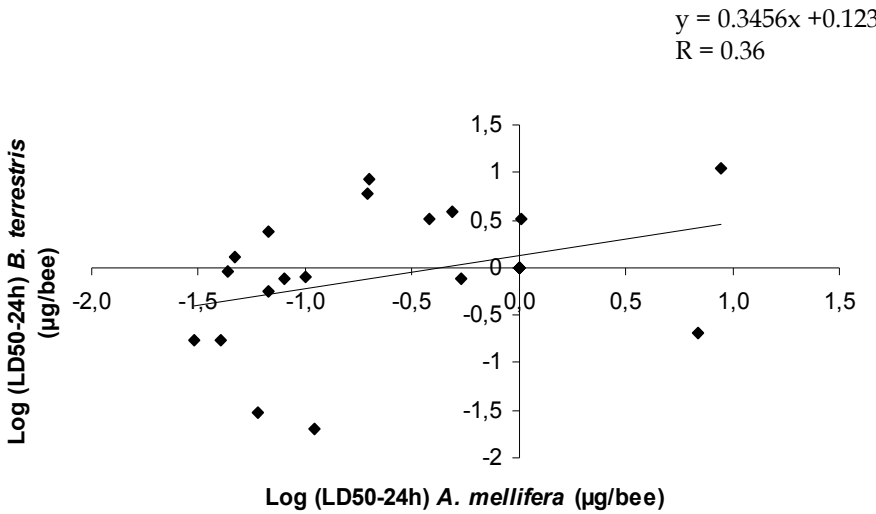

Fig. 4. Sensitivity of pesticide side-effects on bumblebees (*Bombus terrestris*) versus honeybees (*Apis mellifera*). Linear regression analysis was conducted with LD_{50}-24h values after contact and oral exposure for 17 different insecticides (carbamates, pyrethroids, organophophates and neonicotinoids). Data are presented as a mean log (LD_{50}-24h) and originate from Thompson (2001), van der Steen et al. (2008) and Hardstone & Scott (2010).

Although no linear regression could be drawn, this analysis gives a first idea of bumblebee versus honeybee sensitivity for pesticides. It needs to be remarked that the power of this analysis is limited because an LD_{50} was not available for each insecticide. However, based on available data for different IGRs, MACs are safe for both bumblebees and honeybees (Thompson et al., 2005; Mommaerts et al., 2006b), whereas no correlation can be found for the other two classes (CSI and JHA). Indeed, for diflubenzuron (CSIs) the LD_{50}-24 h on larvae showed that *B. terrestris* larvae are more sensitive than *A. mellifera* (LD_{50}-72 h) (Tasei, 2001). For the same compound also Mommaerts et al. (2006a) reported a total loss of *B. terrestris* reproduction, while Thompson et al. (2005) found only short-term effects on *A. mellifera* colonies. In contrast, for the JHA fenoxycarb, *B. terrestris* larvae were less susceptible (LD-24h: >0.650 µg/larvae) than *A. mellifera* larvae (LD_{50}-48h: 0.013 µg/larvae) (Tasei, 2001). Similarly, exposure of micro-colonies to fenoxycarb at its MFRC did not result in negative effects on reproduction (Mommaerts et al., 2006b), while *A. mellifera* colonies started the season slower and queen mating and egg laying were affected after (oral) exposure (Thompson et al., 2005). Based on this information and in order to have a total idea of the pollinator sensitivity towards pesticides, it is recommended that future studies should also evaluate the sensitivity of pesticides on other developmental life-stages. Finally, the pesticide side-effects sensitivity between honeybees and bumblebees is not only different for chemical insecticides. Indeed, for spinosad a biological insecticide comparison showed that

honeybees (LD_{50}-48h: 0.16µg/bee) were 100 times more sensitive than bumblebees (LD_{50}-48h: 19.4 µg/bee) (Halsall & Grey, 1998; Aldershof, 1999).
From the above mentioned results, it is clear that risk assessment bioassays need to evaluate side-effects on species level. The reason for this difference is not only due to a difference in sensitivity, but as already argued by Thompson & Hunt (1999) due to a difference in exposure profile. In this context they identified the following factors: namely the foraging active period, the species of crops visited, and the time of spraying (time on the day and time in the season). For example insecticides belonging to the class of the pyrethroids are applied in the early morning or late evening when they are more toxic and thus perform a higher risk for bumblebees. Similarly, risk assessment measures in honeybees are not useful for bumblebee losses which occur by pesticide applications in March-April, the moment of the year when bumblebee queens emerge and forage to find a nest place in order to start a colony (Thompson & Hunt, 1999).

5. Conclusions and future perspectives

This review gives on overview of the available toxicity data of PPPs on bumblebee species used for the pollination of crops. However, when looking at the obtained data set it is clear that the information is more fragmented in comparison with honeybees. Although in the past efforts have been made to assess risks by developing a variety of methods, we propose to conduct them in a tier approach in order to assess risks in a more complete way. The different levels are: (1) laboratory tests on individual insects ("tier 1"), (2) extended laboratory tests with micro-colonies which include the evaluation of pesticides on key processes such as worker survival, reproduction and behavior ("tier 2"), and (3) semi-field and/or field tests ("tier 3"). Unfortunately, to date most studies do not include semi-field and/or field tests, while it is crucial to make a link between the observed toxicity in the laboratory and the risks under field conditions in order to fully assess the risks. For example laboratory tests ("tier 1 and 2") do not consider pesticide degradation which might occur under field conditions. In addition, the goal of each tier is to classify the PPPs according to their compatibility with bumblebees. However, this point has been overlooked as no guidelines exist for bumblebees and thus these of the IOBC are used without any validation. Proper guidelines are therefore urgently needed which resemble the consequences at colony level by, for example, taking into account the consequences of worker loss according to the size of the colony.
In this review also a wide variety of effects (lethal and sublethal effects) have been reported following pesticide exposure. For lethal effects (worker mortality) the methods used are well defined. However, comparison between pesticide toxicities remains difficult. Therefore we suggest that in the future the already available lethal toxicity tests are more standardized by using a fixed exposure time and worker age and by determining the size of the worker as the length of the bumblebee body is variable. For sublethal effects on adult workers, different endpoints have already been evaluated such as worker life-span, worker biomass, start of oviposition, and this with adequately developed methods. In contrast, sublethal effects on the bumblebee brood have been assessed but these bioassays need to be further improved. Indeed, in honeybees a brood test was recently developed where brood is kept in individual cells without the presence of adults. For bumblebees, the development of such method would benefit from the existing one where side-effects are evaluated by collection of the larvae removed from their cocoon. Moreover, such new test would allow to work

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects				Compatibility	Route	Ref.
								Reproduction		Adult/ Behavior			
				contact	oral sugar water	Oral pollen	contact	oral sugar water	oral pollen				
Insecticides													
Carbamates													
aldicarb	NI	NI	RR								not compatible	s	Biobest
bendiocarb	NI	NI	RR								not compatible	s	Biobest
carbaryl	NI	NI	dose-range		LD50-24h: 3.92 µg/bee; LD50-72h: 3.84 µg/bee								de Wael et al. (1995)
	B. impatiens	field					reduced brood biomass			non irrigated: reduction of colony vitality; reduced worker biomass			Gels et al. (2002)
	NI	NI	RR								remove colonies before product application, retention time 36h	s	Biobest
carbofuran	NI	NI	RR								not compatible	s	Biobest
carbosulfan	NI	NI	RR								not compatible	s	Biobest
ethiofencarb					LD50-24h: 0.205 µg/bee; LD50-48h: 0.78 µg/bee; LD50-72h: 0.158 µg/bee								van der Steen et al. (2008)
	NI	NI	RR								remove colonies before product application, retention time of 48h	s	Biobest
methomyl	*B. terrestris*				LD50-24h: 1.42 µg/bee; LD50-48h: 0.57 µg/bee; LD50-72h: 0.44 µg/bee;								van der Steen et al. (2008)

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality		Sublethal effects			Compatibility	Route	Ref.
					LD50-24h: 3.2 µg/bee; LD50-72h: 2.6 µg/bee						Thompson (2001)
	B. lapidarius				LD50-48h: 2.78 µg/bee; LD50-48h: 2.4 µg/bee; LD50-72h: 2.18 µg/bee						Thompson & Hunt (1999)
	NI	NI	RR						remove colonies before product application, retention time of 72h	s; i	Biobest
methiocarb	NI	NI	RR						not compatible	s	Biobest
oxamyl	NI	NI	RR						not compatible	s	Biobest
pirimicarb	*B. terrestris*				LD50-24h: 8.5 µg/bee						Gretenkord & Dresher (1993)
		cage test				no effect at 900 g/ha					
propoxur					LD50-24h: 3.19 µg/bee; LD50-48h: 2.017 µg/bee; LD50-72h: 1.6 µg/bee						van der steen et al. (2008)
			RR	10-30%							van der steen et al. (2008)
		cage test				no effect at 2400 ml/ha					
	NI	NI	RR						not compatible	s	Biobest
Pyrethroids											
acrinathrin	NI	NI	RR						remove cololies before product application, retention time of 72h	s	Biobest
alphacypermethrin	*B. terrestris*			LD50-24h: 0.17µg/bee	LD50-24h: 0.52 µg /bee						Thompson & Hunt (1999); Thompson (2001)

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality		Sublethal effects				Compatibility	Route	Ref.
				LD50-72h: 0.15 µg/bee	LD50-72h: 0.36 µg/bee							Thompson & Hunt (1999); Thompson (2001)
	NI	NI	RR							not compatible	s	Biobest
bioresmethrin	NI	NI	RR							remove cololies before product application, retention time of 48h	s	Biobest
cyfluthrin				LD50-24h: 0.56 µg/bee	LD50-24h: 0.13 µg/bee							van der Steen et al. (2008)
	NI	NI	RR							not compatible	s	Biobest
cypermethrin	NI	NI	RR							not compatible	s	Biobest
deltamethrin	*B. terrestris*			LD50-48h: 0.9 µg/bee	LD50-24h: 0.6 µg/bee							Thompson (2001)
	B. impatiens	individual bees treated with a spray potter tower	dose-range	LC50-48h: 690 mg/l								Scott-Dupree et al. (2009)
	NI	NI	RR							remove colonies before product appliction, retention time of 72h	s	Biobest
esfenvalerate	NI	NI	RR							not compatible	s	Biobest
fenpropathrin	NI	NI	RR							not compatible	s	Biobest
fenvalerate	NI	NI	RR							not compatible	s	Biobest
flucythrinate	NI	NI	RR							not compatible	s	Biobest
lambda-cyhalothrin				LD50-24h: 0.22 µg/bee; LD50-72h: 0.11 µg/bee	LD50-24h: 0.21 µg/bee; LD50-72h: 0.16 µg/bee							van der Steen et al. (2008)
	B. terrestris	cage test	dose-range						repellent at 400ml/ha			Gretenkord & Dresher (1996)
	NI	NI	RR							not compatible	s	Biobest

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality		Sublethal effects				Compatibility	Route	Ref.
permethrin	*B. terrestris*	NI	dose-range	LD50-24h:0.81 µg/bee								Thompson (2001)
				LD50-72h: 0.82 µg/bee								Thompson (2001)
	NI	NI	RR							not compatible	s	Biobest
resmethrin	NI	NI	RR							remove colonies before product application, retention time of 12h	s	Biobest
tau-fluvalinate					LD50-24h: 0.97 µg/bee; LD50-72h: 0.68 µg/bee							de Wael et al. (1995)
	NI	NI	RR	10-30%								van der Steen et al. (2008)
	NI	NI	RR							remove colonies before product application, retention time of 24h	s	Biobest
Organophosphates												
acephate	*B. terrestris*				LD50-24h: 3.52-135.5µg /bee							Thompson (2001); van der Steen et al. (2008)
					LD50-72h: 3.44-7.37 µg /bee							de Wael et al. (1995); van der Steen et al. (2008)
					LD50-48h: 3.69 µg/bee							van der steen et al. (2008)
	NI	NI	RR							not compatible	s	Biobest
azinfos-methyl	NI	NI	RR							not compatible	s	Biobest
bromophos	NI	NI	RR							not compatible	s	Biobest

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects				Compatibility	Route	Ref.
chlorfenvinphos	NI	NI	RR								remove colonies before product application, retention time of 36h	s	Biobest
chlorpyriphos	*B. terrestris*			LD50-24h: 2.39 µg ai/bee									Thompson (2001)
				LD50-72h: 1.58 µg ai/bee									Thompson (2001)
	NI	NI	RR								not compatible	s	Biobest
	B. impatiens	field					reduced brood biomass			non irigated: reduction of collony vitality; reduced worker biomass			Gels et al. (2002)
chlorpyrifos-methyl				LD50-24h: 0.09 µg/bee; LC50-72h: 0.09 µg/bee	LD50-24h: 0.02-0.38 µg/bee; LC50-48h: 0.05 µg/bee; LD50-72h: 0.051-0.36 µg/bee								de Wael et al. (1995) ; van der Steen et al. (2008)
	NI	NI	RR								not compatible	s	Biobest
chloropyrifos-ethyl	NI	NI	RR								not compatible	s	Biobest
demeton methyl	*B. lucorum*			LD50-24h: 1-2 µg ai/bee									50
				LD50-24h:6-24 µg ai/queen		weakly toxic $	no effect	total loss	50% reduction				Thompson (2001)
	B. pascuorum			LD50-24h: 1-3 µg ai/bee									Thompson (2001)
				LD50-24h: 10-24 µg ai/queen									Thompson (2001)
demeton-S-methyl	*B. terrestris*			LD50-24h: 3.27 µg ai/bee		moderately toxic $	total loss	25-50% reduction	50-75% reduction				Thompson (2001)

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects				Compatibility	Route	Ref.
				LD50-72h: 2.68 µg ai/bee									Thompson (2001)
	NI	NI	RR		non-toxic $	no effect	25-50% reduction	50-75% reduction			not compatible	s	Biobest
diazinon	NI	NI	RR								not compatible	s	Biobest
dichlorvos	NI	NI	RR		highly toxic $	no effect	total loss	total loss			remove colonies before product application, retention time of 36h	s; st	Biobest
dimethoate	*B. terrestris*			LD50-24-72h: 4,7 µg ai/bee	non-toxic $	50% reduction	25-50% reduction	50-75% reduction					Thompson (2001)
				LD50-24-72h: 4.8 µg ai/bee									Thompson (2001)
	B. lucorum			LD50-24h: 2-5 µg ai/bee									Thompson (2001)
				LD50-24h: 5-20 µg ai/queen									Thompson (2001)
	B. pascuorum			LD50-24h:0.5-2 µg ai/bee									Thompson (2001)
				LD50-24h: 1-5 µg/queen	non-toxic $	no effect	25-50% reduction	50-75% reduction					Thompson (2001)
	NI	NI	RR		weakly toxic $	50% reduction	total loss	25-50% reduction			not compatible	s	Biobest
disulfoton	*B. lucorum*			LD50-24h: 2-10 µg/bee									Thompson (2001)
				LD50-24h: > 40 µg/queen	moderately toxic $	no effect	25-50% reduction	50% reduction					Thompson (2001)
	B. pascuorum			LD50-24h: 1-4 µg/bee									Thompson (2001)

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects				Compatibility	Route	Ref.
				LD50-24h: 5-10 µg/queen		weakly toxic $	no effect	25-50% reduction	50-75% reduction				Thompson (2001)
etrimfos	NI	NI	RR								not compatible	s	Biobest
fenitrothion	NI	NI	RR			weakly toxic $	no effect	> 75% reduction	50-75% reduction		not compatible	s	Biobest
malathion	NI	NI	RR								not compatible	s	Biobest
methamidophos	NI	NI	RR								not compatible	s	Biobest
methidathion	NI	NI	RR			weakly toxic $	50% reduction	no effect	> 75% reduction		not compatible	s	Biobest
mevinphos	NI	NI	RR			moderately toxic $	no effect	25-50% reduction	50-75% reduction		remove colonies before product application, retention time of 24h	s	Biobest
naled	NI	NI	RR								remove colonies before product application, retention time of 48h	s	Biobest
	NI	NI	RR			non-toxic $	no effect	no effect			not compatible	f	Biobest
omethoate	NI	NI	RR			moderately toxic $	50-75% reduction	25-50% reduction	no effect		not compatible	s	Biobest
oxy-demeton-methyl	*B. terrestris*	cage test		high mortality at 1200 ml/ha									
	B. terrestris				LD50-24h: 0.75 µg/bee								Thompson & Hunt (1999)
	NI	NI	RR								remove colonies before product application	s	Koppert
parathion	NI	NI	RR								not compatible	s	Biobest
parathion-methyl	NI	NI	RR								not compatible	s	Biobest
phorate	*B. lucorum*			LD50-24h: 1-2 µg/bee									Thompson (2001)
				LD50-24h:6-23 µg/queen									Thompson (2001)

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality		Sublethal effects				Compatibility	Route	Ref.
	B. pascuorum			LD50-24h: 1-2 µg/bee								Thompson (2001)
				LD50-24h: 1-5 µg/quee n								Thompson (2001)
phosalone	*B. terrestris*			LD50-24h: 5.98 µg/bee; LD50-72h: 4.39 µg/bee	LD50-24h: 3.98-60 µg/bee; LD50-72h: 3.98 µg/bee							de Wael et al. (1995)
		cage test										Gretenkord & Dresher (1993)
	NI	NI	RR							cover the colonies before product application	s	Koppert
phosphamidon	NI	NI	RR							not compatible	s	Biobest
pirimiphos-methyl	NI	NI	RR							not compatible	s	Biobest
profenofos	NI	NI	RR							not compatible	s	Biobest
sulfotep	NI	NI	RR							not compatible	f	Biobest
tetrachlorvinphos	NI	NI	RR							not compatible	s	Biobest
triazophos	NI	NI	RR							not compatible	s	Biobest
trichlorfon	NI	NI	RR							not compatible	s	Biobest
vamidothion	NI	NI	RR							not compatible	s	Biobest
Neonicotinoids												
acetamiprid	*B. ignitus*	individual contact test (air dried)	1: 5000 v/v	16days: highly toxic*								Wu et al. (2010)
		individual oral test			LD50-48h: 2.3 mg/bee							Wu et al. (2010)
	B. hypocrita	individual contact test (air dried)	1: 5000 v/v	16days: highly toxic*								Wu et al. (2010)
		individual oral test			LD50-48h: 2.8 mg/bee							Wu et al. (2010)
	B. patagiatus	individual contact test (air dried)	1: 5000 v/v	16days: highly toxic*								Wu et al. (2010)
		individual oral test			LC50-48h: 2.1 mg/bee							Wu et al. (2010)

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects				Compatibility	Route	Ref.
											remove colonies before product application, retention time of 36h	s	Koppert
	NI	NI	RR								compatible	i	Biobest
clothianidin	*B. impatiens*	individual bees treated with potter spray tower	dose range	LC50-48h: 39 mg/l									Scott-Dupree et al. (2009)
imidacloprid	*B.terrestris*	micro-colony	200 (MFRC)		highly toxic $			total loss					Mommaerts et al. (2010b)
			dose-range		LC50-11w: 0.059 mg/l			EC50-11w: 37 µg/l					Mommaerts et al. (2010b)
		micro-colony including foraging	200 (MFRC)		highly toxic $			total loss					Mommaerts et al. (2010b)
			dose-range		20 µg AI/L (LC 50)			EC50-11w: 3.7 µg/l					Mommaerts et al. (2010b)
			dose-range	LD50-24h: 1.3 µg/bee; LD50-48h: 1.15 µg/bee; LD50-72h: 0.05 µg/bee	LD50-24h: 0.02 µg/bee; LD50-48h: 0.02 µg/bee; LD50-72h: 0.23 µg/bee			total loss					van der Steen et al. (2008)
		greenhouse and field	dose-range				no effect on colony development				foraging and homing: no effect		
		field	dose-range								reduced colony life-span 20 µg/l: all workers dead around food area; 10 µg/l: all workers dead in the nest; 2 µg/l safe		Mommaerts et al. (2010b)

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects				Compatibility	Route	Ref.
		chronic feeding test of small colonies	0.002-0.005 (µg/bee)				reduced brood production and a lower number of larvae ejected						Tasei et al. (2000), Thompson (2003)
	B. impatiens	individual bees treated with potter spray tower	dose-range	LC50-48h: 322 mg/l									Scott-Dupree et al. (2009)
				72h: 100% mortality									Gradish et al. (2010)
		micro-colony	0.0192 mg/g						no oviposition	reduced life-span and pollen consumption			Gradish et al. (2010)
		field					when not irrigated reduced brood			when not irrigated reduced colony vitality and worker biomass			Gels et al. (2002)
	NI	NI	RR								not compatible	s; i	Biobest
nicotine	NI	NI	RR								remove colonies before product apllication, retention time of 24h (s) and 12h (f)	s; f	Biobest
thiacloprid	*B.terrestris*	micro-colony	120 (MFRC)		highly toxic $			total loss					Mommaerts et al. (2010b)
			dose-range		LC50-11w:18 mg/l			EC50-11w: 12 mg/l					Mommaerts et al. (2010b)
		micro-colony including foraging	12 mg/l		non-toxic $			> 75% reductio n					Mommaerts et al. (2010b)
	NI	NI	RR								remove colonies before product application, retention time of 24h	s; i	Biobest
thiamethoxam	*B.terrestris*	micro-colony	100 (MFRC)		highly toxic $			total loss					Mommaerts et al. (2010b)

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects				Compatibility	Route	Ref.
					LC50-11w: 0.12 mg/l			EC50-11w: 35 µg/l					Mommaerts et al. (2010b)
		micro-colony including foraging	0.1 mg/l		highly toxic $			> 75% reduction					Mommaerts et al. (2010b)
	NI	NI	RR								not compatible	s	Biobest
IGRs													
CSI													
buprofezin	*B.terrestris*	micro-colony	75 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	25-50% reduction	50% reduction				Mommaerts et al. (2006a)
			dose-range				harmless	LC50-11w: 69 mg/l	LC50-11w: 79 mg/l				Mommaerts et al. (2006a)
	NI	NI	RR								compatible	s	Biobest
chlorfluazuron	NI	NI	RR								remove colonies before product application, retention time of 36h	s	Biobest
cyromazine	*B.terrestris*	micro-colony	100 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	total loss	> 75% reduction				Mommaerts et al. (2006a)
			dose-range				LC50-11w: 636 mg/l	LC50-11w: 1.3 mg/l	LC50-11w: 17 mg/l				Mommaerts et al. (2006a)
	NI	NI	RR								remove colonies before product application, retention time of 36h	s	Biobest
diflubenzuron	*B.terrestris*	micro-colony	288 (MFRC)	non-toxic $	non-toxic $	non-toxic $	total loss	total loss	total loss				Mommaerts et al. (2006a)
			dose-range				LC50-11w: 25 mg/l	LC50-11w: 0.32 mg/l	LC50-11w: 0.95 mg/l	transovarial transport			Mommaerts et al. (2006a)
		individual contact test	24 961 dpm							24h: 44% cuticular penetration			Mommaerts et al. (2006a)
	NI	NI	RR								not compatible	s	Biobest
flucycloxuron	*B.terrestris*	micro-colony	125 (MFRC)	non-toxic $	non-toxic $	non-toxic $	> 75% reduction	no effect	total loss				Mommaerts et al. (2006a)

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects			Compatibility	Route	Ref.
							LC50-11w: 132mg/l	harmless	LC50-11w: 0.78 mg/l			Mommaerts et al. (2006a)
flufenoxuron	*B.terrestris*	micro-colony	50 (MFRC)	non-toxic $	non-toxic $	non-toxic $	50% reduction	total loss	total loss			Mommaerts et al. (2006a)
			dose-range				LC50-11w: 167 mg/l	LC50-11w: 8.6 mg/l	LC50-11w: 9.3 mg/l			Mommaerts et al. (2006a)
		individual contact test	33 876 dpm						24h: 85% cuticular penetration			Mommaerts et al. (2006a)
	NI	NI	RR							not compatible	s	Biobest
hexaflumuron	NI	NI	RR							not compatible	s	Biobest
lufenuron	*B.terrestris*	micro-colony	50 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	no effect	total loss			Mommaerts et al. (2006a)
			dose-range				harmless	harmless	LC50-11w: 218 mg/l			Mommaerts et al. (2006a)
	NI	NI	RR							remove colonies before product application, retention time of 36h	s	Biobest
novaluron	*B.terrestris*	micro-colony	40 (MFRC)	non-toxic $	non-toxic $	non-toxic $	> 75% reduction	total loss	total loss			Mommaerts et al. (2006a)
			dose-range				LC50-11w: 11 mg/l	LC50-11w: 0.99 mg/l	LC50-11w: 6.2 mg/l			Mommaerts et al. (2006a)
	B.impatiens	individual workers treated with a potter spray tower	dose-range	LC50-48h: > 10 000 mg/l								Scott-Dupree et al. (2009)
	NI	NI	RR							remove colonies before product application, retention time of 48h	s	Biobest
teflubenzuron	*B. terrestris*	micro-colony	150 (MFRC)	non-toxic $	non-toxic $	non-toxic $	total loss	total loss	total loss			Mommaerts et al. (2006a)
			dose-range				LC50-11w: 47 mg/l	LC50-11w: 0.27 mg/l	LC50-11w: 1.7 mg/l			Mommaerts et al. (2006a)

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects				Compatibility	Route	Ref.
								arrested egg development, larval mortality		decreased sucrose intake			
		small colonies	30 µg/bee										de Wael et al. (1995); Thompson (2003)
	NI	NI	RR								not compatible	s	Biobest
JHAs													
fenoxycarb	*B. terrestris*	micro-colony	100 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	no effect	no effect				Mommaerts et al. (2006b)
	NI	NI	RR								compatible	s	Biobest
kinoprene	*B. terrestris*	micro-colony	650 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect on male production but higher number of larvae ejected	no effect	no effect on male production but higher number of larvae ejected				Mommaerts et al. (2006b)
			dose-range				LC50-11W: 524 x 106 mg/l	LC50-11w: 28 300 mg/l		2 times longer ovaries with more eggs after contact with 0,065 mg/l			Mommaerts et al. (2006b)
	NI	NI	RR										Biobest
	NI	NI	RR								compatible	s	Biobest
pyriproxyfen	*B. terrestris*	micro-colony	25 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	no effect	no effect on male production but higher number of larvae ejected				Mommaerts et al. (2006b)
		individual contact test	28 907 dpm							24h: 34% cuticular penetration			Mommaerts et al. (2006b)
	NI	NI	RR								compatible	s	Biobest
MACs													
diofenolan	NI	NI	RR								compatible	s	Biobest
halofenozide	*B. terrestris*	individual contact test	5466 dpm							24h: 83% cuticular penetration			Mommaerts et al. (2006b)
methoxyfenozide	*B. terrestris*	micro-colony	96 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	no effect	no effect				Mommaerts et al. (2006b)
	NI	NI	RR								remove colonies before product application, retention time of 24h	s	Koppert

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects			Compatibility	Route	Ref.
				non-toxic $	non-toxic $	non-toxic $	no effect	no effect				
tebufenozide	*B. terrestris*	micro-colony	240 (MFRC)									Mommaerts et al. (2006b)
	NI	NI	RR							compatible	s	Biobest
Biological insecticides												
Adoxophes orana Granulose Virus										compatible	s	Biobest
Bacillus thuringiensis var. aizawai	*B.terrestris*	micro-colony	15000 (MFRC)	non-toxic $	highly toxic $	non-toxic $	no effect	total loss	31% reduction			Mommaerts et al. (2010a)
			1500		non-toxic $		no effect			compatible	s	Biobest
Bacillus thuringiensis var. israelensis		micro-colony including foraging			non-toxic $			no effect		compatible	s	Biobest
Bacillus thuringiensis var. kurstaki	*B.terrestris*	micro-colony	160000 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	no effect				Mommaerts et al. (2010a)
		micro-colony including foraging		non-toxic $	non-toxic $	non-toxic $	no effect	no effect		compatible	s; d	Biobest; Mommaerts et al. (2010a)
Bacillus thuringiensis var. tenebrions										compatible	s	Biobest
Beauveria bassiana	*B.terrestris*	micro-colony	2500000000 (MFRC)	highly toxic $	weakly toxic $	non-toxic $	> 75% reduction	no effect	25% reduction			Mommaerts et al. (2009)
		micro-colony including foraging			non-toxic $			53% reduction of offspring; no effect on number of larvae ejected				Mommaerts et al. (2009)
	B.terrestris	individual workers treated with a potter spray tower	dose-range	54%								Hokkanen et al. (2004)
		flowers treated until drip-off	100000000 CFU/ml	30% after 2h 10% after 48h								Hokkanen et al. (2004)

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality		Sublethal effects				Compatibility	Route	Ref.
				Transfer from infected to non infected bees occurs in the hive								Hokkanen et al. (2004)
		field trial with maximum challenge test		15.4%								Hokkanen et al. (2004)
		field trial with treated flowers	100000000 CFU/ml	7%								Hokkanen et al. (2004)
Cydia pomonella granulovirus	*B.terrestris*	micro-colony	6600000000000 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	no effect				Mommaerts et al. (2009)
Metarhizium anisopliae	*B.terrestris*	maximum challenge test		73%								Hokkanen et al. (2004)
		individual bees treated with a potter spray tower	100000000 CFU/ml	63%								Hokkanen et al. (2004)
Paecilomyces fumosoroseus	*NI*	NI	RR							compatible	s	Biobest
spinetoram	*B.terrestris*	contact test	25 (MFRC)	wet: weakly toxic $; dry:moderately toxic $				total loss				Besard et al. (2011)
			dose-range	LC50-72h dry: 20.8 µg/l; LC50-72h wet: 50 µg/l								Besard et al. (2011)
		micro-colony	dose-range		LC50-72h: 20.8 µg/l; LC50-11w: 2.5 µg/l		no effect at 0.25 µg/l					Besard et al. (2011)

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality		Sublethal effects			Compatibility	Route	Ref.
		micro-colony including foraging	dose-range		LC50-72h: 13.8 µg/l; LC50-7w: 1.9 µg/l		no effect at 0.25 µg/l				Besard et al. (2011)
Spinosad	*B. terrestris*	contact test	dose-range	LC50-72h dry: 40 µg/l; LC50-72h wet:14.3 µg/l							Besard et al. (2011)
		micro-colony	dose-range		LC50-72h: 80 µg/l; LC50-11w: 1.6 µg/l		no effect at 0.4 µg/l				Besard et al. (2011)
		micro-colony including foraging	dose-range		LC50-72h: 44.4 µg/l; LC50-7w: 3.8 µg/l		no effect at 0.4 µg/l				Besard et al. (2011)
	B. impatiens	individual bee treated with a potter spray tower	dose-range	LC50-48h: 895 mg/l					remove colonies before product application, retention time of 24h	s	Scott-Dupree et al. (2009)
		colony	dose-range				at realistic concentrations (0.2-0.8 µg/g) no effect on colony health	no effect on pollen consumption			Morandin et al. (2005)
		colony + flight cage	dose-range					slower foraging at 0.8 µg/g			Morandin et al. (2005)
Spodoptera exigua NPV	NI	NI	RR						compatible	s	Biobest
Verticillium lecanii	NI	NI	RR						compatible	s	Biobest
Others											
azadirachtin	NI	NI	RR						compatible	s	Biobest
chlorantraniliprole	*B. impatiens*	individual bees treated with a potter spray tower	dose-range	72h: harmless							Gradish et al. (2010)
		micro-colony	0.000615 mg/g				no effect on oviposition and number of ejected larvae	no effect on worker life-span and pollen consumption			Gradish et al. (2010)

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality		Sublethal effects				Compatibility	Route	Ref.
					LD50-24h: 3,67 µg/bee; LC50-48h-72h: 1.72 µg/bee							
endosulfan												van der Steen et al. (2008)
	NI	NI	RR							not compatible	s	Biobest
formetanate	NI	NI	RR							not compatible	s	Biobest
indoxacarb	NI	NI	RR							remove colonies before product application, retention time of 3d	s	Biobest
lindane	NI	NI	RR							not compatible	s	Biobest
matrine aqueous solution	*B. ignitus*	individual contact test (air dried)	1: 5000 v/v	16days: highly toxic*								Wu et al. (2010)
		individual oral test			LD50-48h: 0.5 mg/bee							Wu et al. (2010)
	B. hypocrita	individual contact test (air dried)	1: 5000 v/v	16days: highly toxic*								Wu et al. (2010)
		individual oral test			LD50-48h: 1.9 mg/bee							Wu et al. (2010)
	B. patagiatus	individual contact test (air dried)	1: 5000 v/v	16days: highly toxic*								Wu et al. (2010)
mineral oil		individual oral test			LC50-48h: 0.5 mg/bee					remove colonies before product application, retention time of 24h	s	Biobest
metaflumizone	*B. impatiens*	individual bees treated with a potter spray tower	dose-range	72h: moderately harmful								Gradish et al. (2010)
		micro-colony	0.003.32 mg/g				no effect on oviposition and number of ejected larvae		no effect on worker life-span and pollen consumption			Gradish et al. (2010)
Na-salts fatty acids	NI	NI	RR							compatibel	s	Biobest
neemoil	NI	NI	RR							compatible	s	Biobest
propargite	NI	NI	RR							compatible	s	Biobest
pymetrozine	NI	NI	RR							compatible	s; i	Biobest

Table 1. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects				Compatibility	Route	Ref.
pyrethrine	NI	NI	RR								remove colonies before product application, retention time of 24h	s	Biobest
rape seed oil	NI	NI	RR								compatible	s	Biobest
rotenone					LD50-24h: 0.38 µg/bee; LD50-72h: 0.36 µg/bee								de Wael et al. (1995)
	NI	NI	RR								remove colonies before product application, retention time of 12h	s	Biobest
thiocyclam	NI	NI	RR								compatible	s	Biobest
triazamate	NI	NI	RR								compatible	s; i	Biobest

Table 1. Overview of the toxicity of insecticides towards *Bombus* species, (NI: no information; RR: recommended rate; $: toxicity according to the IOBC classification for extended laboratory tests; * toxicity according to the IOBC classification for laboratory studies; £: compatibility according to the side-effect list; Route (s=spraying, st=space treatment, i= irrigation, d=dusting, f=fumigation)

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects				Compatibility	Route	Ref.
								Reproduction		Adult			
				contact	oral sugar water	oral pollen	contact	oral sugar water	oral pollen				
Acaricides													
abamectin	*B. terrestris*	micro-colony	18 (MFRC)	highly toxic $	highly toxic $	moderately toxic $	> 75% reduction		total loss				Besard et al. (2011)
			dose range		LC50-11w: 1.17								Besard et al. (2011)
	B. impatiens	individual bees treated with potter spray tower	10-100-1000	moderately toxic at 100 and 1000 mg/l *									Gradish et al. (2010)
		micro-colony	0.0000038 mg/g			no effect			initiation of oviposition was later ($p<0.05$), no effect on number of ejected larvae	consumed less pollen ($p<0.05$)			Gradish et al. (2010)
				LD50-72h: 0.14	LD50-72h: 0.07 µg/bee								de wael et al. (1995); Marletto et al. (2003)
	NI	NI	RR								remove colonies before product application, retention time of 24h	s	Biobest
acequinocyl	*B. terrestris*	micro-colony	150 (MFRC)	non-toxic $	non-toxic $	weakly toxic $	50% reduction	25-50% reduction	50-75% reduction				Besard et al. (2011)
	NI	NI	RR								remove adults before product application, retention time of 24h	s	Koppert
amitraz	*B. terrestris*	micro-colony	400 (MFRC)	non-toxic $	highly toxic $	moderately toxic $	no effect	total loss	50-75% reduction				Besard et al. (2011)
	NI	NI	RR								compatible	s	Biobest
azocyclotin	*B. terrestris*	micro-colony	750 (MFRC)	non-toxic $	moderately toxic $	moderately toxic $	no effect	25-50% reduction	50-75% reduction				Besard et al. (2011)
				non-toxic	non-toxic								van der Steen et al. (2008)
	NI	NI	RR								remove colonies before product application, retention time of 36h	s	Biobest
benzoximate	NI	NI	RR								compatible	s	Biobest

Table 2. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects			Compatibility	Route	Ref.
				non-toxic $	highly toxic $	weakly toxic $	no effect	total loss	50% reduction			
bifenazate	*B. terrestris*	micro-colony	96 (MFRC)	non-toxic $	highly toxic $	weakly toxic $	no effect	total loss	50% reduction			Besard et al. (2011)
			dose range		LC50-11W: 9,6							Besard et al. (2011)
	NI	NI	RR							compatible	s	Biobest
bifenthrin	*B. terrestris*	micro-colony	30 (MFRC)	highly toxic $	moderately toxic $	moderately toxic $	total loss	25-50% reduction	50-75% reduction			Besard et al. (2011)
			dose range		LC50-11w: 0.36					not compatible	s	Biobest
bromopropylate	*B. terrestris*	micro-colony	500 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	25-50% reduction	50-75% reduction			Besard et al. (2011)
	NI	NI	RR							compatible	s	Biobest
chlorfenapyr	*B. terrestris*	micro-colony	240 (MFRC)	non-toxic $	highly toxic $	highly toxic $	no effect	total loss	total loss			Besard et al. (2011)
clofentazine	*B. terrestris*	micro-colony	150 (MFRC)	non-toxic $	moderately toxic $	non-toxic $	50% reduction	25-50% reduction	50-75% reduction			Besard et al. (2011)
	NI	NI	RR							compatible	s	Biobest
cyhexatin	NI	NI	RR							remove colonies before product application, rentention time of 12h	s	Biobest
diafenthiuron	NI	NI	RR							remove colonies before product application, rentention time of 12h	s	Biobest
dicofol	NI	NI	RR							remove colonies before product application	s	Biobest
dienochlor	*B. terrestris*	micro-colony	500 (MFRC)	non-toxic $	highly toxic $	non-toxic $	no effect	25-50% reduction	50-75% reduction			Besard et al. (2011)
etoxazole	*B. terrestris*	micro-colony	55 (MFRC)	non-toxic $	highly toxic $	weakly toxic $	50% reduction	total loss	25-50% reduction			Besard et al. (2011)
	NI	NI	RR		LC50-11w: 4.4							Besard et al. (2011)
fenazaquin	*B. terrestris*	micro-colony	200 (MFRC)	non-toxic $	weakly toxic $	moderately toxic $	no effect	25-50% reduction	50% reduction			Besard et al. (2011)
	NI	NI	RR							remove colonies before product application, retention time of 12h	s	Biobest
fenbutanin oxide	*B. terrestris*	micro-colony	275 (MFRC)	non-toxic $	weakly toxic $	weakly toxic $	no effect	25-50% reduction	25-50% reduction			Besard et al. (2011)
	NI	NI	RR							compatible	s	Biobest
fenpyroximate	*B. terrestris*	micro-colony	50 (MFRC)	non-toxic $	highly toxic $	weakly toxic $	no effect	> 75% reduction	50-75% reduction			Besard et al. (2011)
	NI	NI	RR							remove colonies before product application, retention time of 36h	s	Koppert

Table 2. Continued

Active ingredient	Bumblebee species	Test method	Test concentration (mg/l)	Worker mortality			Sublethal effects				Compatibility	Route	Ref.
fipronil	NI	NI	RR								not compatible	s; i	Biobest
flucycloxuron	*B. terrestris*	micro-colony	125 (MFRC)	non-toxic $	weakly toxic $	weakly toxic $	50% reduction	no effect	> 75% reduction				Besard et al. (2011)
hexythizox	*B. terrestris*	micro-colony	3 (MFRC)	non-toxic $	non-toxic $	moderately toxic $	no effect	25-50% reduction	50-75% reduction				Besard et al. (2011)
	NI	NI	RR								compatible	s	Biobest
milbemectin	B. terrestris	micro-colony	10 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	no effect					Besard et al. (2011)
pyridaben	B. terrestris	micro-colony	75 (MFRC)	weakly toxic $	highly toxic $	moderately toxic $	50-75% reduction	25-50% reduction	no effect				Besard et al. (2011)
	NI	NI	RR								remove colonies before product application, retention time of 48h	s	Biobest
spirodiclofen	*B. terrestris*	micro-colony	96 (MFRC)	non-toxic $	non-toxic $	weakly toxic $	no effect	25-50% reduction	no effect				Besard et al. (2011)
	NI	NI	RR								not compatible	s	Koppert
spiromesifen	*B. terrestris*	micro-colony	0.8 (MFRC)	non-toxic $	non-toxic $	weakly toxic $	no effect	25-50% reduction	no effect				Besard et al. (2011)
	NI	NI	RR								compatible	s	Koppert
tebufenpyrad	*B. terrestris*	micro-colony	100 (MFRC)	non-toxic $	moderately toxic $	moderately toxic $	no effect	25-50% reduction	50-75% reduction				Besard et al. (2011)
			200 (MFRC)	non-toxic $	moderately toxic $	moderately toxic $	no effect	25-50% reduction	50-75% reduction				Besard et al. (2011)
			10 (MFRC)	non-toxic $	moderately toxic $	moderately toxic $	no effect	25-50% reduction	50-75% reduction				Besard et al. (2011)
			RR	10-30%									van der Steen et al. (2008)
	NI	NI	RR								remove colonies before product application, retention time of 12h	s	Biobest
tetradifon	NI	NI	RR								compatible	s	Biobest

Table 2. Overview of the toxicity of acaricides towards *Bombus* species, (NI: no information; RR: recommended rate; $: toxicity according to the IOBC classification for extended laboratory tests; * toxicity according to the IOBC classification for laboratory studies; £: compatibility according to the side-effect list; Route (s=spraying, i=irrigation)

Active ingredient	Bumblebee species	Test method	Tested concentration	Worker mortality			Sublethal effect				Compatibility	Route	Ref.
							reproduction			adult behaviour			
			(mg/l)	contact	oral sugar water	oral pollen	contact	oral sugar water	oral pollen				
Chemical fungicides													
azoxystrobin	NI	NI	RR								compatible	s	Biobest
benomyl	NI	NI	RR								compatible	s	Biobest
bitertanol	NI	NI	RR								compatible	s	Biobest
boscalid + pyraclostrobin	*B. terrestris*	micro-colony	520 + 134 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	no effect	no effect				own unpublished data
bromuconazole	NI	NI	RR								compatible	s	Biobest
bupirimate	NI	NI	RR								compatible	s	Biobest
captan	NI	NI	RR								compatible	s	Biobest
carbendazim	NI	NI	RR								remove colonies before product application, retention time of 24h	s; d	Biobest
carbendazim + diethofencarb	*B. terrestris*	micro-colony	510 + 510 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	no effect	no effect				own unpublished data
chlorothalonil	NI	NI	RR								compatible	s	Biobest
copper abietate	*B. ignitus*	individual contact test (air-dried)	RR (1:5000 v/v)	30 days:non-toxic									Wu et al. (2010)
	B. patagiatus	individual contact test (air-dried)		30 days: non-toxic									Wu et al. (2010)
	B. hypocrita	individual contact test (air-dried)		30 days: non-toxic									Wu et al. (2010)
copper oxychloride	NI	NI	RR								compatible	s	Biobest
cymoxanil	NI	NI	RR								compatible	s	Biobest
cyproconazole	NI	NI	RR								compatible	s	Biobest
cyprodinil (+ fludioxonil)	*B. impatiens*	individual bees treated with potter spray tower	10-100-1000	non-toxic*									Gradish et al. (2010)

Table 3. Continued

Active ingredient	Bumblebee species	Test method	Tested concentration	Worker mortality				Sublethal effect				Compatibility	Route	Ref.
						no effect				no effect on oviposition and ejected larvea	no effect consumption (pollen)			
		micro-colony				non-toxic $	no effect			no effect				Gradish et al. (2010)
	B. terrestris	micro-colony	375 + 250 (MFRC)	non-toxic $	non-toxic $									own unpublished data
	NI	NI	RR									remove colonies before product application, retention time of 12h	s	Biobest
dichlofluanid	NI	NI	RR									compatible	s	Biobest
difenoconazole	*B. ignitus*	individual contact test (air-dried)	RR (1:1 000 v/v)	30 days:non-toxic										Wu et al. (2010)
	B. patagiatus			30 days: non-toxic										Wu et al. (2010)
	B. hypocrita			30 days: non-toxic										Wu et al. (2010)
	NI	NI	RR									compatible	s	Biobest
dimethomorph	NI	NI	RR									remove colonies before product application, retention time of 24h	s	Biobest
dinocap	NI	NI	RR									compatible	s	Biobest
dithianon	NI	NI	RR									compatible	s	Biobest
dodemorph	NI	NI	RR									compatible	s	Biobest
dodine	NI	NI	RR									remove colonies before product application, retention time of 48h	s	Koppert
ethirimol	NI	NI	RR									compatible	s	Biobest
etridiazole	NI	NI	RR									remove colonies before product application, retention time of 96h	s	Koppert
fenarimol	NI	NI	RR									compatible	s	Biobest
fenbuconazole	NI	NI	RR									compatible	s	Biobest

Table 3. Continued

Active ingredient	Bumblebee species	Test method	Tested concentration	Worker mortality			Sublethal effect				Compatibility	Route	Ref.
				non-toxic $	non-toxic $	non-toxic $	no effect	no effect	no effect				
fenhexamid	*B. terrestris*	micro-colony	750 (MFRC)								cover colonies before product application	s	own unpublished data
	NI	NI	RR								cover colonies before product application	s	Biobest
fenpropimorph	NI	NI	RR								compatible	s	Biobest
flusilazole	NI	NI	RR								compatible	s	Biobest
flutriafol	NI	NI	RR								compatible	s	Biobest
folpet	NI	NI	RR								compatible	s	Biobest
fosetyl-aluminium	NI	NI	RR								remove colonies before product application, retention time of 48h	s	Biobest
hexaconazole	NI	NI	RR								compatible	s	Biobest
imazalil	NI	NI	RR								compatible	s	Biobest
iprodione	*B. terrestris*	micro-colony	1500 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	no effect	no effect				own unpublished data
	NI	NI	RR								compatible	s	Biobest
kresoxim-methyl	NI	NI	RR								cover colonies before product application	s	Koppert
mancozeb	NI	NI	RR								compatible	s	Biobest
maneb	NI	NI	RR								compatible	s; i	Biobest
mepanipyrim	*B. terrestris*	micro-colony	300 (MFRC)	non-toxic $	non-toxic $	non-toxic $	no effect	no effect	no effect				own unpublished data
	NI	NI	RR								compatible	s	Biobest
metalaxyl	NI	NI	RR								compatible	s	Biobest
metiram	NI	NI	RR								compatible	s	Biobest
myclobutanil	*B. impatiens*	individual bees treated with potter spray tower	10-100-1000	non-toxic*									Gradish et al. (2010)
		micro-colony	0.011 mg/g			no effect			no effect on oviposition and ejected larvea	no effect on pollen consumption			Gradish et al. (2010)
	NI	NI	RR								compatible	s	Biobest
nuarimol	NI	NI	RR								compatible	s	Biobest
oxycarboxin	NI	NI	RR								compatible	s	Biobest

Table 3. Continued

Active ingredient	Bumblebee species	Test method	Tested concentration	Worker mortality			Sublethal effect				Compatibility	Route	Ref.
penconazole	NI	NI	RR								remove colonies before product application, retention time of 12h	s	Biobest
potasium bicarbonate	*B. impatiens*	micro-colony	0.081 mg/g			no effect	no effect on oviposition and ejected larvea		no effect on pollen consumption				Gradish et al. (2010)
procymidone	NI	NI	RR								compatible	s	Biobest
propamocarb	NI	NI	RR								compatible	s	Biobest
propiconazole	NI	NI	RR								compatible	s	Biobest
propineb	NI	NI	RR								compatible	s	Biobest
pyrazofos	NI	NI	RR								remove colonies before product application, retention time of 24h	s	Biobest
pyrifenox	NI	NI	RR								compatible	s	Biobest
pyrimethanil	NI	NI	RR								cover colonies before product application	s	Koppert
sulphur	NI	NI	RR								compatible	s; d; f	Biobest
tebuconazole	NI	NI	RR								remove colonies before product application, retention time of 24h	s	Biobest
tetraconazole	NI	NI	RR								compatible	s	Biobest
thiophanate-methyl	NI	NI	RR								compatible	s	Biobest
thiram	NI	NI	RR								compatible	s	Biobest
tolyfluanid	NI	NI	RR								compatible	s	Biobest
triadimefon	NI	NI	RR								compatible	s	Biobest
triadimenol	NI	NI	RR								compatible	s	Biobest
trifloxystrobine	NI	NI	RR								compatible	s	Koppert
triflumizole	NI	NI	RR								compatible	s	Biobest
triforine	NI	NI	RR								compatible	s	Biobest
vinclozolin	NI	NI	RR								compatible	s	Biobest
zineb	NI	NI	RR								compatible	s	Biobest
											not compatible	s; d; di	Biobest

Table 3. Continued

Active ingredient	Bumblebee species	Test method	Tested concentration	Worker mortality			Sublethal effect				Compatibility	Route	Ref.
Biological fungicides													
			CFU/l										
Ampelomyces quisqualis M-10	*B. terrestris*	micro-colony	3,5E+08	weakly toxic $	non-toxic $	non-toxic $	no effect	no effect					Mommaerts et al. (2009)
		micro-colony including foraging			non-toxic $			no effect			no effect		Mommaerts et al. (2009)
Hypocrea parapilulifera + Trichoderma atroviride; 1/1	*B. terrestris*	micro-colony	1,3E+05	non-toxic $	non-toxic $	non-toxic $	no effect	no effect	no effect				Mommaerts et al. (2009)
	B. terrestris	micro-colony	1,3E+05	non-toxic $	non-toxic $	non-toxic $	no effect	no effect	no effect				Mommaerts et al. (2009)
	B. terrestris	micro-colony	1,3E+06	non-toxic $	non-toxic $	non-toxic $	no effect	no effect	no effect				Mommaerts et al. (2009)
		micro-colony including foraging			non-toxic $			no effect			no effect		Mommaerts et al. (2009)
Gliocladium catenulatum J1446	*B. terrestris*	micro-colony	7,5E+08	non-toxic $	non-toxic $	non-toxic $	no effect	no effect	no effect				Mommaerts et al. (2009)
		micro-colony including foraging			non-toxic $			no effect			no effect		Mommaerts et al. (2009)
Bacillus subtilis QST713	*B. terrestris*	micro-colony	7,5E+10	highly-toxic $	highly-toxic $	non-toxic $	total loss	total loss	no effect				Mommaerts et al. (2009)
Trichoderma harzianum T22	*B. terrestris*	micro-colony	6,0E+08	non-toxic $	non-toxic $	non-toxic $	no effect	no effect	no effect				Mommaerts et al. (2009)
		micro-colony including foraging			non-toxic $			no effect			no effect		Mommaerts et al. (2009)

Table 3. Overview of the toxicity of fungicides towards *Bombus* species, (NI: no information; RR: recommended rate; $: toxicity according to the IOBC classification for extended laboratory tests; * toxicity according to the IOBC classification for laboratory studies; £: compatibility according to the side-effect list; Route (s=spraying, i= irrigation, d=dusting, di= dipping; f=fumigation)

standardized (selection of a particular instar for exposure) and to test more concentrations in parallel. Similarly, also the field of behavioral changes lacks proper laboratory methods to assess behavioral changes in a lower tier. Here the development of a PER bioassay would allow to assess the impact on the memory and learning capacity of individual insects already in "tier 1". Furthermore, it is likely that also other endpoints will be identified for risk assessments due to the increasing knowledge of the insect body and its processes and because it is to be expected that new active substance will be found with other modes of action.

The obtained data showed that older insecticides (carbamates, pyrethroids and organophosphates) are more toxic than novel insecticides (IGRs, neonicotinoids and biological insecticides). Also low hazards can be expected based on the data for fungicides, whereas for the acaricides the side-effects are strongly dependent on the route of exposure. In addition, it was clear that over the different groups of PPPs bumblebees are in general less sensitive to pesticide toxicity than honeybees. However, the power of the linear regression between the LD_{50}-24h values of 17 insecticides in *B. terrestris* versus honeybees was poor. In conclusion, the identification and especially the knowledge of the consequences of sublethal effects for populations will lead to the development of IPM programs with low risks for pollinators. Reaching all these goals may be of little help if they are not accompanied by a proper communication with cultivators and farmers in the field.

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7. References

Aldershof, S. (1999). Determination of the acute contact LD_{50} of spinosad (formulated as the 480 G/LSC, NAF-85) for the bumblebee *Bombus terrestris* L. Report GHE-P-7875, Dow AgroSciences, Indianapolis, IN.

Babendreier, D.; Reichhart, B.; Romeis, J. & Bigler, F. (2008). Impact of insecticidal proteins expressed in transgenic plants on bumblebee microcolonies. *Entomologia Experimentalis et Applicata*, Vol. 126, No. 2, (February 2008), pp.148-157, ISSN 1570-7458

Besard, L.; Mommaerts, V.; Vandeven, J.; Cuvelier, X.; Sterk, G. & Smagghe, G. (2010). Compatibility of traditional and novel acaricides with bumblebees (*Bombus terrestris*): a first laboratory assessment of toxicity and sublethal effects. *Pest Management Science*, Vol. 66, No. 7, (July 2010), pp. 786-793, ISSN 1526-4998

Besard, L.; Mommaerts, V.; Abdu-Alla, G. & Smagghe, G. (2011). Laboratory assessment of lethal and sublethal side-effects supports a more benign profile of spinetoram compared to spinosad in the bumblebee *Bombus terrestris. Pest Management Science*, Vol.67, No. 5, (May 2011), pp.541-547, ISSN 1526-498x

Biobest N.V. Side-effect list, 01.04.2011, Available from http://www.biobest.be

Colin, ME.; Le Conte, Y. & Vermandere, JP. (2001). Managing nuclei in insect-proof tunnel as an observation tool for foraging bees, In: *Hazards of Pesticides to Bees* Sublethal

effects of deltamethrin and imidacloprid, Belzunces LP, Pélissier C, Lewis GB (eds.), pp 259-268, INRA, Paris.

de Wael, L.; de Greef, M. & van Laere, O. (1995). Toxicity of pyriproxifen and fenoxycarb to bumble bee brood using a new method for testing insect growth regulators. *Journal of Apicultural Research*, Vol. 34, No.1, (January 1995), pp.3-8, ISSN 2078-6913

Decoutye, A. & Pham-Delègue, MH. (2002). The proboscis extension response: assessing the sublethal effects of pesticides on the honey bee. In: *Honey bees: estimating the environmental impact of chemicals*, Devillers, J. & Pham-Delègue, MH. (eds), pp 67-84, Taylor and Francis, ISBN 041-5275-18-0, London.

Decourtye, A.; Lacassie, E. & Pham-Delègue, MH. (2003). Learning performances of honeybees (*Apis mellifera* L.) are differentially affected by imidacloprid according to the season. *Pest Management Science*, Vol. 59, No. 3, (March 2003), pp. 269-278, ISSN 1526-4998

Decourtye, A.; Armengaud, C.; Renou, M.; Devillers, J.; Cluzeau, S.; Gauthier, M. & Pham-Delègue, MH. (2004a). Imidacloprid impairs memory and brain metabolism in the honeybee (*Apis mellifera* L.). *Pesticide Biochemistry and Physiology*, Vol. 78, No. 2, (February 2004), pp. 83-92, ISSN 0048-3575

Decourtye, A.; Devillers, J.; Cluzeau, S. ; Charaton, M. & Pham-Delègue, MH. (2004b). Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. *Ecotoxicology and Environmental Safety*, Vol. 57, No. 3, (March 2004), pp. 410-419, ISSN 0147-6513

Desneux, N.; Decourtye, A. & Delpuech, JM. (2007). The sublethal effects of pesticides on beneficial arthropods. *Annual Review of Entomology*, Vol. 52, (January 2007), pp. 81-106, ISSN 0066-4170

El Hassani, AK.; Dacher, M.; Garry, V.; Lambin, M.; Gauthier, M. & Armengaud, C. (2008). Effects of sublethal doses of acetamiprid and thiamethoxam on the behavior of the honeybee (*Apis mellifera*). *Archives of Environmental Contamination and Toxicology*, Vol. 54, No. 4, (May 2008), pp. 653-661, ISSN 1432-0703

Gallai, N.; Salles, J-M.; Settele, J. & Vaissière, BE. (2009). Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*, Vol. 68, No. 3, (January 2009), pp. 810-821, ISSN 0921-8009

Gels, JA.; Held, DW. & Potter, DA. (2002). Hazards of insecticides to the bumble bees *Bombus impatiens* (Hymenoptera: Apidae) foraging on flowering white clover in turf. *Journal of Economic Entomology*, Vol. 95, No. 4, (August 2002), pp. 722–728, ISSN 0022-0493

Goulson, D.; Lye, GC. & Darvill, B. (2008). Decline and conservation of bumble bees. *Annual Review of Entomology*, Vol. 53, (January 2008), pp. 191-208, ISSN 0066-4170

Goulson, D. (2010). *Bumblebees behaviour, ecology and conservation*. Oxford University Press, ISBN 019-9553-07-6, New York.

Gradish, AE.; Scott-Dupree, DC.; Shipp, L.; Harris, CR. & Ferguson, G. (2010). Effect of reduced risk pesticides for use in greenhouse vegetable on *Bombus impatiens* (Hymenoptera: Apidae). *Pest Management Science*, Vol. 66, No. 2, (February 2010), pp. 142-146, ISSN 1526-4998

Gretenkord, C. & Dresher, W. (1993). Effects of four pesticides (Decis, Metasystox, Pirimor, Rubitox) on the bumblebee *Bombus terrestris* L.: determination of the oral LD_{50} and

preliminary results with field tests. *Apidologie*, Vol. 24, No. 5, (March 2003), pp. 519-520, ISSN 1297-9678

Gretenkord, K. & Drescher, W. (1996). Laboratory and cage test methods for the evaluation of the effects of insect growth regulators (Insegar, Dimilin) on the brood of *Bombus terrestris*. *Proceedings of 6th International Sympymposium Hazards of Pesticides to Bees*, Braunschweig, Germany, September 1996

Halsall, N. & Grey, AP. (1998). NAF-85 (480 g/l SC of spinosad): acute toxicity to honeybees. Report GHE-T-850, Dow AgroSciences, Indianapolis, IN.

Hardstone, MC. & Scott, JG. (2010). Is *Apis* more sensitive to insecticides than other insects? *Pest Management Science*, Vol. 66, No. 11, (November 2010), pp. 1171-1180, ISSN 1526-498X

Heinrich, B. (1979). *Bumblebee Economics*. Harvard University Press, ISBN 067-4016-39-4, London, England

Hokkanen, H.; Zeng, QQ. & Menzler-Hokkanen, I. (2004). Assessing the impact of *Metarhizium* and *Beauveria* on bumblebees In: *Progress in Biological Control: Environmental Impacts of Microbial Insecticides, Needs and Methods for Risk Assessment*, Hokkanen, H. & Hajek, EA. (eds.), pp 63-72, Kluwer Academic Publishers, ISBN 140-2008-13-9, Dordrecht, The Netherlands

Incerti, F.; Bortolotti, L.; Porrini, C.; Sbrenna, AM. & Sbrenna, G. (2003). An extended laboratory test to evaluate the effects of pesticides on bumblebees. Preliminary results. *Bulletin of Insectology*, Vol. 56, No. 1, pp. 156-164, ISSN 1721-8861

Koppert B.V. Side-effect list, 01.04.2011, Available from http://neveneffecten.koppert.nl/

Marletto, F.; Patetta, A. & Manino, A. (2003). Laboratory assessment of pesticide toxicity to bumblebees. *Bulletin of Insectology*, Vol. 56, No. 1, pp. 155-158, ISSN 1721-8861

Mayes, MA.; Thompson, GD., Husband, B. & Miles, MM. (2003). Spinosad toxicity to pollinators and associated risk. *Reviews of Environmental Contamination and Toxicology*, Vol., 179, pp. 37-71, ISSN 0179-5953

Mommaerts, V.; Sterk, G. & Smagghe, G. (2006a). Hazards and uptake of chitin synthesis inhibitors in bumblebees *Bombus terrestris*. *Pest Management Science*, Vol. 62, No. 8, (August 2006), pp. 752-758, ISSN 1526-4998

Mommaerts, V.; Sterk, G. & Smagghe, G. (2006b). Bumblebees can be used in combination with juvenile hormone analogues and ecdysone agonists. *Ecotoxicology*, Vol. 15, No. 6, (August 2006), pp. 513-521, ISSN 0963-9292

Mommaerts, V.; Platteau, G.; Boulet, J.; Sterk, G. & Smagghe, G. (2008). *Trichoderma*-based biological control agents are compatible with the pollinator *Bombus terrestris*: A laboratory study. *Biological Control*, Vol. 46, No. 3, (September 2008), pp. 463-466, ISSN 1049-9644

Mommaerts, V.; Sterk, G.; Hoffmann, L. & Smagghe, G (2009). A laboratory evaluation to determine the compatibility of microbiological control agents with the pollinator *Bombus terrestris*. *Pest Management Science*, Vol. 65, No. 9, (September 2009), pp. 949-955, ISSN 1526-4998

Mommaerts, V.; Jans, K. & Smagghe, G. (2010a). Impact of *Bacillus thuringiensis* strains on survival, reproduction and foraging behaviour in bumblebees (*Bombus terrestris*). *Pest Management Science*, Vol. 66, No. 5, (May 2010), pp. 520-525, ISSN 1526-4998

Mommaerts, V.; Reynders, S.; Boulet, J.; Besard, L.; Sterk, G. & Smagghe, G. (2010b) Risk assessment for side-effects of neonicotinoids against bumblebees with and without

impairing foraging behaviour. *Ecotoxicology*, Vol. 19, No. 1, (January 2010), pp. 207-215, ISSN 0963-9292

Morandin, LA. & Winston, ML. (2003). Effects of novel pesticides on bumblebee (Hymenoptera: Apidae) colony and foraging ability. *Environmental Entomology*, Vol. 32, No. 3, (June 2003), pp. 555-563, ISSN 0046-225X

Morandin, LA.; Winston, ML.; Franklin, MT. & Abott, VA. (2005). Lethal and sub-lethal effects of spinosad on bumble bees (*Bombus impatiens* Cresson). *Pest Management Science*, Vol. 61, No. 7, (July 2005), pp. 619-626, ISSN 1526-4998

Mullin, CA.; Frazier, M.; Frazier, JL.; Ashcraft, S.; Simonds, R.; van Engelsdorp, D. & Pettis, JS. (2010). High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PLoS ONE*, Vol. 5, No. 3, (March 2010), pp. e9754, ISSN 1932-6203

Riveros, AJ. & Gronenberg, W. (2009). Olfactory learning and memory in the bumblebee *Bombus occidentalis*. *Naturwissenschaften*, Vol. 96, No. 7, (July 2009), pp. 851-856, ISSN 0028-1042

Schaefer, H. & Mülhen, W. (1996). Erste Erfarungen zur Prüfung der Auswirkungen des Pflanzenschuzmittels Alsystin an der erdhummel *Bombus terrestris* L. im freiland. *Apidologie*, Vol. 27, No. 4, pp. 273-275, ISSN 1297-9678

Scott-Dupree, CD.; Conroy, L. & Harris, R. (2009). Impact of currently used or potentially useful insecticides for canola agroecosystems on *Bombus impatiens* (Hymenoptera: Apidae), *Megachile rotundata* (Hymenoptera: Megachilidae), and *Osmia lignaria* (Hymenoptera: Megachilidae). *Journal of Economic Entomology*, Vol. 102, No. 1, (February 2009), pp. 177-182, ISSN 0022-0493

Sechser, B. & Reber, B. (1996). Using a sequential testing scheme under laboratory and field conditions with the bumblebee *Bombus terrestris* to evaluate the safety of different groups of insecticides. *Proceedings of Conference Ecotoxicology Pesticides and Beneficial Organisms*, Cardiff, Wales, UK, October 1996

Skerl, MS.; Bolta, SV.; Cesnik, HB. & Gregorc, A. (2009). Residues of pesticides in honeybee (*Apis mellifera carnica*) bee bread and in pollen loads from treated apple orchards. *Bulletin of Environmental Contamination and Toxicology*, Vol. 83, No. 3, (September 2009), pp. 374-377, ISSN 0007-4861

Tasei, JN.; Sabik, H.; Piratsu, L.; Langiu, E.; Blanche, JM.; Fournier, J. & Taglioni, JP. (1993). A sequential study on the effects of deltamethrin (Decis CE) on bumble bees. *Proceedings of the 5th International Symposium Hazard of Pesticides to Bees*, Wageningen, The Netherlands, October 1993.

Tasei, JN.; Sabik, H. & Pirastru, L. (1994). Effects of sublethal doses of deltamethrin (Decis-CE) on *Bombus terrestris*. *Journal of Apicultural Research*, Vol. 33, No. 3, (1994), pp. 129-135, ISSN 0021-8839

Tasei, JN.; Lerin, J. & Ripault, G. (2000). Sub-lethal effects of imidacloprid on bumblebees, *Bombus terrestris* (Hymenoptera: Apidae), during a laboratory feeding test. *Pest Management Science*, Vol. 56, No. 9, (September 2000), pp. 784-788, ISSN 1526-498X

Tasei, JN.; Ripault, G. & Rivault, E. (2001). Hazards of imidacloprid seed coating to *Bombus terrestris* (Hymenoptera: Apidae) when applied to sunflower. *Journal of Economic Entomology*, Vol. 94, No. 3, (June 2001), pp. 623-627, ISSN 0022-0493

Tasei, JN. (2001). Effects of insect growth regulators on honey bees and non-*Apis* bees. A review. *Apidologie*, Vol. 32, No. 6, (November-December 2001), pp. 527-545, ISSN 0044-8435

Thompson, MH. & Hunt, LV. (1999). Extrapolating from honeybees to bumblebees in pesticide risk assessments. *Ecotoxicology*, Vol. 8, No. 3, (June 1999), pp. 147-166, ISSN 0963-9292

Thompson, HM. (2001). Assessing the exposure and toxicity of pesticides to bumblebees (*Bombus* sp.) *Apidologie*, Vol. 32, No. 4, (July-August 2001), pp. 305-321, ISSN 0044-8435

Thompson, HM. (2003). Behavioural effects of pesticides in bees-their potential for use in risk assessment. *Ecotoxicology*, Vol. 12, No. 1-4, (February-August 2003), pp. 317-330, ISSN 0963-9292

Thompson, HM.; Wilkins, S.; Battersby, AH.; Waite; RJ. & Wilkinson, D. (2005). The effects of four insect growth-regulating (IGR) insecticides on honeybee (*Apis mellifera* L.) colony development, queen rearing and drone sperm production. *Ecotoxicology*, Vol. 14, No. 7, (October 2005), pp. 757-769, ISSN 0963-9292

Toxicity data of PPPs for honeybees used in figure 3, 31.03.2011, Available from http://sitem.herts.ac.uk/aeru/footprint/en/index.htm

van der Steen, JJM. (1994). Method development for the determination of the contact LD_{50} of pesticides to bumblebees (*Bombus terrestris* L.). *Apidologie*, Vol. 24, No. 5, (1993), pp. 463-465, ISSN 1297-9678

van der Steen, JM. (2001) Review of the methods to determine hazards and toxicity of pesticides to bumblebees. *Apidologie*, Vol. 32, No. 5, (September-October 2001), pp. 399-406, ISSN 0044-8435

van der Steen, JJM. (2005). Bumble bee brood subgroup report. *9th International Symposium Hazard of Pesticides to Bees*, York, UK, October 2005 (abstract).

van der Steen, JJM.; Bortolotti, L. & Chauzat, MP. (2008). Can pesticide acute toxicity for bumblebees be derived from honeybees LD_{50}-values? *10th International Symposium Hazard of Pesticides to Bees*, Bucharest, Romania October 2008 (Abstract).

Van Engelsdorp, D.; Evans, J.; Saergerman, C.; Mullin, C.; Haubruge, E.; Nguyen, BK.; Frazier, M.; Frazier, J.; Cox-Foster, D.; Chen, YP.; Underwood, R.; Tarpy, DR. & Pettis, JS. (2009). Colony collapse disorder: a descriptive study. *PLoS ONE*, Vol. 4, No. (August 2009), pp. e6481, ISSN 1932-6203

Velthuis, HHW. & van Doorn, A. (2006). A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie*, Vol. 37, No. 4, (July-August 2006), pp. 421-451, ISSN 0044-8435

Wu, J.; Li, j-M.; Peng, W-J. & Hu, F-L. (2010). Sensitivities of three bumblebee species to four pesticides applied commonly in greenhouses in China. *Insect Science*, Vol. 17, No. 1, (February 2010), pp. 67-72, ISSN 1672-9609

Wu, JY.; Anelli, CM. & Sheppard, WS. (2011). Sub-lethal effects of pesticide residues in brood comb on worker honey bee (*Apis mellifera*) development and longevity. *PLoS ONE*, Vol. 6, No. 2, (February 2011), pp. e14720, ISSN 1932-6203

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Chemical Control of Spiders and Scorpions in Urban Areas

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Brazil

1. Introduction

The chemical control of invertebrates that are regarded as pests has always been governed by the development of molecules with insects as their main target. The group of arachnids, which comprise spiders and scorpions among other groups, has received little attention, with the exception of mites which are considered to be agricultural pests. In this chapter, we make a critical review of the chemical strategies used to control synanthropic (ecologically associated with humans) spiders and scorpions that are considered public health problems and also make suggestions regarding future lines of research in this area.

2. Biology of spiders and scorpions – basic aspects

Spiders (Araneae) and scorpions (Scorpiones) constitute, within the Orders of Class Arachnida - together with mites (Acari), - the pests of greatest economic and public health concern. The other orders of arachnids – Schizomida, Thelyphonida, Amblypygi, Palpigradi, Ricinulei, Opiliones, Solifuga and Pseudoscorpiones – representing animals that are mainly found in the natural environment and do not cause any significant economic damage or health problems to humans.

2.1 Biology of spiders

Spiders constitute a diversified group of organisms with currently 42,473 officially recognized species, grouped into 110 families [1]. Spiders are widespread throughout the world and have conquered all of the ecological environments except for the air and oceans. Big tarantulas can have a large body length (*Theraphosa blondi* from Amazonia can have a leg span of up to 28 cm) but most spiders are small (with a body length of 2-10 mm) [2]. Practically all the spiders capture prey for food and the main prey group for most species consists of insects, although a wide range of invertebrates including spiders (Mimetidae spiders specialized in hunting spiders) and even small vertebrates are included [2]. Spiders are beneficial to man in various ways, and generally act as a form of insect control in the natural ecosystem [3]. Their practical use as possible agents of biological control has been confirmed in several cases [4]. Many species of tarantulas are considered pets [5]. Among

the different species of spiders, only a few genera are regarded as a risk to human or animal health.

Spiders (Figure 1) have the body divided into two main regions, the prosoma or cephalothorax and the opisthosoma or abdomen. The prosoma serves as the attachment site for six pairs of extremities: one pair of biting chelicerae, one pair of leg-like pedipalps and four pairs of walking legs. The opisthosoma chiefly carries out vegetative tasks: digestion, circulation, respiration, excretion, reproduction and silk production. In contrast to the prosoma which is hard, the abdomen is rather soft and saclike; the spinnerets are located at its posterior end.

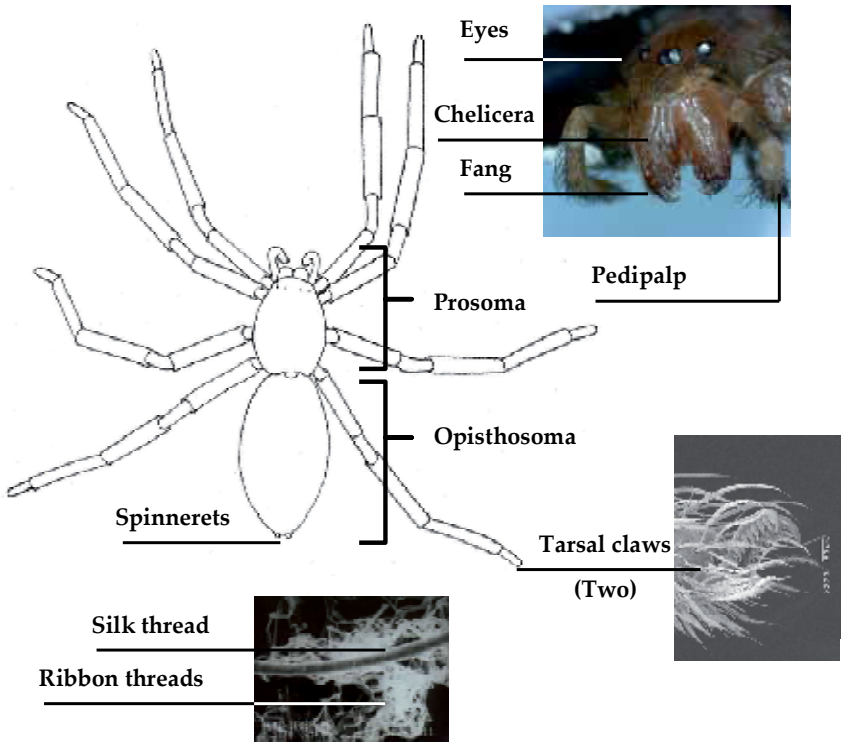

Fig. 1. External appearance of a spider´s body, showing some anatomic details of the brown spider *Loxosceles intermedia.*

Almost all species of spider use venom stored in venom glands which open at the tips of the cheliceral fangs, where it is injected into their prey. Spider venom may contain many different substances. It is mainly a mixture of large neurotoxic polypeptides (molecular weight 5,000–13,000) and smaller biogenic amines and aminoacids; proteolytic enzymes may also be present [2]. Venom composition is highly species-specific and depends on many factors including sex, nutrition, natural habitat, climate, etc. [6]**.** Spiders have been held culpable as a cause of human suffering for centuries, but there are few species in the world that cause medically significant envenomation [7] (Table 2). In several larger species (*Phoneutria* spp., *Atrax* spp.), the venom may have evolved for defense against mammals

(indicated by immediate painful bite due to venom components or to mechanical damage inflicted by large fangs). However, in recluse spiders, an initially painless bite at fang penetration with effects only manifesting hours later must be perceived as a mere evolutionary vagary of venom chemistry that such toxic components have effects on humans [8]. Most other spiders involved in bites that have been verified only have minor, transient effects. Several species of tarantulas have urticating hairs that can cause some health problems [5, 7, 8, 9]. Many spiders which have been blamed for causing medical ailments, have been elevated to medical significance as a result of circumstantial evidence, poor reporting, and repetitive citation in the literature; several species have been shown to be harmless when alleged bites in humans have been subjected to more stringent scientific evidence [7, 8].

Spiders are not the only organisms that spin webs but the importance and various ways that spiders make use of webs has no parallel in any other group of animals [2]. Currently, webs similar to those of spiders can be produced through synthetic means and a wide range of technological applications are envisaged in the future [10]. The webs are only produced in the spinnerets located at the posterior end of the abdomen. Most species of spiders leave a dragline of thread secreted from its spinnerets which is fixed to the substrate at regular intervals and can serve as a safety line or as a means of intraspecific communication [2]. Of the venomous spiders which are found in the urban environment, only *Loxosceles* species do not secrete a dragline. The spiders that spin orbicular webs are not included in the species that are confirmed to be of risk to human health [8]. Spiders can have two or three small claws, called tarsal claws, at the extreme ends of their legs (Figure 1). Some spiders have dense cushions of extremely fine hair lying directly under the claws, called claw tufts [2]. All spiders that have claw tufts on the tips of their legs can walk with ease on smooth vertical walls, and even on window panes [2]. Spiders without claw tufts such as *Loxosceles* and *Latrodectus* depend on substrates as rough surfaces where the claws can be given support. *Phoneutria* spiders manage to climb up smooth substrates, in spite of the considerable size of the adult spiders. Spiders employ a hydraulic mechanism mediated by the prosoma to assist the movement of its legs and compared to insects, have a much lower ratio of muscles to body volume [2]. Owing to this particular feature spiders are very sensitive to water loss, as the loss of body fluid causes serious locomotor difficulties. The spiders that records periods without food or water are included on the *Loxosceles* genus, which can resist more than one year without food or water. At the same time, most spiders given the chance to obtain water, can survive periods of several months without food. The use of pesticides can thus significantly reduce the availability of prey but this alone will not lead to the deaths of spiders. Some studies have shown that spiders can be fed on insects killed by pyrethroids, without apparently being affected [11, 12]. The pedipalps (Figure 1) of adult male spiders are copulatory organs. In general, male spiders are smaller than females and have a shorter life cycle. In black widow spiders, for example, the weight of the small males represents only 1-2% of the female mass. In contrast to females, most male spiders change radically their habits after their final molt. They leave their retreats and webs and start searching for females; often they no longer even catch prey. Due to the risk of intraspecific predation, spiders have a specific courtship behavior that generally precedes mating. The common belief that male spiders are eaten by the females during or after copulation only applies to a few species. Fertilized females lay eggs and build an egg case (also know as egg sac or cocoon) made of silk [2]. Some spiders can build several egg cases before they die. Large spiders may have cocoons with thousands of eggs. Generally there is no maternal care after

the spiderlings leave the egg sac. The young spiders molt several times until they become adults. With most Araneomorphae, no further ecdysis occur after sexual maturity but in the case of Mygalomorphae molts continue to occur in the adult stage [5]. The length of intermolt intervals depends on nutritional conditions and the number of molts on the ultimate body size. Small spiders only need a few molts (about five) whereas large spiders pass through about ten molts before reaching the adult stage. Small males achieve maturity with one or two fewer molts than large females [2].

2.2 Biology of scorpions

Scorpions originated approximately 400 millions years ago and were among the first arthropods to occupy terrestrial environments. Today they can be found worldwide, except in Antarctica, and the presence of some species in urban areas is well-known [13]. Scorpions are commonly thought of as desert creatures, but in fact, they occur in many other habitats, including grasslands and savannahs, deciduous forests, mountain pine forests, intertidal zones, rain forests and caves. A matter of historical curiosity is the fact that nearly 2,000 years ago, live scorpions were used by the Parthians as military weapons in the form of "scorpion bombs". Terracotta pots have been found at the desert fortress of Hatra near modern Mosul, Iraq, where scorpion bombs were used to successfully repulse Roman besiegers in AD198 [14]. Approximately 1,500 species of scorpions have been described in the world, which are currently divided into 18 families [15]. There is considerable disagreement about the best way to classify scorpions [15, 16]. A few species possess potent toxins capable of killing human beings (Table 1). All scorpion species with highly potent, mammal-specific neurotoxins, except for Scorpionidae, belong to the family Buthidae, including the genera *Androctonus*, *Buthacus*, *Buthus*, *Centruroides*, *Leiurus*, *Mesobuthus*, *Parabuthus*, and *Tityus* [13, 17, 18]. However, the size of the scorpion seems to play an important part: for the genera considered as dangerous (especially *Tityus* and *Leiurus*), species exceeding 5 cm must be regarded as potentially dangerous for humans, even if they do not appear in Table 1 [18]. Buthidae is the largest family of scorpions: Fet & Lowe [19] listed 73 genera and 529 species in the "Catalog of Scorpions of the World", and it is likely that many more species are still to be discovered. This group is ecologically diverse and became widespread across the globe [20].

Like all arachnids, scorpions have mouthparts called chelicerae, a pair of pedipalps, and four pairs of legs. The pincer-like pedipalps are used primarily for capturing prey and defense, but are also covered with various types of sensory hairs. The body is divided into two main regions, the cephalothorax and the abdomen (Figure 2). The cephalothorax is covered by a carapace that usually bears a pair of median eyes and 2 to 5 pairs of lateral eyes at its front corners. The abdomen consists of 12 distinct segments, with the last five forming the metasoma which most people refer to as the "tail". At the end of the abdomen is the telson, which is a bulb-shaped structure containing the venom glands and a sharp, curved stinger to deliver venom (Figure 2). Scorpions experience great difficulty in climbing up smooth surfaces and one of the recommendations for their control is to cover the foundations of buildings with surfaces that are metallic or made of other smooth materials which can prevent the creatures from ascending. Inside the dwellings, the furniture, such as the beds, should have smooth bases and be kept away from walls to deny the scorpions access. On its underside, the scorpion bears a pair of unique comb-like sense organs called the pectins (Figure 2); these are usually larger and bear more teeth in the male and are used

to sense the texture and vibration of surfaces. They also serve as chemoreceptors (chemical sensors) to detect pheromones (communication chemicals) [13, 21]. By means of their pectins, scorpions can detect various kinds of pesticide applied to the substrate, as will be seen later in this chapter, and are able to take action to avoid molecules, particularly non-microencapsulated formulas.

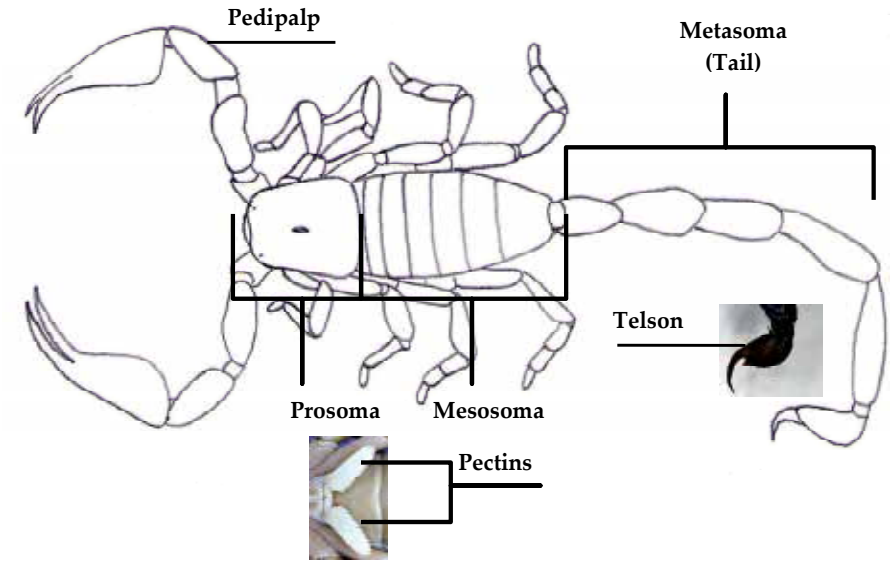

Fig. 2. External appearance of a scorpion's body, showing some anatomic details of *Tityus costatus*.

Scorpions fluoresce or glow strongly under ultra-violet light [13, 21] so they are easy to find with the aid of a black light during the night. Currently UV LED models are available, and the night is the best time for scorpion hunting as they are generally more active in low luminosity. All the species are predatory creatures that feed on a variety of insects, spiders, centipedes and other scorpions, thus, areas with an abundance of prey, such as cockroaches, can expect to have high-density levels of scorpions. The larger scorpions occasionally feed on vertebrates, such as small lizards, snakes, and mice. Most scorpions are ambush predators who detect prey when it comes within reach. As with many predators, scorpions tend to forage in distinct and separate territories, returning to the same area each night. They may enter homes and buildings when their territory has been disrupted by construction, tree removal or floods, etc. Although they are equipped with venom for defense and prey acquisition, scorpions themselves fall prey to many types of creatures, such as centipedes, tarantulas, insectivorous lizards, snakes, birds (owls and chicken), and mammals (including shrews, grasshopper mice, and bats) [13]. Most scorpions are active at night, and spend their days where it is cool and moist under rocks, wood, tree bark or in burrows. Although scorpions have been seen drinking directly from water reservoirs, they derive most of their water from their food, but this varies according to species.

Scorpions have a complex mating ritual in which the male uses his pedipalps to grasp the female's pedipalps. The male then leads her in a "courtship dance". The details of courtship vary from species to species. The sperm from the male is contained within a structure called

Species and Distribution	
Androctonus	
A. aeneas	Africa, North of Sahara, Saharan oases and African Sahel
A. australis	From Algeria to Egypt, Saharan oases
A. crassicauda	From North Africa to Saudi Arabia and Turkey
A. mauretanicus	Morocco
A. hoggarensis	Saharan mountains
Hottentota	
H. franzwerneri	Morocco
H. tamulus	India
Buthus	
B. occitanus	East Mediterranean basin and African Sahel
Leiurus	
L. quinquestriatus	Africa, Middle-East
Parabuthus	
P. granulatus	South Africa
P. transvaalicus	South Africa, Zimbabwe
P. villosus	South Africa, Namibia
P. liosoma	Saudi Arabia
Hemiscorpius	
H. lepturus	Iran, Iraq
Mesobuthus	
M eupeus	Turkey, Caucasus, Iran, Afghanistan
Centruroides	
C. exilicauda	South of the United States
C. infamatus	South of the United States, Mexico
C. elegans	Mexico
C. noxius	Mexico
C. suffuses	Mexico
C. limpidus	Mexico
C. gracilis	Colombia
Tityus	
T. pachyurus	Colombia
T. trinitatis	Trinitad
T. discrepans	Amazonian basin
T. cambridgei	Amazonian basin
T. caripitensis	Venezuela
T. surorientalis	Venezuela
T. arellanoparrai	Venezuela
T. bahiensis	Brazil
T. brazilae	Brazil
T. serrulatus	Brazil
T. stigmurus	Brazil
T. trivittatus	Argentina

Table 1. Distribution of a selection of medically important scorpion species based on Keegan [17] and Chippaux and Goyffon [18].

a spermatophore, which is deposited by the male on a surface over which the female is dragged. The male sweeps his pectins over the ground surface to help locate a suitable place to deposit his spermatophore. The female draws the sperm into her genital pore, which is located near the front ventral side of her abdomen. However, several species, like *Tityus serrulatus*, are represented only by females and reproduce by parthenogenesis. This reproductive strategy undoubtedly greatly increases the opportunities for dispersion and invasion of anthropogenically-impacted habitats. The gestation period is long, from several months to over a year, depending on the species. The young develop as embryos in the female. The young are born live and ascend their mother's back. Generally, a female gives birth to about 15-35 young [13]. They remain on her back until they molt for the first time, or even for some more weeks. Typically five or six molts over two to six years are required for the scorpion to reach maturity. The average scorpion probably lives for three to five years, but some species may live up to 25 years. A few scorpions exhibit social behavior that goes beyond that of the mother-young relationship, such as forming over-wintering aggregations, colonial burrowing, and perhaps even living in extended family groups that share burrows and food [13, 21]. All scorpions possess venom and can sting, but their natural tendency in a confrontation situation is to hide or attempt to escape. As they are able to control the venom flow, some sting incidents are venomless or only mild envenomations. In view of the medical and biological importance of scorpions, it is unfortunate that we are still relatively ignorant of their characteristics. Obviously, more research needs to be conducted on almost all aspects of scorpion biology. For example, we know very little about the natural history or field behavior of most of the deadly species [21].

3. Control of spiders and scorpions - history, context and key initiatives

The organochlorine pesticides were used extensively until the 1970s to combat invertebrate pests. In the 1970s its use was restricted in the United States and later in other countries, as a consequence of its power to bioaccumulate in the environment, causing major environmental impacts. Organochlorines were classified as Persistent Organic Pollutants (POPs), which led to the development and use of other classes of compounds for pest control. So part of the literature until the 1970s lists organochlorine pesticides among the products indicated for chemical control, but other molecules in the following period were tested as possible agents of pest control. The Stockholm Convention on Persistent Organic Pollutants, a global treaty focused on POPs, adopted in 2001 and entered into force in 2004, requires parties to take actions to eliminate or reduce the release of POPs into the environment. The mandate to draw up the Treaty clearly stated that none of its measures should adversely affect public health; therefore, the use of DDT in disease vector control was given special status, as this is considered an acceptable purpose. Nevertheless, the Treaty imposes certain requirements on parties that use DDT [22]. It was observed a general shift from organophosphorus to carbamate insecticides; then a shift to pyrethroids, starting in the 1980s [22].

In this chapter we reviewed information about species of spiders and scorpions that are pests of public health interest, as well as bioassays of pesticides on these target species and field tested control initiatives. We searched for books, and papers in peer reviewed journals through databases (Medline and Scielo mainly) and the bibliographical references contained therein, as well as official documents of the WHO and manuals or bulletins made available online by Universities or Governments. A major obstacle was the almost total lack of

bioassays published in peer reviewed journals for some species. Therefore, several documents relating to congress presentations, masters and doctoral thesis and other materials, were included when considered relevant. We defined the magnitude of the problem, mainly related to public health, and then focused on selected species.

3.1 Spiders - basic epidemiologic data and main control efforts

Spiders arouse feelings of aversion in people and of all the invertebrates constitute one of the main causes of phobic reactions [2]. A spider bite often inspires dread and is associated with a good deal of mythology resulting from the media attention that is given to the rare cases of fatalities [7, 8]. There is usually an extremely low degree of tolerance among people of spiders in dwellings, even of those that do not pose any risk to human health or are not very aggressive. In general, spiders around the world are erroneously blamed for causing dermonecrotic and other lesions when the evidence points in many other directions. [7, 8]. Such misdiagnoses can lead to serious medical complications if the correct diagnosis is delayed or the treatment is inappropriate or ineffective [7, 8]. Since most studies are retrospective and few data on spider bites have been verified, the literature on spider envenomation is often based on inference from animal models in toxicology; circumstantial evidence; poorly designed or otherwise inferior clinical studies; and unfortunately, considerable hyperbole [8]. There are no international registries for spider bites, but the American Association of Poison Control Centers (AAPCC), the Australian Poison Information Center (PIC), and several academic and governmental health centers in Australia, Brazil and Chile for example, report descriptive data. The spiders whose venom is harmful to humans include the *Phoneutria*, *Atrax*, *Latrodectus* and *Loxosceles* genera (Table 2).

Spider taxa	Main active components of venom	Symptoms after bite
"Widow" spiders of the genus *Latrodectus* (Theridiidae)	Alpha-latrotoxin, protein neurotoxin, causes exhaustive release of neuromediators	Pain lasting from hours to days with potentially lethal nonspecific systemic effects
"Brown" or "recluse" spiders of the genus *Loxosceles* (Sicariidae)	Enzyme phospholipase D (sphingomyelinase D), necrotic toxin	Skin injuries of different severity from slight irritation to serious ulcers and possible development of systemic hemolysis leading to death
"Armed" spiders of the genus *Phoneutria* (Ctenidae)	Peptide neurotoxins affecting sodium channels	Pain at site of sting, priapism, possible life-threatening hypotension
Australian "funnel-web"spiders of genera *Atrax* and *Hadronyche* (Hexathelidae)	Delta-atracotoxins, peptide neurotoxins affecting sodium channels	Local and systemic effects of different severity: pain at site of sting, paresthesia, muscular spasm, general excitation, hypertension, disturbance of cardiac rhythm (arrhythmia), coma

Table 2. Spiders with venoms that constitute significant threat for human health. Based on Vassilevski *et al.* [6].

In fact, these four genera comprise the only spiders whose toxicity has been proved. Many other species have been linked to cases of poisoning (e.g. *Tegenaria agrestis*, *Cheiracanthium* spp., *Lampona* spp., *Steatoda* spp., Lycosidae) but in every case with proof that is not convincing and in most cases has been subsequently refuted [8]. For example, in South America, experimentally induced necrosis and poor clinical reporting have led to harmless wolf spiders (family Lycosidae) to be blamed for causing skin lesions [23]. Wolf spider antivenin was developed and used for decades. Subsequent tracking of 515 documented wolf-spider bites in humans with no necrosis showed the attribution to be erroneous and the treatment unwarranted [23]. In another example, largely on the basis of scraps of unsubstantiated evidence, white-tailed spiders (*Lampona* spp.) were erroneously assumed to be the etiologic agents of human dermonecrosis in Australia. This led to hyperbole in the popular and medical press, creating an urban myth [24]. Several American states report dozens to hundreds of loxoscelism diagnoses annually, even though brown recluse spiders are extremely rare or have never been found in those states or regions [8]. It is important to determine what constitutes reliable evidence in clinical toxinology; a definite spider bite case requires all the following criteria to be met: (*a*) clinical effects at the time of the bite; (*b*) the spider being caught immediately at the time of the bite; and (*c*) expert identification of the spider [7, 8, 25].

With regard to the chemical control of spiders, on the basis of the review of the literature, it is evident that a large number of the suggestions about the molecules that should be used, as well as the forms of application, are based on subjective experience in the field, on the recommendations of the manufacturers or on methods designed to insect control. Much less frequent are bioassays carefully carried out with spiders, or field trials of these products and their methods of application [11]. Spiders are not only considered targets of pest control programs. They can be used for the control of insects in integrated agricultural pest management programs [3, 26, 27]. Most of the studies on the susceptibility of spiders to pesticides are based on an evaluation of agricultural products used in the field. Laboratory studies have demonstrated that broad-spectrum insecticides such as organophosphates, carbamates and organochlorines have significant lethal effects on spiders in general [28]. In Israel, laboratory residue studies, showed that the organophosphate chlorpyrifos was highly toxic to *Cheiracanthium mildei*, a hunting spider known to occur in large numbers in citrus orchards [29], whereas natural products (i.e. *Bacillus thuringiensis* and neem extracts) were virtually non-toxic to spiders [30]. Fungicides have been shown to have little or no toxicity against spiders [31]. The effects of exposure to a single sublethal dose of the pesticide malathion on the mating behavior of the lycosid *Rabidosa rabida* were such that it was severely disrupted and resulted in the most heavily dosed males being killed by the females without achieving copulation. The spiders were tested in combinations where one or both sexes were exposed to the insecticide. The data indicate that while there was no effect on the patterning of courtship behavior, the control males initiated courtship more rapidly than those that had been exposed [32].There was also an alteration in the walking patterns, generally leading to an increase in locomotion [33]. The insect growth regulators tebufenozide and fenoxycarb are safe for spiders and predatory mites [34]. Field experiments have revealed that some species of spiders are more sensitive to insecticides than others [35]. Among many factors influencing their susceptibility, foraging mode seems to play an important role. Aspects of foraging mode that appear to be relevant are whether the spider is diurnal or nocturnal, a hunter or a web-maker [35]. Another source of data on the effects of pesticides on spiders has emerged from the mosquito management campaigns

both in the United States and globally, for the effective control of the mosquito vectors of the West Nile virus and many other diseases. Davis and Peterson [36] carried out studies with adulticides (permethrin and d-phenothrin) and larvicides (*Bacillus thuringiensis israelensis* and methoprene) on non-target aquatic and terrestrial arthropods (including spiders) after single or multiple/repeated applications. Nearly all the responses evaluated registered few, if any, deleterious effects from the application of the insecticide.

In this Chapter, different categories will be presented and adopted concerning the situations with varying degrees of relevance and urgency in the need to control spider populations. Some genera, such as *Latrodectus* and *Phoneutria*, can be included in more than one category, depending on the species involved and the situation under consideration:

1. The presence of species of spiders representing a potential risk to human health and able to effectively establish populations inside dwellings or in the peridomicile for several generations. Also being capable of spreading out to neighboring areas, with greater or less speed, depending on their method of dispersion and reproductive capacity. They thus have a real potential to become a public health hazard. Currently, several species of the *Loxosceles* genus are those that best fit this citerion. They may prefer the intradomicile or peridomicile areas, depending on the species involved, and are regarded as significant public health problems, particularly in Chile and Brazil. Some species of the *Latrodectus* genus are mainly concentrated in the peridomicile area while others conform to the pattern of the item **5** below.
2. The presence of species which pose a risk to human health but become much more frequent at certain specific times of the year. Usually in the reproductive season or the warmer periods, when there is an increased mobility and they can occasionally invade dwellings, though without normally establishing permanent intradomiciliar populations. One example being the armed spiders *Phoneutria nigriventer*, which generally involve attacks caused by males wandering about in search of females during the reproductive season [37].
3. The presence of species that do not represent a significant risk to human health and which can occasionally be found in dwellings and even establish stable populations. The spiders that are sometimes found in the dwellings form a part of the fauna of invertebrates in the peridomicile or surroundings of the dwelling, usually in areas of natural or disturbed vegetation nearby. Mention should be made of the cursorial spiders of the families Lycosidae (wolf spiders), Salticidae (jumping spiders) and also the males of the Mygalomorphs (tarantulas). These spiders, especially those of greatest size, can cause considerable discomfort to the residents and even lead to cases of bites, though without serious implications for human health. Other spiders occupy the intradomiciliar region on a permanent basis, like the cosmopolitan *Achaearanea tepidariorum*, which spins cobwebs in corners of rooms, windows and similar places. The southern house spider *Kukulcania hibernalis* is found in many places in the world and can spin webs in various items of constructions. The pholcids or daddy longlegs spiders, like *Pholcus phalangioides*, are also common in various parts of the world, as well as *Metaltella simoni* (Amaurobiidae) Residents of dwellings can be made uncomfortable by the presence of these spiders, particularly on account of their visible webs and they can become the target of control measures.
4. In some specific industrial or business areas, a situation like that of items **1**, **2** or **3** could prevail and there is a low tolerance level to the presence of spiders on the part of clients, employees or even by the quality control standards. This is the case with shopping

centers, and factories which have strict standards of hygiene, like the food industry and others. There is a need to carry out effective control measures more often, regardless of the risk of injuries from bites - this is usually only done by pest control companies. A lot of spiders that spin webs, like *K. hibernalis* and *A. tepidariorum* can be included as targets in this category, together with many araneids with orbicular webs and also other pholcids and theridiids. These webs can accumulate detritus and periodically it maybe necessary to carry out a mechanical removal. A novel case that involved industrial products was found in the car industry. In 2011, Mazda will be recalling 65,000 vehicles in the United States, Canada and Mexico so that they can be fitted with special screens to keep out spiders. There is concern that the webs could block the air flow in the fuel tank vent and this could cause the tank to leak or even catch fire. The spider involved was identified as the yellow sac spider *Cheiracanthium inclusum* [38].

5. Some venomous spiders that are found in cultivars in nature, can travel together with the products that have been gathered or harvested and then be introduced into private dwellings and commercial premises in the country of origin or even in other countries. Ctenids such as *P. nigriventer* occur in banana plantations in Brazil. The widow *Latrodectus hesperus* have become a pest due to their unwanted presence in grape clusters in the table grape vineyards of San Joaquim Valley, California, USA [39]. In 2001, New Zealand banned the imports of California table grapes after four *L. hesperus* individuals were found in table grape clusters [40].
6. Recent papers have drawn attention to the potential occupational and contact allergies caused by spiders such as *Tegenaria domestica* [41, 42], and even to a new asthma-causing allergen from the cellar spider *Holocnemus pluchei* [43].

In this Chapter we will focus on the *Loxosceles* and *Latrodectus* genera (which fit into item **1** in our classification), because of their medical importance in several parts of the world, the synanthropy of various species of the genera and from the literature available on their chemical and other control measures. *Phoneutria* will not be included, as it is in the items **2** and **5** of our classification. The *Atrax* genus will not be included because they are not common in urban areas and severe envenoming is rare with this species with only 5-10 cases recorded annually, largely confined to a restricted region in Eastern Australia [8].

Latrodectus – The species of the genus *Latrodectus* (Theridiidae) are commonly known as "widow" spiders. The name of the black widow spider, *Latrodectus mactans*, is a combination of Latin and Greek, meaning "deadly biting robber". European authors have documented widow spider bites for over 2000 years [44]. From the 13th to 17th centuries in Europe, the tarantella was danced to ward off the effects of spider envenomation. The spider involved was almost certainly the endemic widow spider, *L. tredecimguttatus* [44]. In the late 1800s, American and Australian entomologists linked these spiders to severe illness and death [8]. There are currently 30 recognized *Latrodectus* species [1], commonly known as black widow spiders (e.g., the North American *L. mactans* and *L. tredecimguttatus* in Europe), which are often recognized by their red abdominal ''hour-glass'' mark, as well as the Australian red-back spider (*L. hasselti*) and the cosmopolitan brown widow (*L. geometricus*). They are widespread throughout Africa, the Americas, Europe, Southeast Asia, and Australasia [8]. Phylogenic work has confirmed that there are two main groupings (*mactans* clade and *geometricus* clade) [45]. Members of the genus are notorious for the highly potent neurotoxin alpha-latrotoxin contained in their venom, which triggers a massive neurotransmitter release upon injection in vertebrates [46]. People may suffer eye injuries from contact with the bodily fluids of a widow spider, when the spider is crushed with a tool such as a

hammer [47]. Another possible source of envenomation is a toxin in *Latrodectus* eggs that has a different mammalian toxicity expression from that of venom; its effects on humans are unknown [48]. The insecticidal components of *Latrodectus* venom (latroinsectotoxins) have been investigated by the chemical industry for their potential use in pesticides [49]. Several *Latrodectus* species are synanthropic and often found in urban areas around houses, garden sheds, and barns [50], as well as in agricultural areas [38, 50]. They are generalist web predators which are known to feed on insects, crustaceans, other arachnids, and small vertebrates including lizards, geckos, and mice [51], and this broad diet may in part explain the presence of a vertebrate specific toxin in their venom. The threads of the black widow spider web are so strong that they were used as cross hairs on the gunsights of World War II American naval ships [52]. The treatment of widow spider bites varies in different regions of the world and is based on the availability of antivenom, its perceived effectiveness, and the degree of support given to other forms of treatment [8]. The limited evidence currently available of the efficacy and safety of alternative treatment, lends support to the use of antivenom in cases of latrodectism which cause severe or persistent pain or have systemic effects [8]. *Steatoda* spiders (Theridiidae) often bear a resemblance in body form and dark coloration to widow spiders and cause mild latrodectism effects. In one case, black widow antivenom was given for a *Steatoda* bite and appeared to relieve bite symptoms [53].

Human transport has undoubtedly widened the range of some species in the genus [45]. However, there continue to be poor estimates of the number of bites caused by *Latrodectus* worldwide [7,8]. In peridomestic situations, widow spiders are found in dark or dry places; bites are common when people put on shoes, bike helmets, or gardening gloves, and when spiders are clutched by fingers under outdoor furniture, in potted plants, or in dry storage areas [7,8]. In Mediterranean agricultural conditions, *L. tredecimguttatus* is found in wheat, with bites being an occupational hazard of farmworkers [44]. In natural environments, *Latrodectus* spiders live under stones, in small mammal burrows, under logs, in shrubs, and even in trees, depending on the species [54]. Some species are not found in synanthropic association [44]. In the past, many bites, predominantly on the buttocks and genitalia, occurred in outdoor toilets [55]. The decline in the use of outdoor privies and an increased public knowledge of the black widow has resulted in a decline in the number of bites by this species in the United States. Most bites are caused by female spiders. Bites generally occur during warmer months. In the case of *L. hasselti* [56], there was no significant difference between the effects of bites by juvenile and adult females. The adult male only caused mild, short-lived, pain. The subadult male caused severe pain for 12 hours, but with no systemic effects [56]. In a 10-year (1980−1990) retrospective analysis of latrodectism in Brazil, Lira da Silva et al. [57] reported that most widow bites occurred in cities, affected predominantly men, and were most commonly inflicted by *L. curacaviensis*. Certain members of the genus are increasingly being detected in new and widely separated localities. For example, it appears certain that several species have recently been introduced to Hawaii [58], Japan [59], Australia [60] and New Zealand, where they have been intercepted in consignments of imported goods arriving from different countries at post-border quarantine facilities [39]. While it is clear that *L. geometricus* has been recently introduced to many localities around the world [45], it is uncertain what proportion of its remaining distribution (including Africa, parts of North America, and South America) constitutes its native range, as it was first documented in both South America and Africa at the time of its scientific description in 1841 [45]. Many spiders (including theridiids) can disperse over long distances by ballooning, when they are juveniles, travelling long distances [2]. Thus it is plausible to hypothesize that in the course of their

evolution *Latrodectus* spiders have occasionally colonized distant landmasses within which subsequent lineage diversification has occurred [45].

Some interesting records of biological control of *Latrodectus* are found in old publications. *Latrodectus* spiders invaded Hawaii around 1935 and became a problem in the warmer drier sections of the islands [61]. An egg parasitoid, *Baeus latrodecti*, was discovered parasitizing black widows in a small area characterized by sand dunes in Southern California [62]. Parasitized eggs were shipped to Hawaii, where a rearing program was initiated and over 32,000 *B. latrodecti* were liberated during 1939-40 [61, 62]. Bianchi [63] stated that a search on the island of Hawaii failed to reveal the parasitoid, but it was later found to be established on Maui. *Eurytoma latrodecti* was introduced from Hawaii to Kwajalein in 1950 to control the black widow spider, and there were reports that it had become established [63]. Some Spechidae wasps such as *Chalybion californicum* are regarded as effective predators of *Latrodectus* spiders, especially immature spiders [64].

In the older literature of the United States [52, 54] the periodical eradication of the spiders and their egg masses by mechanical means was considered the most satisfactory method of control. "At night the spiders can be easily detected with the aid of a headlamp or flashlight, and destroyed by hand. Everyone should know this brilliantly marked spider by sight, and avoid contact with it" [52] (*L. mactans*). A reasonable degree of orderliness in the storage of equipment and disposal of rubbish would reduce the available sites for nests and webs. Outdoor privies were common sites for attacks by *Latrodectus*; most victims were men and the injury was centered on the external genitalia [55]. A periodic examination of the outdoor privies was recommended and it was suggested to paint the undersides of the seats with creosote, crude oil, or some other repellant. [52]. Recently Hernandez [39] evaluated several pesticides against *L. Hesperus* in table grape vineyards. Of the tested pesticides, methomyl, fenpropathrin, and chlorpyrifos provided 100% control of adult male and female black widows by direct exposure. Only chlorpyrifos and fenpropathrin provided control of adult females, within 10 days post-treatment, when the spiders were exposed to treated vine bark. Methomyl was the most toxic to adult females while fenpropathrin was the most toxic chemical to the immature stages. In 2008 [65], another work examined ultralow oxygen treatment (ULO) for control of *L. hesperus* on harvested table grapes and achieved complete control of the spiders with no negative effects on grape quality. Owing to the relatively short treatment time required, its effectiveness at low storage temperature and the easily attained oxygen level, the ULO treatment has the potential to be implemented commercially for the control of black widow spiders on harvested table grapes [65]. The Extension Manuals of the University of California (UCA) [66] and others recommend taking preventive measures of physical control to reduce the occurrence of black widow spiders; these include cleaning areas of rubble, scrap and lumber materials or old machinery that has been disused for a long period of time, destroying webs, egg sacs and spiders by brushing or vacuuming and caulking or sealing openings to prevent entry by both insects and spiders. The ability of spiderlings of *L. hesperus* to pass through different sized mesh screen and the implications for exclusion from air intake ducts and greenhouses has recently been evaluated [67]. The purpose was to determine whether immature individuals of these spiders could be excluded from buildings, and it was found that the mesh size sufficient for exclusion is too small for practical use in most cases, although there are special situations where this small mesh might be useful [67]. According to the UCA extension manual [66] the chemical control should use sorptive dusts containing amorphous silica gel (silica aerogel). Pyrethrins may be useful in certain indoor situations. According to the UAK extension manual [68] when

applied as a dustlike film and left in place, a sorptive dust provides permanent protection against spiders. The dust is most advantageously used in cracks and crevices and in attics, wall voids, and other enclosed or unused places. As was the case with other spiders and scorpions, most of the suggestions for control of widows are based on the recommendations on the labels of the products, with few bioassays being referred to in the literature. In 2005 [69] a bioassay using few spiders tested three management strategies against *L. Hesperus* with chemicals being directly applied to the substrate, spider webbing and spider; three classes of chemicals were tested phenyl pyrazole (fipronil); pyrethroid (cyfluthrin); and neonicotinoid (imidacloprid). Fipronil controlled 96% of the spiders after 8 days, cyfluthrin controlled 70% after 5 days and imidacloprid controlled 18% of the spiders after 14 days. A higher level of control was obtained with the application of fipronil and cyfluthrin to female *L. hesperus* and male *Tegenaria agrestis* than to female *T. agrestis* The application methods showed similar results.

If necessary to control synanthopic *Latrodectus* species, it is important to keep in mind that the widow spiders are less abundant in the indoor than the outdoor environment, they are not cursorial spiders and that they have distinctly visible webs. The removal of webs egg sacs and spiders by vacuuming at indoor environments can thus be the first line of defence. Chemical control should be used mostly indoors. In the outdoor areas, the residual effect will always be lower than that of the indoor environments.

Loxosceles - The Sicariidae family is composed of the *Sicarius* and *Loxosceles* genera [1]. The name *Loxosceles* means slanted legs due to the way the spider holds its legs at rest [70]. These spiders are colloquially known as recluse spiders, violin spiders, and fiddleback spiders in North America. In South America it is known in Brazil as "aranha-marrom", in Chile as "araña de los rincones". There are about 100 species concentrated in tropical and subtropical portions of the world, with a few species reaching temperate areas; the highest concentration of species is in the Western Hemisphere [1]. Many species have remote distributions, being known from less than 10 specimens in museum collections [71], so their medical importance is very low because they rarely interact with humans. In Brazil species of *Loxosceles* are frequent in karstic caves [72]. Brown spiders *L. adelaida* are found near and inside the caves in a State Park, in São Paulo, Brazil, which are visited by thousands of tourists every year [73]. The venom of this species is potentially able to cause envenomation with the same gravity of those produced by synanthropic species [74], but no bite was reported. The *Loxosceles* spiders are among the most enduring of the suborder Araneomorphae [75] and also have the lowest rate of water loss recorded on spiders [75]. One feature that facilitates the identification of spiders involved in possible loxoscelic accidents is that all *Loxosceles* species have six eyes, arranged in three dyads forming a curved line (Figure 1) [71]. They build webs covering the substrate in an irregular manner, described as having the appearance of frayed cotton [76, 77, 78]. It is common to find prey of many different taxa in their webs [76]. *Loxosceles* produces two types of silk threads: a cylindrical, typical of most spiders, and another in a "ribbon" shape, apparently unique to this genus [76, 77] (Figure 1). As it was noticed that the dry ribbons were highly electrostatic, it is suggested that the electrostatic interaction plays an important role in prey capture for *Loxosceles* [78]. Preferred habitats includes rock piles, wood piles, rat holes, attic and basement crawl spaces, indoor trash and clothing piles, cardboard boxes, and storage sheds. *Sicarius* and *Loxosceles* are the only known spiders with venom able to cause necrotic arachnidism in humans [79]. Some biotechnological applications of *Loxosceles* genus venom

toxins are being devised [80]. Although several toxins have been isolated from *Loxosceles* venom and various enzymatic activities have been identified, the medically important component appears to be sphingomyelinase D [79]. This dermonecrotic venom component has been identified in all species thus far tested, and it would not be surprising if all *Loxosceles* spiders are capable of causing necrotic skin lesions [74, 79]. Among the spiders that can cause serious health risks to humans, the brown spiders -particularly *L. reclusa*, *L. laeta* and *L.intermedia* - were the targets of the major control initiatives in the world.

The loxoscelism was first described in 1937 by the Chilean physician Atilio Macchiavello Varas [81, 82], who demonstrated that *L. laeta* bite caused the "arañismo cutáneo-gangrenoso y hemolitico do Chile". In 1957 it was described in North America [8]. Four categories of loxoscelism exist: (*a*) no effects, (*b*) minor injury with edema and erythema, healing without supportive care, (*c*) dermonecrotic injury with development of a hardened ulcer that sloughs off, leaving a scar, and (*d*) systemic effects with hemolysis sometimes leading to disseminated intravascular coagulation and, in rare cases, renal failure and death, reported to occur mostly in children [8]. Systemic loxoscelism is rare in the United States, with a 3% incidence rate and no deaths in a population of 111 patients with expert confirmed brown recluse *L. reclusa* bites in a 1997 survey [83]. In the State of Paraná, Brazil, where *L. intermedia* is the most abundant species, systemic loxoscelism is also rare [84]. Systemic loxoscelism seems to be much more common following *L. laeta* bites, with a case fatality rate of 3.7% in 216 bites [85]. Most bites occur when spiders are trapped against human skin and an object, such as a person rolling over in bed or getting dressed, in which spiders have crawled into clothing or shoes. *Loxosceles* females are more venomous and larger than males, which rarely inflict severe envenoming bites [86]. Like *Latrodectus* spiders, *Loxosceles* spiders are generally nonaggressive, reclusive, prefer to retreat when threatened, and bite only if handled or trapped in garments or bed linens. It continues to be difficult to define the true epidemiology of loxoscelism, because strict criteria for inclusion of definite bites are rarely used, and because many cases of local tissue injury are incorrectly attributed to loxoscelism [87]. Although *Loxosceles* spiders were not considered medically important until the midtwentieth century, in the following decades, their reputation spread so profusely throughout North America that brown recluse spider bites were diagnosed by medical professionals in regions of the continent proportionally greater than the number of *Loxosceles* spiders known from the area and, in some cases, where none have ever been found [87]. Medical toxicologists and arachnologists have assembled a still-growing list of many medical maladies mistaken for *Loxosceles* envenomation [8]. For most *Loxosceles* bites, RICE (rest, ice, compression, elevation) therapy is considered proper remedy because most events are minor; therefore, conservative treatment is advocated in most cases [8, 87]. For more dramatic ulcers or skin damage, regular and intense wound care is required; other more specific treatments are still controversial and unproven [8, 87]. In Brazil [88] and Chile [85], *Loxosceles*-specific antivenoms are produced and distributed by the government to be used on severe envenomations. New antiloxoscelic serum approaches are under investigation [89].

The brown recluse spider, *L. reclusa*, is the most common recluse spider in North America The species is synanthropic over much of its range in the United States, being primarily found in the South-Central states [90] and, as such, is commonly misconstrued as being ubiquitous throughout the country, particularly by medical professionals [8, 87]. Infestations of thousands of *L. reclusa* spiders in homes in Kansas were reported with no incidents of

bites to human occupants [91]. These reports suggest that even in cases of high densities of *L. reclusa* spiders, bites are unlikely, and therefore in nonendemic areas, envenomations by this species should be considered highly improbable [8, 87]. *L. reclusa* prefers dry, dark areas, and outside of human habitation, is often found under stones and within the bark of dead trees. The Mediterranean recluse, *L. rufescens*, has dispersed to many other countries, originating from the circum-Mediterranean region [71]. It has been collected in many localities in the United States. In nonendemic *Loxosceles* areas in North America, it is more likely to find a spot infestation of the non-native *L. rufescens* than the native *L. reclusa* [87]. The Mediterranean recluse has also become established in Australia [92]. Gertsch [71] states that there are no valid specimens of *L. rufescens* from South America. In Europe *L. rufescens* is the most abundant species [71]. In Portugal, any loxoscelic accident was proven until recently [93]; some reported cases could be due to mistaken diagnosis of serious infections by strains of *Streptococcus* or *Staphylococcus aureus* [93]. In France [94] and Greece [95] loxoscelism is also is very rare, with few probable cases. In Israel, a retrospective single-centre study included 52 patients with necrotic arachnidism hospitalized in the dermatology department between 1997 and 2004 [96]. Although *Loxosceles* was introduced to a small part of Australia [92], a prospective analysis of 750 definite spider bites in Australia over a 27-month period reported no evidence of necrotic araneism in the Australian experience [25, 97]. From South Africa, *L. parrami* was reported as medically important [98]. Accidents by *Loxosceles* represent around 4% of the total number produced by venomous animals in Argentina [99]. Despite the overreporting of accidents related to *Loxosceles* in certain localities such as the United States [87], hundreds or thousands of bites per year really occur every year in other regions of the World. This is the case for Chile and Southern Brazil. Chile, the country where loxoscelism was first confirmed, reported many bites by *L. laeta* [99, 100]. In a retrospective analysis of 1,348 suspected spider bites in Chile over a 40-year period, 16.6% of the dermonecrotic lesions were caused by *Loxosceles* spiders [100]. In Brazil, most accidents are recorded in the Paraná State, where loxoscelism is considered a serious public health problem. The species responsible for the majority of the reported accidents is *L. intermedia* [84, 102]. This species prefers indoor environments [103] and wanders extensively, increasing its chances of human contact, with accidents being more frequent in the hottest months of the year [84]. In Santa Catarina State, adjacent to Paraná, many bites have also being reported [104, 105]. A clinical and epidemiological study of 267 cases of envenomation by *L. laeta* and *L. intermedia*, reported that 4 patients (1.5%) died, all of whom were children under 14 years old [104].

Loxosceles spiders are considered a challenging target for control in urban areas, due to specific features of their biology [11, 87]. Their morphology is homogeneous, sometimes making the separation of species difficult [71]. Although few groups of this genus have been studied until now, the behavior seems to be more variable between species than previously supposed [106]. Species seem to differ especially in their foraging strategies; with either more investment in the construction of webs and consequently a sit-and-wait behavior, or lower investment in the construction of webs and a tendency to active hunting [106]. This difference between species in foraging behavior is directly linked to the likelihood of loxoscelic accidents [106]. However several features that are common to the species of *Loxosceles* are very important for their successful adaptation to domestic and peridomestic environments [87]. One factor is their exceptional longevity, considering body size. The average life spans for *L .reclusa* [107] males is 897 days and for females 794 days, with 25% of the females living over 1,000 days, one surviving 4.8 years. To *L. laeta* it was reported [108,

109] for females the average of 2.1 years to mature and another 4.8 years as adults. For *L. intermedia* [110] longevities of 1,176±478 days for females and 557±87 days for males were reported. Probably linked to the long life span, the female *Loxosceles* spiders are able to produce several egg sacs with a variable period of time between subsequent ones, instead of concentrating all the reproductive effort on a single egg sac, as other spiders with shorter life spans [2]. So, when egg cases and webs are removed but the females are not removed, new egg sacs can be readily produced, mostly if the female mated several times, as is common for this genus [87, 111]. *L. reclusa* average 50 eggs per egg sac and 2.7 egg sacs per female with a 48% hatch rate [112]. For laboratory-reared *L. intermedia* restricted to one mating, egg sacs contained approximately 30 eggs where 70% hatched, however, the egg sacs of field collected females of unknown mating history averaged around 50 eggs with 80% hatch [111]. Similar fecundity numbers are presented for other species: *L. laeta* – mean of 88.4 eggs per sac [113], *L. gaucho* – mean of 61.3 eggs per sac [114]. Laboratory reared spiders, if not exposed to high temperatures, can obtain all the water they need from prey, as we could observe during the several years we kept *L. intermedia, L. laeta* and *L. gaucho*. We were able to keep females of these species for one year in plastic containers, without food and water, and these spiders after fed with insects, lived up to two more years (unpublished data). Eskafi et al. [75] purposely starved field-collected *L. reclusa* at different temperatures and relative humidities. Spiders at 5ºC survived 4 to 7 months whereas this dropped to 1 to 2 months at 30°C and less than 2 weeks at 40°C. Starved mature *L. laeta* [108] took an average of 1.2 year to succumb. The dispersal capability of *Loxosceles* species is low, because they do not use ballooning in earlier stages as *Latrodectus* species [45]. So their dispersal to long distances depends on human activity. *Loxosceles* spiders can be found in very high density in synanthropic situations. A Kansas family collected 2,055 *L. reclusa* spiders in their home in 6 months [91] and a survey in Kansas showed that 22 of 25 homes had *L. reclusa* with an average of 83.5±114.9 spiders per home (range 1 to 526) [115]. In a Chilean survey, more urban (40.6%) than rural (24.4%) houses were infested, but a higher density of spiders was found in rural (11.9) than in urban (3.9) houses [116]. In Curitiba, Paraná State, Brazil, *L. intermedia* and/or *L. laeta* were found in 97% of the dwellings visited [117]. Brown spiders are not social spiders in the sense of sharing webs, prey capture and defense such as and other social or cooperative spiders [2] but there is intraspecific and interespecific species recognition [118] that either reduces aggressive interactions and/or allows escape to a safe distance to avoid predation such as exists for *L. gaucho* in female-female [119] and male-male interactions [120] and for *L. intermedia* in male-female interactions, when acoustic signals are also involved [118] As long as there is enough prey and microhabitats (crevices, etc.) available, cannibalism seems to be rare among spiderlings [121] and adults. The upper and lower limits for temperature tolerance have been reported for some species. For *L. reclusa* it was reported that the activity limits were of 4.5°C to 43°C; with 4-hour exposures, there was 47% mortality at 27°C and 21°C; with 30-days exposure all spiders survived at 0°C but none at 25°C [122]. With 1-hour exposures at constant temperatures, it is reported [123] an upper LT_{50} (median lethal temperature) of 35°C for *L. intermedia* and of 32°C for and *L. laeta*; the lower LT_{50} was 27°C for both species. A recent paper addressed *L. reclusa* distribution, employing ecological niche modeling to investigate the present and future distributional potential of this species, and demonstrated that under future climate change scenarios, the spider's distribution may expand northward, invading previously unaffected regions of the USA [90]. *Loxosceles* spiders make irregular predomimantly 2-D or 3-D webs, depending on the microhabitat available. *L. reclusa* in laboratory conditions preferred crevice widths of 9

mm or bigger with no correlation of body size to crevice width, whereas *L. laeta* preferred crevice sizes of 6.4 mm or bigger [121], with a marginally significant correlation between crevice width and body size. Both species preferred vertical instead of horizontal-oriented refugia and refugia with conspecific silk compared with previously uninhabited refugia. There was no significant difference between the species in their propensity to move among refugia in the 30-days trial; however, both species had individuals that were always found in the same refugium for the entire assay and individuals changing refugia every 2-3 days. The propensity to switch refugia was not affected by the degree of starvation for the period tested [121]. Another work [124] investigated why *L. laeta* and *L. recluse* preferred small cardboard refugia covered with conspecific silk compared with never-occupied refugia. When the two *Loxosceles* species were given choices between refugia previously occupied by their own and by the congeneric species, neither showed a species-specific preference; however, each chose refugia coated with conspecific silk rather than those previously inhabited by a distantly related cribellate spider, *Metaltella simoni.* Considering the inability to show attraction to chemical aspects of fresh silk, it seems that physical attributes may be more important for selection and that there might be repellency to silk of a recently vacated spider [124]. Some species, such as *L. laeta*, seem to invest more energy on web building, defends aggressively their webs from conspecifics and other spiders and are rarely seem much distant from the webs. We found evidence, from molts and very dense webs that it is common in undisturbed areas to find *L. laeta* females that spent their entire life in a single location. This sedentary behavior is certainly linked to the low number of accidents observed in houses with many *L. laeta* [116], although these spiders are probably the most aggressive synanthropic *Loxosceles.* In the other extreme we have *L. intermedia*, the species that probably causes more accidents with humans in the genus. These spiders do not invest much energy in webs, are frequently found at big distances from the webs, and may change locations frequently during lifetime (unpublished data). This vagant, active hunting behavior certainly increase the risks of accidents, and we have bites recorded in houses with few spiders [106, unpublished data]. One publication [125] states that *L. reclusa* is a scavenger, and exhibits a clear preference for dead prey over live prey. However, a more detailed study [126] suggests that scavenging is an opportunistic behavior in recluses that requires specific circumstances that may rarely occur in nature. In natural habitats, *Loxosceles* spiders can be found under rocks and the loose bark of dead trees; *L. intermedia* might be an exception, as it is almost impossible to find in these microhabitats, far form urban areas. In synanthropic environments, *Loxosceles* spiders are found in cardboard boxes especially under folded flaps, in cupboards, behind bookcases and dressers, in trash, under broken concrete and asphalt and, of medical concern, in shoes and clothes left out on the floor or stored in closets and garages. There is a propensity for *L. laeta* and *L. intermedia* to be found frequently in association with rough surfaces such as cardboard, construction material, wood and cloth and less so with smooth surfaces such as metal and ceramic [102]. This fact is probably linked to the absence of subungueal tufts on *Loxosceles* species and consequent need to use rough surfaces to climb.

As mentioned above, *Loxosceles* spiders have proven to be a challenge to pesticide professionals, pesticide manufacturers, and homeowners [127]. Although several attempts have been made to control and eradicate populations in homes, hospitals, warehouses and other human dwellings and workplaces, most eradication efforts failed and sometimes it is considered an unlikely, if not an impossible task [127,128]. However, in 1966 [129] a focus of *L. laeta* in a large museum building at Harvard University in a dense indoor infestation was

controlled by applications of lindane and chlordane (currently banned products). It was considered later [130] that *L. reclusa* infestations can be managed with physical control and a combination of residual applications plus aerosolisizing contact materials. In 1968 [131] *L. laeta* was found in Southern California, in a building in Sierra Madre, close to Los Angeles. The media coverage created a panic on the population. A thorough search by local and state health and vector control officials eventually turned up that several parts of L. A. County and virtually hundreds of buildings that were infested with *Loxosceles* spiders. Following the discovery, a thorough eradication program was enacted. The building where the first infestation was discovered, a restored adobe structure, was tented and fumigated. Several years later, it was observed [131] that most of the control programs were unsuccessful. Within about a week following applications, some places have been reinfested by additional spiders. It was suggested then that repeated applications over long periods of time would be necessary to obtain control [131]. Previous evaluations of insecticides on *L. reclusa* indicated that a mixture of DDT (2 mg/kg) and chlordane (2 mg/kg) was effective and that lindane is an adequate insecticide for infested areas, with a median lethal dose (LD_{50}) of 85 µg/g spider [132]. Using insecticide-impregnated filter paper, Levi and Spielmann [129] evaluated DDT (4 mg/kg), dieldrin (1.6 mg/kg), lindane (0.5 mg/kg), chlordane (4 mg/kg) and mixtures of these products on *L. laeta*, pointing out lindane and chlordane as the most efficient products. Hite et al. [112] analyzed 13 insecticides applied directly on the body of *L. reclusa,* also selecting lindane with the best performance. Technical grade lindane and diazinon (1 mg/kg diluted) were also evaluated [133] by using filter paper as the substrate in tests with *L. reclusa* allowing a residual effect for up to 1 week. Gladney and Dawkins [134] evaluated the insecticides resmethrin, dichlorvos, methoxychlor and ronnel (fenchlorphos) by direct application on the dorsal surface of *L. reclusa.* Only the first product allowed a low median lethal dose, expressed as micrograms of product per gram of spider. In addition to the methodological variability found among the works cited, there was an important recognition of the carcinogenic effects of some of these products, many of which are currently restricted, severely restricted or banned in many countries. Recently [11] we designed a bioassay involving hundreds of females of *L. intermedia*, followed by a field test of the best performing molecule. Qualitative filter paper sheets were individually impregnated with 2 mL of the given insecticide aqueous solutions at the desired concentrations and allowed to air dry for 24 h. A previous assay was carried out with 12 concentrations of each insecticide, using four repetitions of three spiders per concentration, plus the respective controls (spiders placed on filter paper with no insecticide added). Mortality was assessed at 2, 4, 12, and 24 h after the contact of the spiders with the products, and seven concentrations were finally established for each product as causing between 1 and 99% mortality. Bioassays to determine the median lethal concentrations (LC_{50}) were conducted in the same way, with mortality being assessed 24 h after introducing the spiders into the containers. In laboratory tests, the most active pesticides in descending order were microencapsulated (ME) lambdacyhalothrin (LC_{50}=0.023 mg/kg), nonmicroencapsulated lambdacyhalothrin (LC_{50}=0.047 mg/kg), deltamethrin (LC_{50}=0.26 mg/kg), and cypermethrin (LC_{50}=1.38 mg/kg). Cockroaches, *Phoetalia circumvagans*, killed with ME lambdacyalothrin, were offered to the spiders. *L. intermedia* fed on 63.3% of the dead cockroaches during the first 6 h of experiment; none of the spiders died during the subsequent 15 days. Microencapsulated lambdacyalothrin was chosen for application in two contiguous houses. The mean volume applied was 22.8 mg a. i./m^2. Dead spiders were

found during all the inspections up to 60 days after the initial application. In total, 297 dead spiders were collected; 65.7% in the attic shared by the two homes.

Based on field and laboratory observations, Sandidge [135, 136] proposed protocols for the control of *L. reclusa*, to be used by pest control campanies. It involved [136] preliminary search for spiders, locating favorite hiding places in the house, as one major problem with brown recluse management is finding where spiders reside and where they retreat when pest applications are performed. A second problem is how to access and treat many of these areas. Sticky insect monitoring traps should be placed during the preliminary search to detect spider travel routes, invasion points into the house and access areas into and out of different rooms. The home should be revisited after several days to examine the contents of all traps. The age structure of the captured spiders—older adults, juveniles, or spiderlings could be determined. Insects trapped should also be examined, as if prey can enter houses, spiders can also enter and leave freely and structural changes (mechanical exclusion) may be necessary to correct the problem. Later it should be devised an approach to control each specific population. The age, size, sex, and condition of spiders will be a guide in later pesticide application. Sandidge [135] states that males travel more frequently and are more aggressive than females. However *L. laeta, L.intermedia* and *L. gaucho* [106] males are not aggressive at all and generally the male bite is considered less dangerous than female bites [86, 87]. For *L. reclusa* [135], according to Sandidge, areas with a large number of males put the homeowner at a greater risk of spider bite. The areas for traversing spiders should be treated using sticky insect monitoring traps and low toxicity chemicals with high residuals and/or products that cause mechanical injury. Males are highly active throughout the summer and are more likely to contact and therefore be killed by aqueous applications, dusts, or crack and crevice products. Areas containing a large number of females are more likely to provide the optimal environmental conditions and low disturbance necessary for egg and spiderling development. It is suggested to treat these areas with aqueous sprays for a fast knockdown, and consider fumigation or fogging for heavy infestations [136]. Many chemicals may not kill the spider, but will disrupt the nervous system and other bodily functions, causing the spiders to be extremely agitated and aggressive. Eggs and egg sacs are resistant to pesticide treatments, so they must be located and removed. Juvenile spiders are found in large numbers throughout the house at certain times of the year [135]. In [136], the details of construction favourable to *L. reclusa* are analyzed, and the attics are considered the most common female breeding site. This is true also for *L. intermedia* [11] and other species of brown spiders. The use of pesticide dusts is recommended [135, 136], but for *L. intermedia* ME lambdacyalothrin was efficient in controlling spiders in attics [11]. In Chile, in recent years, a multinational company [137] promoted a house paint formulation with supposed repellent properties against *L. laeta*, in the commerce. However, in laboratory tests, the paint does not have a repellent action against the spider [137].

The integrated pest management (IPM) concept to reduce *Loxosceles* populations in homes may rely upon many strategies. *Hemidactylus mabouia* geckos [138] may be efficient predators of *L. intermedia* in domestic environments [138]. Interspecific predation of three cosmopolitan house spiders, *Achearanea tepidariorum* (Theridiidae), *Steatoda triangulosa* (Theridiidae), and *Pholcus phalangioides* (Pholcidae), and *L. reclusa* [139] were examined to evaluate transitive predatory relationships and to explore the potential use of cosmopolitan spiders as effective biological control agents. Fifty houses were visually inspected. Although statistical tests showed a decrease in *L. reclusa* population densities with increased population densities of two cosmopolitan species, alluding to a potential beneficial

interaction for biological control, observations of spider behavior, web positioning (niche partitioning), and predation showed little possibility of biological control capabilities [139]. The use of appropriate vacuum cleaners showed to be efficient on removing and killing *L. intermedia* at all developmental stages, and it should be valid for other species of *Loxosceles* also [140]. Measures aimed to educate the population about behaviors that may reduce the likeliness of accidents, of how to recognize brown-spider webs [76], and which microhabitats are preferred by the spider doubtlessly can contribute toward minimizing the problem. However, chemical control continues to be the main or unique tool used by pest control companies and is the object of great interest for the population.

The control program designed to combat *L. reclusa* in the United States [135, 136] is detailed and based on field experience. However this program is designed to pest control companies, and involves the indoor application of several pesticides. In our field experience in Brazil [11, 138, 140] mostly with *L .intermedia,* we suggest to homeowners the frequent use of vacuum cleaners [140], and the tolerance to the presence of geckos in their homes, as they are probably the only indoor predator possibly tolerated by people and capable of removing adult and immature *Loxosceles* individuals [138]. As the gecko in considered a sit-and-wait predator, vagant spiders are more likely to be captured. In laboratory conditions, one *H. mabouia* gecko ingested three *L. intermedia* female in 30 minutes, and six in one day. A study on the feeding habits of *H. mabouia* in natural habitats [141] revealed that spiders are the most important item in its diet. We also suggest the elimination of cracks, crevices and other refugia in houses, that would be occupied by spiders in several developmental stages. We evaluated the effect of this minimization of refugia in dwellings (paper in preparation), and it was found to be cause significant reduction on the absolute number of spiders found and also facilitating the location and removal of spiders with the vacuum cleaner or other method. The attic of houses is a place were more adult females are generally found [11], so it is essential to remove these females from the population in order to reduce the population growth and also migration of spiders from the attic to other places of houses, mostly in days with temperatures above 30°C temperatures [142]. Other physical control measures such as moving beds away from corners and walls, and careful storage of clothing and linens, particularly soiled clothing and linens avoiding will reduce the chance of indoor spider bites, especially when dressing, grooming, or sleeping. In addition, checking shoes, socks, gloves, hats, sheets, and towels and all clothing and linens before donning or using will also expose hiding spiders that might bite when squeezed in clothing, towels, or bed linens. Indoor spider bites may also be prevented by properly insulating homes, especially all windows and exterior doors, attics and basement crawl spaces. Also avoiding the storage of material in paper boxes, using plastic boxes or bags instead. Chemical control is recommended when high infestations are found, or if there is a significant phobic reaction toward brown spiders and/or if people with special health conditions such as diabetes are residents. Very old residents or small children can also be considered on the chemical control recommendation. We suggest, for *L. intermedia* [11], the use of ME lambdacyhalothrin only in attics, using label recommendations and by pest control operators, if people are allergic to pyrethroids or afraid of pesticide use. If this is not the case, indoor aplicaton of ME lambdacyhalothrin can be performed also inside the house. Residents should leave the house for at least 12 hours, but we recommend 24 hours, in order to reduce the risk of loxoscelic accidents by affected spiders. We suggest the application of the pesticide during colder months, to reduce risk of accidents post treatment.

3.2 Scorpions – basic epidemiologic data and main control efforts

With the exception of snakes and bees, scorpions are responsible for more human deaths every year than any other nonparasitic group of animals [13]. Scorpion stings (scorpionism) are the most important cause of arachnid envenomation and are responsible for significant morbidity and pediatric mortality in many parts of the world. This is because either the incidence or severity of envenomations is high (or both factors at the same time), and are difficult for the health services to cope with [18]. The treatment of scorpion envenomation is complex and controversial, in particular regarding the effectiveness of antivenoms and associated symptomatic treatments [18]. In general, the degree of seriousness of scorpion stings depends on factors such as the size of the scorpion, the amount of venom injected, the body mass of the victim and the sensitivity of the patient to venom [18]. Most of these symptoms and clinical signs are caused by the release of adrenaline, noradrenaline and acetylcholine arising from the presence of the toxic venom in particular sites on sodium channels [18]. Other factors such as the interval of time between the bite and the administration of intravenous infusions and the maintenance of vital functions, can affect the initial progression of the illness and the early diagnosis. According to the most recent studies, seven areas in the World can be identified as being at risk: north-Saharan Africa, Sahelian Africa, South Africa, Near and Middle-East, South India, Mexico and South Latin America, east of the Andes [18] (Figure 3). This means that 2.3 billion people are at risk. The estimated annual number of scorpion stings worldwide exceeds 1.2 million and lead to more than 3,250 deaths (0.27%) [18]. The epidemiology of scorpionism in the world is poorly understood, because, among other reasons, many cases are not brought to medical attention. Effective treatment for these conditions is critically dependent on therapeutic sera, which is often unavailable or unaffordable in some of the countries where it is most needed. In 2007, the WHO created a five-year plan to boost antivenom production in developing countries, help authorities forecast market needs and strengthen their regulatory capacity [143]. Over

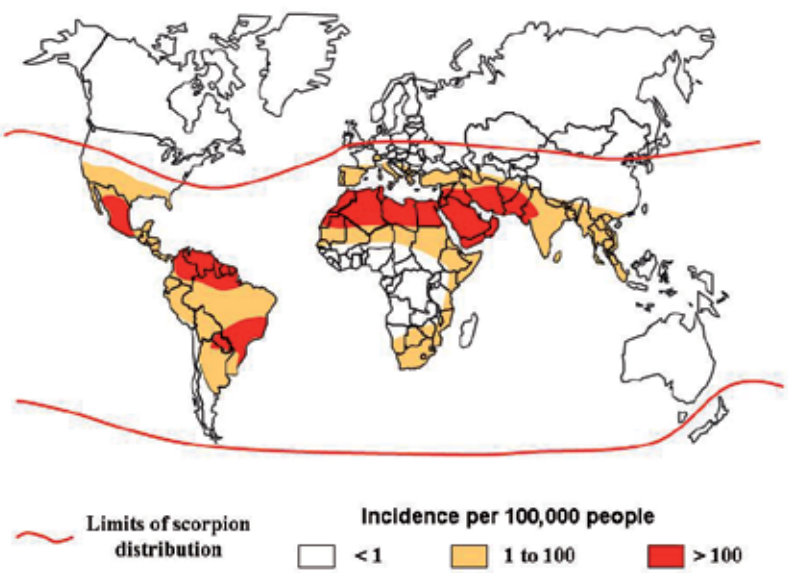

Fig. 3. Scorpionism areas of the world. Reproduced with permission from Chippaux and Goyffon [18].

10 million vials of antivenom sera are needed to treat snake and scorpion bites worldwide, with an estimated 2 million vials required for Africa alone [143]. In Mexico ca. 250,000 scorpion stings were reported yearly, but fatalities have declined from 2,000 to less than 50 per year following widespread distribution of antivenoms [143]. However, there is no mention in the WHO directive regarding the stimulus to the bioassays of existing pesticides or the development of new strategies and molecules for scorpion control [143].

Mexico referred to above, is the country with the highest incidence of scorpion stings. We list examples of the scale of the problem of scorpionism in other countries. *Hottentota tamulus* ("red scorpion") is the principal species of scorpion responsible for serious envenomation in India [144]. There is a lack of epidemiologic data concerning its incidence and mortality rate in India, although fatalities are regularly mentioned [144]. In Tunisia up to 40,000 stings, 1,000 hospital admissions and 100 deaths were former reported annually [145]. In Khuzestan, south-west Iran, where scorpion stings are the fourth main cause of death, 12% of the 25,000 stings treated each year and more than 95% of the fatalities are attributable to *Hemiscorpius lepturus* [146]. In Morocco, scorpion attacks are the primary source of envenomation and represent between 30 and 50% of all cases reported to the Moroccan Poison Control Center. *Androctonus* and *Buthus* are the genera most frequently responsible for these stings in the country [147]. In Saudi Arabia a total of 72,168 cases of scorpion stings were recorded between 1993 and 1997 [148]; *Leiurus quinquestriatus*, *Androctonus crassicauda*, and *Apistobuthus pterygocercus* were responsible for most of the stings. The study also shows that there was a low threat to life despite the high number of stings; this is due to the availability of medical facilities and the use of multi-center antivenom in different parts of Saudi Arabia [148]. In turkey, Scorpion envenomation cases are a considerable public health problem in all regions of the country, and important health-threatening scorpions in Turkey are *A. crassicauda*, *L. quinquestriatus*, *Mesobuthus gibbosus* and *M. eupeus* [149]. In Algeria [150], scorpion envenomation is a real public health problem; in 2000, 47,521 people were stung by scorpions and 108 deaths were recorded. Since the setting up of the National Committee to combat Scorpion Envenomations, several steps have been taken to deal with this problem [150]. In Brazil, 37,000 scorpion stings and 50 deaths were reported in 2005 and scorpion stings by *Tityus* spp. are an increasing health problem, due to the invasion of the urban environment by some scorpion species [151]. In Argentina, most injuries are caused by *Tityus trivittatus* [152]. Scorpionism in Argentina is a public health problem that is under control due to its relatively low incidence and the accessibility of specific antivenoms. In Venezuela, envenomation by *Tityus* scorpions is a common public health hazard [153]. In Colombia, scorpionism by *T. asthenes* is prevalent [154]. In the South of Europe (Portugal, Spain, Mediterranean France, Italy, and Greece), only small sized scorpions are found, the venoms of which are not usually dangerous to humans (*Euscorpius* sp., *Buthus occitanus*, *Mesobuthus* sp.). However, epidemiological data are limited and scarce [18]. In the South of France, the annual incidence is about five scorpion stings per 100,000 inhabitants, and the symptoms are mild. In Spain, less than 1% of the scorpion stings can be regarded as severe and no deaths have been recorded [18]. Scorpionism is low in Australia too; the stings from Australian buthid scorpions have more severe effects than those from the larger species in the Urodacidae (genus *Urodacus*) and Liochelidae (genus *Liocheles*) families [155]. In the United States, deaths from scorpionism are extremely rare [17, 18].

Scorpions represent a huge challenge in pest control because there is practically zero tolerance of their presence among residents of dwellings, even when species that do not

pose a significant risk to people's health are involved – the sting is almost always very painful, as well as being psychologically traumatic. In addition, as well as occupying intra and peri-domiciliary areas, several species of scorpions can also make use of the rainwater and drainage systems of the cities. In these sites they usually find abundant food supplies of insects such as cockroaches, and large colonies can become established and infest or reinfest many parts of the city. Scorpions can also detect contact pesticides through their pectens. Areas with inadequate sanitation are particularly susceptible to infestation by scorpions. The literature mentions that before the 1980s, organochlorine pesticides were used as a strategy of control, such as, for example, chlordane (2%) [17], which at present is only permitted for termites and is illegal in the United States if used by anyone other than a licensed pest control operator. Currently, only pyrethroids (principally) and in certain cases, organophosphates and carbamates will be among the molecules recommended to be effectively employed in public health control programs involving the application of products in the household, as will be seen in what follows. One of the main problems of scorpionism is how to achieve scorpion control through an effective participation of local communities. Although the use of chemical insecticides is one of the main measures adopted for controlling scorpions, few studies have focused on the efficacy and viability of this approach. The main measures taken for the control of scorpions naturally occur in the localities where they are regarded as a serious hazard to public health. In this Chapter, our aim is to consider studies and measures for controlling scorpion species that proved to be capable of rapidly forming large colonies in urban areas and spreading over great distances in the anthropic environment and about which references on bioassays and field application of pesticides are available. We will focus on Mexico and Brazil, countries which have serious problems with scorpionism in several areas but which adopt different approaches regarding the control strategies recommended.

Mexico - This country has had the largest number of deaths from scorpionism recorded in the world. The highest annual mortality rate (up to 1,944 deaths) was reported in 1944 [156]. Between 1979 and 2003, 6,077 deaths from scorpion stings were recorded [157, 158]. In 2005, 247,796 cases of scorpion poisoning were recorded, a figure that rose to 269,149 in 2006. *Centruroides* (Buthidae) is envolved in the majority of the recorded accidents. The most dangerous species are *C. noxius, C. limpidus limpidus, C. suffusus suffusus, C. infamatus, C. pallidiceps* and *C. elegans* [157]. In some states of West Central Mexico, such as Colima, a small State of the Pacific coast, the annual incidence reaches 1,350 scorpion stings per 100,000 inhabitants [158], and this figure rises to as much as 2,050 stings per 100,000 in some communities of Morelos, in the South-West of Mexico City. This figure is probably an underestimate, especially as the effectiveness of the treatment has dramatically decreased the mortality rate. All the authors are in agreement that adults are stung more often than children and that most scorpion stings occur during the night at home. The annual mortality rate following scorpion stings was approximately 0.6 deaths per 100,000 inhabitants in the 1970s, and it dropped dramatically to 0.07 in the beginning of the 21st century [157] thanks to a wider use of antivenom. The mortality rate remains rather high among children, in particular those younger than 5 years old [157]. The seasonal peak of stings lasts from March to June, i.e. at the end of the dry season, when the climate is hot [158, 159]. Climate is an essential predictor: in Colima State, there is very little rain and there are few stings in the winter when the minimum temperature is below about 16 °C. The number of scorpion stings is independent of the actual rainfall when this is above 30 mm/month [159]. The demographic factor is also significant. In Guanajuato state, 92% of the stings take place in an

urban environment. However, in communities with less than 2,500 inhabitants, the risk is nearly 12 times higher than in cities with more than 20,000 inhabitants [157].
At the beginning of the 20th Century, the Mexican authorities decided to offer prizes for the capture of scorpions and in just one summer, between 80,000 and 100,000 *Centruroides* scorpions were collected [160]. In 1962, a pest control scheme was put into effect in several parts of Mexico, which involved, among other non-chemical control recommendations, placing ceramic tiles and metal sheets around the foundations of houses [161, 162]. The premises were treated, indoors and peridomestically, with organochlorine lindane, but the results were unsatisfactory [156]. The North American Free Trade Agreement (NAFTA) in 1994 issued directives for phasing out organochlorines, and the use of Lindane was also banned in Mexico after 1999 [162]. New guidelines from the Secretariat of Health gave priority to alternative pesticides: carbamates and pyrethroids [163]. However, there was a fear that, as pyretrhroids tend to irritate and stimulate the motor activity of certain arthropods, its use would cause more stings. Field trials of pyrethroid pesticides were undertaken in Morelos during 1998-2000 at the village of Chalcatzingo (population initially 2,760 inhabitants, 530 dwellings) [162]. This study was taken into account by the Mexican Health Secretariat, when it officially recommended the use of pyrethroids in scorpion control [162, 163]. Pre-intervention surveys detected scorpions (Scorpiones: Buthidae) of two species in the majority of the houses: *C. limpidus limpidus* outnumbering *Vaejovis mexicanus smithi*. The prevalence of scorpions was assessed, both before and after the spraying, through direct searches (40 min/house) and by householder reports of sightings inside the houses. Pyrethroids and residual treatments were evaluated in different sectors of Chalcatzingo, with almost complete coverage indoors and peridomestically, using the following four formulations: bifenthrin 10% wettable powder (WP) applied at 50 mg a.i./ m^2, cyfluthrin 10% WP (Solfac 10 WP) at 44-55 mg a.i./m^2, deltamethrin 2.5% suspension concentrate (Biothrin 25 SC) at 11 mg a.i./ m^2 and 5% WP (K-Othrine 50 WP) at 35 mg a.i./ m^2. Phase 1 compared bifenthrin 10 WP, Solfac 10 WP and Biothrin 25 SC sprayed in December 1998; phase 2 compared Solfac 10 WP and K-Othrine 50 WP sprayed in June and again in December 2000, with follow-up surveys of scorpions one month post-spray and subsequently. The prevalence of scorpions was reduced by 64-77% peridomestically one month post-spray and by 83, 46 and 15% in houses sprayed with cyfluthrin WP, bifenthrin WP or deltamethrin SC, respectively. Householder reports of sighting scorpions indoors were 33-85% below pre-intervention levels. The cumulative effects of the three spray-rounds over 3 years reduced scorpion prevalence by approximately 60% in the deltamethrin WP re-sprayed area and approximately 90% in the cyfluthrin WP re-sprayed area. The number of householder sightings also fell by 67 and 28% in the cyfluthrin and deltamethrin re-sprayed areas, respectively. The operational efficacy of these products against scorpions at the dosages applied was ranked in the following order: cyfluthrin WP > bifenthrin WP > deltamethrin SC > WP. The reported cases of scorpion sting intoxication fell by 17% during this study after having risen by approximately 40% in the previous four years. When scorpions were exposed to lethal doses of the four pyrethroid formulations, none of them registered an increase in tail wagging, movement or activity, during the field observations. Thus, this study found no contra-indications for the use of these pyrethroids for scorpion control, apart from transient side-effects such as sneezing and itching if the sprayers were inadvertently exposed. According to the study authors, failure to completely eliminate scorpions from houses and the peridomicile can be attributed to the difficulty of treating all their hiding-places, rather than to the ineffectiveness of the pyrethroid [162]

A publication [164] by the National Institute of Public Health of the Mexican Secretary of Health which is designed for medical personnel and other interested parties, recommends a number of control methods and preventative measures, based on the governmental official norms for scorpion control [163] which are outlined as follows:

- The avoidance of children´s games or manual handling that risk exposure to scorpions (lifting up stones, bricks and bulky objects, exploring bushes or sliding one's hands along walls, walking barefoot or leaving clothes on the ground).
- Examining and shaking out one's clothes and shoes before putting them on.
- Checking the angles of walls, doors and windows. The leveling and patching of roofs, walls and floors is recommended.
- Putting some form of protection on the doors and windows and underneath the roofs, using materials such as tiles for the interior and exterior skirting-boards, polished cement, and galvanized sheet metal in the surrounding area of the dwelling.
- Using canopies for the cots and beds of children and immersing the bed legs in containers filled with water. The bed must be placed 10 cm away from the wall and care should be taken to make sure that clothes are not in contact with the floor.
- Disposal of any garbage, stones or wood in the surrounding area of the dwelling.
- The use of intra- and extra-domiciliary insecticides. Pyrethroids: lamda-cyhalothrin and cyfluthrin. Carbomates: propoxur. In the Mexican Official Norm (MON) regarding scorpions [164] the pesticide use is detailed (itens 7.4.1.1 and 7.4.4.2). Preference is given to pyrethroids lambdacyhalothrin (0.03 g/m^2) and Cyfluthrin (0.04 to 0.08 g/m^2), with estimated residual effects lasting about 6 months. The second recommendation is for carbamates like bendiocarb (20 mg/m^2) and propoxur (30 mg/m^2), with residual effects lasting six months. The recommendations are for intradomestic and peridomiciliar applications.
- The NOM [164] also recommends whitewashing fences, walls and trees near the house. The lime paint, with or without some local plants added during preparation, has a repellent action on *Centruroides* and possibly other scorpions.

Brazil - Scorpionism constitutes a public health hazard in many parts of Brazil [151, 165, 166, 167, 168], particularly in urban areas. The effects of human activity and environmental changes have allowed the expansion of some species that invade human dwellings and displace less dangerous autochthonous species [168]. In 1988, when a report was being prepared on the number of scorpion attacks in the country, it was found that there had been a significant increase in the number of cases. Most of these were benign with only a 0.58% fatality rate. The deaths were linked to stings caused by *T. serrulatus*, and most often occurred among children less than 14 years old [151]. From a public health perspective, the increase of the dispersion of *T. serrulatus* through the country was a cause for concern. This species, which originated in the state of Minas Gerais, is showing a progressive dispersion, and its presence has now been recorded in the Brazilian southeast, south and middle-west regions [151]. It has a bright yellow color from which it derives its name – "yellow scorpion". It measures up to 7 cm in length. Its colonies are only formed by females whose reproduction occurs through parthenogenesis [169]. In some studies, *T. serrulatus* is regarded as being a component of a complex that includes *T. stigmurus* [168]. Although traditionally inhabiting the savanna regions and open plains, this species has become well adapted to the life of urban households. In urban areas this species and others of scorpions and synanthropic animals have access to shelters containing garbage, debris, piles of bricks, and an abundance of food such as cockroaches and other insects. The lack of competitors

and predators such as monkeys, coatis, seriemas, toads and frogs, also allows the scorpions to proliferate rapidly [168]. The preferred temperature of the yellow scorpion *T. serrulatus* was investigated [170] and it was concluded that this species does not have a specific preferred temperature within the range of 14°C - 38°C and may even tolerate temperatures below 8°C, when in a torpid state. This fact suggests that the species is highly adaptable to different thermal zones. Stutz (personal communication) notes that most of the *T. serrulatus* in the town of Uberlândia, Minas Gerais are established in the drainage system and thus are able to enter the residential dwellings, which makes it difficult to control the species by chemical means.

More than 130 species of scorpions are known to exist in Brazil. Although most accidents are caused by the *T. serrulatus*, *T. bahiensi*s and *T. stigmurus* species are also of great public health concern [168]. The brown scorpion *T. bahiensis* has less toxic venom than *T. serrulatus* and is responsible for most of the attacks recorded in São Paulo [165]. In the north-east of the country, *T. stigmurus* causes most of the stings, some of which are very serious [167]. Other species of this genus are the cause of attacks of varying degrees of seriousness in several parts of Brazil and South America [18] and it is probable that all the species of this genus have a venom that can affect people [18, 171]. Apart from this genus, sporadic attacks have been recorded in Brazil with some species from the genera *Bothriurus*, *Rhopalurus* and *Brotheus* but none of them were serious (168, 171].

At the beginning of the 20th Century scorpionism by *T. serrulatus* and *T. bahiensis* in the the State of Minas Gerais was established as a serious public health issue. The Ezequiel Dias State Institute began to produce serum, and also offered financial rewards for the capture of scorpions so that the venom could be extracted to produce anti-scorpion serum; this inducement led to the capture of 107,533 specimens in 6 years [160]. Dias, in 1924 [160] already foresaw the need: a) to test molecules for chemical control b) to construct buildings that were unsuitable for the life and procreation of scorpions c) to take supplementary measures. Unfortunately, not many of his recommendations were put into effect by the authorities in the years that followed. Preliminary experiments were carried out in the laboratory by the same author with *T. bahiensis* which were exposed to many substances, including xylol, chloroform, hydrocyanic acid, gasoline, naphthalin, coal gases, carbon sulfurate and sulfurous gas. The following gases were also tested in an experimental room: acetylene gas, creolin, naphthalin, tar, coal and chlorine. All these substances were lethal to the scorpions in the laboratory but showed negative effects in larger spaces. In the later stage of the field study, the laboratory assistants used sulfurous gas (obtained by burning sulfur) for fumigating the buildings and dwellings in the ratio of 20 g/m^3. In many cases this led to positive results. The use of xylol in furniture to control scorpions was also tested. Dias stated that, however effective the chemical control method might be, this should only be adopted in an emergency, since given the same conditions, reinfestation will occur. Some time after this pioneering work, the use of sulfurous gas and xylol was forbidden because as well as being extremely difficult to employ, it posed great risks to health and the natural environment. Dias [160] drew up a list of 18 construction features to reduce the risk of infestation by scorpions, which should be required by the sanitary authorities, together with those responsible for giving approval for new buildings. He paid special attention to the need to forbid woodsheds to be built in areas (closed or otherwise) near to dwellings. In the case of backyards, he advised keeping hens to control the scorpions because they are able to ingest a lot of *T. serrulatus* very quickly. In rich districts, he advised the purchase of

scorpions as a strategy for reducing their numbers [169]. According to Magalhães [172] Brazil, in the State of Minas Gerais, carried out the first systematic campaign against scorpions in the world. This author describes tests that were conducted with the organochlorine DDT in various formulas, with bioassays that involved *T. bahiensis* and *T. serrulatus*, and records the repellent activity of the powder derived from this product against the scorpions. According to Bücherl [173, 174], contact insecticides (organochlorates at that time) only have satisfactory results when sprinkled in all areas at the same time and if the supposedly infected area is treated at least three times per month, for three uninterrupted months. Later, a ban on the further use of organochlorates was imposed in Brazil which is a signatory to the Stockholm convention and other agreements concerning Persistent Organic Pollutants (POPs). Pyrethroids and organophosphates were used to combat these arachnids, particularly in situations where alternative control measures like the elimination of breeding sites, woodpiles, rocks and debris from areas around homes, have been difficult to apply or been shown to be unsuitable [175].

The "Manual de Diretrizes para Atividades de Controle de Escorpiões", published by the São Paulo Secretariat of Health in 1994 [176], provides guidelines to be used on scorpion control programs. The manual listed ME pesticides as a possible adjuvant means of controlling infestation by scorpions [176]. On the basis of empirical studies and observations, some investigators stated that *Tityus* scorpions can make use of their pectens to detect non-microencapsulated formulas applied to the substrate such as organochlorates, pyrethroids, organophosphates and carbamates [177, 178, 179, 180]. After being exposed to these, the scorpions might show an increased activity and thus intensify the risk of attacks involving human beings. One of the studies on the effectiveness of three insecticides (bendiocarb, deltamethrin, and lambdacyalothrin ME) [177] for controlling *T. serrulatus,* stated based on preliminary laboratory bioassays, that exposing the specimens to the insecticides tested, did not significantly affect their survival. The scorpions were exposed for 15 minutes, after they had been put in the containers without the product. The exposures were carried out in distinct groups on days 1, 15 and 30 after the treatment. The pesticides were spread on the tile surfaces in accordance with the amount recommended by the manufacturer. No knockdown effect was reported in the 15 minutes of exposure and the bendiocarb caused the least irritation, followed by the lambdacyalothrin ME and the deltamethrin. The effect of irritation was only observed during the period of exposure to the surface that had been treated by the pyrethroids and disappeared when the specimens were taken to the other substrates. The authors concluded that the amounts recommended by the manufacturers for the pyrethroids tested did not show results that were favorable to the control of scorpion colonies. One of the preliminary laboratory assessments used glass bowls treated with 5 mL of prepared solution with Diazinon ME (0.3%) with exposure to *T. serrulatus* for one hour [181]. In these conditions, after exposure the animals died in periods no longer than 24 h and the treated substrate was shown to be capable of bringing about mortality up to 62 days after its application. One test which could only be partly completed was conducted in the town of Aparecida, São Paulo state, where there is a predominance of *T. serrulatus* [178] and at that time experienced about 45 attacks/100,000 inhabitants per year. Spraying of diazinon ME (30%) (6g/m^2) was undertaken, in 360 household units in the peridomicile and domicile areas, as well as in a cemetery where it was sprayed together with 1% dichlorvos. The cemetery application with dichlorvos had a significant repellent effect, and its use its use was interrupted due to the intense migration of the scorpions to

neighboring areas, alarming the population. The live individuals present in areas treated only with diazinon ME, were collected and kept on laboratory in containers without pesticides. The average time that it took for the specimens sent to the laboratory to die was 37 days; in this same period, no fatalities were observed in the control group. In the same town, a project for the control of scorpions was planned in 1995 [182]. Studies were carried out of the main sites where scorpions could be found in the fields or town, as well as of the environmental conditions of the urban habitat that could facilitate the procreation and dispersion of scorpions. The basic problems of urban infra-structure were assessed, such as the means of packing and collecting public and domestic urban garbage, basic sanitation (drainage systems and pluvial water galleries) and the situation regarding the wastelands and building construction in the urban areas. After the epidemiological study, a number of educational measures were taken which comprised the preparation and distribution of leaflets, collective cleaning activities, domiciliary visits and the involvement of teachers and students in the public and private teaching network that formed a part of the campaign. In focal points of high risk, in particular in the kindergartens, the use of chemical control was suggested which involved deltamethrin-based pyrethroids. Within the sanitary norms that prevailed in the urban zone, a further suggestion was the use of natural predators like domestic hens to combat scorpionism. A further idea was to introduce a scheme where one week each year would be devoted to the study of scorpionism within the schools of the town where the problem prevails; this would be an educational measure that could help prevent attacks and improve scorpion control.

In the state of Pernambuco, the records on scorpionism by *T. stigmurus* constitute one of the highest incidences of scorpionism in the country, with 43.5 cases/100,000 habitants [183]. In natural environments, this species is found under stones, in cracks and in decomposing trees, especially in environments where the ground is very damp [183]. However, in urban areas, *T. stigmurus* has been found invading human dwellings, and living in roofs or among debris in backyards. An investigation in Recife, capital of the State of Pernambuco, analyzed the events that followed the application of the insecticide Demand 2.5 CS (lambdacyalothrin ME) in the field, to control *T. stigmurus* [183]. During three months, 69 premises were monitored on different days after being treated with insecticide, to determine the degree of scorpion detection frequency and mortality. Spraying was preferentially performed outdoors around the foundations of the buildings and potential breeding sites such as debris and sewers. Indoor application was only carried out in premises where there had been constant sightings of these animals inside the house. The treated premises were monitored for one month following the application of the insecticide. During this period, the premises were visited on the following days: 1, 7, 15 and 30 days after the treatment. The results showed that 42% of the premises had the presence of scorpions, with an average of three specimens per house. The highest incidence was recorded during the first week following the treatment. Only 7% of the specimens were found dead. Most (72%) of the inhabitants of the premises showed a knowledge of prevention and control measures. Despite this, 100% of the premises revealed breeding sites, mainly (79.7%) waste material (rubble, debris, newspapers, plastic receptacles and sticks. However, most (56.5%) of the people showed little interest in adopting preventative measures. These results provide evidence that the scorpion control method used by the health agents during this investigation was inefficient, and suggest that the method may have had a dispersive effect on these animals, as also suggested by other study in Belo Horizonte, Minas Gerais state, Brazil [180]. The treatment

with Demand 2.5 CS took place between May and June, coinciding with the period of highest rainfall in this region [183]. This may have intensified the dispersion of the scorpions away from their hiding places due to habitat overflow. Moreover, the rain may also have reduced the effect of the insecticide, by washing it away, since it was mainly applied in the areas surrounding the homes. The results shown in this study [183] may have been influenced by the way the infected area was treated, which involved a single application, compared to three applications in another study carried out in Mexico [162].

Measures taken to control scorpions excluding use of pesticides included in the program were first implemented in Bandeirantes, a town of about 32,000 inhabitants in the State of Paraná [151, 185]. The activities which were set in motion by the State Secretary of Health, in partnership with the municipal authorities, have succeeded in achieving concrete results without the use of pesticides. The project consisted of analyzing the situation in the town, determining the spatial distribution of the scorpions, planning measures for intervention and training a municipal team to undertake the work. In two years (2006-2008), the program led to the capture of thousands of scorpions in the town. The municipal work consists of going directly to the key sites where the scorpions are located, removing them and making the residents of the dwellings aware of how to avoid a new infestation. This methodology is recommended in the publication "Manual de Controle de Escorpiões" (Scorpion Control Manual), published by the Brazilian Secretariat of Health in 2009 [151]. Some other cities [186] halted the use of pesticides on scorpion control, following the new Brazilian official guidelines [151], although it is still in use in many cities. The Scorpion Control Manual [151] lists as reasons which make chemical treatment ineffective to control of *Tityus* scorpions the habit of these animals to shelter in the cracks of walls, underneath cardboard boxes, and in piles of bricks, tiles, pieces of wood, and fissures or crevices in the ground, together with their ability to remain for months without moving. As well as this, they have the supposed ability to remain with their pulmonary stigmata closed for a long period. The products whose use is not recommended, by the Manual are chemical domestic sanitation items consisting of formaldehyde, cresols and para-chlorobenzenes and products used as insecticides, raticides, cockroach-killers or repellents of the pyrethroid and organophosphate groups. This is because, according to the Manual, the displacement and consequent dispersion of the scorpions from the places previously not exposed to the effects of these products increases the risk of attacks. As well as this, it could create a false feeling of being protected on the part of the residents, who will be led to believe that the problem has been overcome and prevent them from coming to terms with their environment. The Scorpion Control Manual state that, up till now, the effectiveness of the chemical products for scorpion control in the natural environment has not been defined scientifically [151]. When there is a need to control cockroaches in places where scorpions are present, the use of formulas with gel or powder is recommended.

In the pest control manuals produced after the banning of the organochlorine pesticides, which were made available to American universities, it is suggested that scorpions are difficult to control by just relying on pesticides [187]. Thus, the first control strategy is to make alterations to the area surrounding a house or structure. [187, 188]. As regards pesticides [188], it is suggested that wettable powder formulations provide a better residual control of crawling pests when applying perimeter sprays. When using pyrethroids or other insecticides designed for scorpion control, the highest permissible label rate should be used. Scorpion control pesticides registered in October 2000 in the United States include the following: permethrin,

cyfluthrin, cypermethrin, lambda-cyhalothrin ME, deltamethrin. Daytime spraying is largely ineffective. The most effective scorpion management method would be nightime blacklight collecting. [187]. Daar [189] suggests that spraying the perimeters of buildings is both unnecessary and ineffective since scorpions are able to tolerate a great deal of pesticide in their environment. Moreover, the use of physical controls along with education to reduce the fear of scorpions will help prevent the application of unnecessary chemical treatments.

4. The methodological problem of the bioassays and recommendations for control

The development of specific molecules to control spiders and scorpions has been neglected, probably due to the limited demand for control measures. As a result, pesticides such as pyrethroids, carbamates and organophosphates have been adapted and used for arachnids in label amounts generally above those recommended for insects, thus causing potential environmental liabilities with regard to their use. In Mexico, a field study cited above [162] in a small community tested the use of pyrethroids for the control of scorpions with monitoring over a period of three consecutive years, presenting positive results and no significant repellent effects of the pytrethroid molecules tested. The mexican official norm NOM-033-SSA2-2002, for the surveillance, prevention and control of scorpionism [163], published in 2003 and focused on the control of *Centruroides,* included pyrethroids and carbamates among the products listed for used as part of scorpion control programs. In Brazil, the most recent official document on scorpion control [151] recommends the avoidance of chemical control with scorpions of the genus *Tityus* and supposes that the same molecules used in Mexico to control *Centruroides* spp. scorpions are not effective against *Tityus* spp. There exists on *Tityus* information of a small-scale field test where there was a single use of pyrethroids ME without good results [179] but there are also accounts of the effective use of carbamates in field scorpion populations [178]. Certainly, there are differences in the susceptibility, the habitats, the behavior and other aspects of *Tityus* and *Centruroides* scorpions. However we have not found any mention to published laboratory bioassays, based on methodology that follows the guidelines of WHO or others widely accepted by the scientific community, aiming at scorpions found in Mexico, Brazil or other countries. Even the manuals issued by the American University Extension Services [187,188] adopt the recommendations that can be found on the labels of the products. There is thus a serious gap in this area since the lack of rigorous experimental approach might lead to public policies that are not optimized to reduce scorpion populations in urban areas. The WHO [22] recommends a series of procedures for assessing the pesticides that are used to control animals that represent a public health concern (Figure 4). Among the successful measures that have been taken on the basis of this protocol, and which have led to a reduction of infestations and transmission of diseases, it is worth mentioning the chemical control of vectors of the Chagas disease and the use of mosquito nets impregnated with pesticides to combat malaria in Africa [22]. However, this scheme was not adopted to address the problem of spiders and scorpions of public concern. The initial phase of laboratory studies was even more neglected and field studies were generally conducted on the basis of the manufacturers´ recommendations. In reality, what is still needed is the development of an experimental design to make an evaluation of the molecules specific to spiders and scorpions, with good reproducibility. So the comparison of results for different species, or the same species in different regions can be made feasible. Thus, the evaluation of

the methods for testing the susceptibility of the brown spider *L .intermedia* to pyrethroids [11], represents a starting point for improving methods of evaluating the susceptibility of spider and scorpions to pesticides. Here we outline some of the factors that are regarded as necessary for a rigorous validation of a methodology for evaluation of pesticides candidates to use for chemical control of spiders and scorpions in the urban environment.

A) The introduction and maintenance of a reference colony of the target species. In the process of evaluating the susceptibility of a population of organisms, it is strategic to stablish a population sensitive to reduced concentrations of the active principles of biocide molecules. This standard population would allow geographical and temporal comparisons among different regions, evaluating the degree of sensitivity or resistance to a particular product and other parameters. The laboratory colony is vital to carry out evaluations which involves comparing the susceptibility of colonies in the field with that of the laboratory colony and thus, enabling for example, calculation of the resistance ratio (the difference between the LC_{50} of the field population and the LC_{50} of the laboratory population) which has been widely used in studies of the *Aedes Aegypti* populations (Diptera: Culicidae) [190]. In the case of *Loxosceles* and *Latrodectus* spiders, and *Tityus* and other scorpions genera of Buthidae scorpions, the stablishment of these colonies is feasible and of relatively low cost.

B) The age of the population being evaluated. The development stage and age of the organism can result in differences of response to a particular substance. In the lack of availability of a reference colony, it is important to ensure that the organisms being evaluated are at the same stage of development, making replications possible. A suitable number of individuals must be obtained by means of preliminary evaluations. In the case of field populations, an active search for specimens of approximately the same age can be considerably difficult. Obtaining an F_1 generation under laboratory conditions would be a way of overcoming this problem. In both cases, previous studies of the biology of the targeted species being analyzed in the field and laboratory is essential to ensure the reliability of the results obtained in determining the susceptibility of a population to an active principle.

C) The basic information needed in evaluating the potential benefits of products to control spiders and scorpions. The adequate knowledge of the biology, physiology and behavior of the targeted species is useful both for the preparation of analytical experiments under laboratory conditions and in the integrated program control of populations of scorpions and spiders in urban habitats. This information can reduce the likelihood of spiders and scorpions coming into contact with humans in the home environment. It is necessary also to acquire correct understanding of the mechanisms involved and the physiological effects of the substances on the arachnids.

D) Standartization. When a bioassay is conducted to assess the toxicity of molecules on target species creatures such as spiders or scorpions, the results are most suitably expressed not in units of mass, volume or the percentage of the active principle needed to cause lethality, but rather by using solutions of these molecules with concentrations expressed in number of moles per litre, also known as Molarity. If this procedure could become standardized, it would provide an accurate idea of the number of molecules used in each test, information of much greater value than the mass or volume of the pesticide needed to cause the death of these creatures.

The control of organism considered as pests always raises the possibility of analogies with warfare [191] and the pesticides have very often been used in an analogous situation to the use of weapons for the destruction of enemies. However, at present it is clear that practically no pest that is a cause of concern to public health is faced with the prospect of having all its

populations eradicated by man and even less, by means of chemical control only. The damage caused to human health is an aggravating factor that justifies all the attention paid to this issue but it should be stressed that all the organisms regarded as agricultural or urban pests have been able to escape from natural predators and have had access to some form of resource – usually made available by man – which has allowed a rapid growth in population to occur. In the light of this, chemical control is one of the strategies to be employed in integrated pest management programs, with a varying degree of importance that depends on the ecology and behavior of each species and the risks involved to human and environmental health. In the case of spiders and scorpions, as mentioned above, researchers and universities have traditionally tended to downplay the importance of research studies about the ecology and behavior of spiders and scorpions considered to be dangerous to man [192]. In many cases there are not enough rigorous laboratory bioassays to evaluate the effectiveness of pesticides on these organisms. Public health personnel, with a lack of reliable scientific knowledge about the ecology, biology and susceptibility if the target species to pesticides faces the sometimes urgent need to stablish pest management programs to control spiders or scorpions in urban areas. The scientific knowledge and novel management tools are part of the activities that universities and research institutes should be developing [193]. In our view, interaction between the universities and public health authorities is essential to optimize integrated vector management, being chemical control part of these programs.

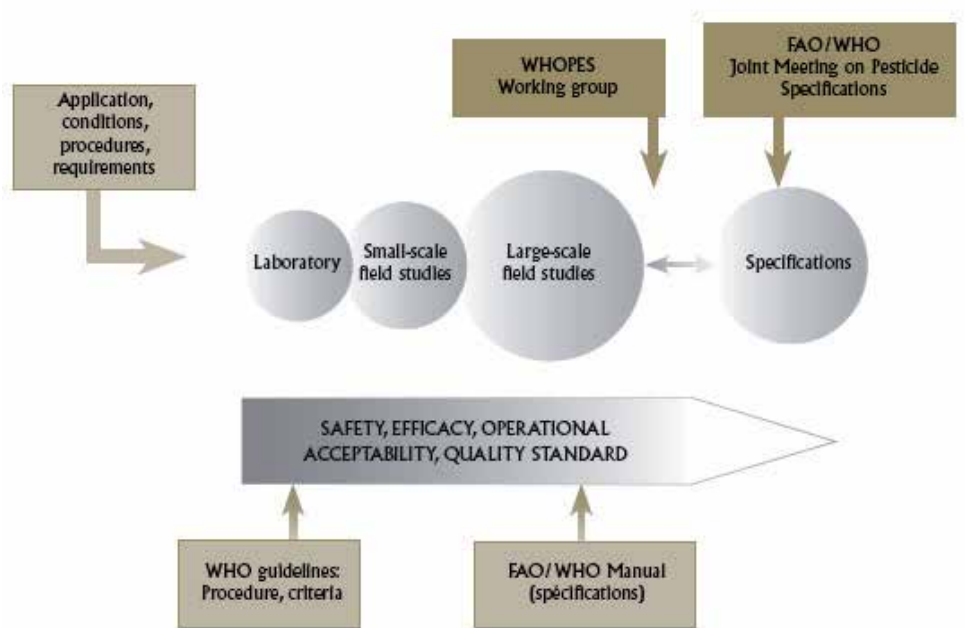

Fig. 4. World Health Organization Pesticide Evaluation Scheme (WHOPES).

5. Perspectives

In countries where synanthropic venomous species of spiders and scorpions that constitute a risk to public health are found, it is urgent the stablishment of a productive, long term form of interaction between the universities and the health managers in the design of bioassays in the laboratory and various small to large scale field studies in the field (Figure

4) [22, 192, 193]. One area still to be explored is the knowledge of mechanisms involved in determining the resistance of spiders and scorpions to pesticides used in urban areas, and to check the possible occurrence of cross resistance with pesticides eventually used in these areas to combat other pests like cockroaches and several species of vector mosquitoes (Dengue, West Nile virus, Malaria, Chagas disease, filariosis) [22]. As has occurred throughout history, in the future the control of spiders and scorpions can continue to benefit from techniques being developed for the control of insect pests and subsequently adapted to these organisms. It has been recognized that RNA interference (RNAi) might be used to ensure that only the target animals are killed, which can be effected by down-regulating the essential gene functions in arthropods [194]. This method may lead to the development of a new generation of species-specific pesticides. Overcoming the specific delivery of dsRNA or siRNA into the cytoplasm of the target cells is still an important issue in the use of RNAi for insecticides [194]. With the increase in the number of genome projects, the RNAi method will be more useful for genome-wide analyses of gene functions. We will then have access to a large pool of info on various insect and other invertebrate genes that may help on the control of arthropods in the real world [195]. Although the use of pheromones already constitutes a very well established area which is recognized for its effectiveness in the integrated control of many insect pests, it has never been used in the control of spiders and scorpions. In spite of ample behavioral evidence of the use of semiochemicals for many species of spiders and scorpions [2, 13, 21], the chemical ecology of the arachnids is still in its initial stage. Only some pheromones have been described for spiders [196] and in the case of scorpions, no substance has been definitively isolated as a semiochemical. However, the sensory apparatus of spiders and scorpions suggests that traps could attract the creatures to relatively short distances, without marked directionality to the olfactory stimulus. In the field of chemical control, several repellents are described for insects. Nonetheless, particularly in the case of scorpions, there are some substances that can act as important chemical barriers to their free passage and also prevent the reinfestation of risk areas. The lack of information and knowledge about the use of these techniques for spiders and scorpions is an impediment in the design of control strategies. In order to identify how the distribution of spiders and scorpions might change as a result of climate warming trend, and to plan vector control strategies, the ecological niche modelling technique can be used, as recently made for *L. reclusa* in the United States [90].

6. Conclusion

The presence of venomous scorpions and spiders in the urban environment can be regarded as a public health hazard that is still being neglected. It is necessary the construction of knowledge in a collaborative network with regard to several factors such as, for example, the biology of the targeted species, the determination of the most suitable molecules, and widely accepted predictive indices as trigger alerts of control measures. The use of microencapsulated formulas seems to be nowadays the most recommended targeting these arachnids, due to lower probability of significant repellency to the animals.

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8. References

[1] N. I. Platnick (2011). The World Spider Catalog, version 12.0. American Museum of Natural History. [Online]. Available: http://research.amnh.org/iz/spiders/catalog

[2] R. Foelix, *Biology of Spiders*. 3rd ed. New York: Oxford University Press, 2010.

[3] D. H. Wise, *Spiders in Ecological Webs*. 1st ed. Cambridge: Cambridge University Press, 1993.

[4] S. E. Riechert, and T. Lockley, "Spiders as Biological Control Agents," *Annual Review of Entomology,* vol. 29, pp. 299-320, Jan. 1984.

[5] S. A. Schultz and M. J. Schultz, *The Tarantula Keeper´s Guide*. 2nd ed. New York: Barron´s Educational Series, 2009.

[6] A. A. Vassilevski, S. A. Kozlov and E. V. Grishin, "Molecular Diversity of Spider Venom," *Biochemistry (Moscow*), vol. 74, no. 13, pp. 1505-1534, 2009.

[7] J. H. Diaz, "The Global Epidemiology, Syndromic Classification, Management and Prevention of Spider Bites," *American Journal of Tropical Medicine and Hygiene,* vol. 71, no. *2,* pp. 239–250, 2004.

[8] R. S. Vetter and G. K. Isbister, "Medical Aspects of Spider Bites,"*Annual Review of Entomology*, vol. 53, pp. 409–29, 2008.

[9] A. Battisti, G. Holm, B. Fagrell and S. Larsson, "Urticating Hairs in Arthropods: Their Nature and Medical Significance," *Annual Review of Entomology*, vol. 56, pp.203-220, Jan. 2011.

[10] J. A. Kluge, O. Rabotyagova and D. L. Kaplan. "Spider Silks and their Applications," *Trends in Biotechnology*, vol. 26, no.5, pp244-251, May 2008.

[11] M. A. Navarro-Silva, J. E .L. Duque, E. N. Ramires, C. F. S. Andrade, E. Marques-da-Silva, F. A. Marques, C. E. Delay, J. D. Fontana, A. C. S. Silva and G. M. Fraguas, "Chemical Control of *Loxosceles intermedia* (Araneae: Sicariidae) with Pyrethroids: Field and Laboratory Evaluation," *Journal of Economic Entomology*, vol.103, no 1, pp. 166-171, Feb. 2010.

[12] J. S. Sandidge, "Arachnology: Scavenging by Brown Recluse Spiders," *Nature,* vol. 426, no. 6962, p. 30, Nov. 2003.

[13] G. A. Polis, *The Biology of Scorpions*. Stanford: Stanford University Press, 1990.

[14] A. Mayor, *Greek Fire, Poison Arrows and Scorpion Bombs - Biological and Chemical Warfare in the Ancient World*. New York: The Overlook Press, 2008.

[15] L. Prendini and W. C. Wheeler, "Scorpion Higher Phylogeny and Classification, Taxonomic Anarchy, and Standards for Peer Review in Online Publishing," *Cladistics*, vol. 21, pp. 446–494, 2005.

[16] W. R. Lourenço, "The Scorpion Families and their Geographical Distribution," *Journal of Venomous Animals and Toxins*, vol. 7, no. 1, pp. 3-23, 2001.

[17] H. Keegan, *Scorpions of Medical Importance.* Jackson: University Press of Mississippi, 1980.

[18] J. P. Chippaux and M. Goyffon, "Epidemiology of Scorpionism: a Global Appraisal," *Acta Tropica*, vol. 107, no. 2, pp. 71-79, Aug. 2008.

[19] V. Fet, W. D. Sissom, G. J. Lowe and M. E. Braunwalder, *Catalog of the Scorpions of the World (1758-1998)*. New York: New York Entomological Society, 2000.

[20] V. Fet, B. Gantenbein, A. V. Gromov, G. Lowe and W. R. Lourenço, "The First Molecular Phylogeny of Buthidae (Scorpiones)," *Euscorpius,* vol. 4, pp1-10, Feb. 2003.

[21] P. Brownell and G. Polis, *Scorpion Biology and Research*. New York: Oxford University Press, 2001.

[22] World Health Organization, *WHO Pesticide Evaluation Scheme: 50 Years of Global Leadership*. Geneva, WHO Press, 2010.

[23] L. A. Ribeiro, M. T. Jorge, R. V. Piesco and S. A. Nishioka, "Wolf Spider Bites in São Paulo, Brazil: a Clinical and Epidemiological Study of 515 cases," *Toxicon*, vol .28, pp. 715–717, 1990.

[24] G. K. Isbister and M. R. Gray, "White-tail Spider Bite: a Prospective Study of 130 Definite Bites by *Lampona* Species," *Medical Journal of Australia*, vol. 179, pp. 199–202, 2003.

[25] G. K. Isbister, "Data Collection in Clinical Toxinology: Debunking Myths and Developing Diagnostic Algorithms," *Journal of Toxicology and Clinical Toxicology*, vol. 40, pp. 231–237, 2002.

[26] S. E. Riechert and T. Lockley, "Spiders as Biological Control Agents," *Annual Review of Entomology,* vol. 29, pp. 299-320, Jan. 1984.

[27] M. Alderweireldt, "Habitat Manipulations Increasing Spider Densities in Agroecosystems: Possibilities for Biological Control," *Journal of Applied Entomology,* vol. 118, pp. 10–16, 1994.

[28] D. M. Amalin, J. E. Peña, S. J. Yu and R. McSorley, "Selective Toxicity of Some Pesticides to *Hibana Velox* (Araneae: Anyphaenidae), a Predator of Citrus Leafminer," *Florida Entomologist,* vol. 83, no. 3, pp. 254-262, Sep. 2000.

[29] F. Mansour, "A Malathion-Tolerant Strain of the Spider *Cheiracanthium mildei* and its Response to Chlorpyrifos," *Phytoparasitica,* vol 12, no. 3-4, pp. 163-166, 1984.

[30] F. A.Mansour, K. R. A. Ascher and F. Abo-moch, "Effects of Margosan-o™, Azatin™ and RD9-Repelin® on Spiders, and on Predacious and Phytophagous mites," *Phytoparasitica,* vol 21, no. 3, pp. 205-211, 1993.

[31] J. D. Stark, P. C. Jepson and C. F. G.Thomas, "The Effects of Pesticides on Spiders from the Lab to the Landscape," *Reviews in Pesticide Toxicology*, vol. 3, pp. 83-110, 1995.

[32] W. J. Tietjen, "Pesticides Affect the Mating Behavior of *Rabidosa rabida* (Araneae, Lycosidae)," *The Journal of Arachnology*, vol 34, pp. 285–288, 2006.

[33] W. J. Tietjen and A. B. Cady, "Sublethal Exposure to a Neurotoxic Pesticide Affects Activity Rhythms and Patterns of Four Spider Species", *The Journal of Arachnology,* vol. 35, pp. 396–406, 2007.

[34] B. J. Valentine, *G. M.* Gurr and W. G. Thwaite, "Efficacy of the Insect Growth Regulators Tebufenozide and Fenoxycarb for Lepidopteran Pest Control in Apples, and their Compatibility with Biological Control for Integrated Pest Management," *Australian Journal of Experimental Agriculture*, vol. 36, no. 4, pp. 501-506, 1996.

[35] S. Pekar, "Foraging Mode: a Factor Affecting the Susceptibility of Spiders (Araneae) to Insecticide Applications," *Pesticide Science*, vol. 55, pp. 1077-1082, 1999.

[36] R. S. Davis and R. K. D. Peterson, "Effects of Single and Multiple Applications of Mosquito Insecticides on Nontarget Arthropods," *Journal of the American Mosquito Control Association*, vol. 24, no. 2, pp. 270–280, 2008.

[37] F. Bucaretchi, C. R. R. Deus, S. Hyslop, P. R. Madureira, E. M. De Capitani and R. J. Vieira, "A Clinico-Epidemiological Study of Bites by Spiders of the Genus *Phoneutria*," *Revista do Instituto de Medicina Tropical de São Paulo*, vol. 42, pp.17–21, 2000.

[38] T. Hsu, (2011, March 03). Mazda Recalls 65,000 Cars for Spider Problem. *Los Angeles Times* [Online] Available: http://articles.latimes.com/2011/mar/03/business/la-fimazda-spider-20110304

[39] P. Hernandez, "Population Dynamics and Insecticidal Control of the Black Widow Spider *Latrodectus Hesperus* (Araneae: Theriididae) From California's San Joaquin Valley Table Grape Vineyards," MSc. Thesis, College of Agricultural Sciences and Technology, California State University, 1996.

[40] G. Wycherley (2001, November 7). Black Widow Threat Forces Grapes Ban. *The New Zealand Herald* [Online] Available: http://www.nzherald.co.nz/nz/news/article.cfm?c_id=1&objectid=226988

[41] A. Martínez, J. Martínez, R. Palacios and R. Panzani, "Importance of Tropomyosin in the Allergy to Household Arthropods. Cross-reactivity with other Invertebrate Extracts,"*Allergologia et Immunopathologia*, vol. 25, no. 3, pp. 118-126, 1997.

[42] T. Hasan, S. Mäkinen-Kiljunen, H. Brummer-Korvenkontio, T. Pajunen and T. Reunala, "Occupational IgE-Mediated Allergy to a Common House Spider (*Tegenaria domestica*)," *Allergy*, vol. 60, pp. 1455–1457, 2005.

[43] I. Bobolea, P. Barranco, C. Pastor-Vargas, V. Iraola, F. Vivanco and S. Quirce, "Arginine Kinase from the Cellar Spider (*Holocnemus pluchei*): A New Asthma-Causing Allergen," *International Archives of Allergy and Immunology,* vol. 155, no.2, pp. 180-186, 2011.

[44] Z. Maretic and D. Lebez, *Araneism with Special Reference to Europe*. Ljubljana: Nolit Publishing, 1979.

[45] J. E. Garb, A. Gonzalez and R. G. Gillespie,"The Black Widow Spider Genus *Latrodectus* (Araneae: Theridiidae): Phylogeny, Biogeography, and Invasion History," *Molecular Phylogenetics and Evolution,* vol. 31, pp. 1127–1142, 2004.

[46] M. Reyes-Lugo, T. Sánchez, H. J. Finol, E. E. Sánchez, J. A. Suárez, B. Guerrero and A. Rodríguez-Acosta, "Neurotoxic Activity and Ultrastructural Changes in Muscles Caused by the Brown Widow Spider *Latrodectus geometricus* Venom," *Revista do Instituto de Medicina Tropical de São Paulo*, vol. 51, no. 2, pp. 95-101, 2009.

[47] G. K. Isbister, "Acute Conjunctival Inflammation Following Contact with Squashed Spider Contents," ," *American Journal of Ophthalmology,* vol. 136, pp. 563–564, 2003.

[48] D. C. Buffkin, F. E. Russell and A. Deshmukh, "Preliminary Studies on the Toxicity of Black Widow Spider Eggs." *Toxicon,* vol.9, pp. 393–402, 1971.

[49] A. Rohou, J. Nield and Y.A. Ushkaryov, "Insecticidal Toxins from Black Widow Spider Venom," *Toxicon,* vol. 49, no. 4-5, pp. 531–549, Mar.2007.

[50] G. J Muller, "Black and Brown Widow Spider Bites in South Africa – a Series of 45 Cases," *South African Medical Journal,* vol. 83, pp. 399–405, 1993.

[51] J. A. Hódar and F. Sánchez-Piñero, "Feeding Habits of the Black Widow Spider *Latrodectus lilianae* (Araneae: Theridiidae) in an Arid Zone of South-East Spain," *Journal of Zoology (London)*, vol. 257, pp. 101–109, 2002.

[52] W. J. Gertsch, *American Spiders*. New York: DeVan Nostrand Inc., 1949.

[53] G. K. Isbister and M. R. Gray, "Effects of Envenoming by Comb-footed Spiders of the Genera *Steatoda* and *Achaearanea* (family Theridiidae: Araneae) in Australia," *Journal of Toxicology and Clinical Toxicology*, vol. 41, pp. 809-819, 2003.

[54] B. J. Kaston, "Comparative Biology of American Black Widow Spiders," *Transactions of the San Diego Society of Natural History,* vol. 16, pp. 33–82, 1970.

[55] H. T. Kirby-Smith, "Black Widow Spider Bite," *Annals of Surgery,* vol. 115, pp 249–257, 1942.

[56] G. K. Isbister and M. R. Gray, "Latrodectism: a Prospective Cohort Study of Bites by Formally Identified Redback Spiders, "*Medical Journal of Australia,* vol. 179, pp. 88–91, 2003.

[57] R. M. Lira-da-Silva, G. B. Matos, R. O. Sampaio and T. B. Nunes, "Estudo Retrospectivo de Latrodectismo na Bahia, Brasil," *Revista da Sociedade Brasileira de Medicina Tropical,* vol. 28, pp. 205–210, 1995.

[58] L. W. Pinter, "The Widow Spiders of Hawaii," *Proceedings of the Third Conference in Natural Sciences Hawaii Volcanoes National Park, 1980,* p. 265.

[59] H. Ono, "Records of *Latrodectus geometricus* (Araneae: Theridiidae) from Japan," *Acta Arachnologica*, vol.44, pp.167–170, 1995.

[60] L. M. Forster, "The Stereotyped Behaviour of Sexual Cannibalism in *Latrodectus hasselti* Thorell (Araneae: Theridiidae), the Australian Redback Spider," *Australian Journal of Zoology*, vol. 40, pp. 1–11, 1992.

[61] C. E. Pemberton, "History of the Entomology Department Experiment Station, H.S.P.A. 1904-1945," *Hawaiian Planters' Record*, vol. 52, pp. 53-90, 1948.

[62] W. D. Pierce, "Utilization of the Black Widow Parasite, and Further Data on Spiders and Parasites," *Southern California. Academy of Sciences Bulletin*, vol. 41, pp. 14-28, 1942.

[63] F. A Bianchi, "Notes on the Abundance of the Spiders *Lactrodectus mactans*, *L. geometricus* and *Argiope avara*, and of their Parasites on the Island of Hawaii, " *Hawaiian Entomological Society Proceedings*, vol. 12, pp. 245-24, 1945.

[64] H. L. Sweetman, *The Principles of Biological Control*. Dubuque: Brown Publishers, 1958.

[65] Y. Liu, K. M. Daane, J. S. Tebbets and L. J. Bettiga, "Ultralow Oxygen Treatment for Control of *Latrodectus hesperus* (Araneae: Theridiidae) on Harvested Table Grapes," *Journal of Economic Entomology*, vol. 101, no. 5, pp. 1515-1518, 2009.

[66] R. Vetter, P. O'Connor-Marer, E. Mussen, L. Allen, K. Daane, G. Hickman, A. Slater, P. Phillips and R. Hanna (2001). *Pest Notes – Spiders, Publication 7442*. University of California Division of Agriculture and Natural Resources. [Online]. Available: http://www.ipm.ucdavis.edu

[67] R. S. Vetter, C. P. Flanders and M. K. Rust, "The Ability of Spiderlings of the Widow Spider *Latrodectus hesperus* (Araneae: Theridiidae) to Pass Through Different Size Mesh Screen: Implications for Exclusion from Air Intake Ducts and Greenhouses," *Journal of Economic Entomology*, vol. 102, no. 3, pp. 1396-1398, Jun. 2009.

[68] J. D. Hopkins, G. Lorenz, G. Studebaker and K. M. Loftin. *Biology and Control of Spiders, Scorpions, Centipedes and Millipedes - FSA7018*. University of Arkansas, Divivision of Agriculture, Coopertive Extension service. [Online]. Available: www.uaex.edu/other_areas/publications/pdf/fsa-7018.pdf

[69] M. M. Gaver and L. D. Hansen, "Efficacy of Insecticides to Control T*egenaria agrestis* and *Latrodectus hesperus* (Araneae: Agelenidae and Theridiidae). *Proceedings of the Fifth International Conference on Urban Pests,* 2005, pp.375-379.

[70] H. D. Cameron, "An Etymological Dictionary of North American Spider Genus Names." In *Spiders of North America: an Identification Manual*, American Arachnological Society, 2005, pp. 274–330.

[71] W. J. Gertsch and F. Ennik, "The Spider Genus *Loxosceles* in North America, Central America and the West Indies (Araneae Loxoscelidae)," *Bulletin of the American Museum of Natural History,* vol. 175, pp. 264– 360, 1983.

[72] P. Gnaspini and E. Trajano, "Brazilian Cave Invertebrates with a Checklist of Troglomorphic Taxa,". *Revista Brasileira de Entomologia,* vol. 38, pp. 549-584, 1994.

[73] R. M. Gonçalves-de-Andrade, F. D. Pretel and D.V. Tambourgi, " The Spider *Loxosceles adelaida* Gerstch, 1967 (Araneae, Sicariidae) in the Karstic Area of Ribeira Valley, PETAR, São Paulo, Brazil,"*Journal of Entomology,* vol. 4, no. 1, pp. 46-50, 2007.

[74] F. Pretel, R. M. Gonçalves-de-Andrade, F.C. Magnoli, M. E. da Silva, J. M. Jr. Ferreira, C.W. van den Berg and D. V. Tambourgi, "Analysis of the Toxic Potential of Venom from *Loxosceles adelaida*, a Brazilian Brown Spider from Karstic Areas," *Toxicon*, vol. 45, no. 4, pp.449-458, Mar. 2005.

[75] F. M. Eskafi, J. L. Frazier, R. R. Hocking and B.R. Norment, "Influence of Environmental Factors on Longevity of the Brown Recluse Spider," *Journal of Medical Entomology*, vol. 14, pp. 221–228, 1977.

[76] E. N. Ramires, "Produção de Fios de Seda em Forma de Fita em Aranhas do Gênero *Loxosceles* (Sicariidae)," *Tuiuti: Ciência e Cultura*, vol. 15, pp. 12-16, Fev. 2000.

[77] E. N. Ramires (2001, Mar.). "Ribbon-threads" in the Webs of Brown Spiders (*Loxosceles* spp). *Micscape Magazine*. [Online] pp. 1-3. Available: http://www.microscopy-uk.org.uk/mag//artmar02/erspidersilk.html

[78] J. A. Coddington, H. D. Chanzy, C. L. Jackson, G. Raty and K. H. Gardner, "The Unique Ribbon Morphology of the Major Ampullate Silk of Spiders from the Genus *Loxosceles* (Recluse spiders)," *Biomacromolecules,* vol. 3, pp. 5–8, 2002.

[79] G. J. Binford, M. R. Bodner, M. H. J. Cordes, K. L. Baldwin, M. R. Rynerson, S. N. Burns and P. Zobel-Thropp, "Molecular Evolution, Functional Variation and Proposed Nomenclature of the Gene Family that Includes Sphingomyelinase D in Sicariid Spider Venoms, " *Molecular Biology and Evolution,* vol. 26, no. 3, pp. 547-566, 2009.

[80] A. Senff-Ribeiro, P. Henrique da Silva, O. M. Chaim, L. H. Gremski, K. S. Paludo, R. Bertoni da Silveira, W. Gremski, O. C. Mangili and S.S. Veiga , "Biotechnological

Applications of Brown Spider (*Loxosceles* genus) Venom Toxins," *Biotechnology Advances*, vol. 26, no.3, pp. 210-218, 2008.

[81] A. V. Macchiavello, "La *Loxosceles laeta* Causa de Aracnoidismo Cutaneo o Mancha Gangrenosa de Chile," *Revista Chilena de Historia Natural,* vol. 41, pp. 11-19, 1937.

[82] W. Bücherl, " Aranhas do Gênero *Loxosceles* e Loxoscelismo na América," *Ciência e Cultura,* vol. 13, pp. 213-224, 1961.

[83] S. W. Wright, K. D. Wrenn, L. Murray L and D. Seger, "Clinical Presentation and Outcome of Brown Recluse Spider Bite," *Annals of Emergency Medicine,* vol. 30, pp. 28–32, 1997.

[84] E. Marques-da-Silva, R. Souza-Santos, M. L. Fischer and G. B. G. Rubio, "*Loxosceles* Spider Bites in the State of Paraná, Brazil: 1993-2000," *Journal of Venomous Animals and Toxins Including Tropical Disea*ses, vol. 12, pp. 110-123, 2006.

[85] H. Schenone , T. Saavedra, A. Rojas and F. R. Villarroel, "Loxoscelism in Chile. Epidemiologic, Clinical and Experimental Studies," *Revista do Instituto de Medicina Tropical de São Paulo*, vol. 31, no. 6, pp. 403-415, 1989.

[86] K. C. de Oliveira, R. M. Gonçalves de Andrade, R. M. Piazza, J. M. Jr. Ferreira, C. W. van den Berg and D. V. Tambourgi, "Variations in *Loxosceles* Spider Venom Composition and Toxicity Contribute to the Severity of Envenomation," *Toxicon*, vol. 45, no. 4, pp. 421-429, Mar. 2005.

[87] R. S. Vetter, "Spiders of the genus *Loxosceles* (Araneae, Sicariidae): a Review of Biological, Medical and Psychological Aspects Regarding Envenomations," *The Journal of Arachnology,* vol. 36, pp. 150–163, 2008.

[88] I. Pauli, J. C. Minozzo , P. H. da Silva, O. M. Chaim and S. S. Veiga, "Analysis of Therapeutic Benefits of Antivenin at Different Time Intervals after Experimental Envenomation in Rabbits by Venom of the Brown Spider (*Loxosceles intermedia*)," *Toxicon*, vol. 53, no. 6, pp. 660-671, May 2009.

[89] D. M. de Almeida, M. F. Fernandes-Pedrosa, R. M. de Andrade, J. R. Marcelino, H. Gondo-Higashi H, I. L. de Azevedo, P. L. Ho, C. van den Berg C and D. V. Tambourgi, "A New Anti-Loxoscelic Serum Produced Against Recombinant Sphingomyelinase D: Results of Preclinical Trials," *American Journal of Tropical Medicine and Hygiene*, vol. 79, no. 3, pp. 463-470, Sep. 2008.

[90] E. E. Saupe, M. Papes, P. A. Selden and R. S. Vetter, "Tracking a Medically Important Spider: Climate Change, Ecological Niche Modeling, and the Brown Recluse (*Loxosceles reclusa*)," *PLoS ONE,* vol. 6, no. 3, pp. 17-31, 2011.

[91] R. S. Vetter and D. K. Barger, "An Infestation of 2055 Brown Recluse Spiders (Araneae: Sicariidae) and no Envenomations in a Kansas Home: Implications for Bite Diagnoses in Nonendemic Areas," *Journal of Medical Entomology*, vol. 39, pp. 948–51, 2002.

[92] R. V. Southcott, "Spiders of the Genus *Loxosceles* in Australia," *Medical Journal of Australia,* vol. 1, pp. 406–408, 1976.

[93] P. Cardoso and A. P. G. Almeida, "Envenenamento por Aranhas em Portugal Verdade ou Mito?,"*Acta Medica Portuguesa*, vol. 23, pp. 33-38, 2010.

[94] C. Pernet, M. Dandurand, L. Meunier and P. E. Stoebner, "Necrotic Arachnidism in the South of France: Two Clinical Cases of Loxoscelism," *Annals of Dermatology and Venereology,* vol. 137, no.12, pp.808-821, Dec. 2010.

[95] M. P. Stefanidou, M. Chatzaki, K. G. Lasithiotakis, D. J. Ioannidou and A. D. Tosca, "Necrotic Arachnidism from *Loxosceles rufescens* Harboured in Crete, Greece,".*Journal of the European Academy of Dermatology and Venereology*, vol. 20, no. 4, pp. 486-487, Apr. 2006.

[96] P. Dyachenko, M. Ziv and D. J. Rozenman, "Epidemiological and Clinical Manifestations of Patients Hospitalized with Brown Recluse Spider Bite," *Journal of the European Academy of Dermatology and Venereology,* vol. 20, no. 9, pp. 1121-1125, Oct. 2006.

[97] G. K. Isbister and M. R. Gray, "A Prospective Study of 750 Definite Spider Bites, with Expert Spider Identification," *Quartertly Journal of Medicine,* vol. 95, pp. 723–731, 2002.

[98] G. Newlands and P. Atkinson, "Behavioural and Epidemiological Considerations Pertaining to Necrotic Araneism in Southern Africa," *South African Medical Journal,* vol. 77, pp. 92–95, 1990.

[99] A. R. De Roodt, O. D. Salomón, S.C. Lloveras and T. A. M. Orduna, "Poisoning by Spiders of *Loxosceles* Genus," *Medicina (Buenos Aires),* vol. 62, no. 1, 83-94, 2002.

[100] J. C. Ríos, M. Pérez, P. Sánchez, M. Bettini, J. J. Mieres and E. Paris, "Prevalence and Epidemiology of *Loxosceles laeta* Bite: Analysis of Consultations to a Poison Control Center," *Revista Medica de Chile*, vol. 135, no. 9, pp. 1160-1165, Nov. 2007.

[101] J. J. M. Moreno and S. V. Silva, "Loxoscelismo Cutáneo y Cutáneo-Visceral: Revisión Sistemática. *Revista Chilena de Infectología,* vol. 26, n.5, pp. 420-432, 2009.

[102] E. Marques-da-Silva and M. L. Fischer, "Distribuição das Espécies do Gênero *Loxosceles* Heinecken & Lowe, 1835 (Araneae: Sicariidae) no Estado do Paraná,'´ *Revista da Sociedade Brasileira de Medicina. Tropical*, vol. 38, pp. 331-335, 2005.

[103] M. L. Fischer and J. Vasconcellos-Neto, "Microhabitats Occupied by *Loxosceles intermedia* and *Loxosceles laeta* (Araneae: Sicariidae) in Curitiba, Paraná, Brasil," *Journal of Medical Entomology*, vol. 42, pp. 756-765, 2005.

[104] U. M. Sezerino, M. Zannin, L. K. Coelho, J. Gonçalves Júnior, M. Grando , S. G. Mattosinho, J. L. Cardoso, V. R. von Eickstedt, F. O. França, K. C. Barbaro and H. W. Fan, " A Clinical and Epidemiological Study of *Loxosceles* Spider Envenoming in Santa Catarina, Brazil," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 92, no. 5, pp. 546-48, 1998.

[105] M. P. Cristiano, D. C. Cardoso and M. S. Raymundo, "Contextual Analysis and Epidemiology of Spider Bite in Southern Santa Catarina State, Brazil," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 103, no. 9, 943-948, Sep. 2009.

[106] E. N. Ramires, "Uma Abordagem Comparativa ao Comportamento Defensivo, Agonístico e Locomotor de Três Espécies de Aranhas do Gênero *Loxosceles* (Sicariidae)," PhD. Thesis, Institute of Psychology, São Paulo University, 1999.

[107] R. J. Elzinga, "Observations on the Longevity of the Brown Recluse Spider, *Loxosceles reclusa* Gertsch & Mulaik," *Journal of the Kansas Entomological Society*, vol. 50, pp. 187–188, 1977.

[108] D. C. Lowrie, "Starvation Longevity of *Loxosceles laeta* (Nicolet) (Araneae)," *Entomological News*, vol. 91, pp. 130–132, 1980.

[109] D. C. Lowrie, "Effects of Diet on the Development of *Loxosceles laeta* (Nicolet) (Araneae, Loxoscelidae)," *The Journal of Arachnology*, vol. 15, pp. 303-308, 1987.

[110] M. L. Fischer and J. Vasconcellos-Neto, "Development and Life Tables of *Loxosceles intermedia* Mello-Leitão 1934 (Araneae, Sicariidae)," *The Journal of Arachnology*, vol. 33, pp. 758–766, 2005.

[111] M. L Fischer and J. Vasconcellos-Neto, "Parameters Affecting Fecundity of *Loxosceles intermedia* Mello-Leitão 1934 (Araneae, Sicariidae)," *The Journal of Arachnology,* vol. 33, pp. 670–680, 2005.

[112] J. M. Hite, W. J. Gladney, J. L. Lancaster and W.H.Whitcomb, "The Biology of the Brown Recluse Spider," *University of Arkansas, Fayetteville Agricultural Experiment Station Bulletin* No. 711. 26 pp. 1966.

[113] M. E. Galiano, "Ciclo Biológico y Desarollo de *Loxosceles laeta* (Nicolet, 1849)," *Acta Zoologica Lilloana* vol. 23, pp. 431–464, 1967.

[114] I. M. P. Rinaldi, L.C. Forti and A.A. Stropa, "On the Development of the Brown Spider *Loxosceles gaucho* Gertsch (Araneae, Sicariidae): the Nympho-Imaginal Period," *Revista Brasileira de Zoologia,* vol. 14, pp. 697–706, 1997.

[115] J. S. Sandidge, "Predation by Cosmopolitan Spiders on the Medically Significant Pest Species *Loxosceles reclusa* (Araneae: Sicariidae): Limited Possibilities for Biological Control," *Journal of Economic Entomology,* vol. 97, pp. 230–234, 2004.

[116] H. Schenone, A. Rojas, H. Reyes, F. Villarroel and G. Suarez, "Prevalence of *Loxosceles laeta* in Houses in Central Chile," *American Journal of Tropical Medicine and Hygiene*, vol. 19, pp. 564–567, 1970.

[117] M. L Fischer, "Utilização do Habitat por *Loxosceles intermedia* Mello-Leitão, 1934 e *L. laeta* (Nicolet, 1849) no Município de Curitiba, PR: uma Abordagem Experimental sobre Aspectos Ecológicos e Comportamentais," PhD. Thesis, Zoology Department, Federal University of Paraná, 2002.

[118] M. L. Fischer, A. Cokl, E. N. Ramires, E. Marques-da-Silva, C. Delay, J. D. Fontana, L. Donatti, V. F. Schneider and F. A. Marques, "Sound is Involved in Multimodal Communication of *Loxosceles intermedia* Mello-Leitão, 1934 (Araneae; Sicariidae)," *Behavioral Processes,* vol. 82, no. 3, pp. 236-43, 2009.

[119] A. A. Stropa and I.M.P. Rinaldi, "Relative Tolerance and Communication in Agonistic behaviour Between Females of *Loxosceles gaucho* (Araneae, Sicariidae), " *Bulletin of the British Arachnological Society*, vol. 12, pp. 41–45, 2001.

[120] A. A. Stropa, "Social Encounters between Male Brown Spiders, *Loxosceles gaucho* (Araneae, Sicariidae)," *The Journal of Arachnology*, vol. 35, pp. 493–498, 2007.

[121] R. S.Vetter and M. K. Rust, "Refugia Preferences by the Spiders *Loxosceles reclusa* and *Loxosceles laeta* (Araneae: Sicariidae)," *Journal of Medical Entomology*, vol. 45, pp. 36–41, 2008.

[122] K. L. Cramer and A.V. Mayright, "Cold Temperature Tolerance and Distribution of the Brown Recluse Spider *Loxosceles reclusa* (Araneae, Sicariidae) in Illinois," *The Journal of Arachnology,* vol. 36, pp. 136–139, 2008.

[123] M. L. Fischer and J. Vasconcellos-Neto, "Determination of the Maximum and Minimum Lethal Temperatures (LT_{50}) for *Loxosceles intermedia* Mello-Leitão, 1934 and *L. laeta* (Nicolet, 1849) (Araneae, Sicariidae)," *Journal of Thermal Biology*, vol. 28, no. 8, pp. 563-570, Nov. 2003.

[124] R. S. Vetter, M. K. Rust, "Influence of Spider Silk on Refugia Preferences of the Recluse Spiders *Loxosceles reclusa* and *Loxosceles laeta* (Araneae: Sicariidae)," *Journal of Economical Entomology*, vol. 103, no. 3, pp. 808-815, Jun. 2010.

[125] J. S. Sandidge, "Scavenging in Brown Recluse Spiders," *Nature*, vol. 426, p. 30, 2003.

[126] K. L. Cramer, "Are Brown Recluse Spiders, *Loxosceles reclusa* (Araneae, Sicariidae) Scavengers? The Influence of Predator Satiation, Prey Size, and Prey Quality," *The Journal of Arachnology*, vol. 36, no. 1, pp. 140-144, 2008.

[127] R. D. Hall and P. C. Anderson, "Brown Recluse Spider Bites: Can they be Prevented?," *Missouri Medicine,* vol. 78, no.5, pp. 243–245, 1981.

[128] R. J. Gorham, "The Brown Recluse Spider *Loxosceles reclusa* and Necrotic Spider Bite - a New Public Health Problem in the United States," *Journal of Environmental Health,* vol. 31, pp. 138–145, 1968.

[129] H. W. Levi and A. Spielmann, "The Biology and Control of the South American Brown Spider *Loxosceles laeta* (Nicolet) in a North American Focus," *American Journal of Tropical Medicine and Hygiene*, vol. 13, pp.132-136, 1964:

[130] J. Paige, "Brown Recluse, a Dangerous but Shy Insect, "*Pest Control* vol. 55, no. 5, p.52, May 1987.

[131] B. Hebert, "Ouch," *Pest Control Technology*, pp. 72-74, Oct, 1993.

[132] C. W. Wingo, "The status of *Loxosceles reclusa,* the Brown Recluse Spider, as a Public Health Problem," *Proceedings of the North Central States Branch Entomological Society of America,* vol. 19, pp. 15-18, 1964.

[133] R. B. Norment and and T. L. Pate, "Residual Activity of Diazinon and Lindane for Control of *Loxosceles reclusa," Journal of Economical Entomology*, vol. 61, pp 574-575, 1968.

[134] W. J. Gladney and C. C. Dawkins, "Insecticidal Tests against the Brown Recluse Spider," *Journal of Economical Entomology*, vol. 65, pp 574-575, 1972.

[135] J. S. Sandidge and J. L. Hopwood, "Brown Recluse Spiders: a Review of Biology, Life History and Pest Management," *Transactions of the Kansas Academy of Sciences*, vol. 108, pp. 99-108, 2005.

[136] J. S. Sandidge, *Brown Recluse Spiders. A Knowledge Based Guide to Control and Elimination*. Author´s edition, 2009.

[137] A. Catalán, J. E. Araya, H. Varela, W. Cortés, H. Sagua and J. González, "Effectiveness of a Repellent Paint against the Spider *Loxosceles laeta," Revista Médica de Chile*, vol. 137, pp. 240-245, 2009.

[138] E. N. Ramires and G. M. Fraguas, "Tropical House Gecko (*Hemidactylus mabouia*) Predation on Brown Spiders (*Loxosceles intermedia*)," *Journal of Venomous Animals and Toxins Including Tropical Diseases*, vol. 10, pp. 185-190, 2004.

[139] J. Sandidge, "Predation by Cosmopolitan Spiders upon the Medically Significant Pest Species *Loxosceles reclusa* (Araneae: Sicariidae): Limited Possibilities for Biological Control," *Journal of Economic Entomology*, vol. 97, no. 2, pp. 230-234, Apr. 2004.

[140] E. N. Ramires, A. V. L. Retzlaff, L. R. Deconto, J. D. Fontana, F. A. Marques and E. Marques-da-Silva, "Evaluation of the Efficacy of Vacuum Cleaners for the Integrated Control of Brown Spider *Loxosceles intermedia," Journal of Venomous Animals and Toxins Including Tropical Diseases*, vol. 13, pp. 607-619, 2007.

[141] C. Zamprogno and R. L. C., Teixeira, "Hábitos Alimentares da Lagartixa-de-Parede *Hemidactylus mabouia* (Reptilia, Gekkonidae) da Planície Litorânea do Norte do Espirito Santo, Brasil," *Revista Brasileira de Biologia,* vol. 58, pp. 143-150, 1998.

[142]] L. R. Pinto, F. Mendonça and W. M. Araujo, "A Influência das Variações Térmicas nos Acidentes Loxoscélico em Curitiba/PR," *Revista Brasileira de Climatologia*, vol. 5, pp. 1-5, Sep. 2009.

[143] World Health Organization, *Rabies and Envenoming: A Neglected Public Health Issue*. Geneva, WHO Press, 2007.

[144] H. S. Bawaskar and P. H. Bawaskar, "Utility of Scorpion Antivenin vs Prazosin in the Management of Severe *Mesobuthus tamulus* (Indian Red Scorpion) Envenoming at Rural Setting," *Journal of the Association of Physicians of India*, vol. 55, pp. 14-21, Jan. 2007.

[145] N. Mansour, "Délai et Caractéristiques de la Prise en Charge des Piqués par Scorpion dans la Région de Sidi-Bouzid," *Archives de l'Institut. Pasteur deTunis*, vol. 78, pp. 25–31, 2001.

[146] M. H. Pipelzadeh, A. Jalali, M. Taraz, R. Pourabbas and A. Zaremirakabadi, "An Epidemiological and a Clinical Study on Scorpionism by the Iranian Scorpion *Hemiscorpius lepturus,*" *Toxicon*, vol. 50, pp. 984–992. 2007.

[147] R. B. Soulaymani, Z. Faraj, I. Semlali, L. Ouammi and M. Badri, "National Strategy in the Battle Against Scorpion Stings and Envenomations. Application and Evaluation," *Bulletin de la Societé de Pathologie Exotique*, vol. 96, no.4, pp. 317-319, Nov. 2003.

[148] M. K. Al-Sadoon and B. M. Jarrar, "Epidemiological Study of Scorpion Stings in Saudi Arabia Between 1993 and 1997," *Journal of Venomous Animals and Toxins Including Tropical Diseases*, vol. 9, pp. 54-64, 2003.

[149] O. Ozkan, S. Adigüzel, S. Yaki_Tiran, Y. Cesaretli, M. Orman and K. Z. Karaer, "*Androctonus crassicauda* (Olivier 1807) Scorpionism in the Sanliurfa Provinces of Turkey," *Turkiye Parazitolojii Dergisi*, vol. 30 pp. 239–245, 2006.

[150] A. C. Benguedda, F. Laraba-Djébari, M. Ouahdi, H. Hellal, L. Griene, M. Guerenik and Y. Laid, "C.N.L.E.S., 2002. Expérience de Quinze Années de Lutte contre l'Envenimation Scorpionique en Algerie, " *Bulletin de la Societé de Pathologie Exotique*, vol. 95, pp. 205-208, 2002.

[151] Brasil, *Manual de Controle de Escorpiões.* Ministério da Saúde, Secretaria de Vigilância em Saúde, Departamento de Vigilância Epidemiológica – Brasília: Ministério da Saúde, 2009.

[152] O. D. Salomón and A.R. de Roodt, "Escorpiones: Denuncia Espontánea en Dos Centros de Referencia en la Ciudad de Buenos Aires, 1997–2000," *Medicina (Buenos Aires)*, vol. 61, pp. 391–396, 2001.

[153] A. Borges, F. J. Rojas-Runjaic, N. Diez, J. G. Faks, H. J. Op den Camp and L. De Sousa, "Envenomation by the Scorpion *Tityus breweri* in the Guayana Shield, Venezuela: Report of a Case, Efficacy and Reactivity of Antivenom, and Proposal for a Toxinological Partitioning of the Venezuelan Scorpion Fauna.," *Wilderness & Environmental Medicine*, vol. 21, no. 4, pp. 282-290, Dec. 2010.

[154] J. C. Quintana, P. Arbeláez, J. Fernández, J. F. Silva, J. Barona, J. C. Gutiérrez, A. Díaz, R. Otero and J. P. Gómez, "*Tityus asthenes* Scorpion Stings: Epidemiological, Clinical and Toxicological Aspects, " *Biomedica*, vol. 30, no. 1, pp. 126-139, Jan. 2010.

[155] G. K. Isbister, E. S. Volschenk and J. E. Seymour, "Scorpion Stings in Australia: Five Definite Stings and a Review," *Internal Medicine Journal*, vol. 34, no. 7, pp. 427-430, Jul. 2004.

[156] L. Mazotti, "Medidas Complementarias en Relación con la Protección Mecánica de los Edificios contra los Alacranes," *Revista del Instituto de Salubridad y Enfermedades Tropicales*, vol. 24, no. 1-4., 1964.

[157] A. Celis, R. Gaxiola-Robles, E. Sevilla-Godínez, M. J Orozco Valerio and J. Armas, "Trends in Mortality from Scorpion Stings in Mexico, 1979-2003," *Revista Panamericana de Salud Publica.*, vol. 21, no. 6, 373-380, 2007.

[158] G. Chowell, P. Díaz-Dueñas, R. Bustos-Saldaña,A. Alemán Mireles and V. Fet, "Epidemiological and Clinical Characteristics of Scorpionism in Colima, Mexico (2000–2001)," *Toxicon*, vol. 47, no. 7, pp. 753-758, 2006.

[159] G. Chowell, J. M. Hyman, P. Díaz-Dueñas and N. W. Hengartner, "Predicting Scorpion Sting Incidence in an Endemic Region Using Climatological Variables. *International Journal of Environmental Health Research*, vol. 15, no.6, pp. 425-435, Dec. 2005.

[160] E. Dias, S. Libanio and M. Lisbôa, "Lucta Contra os Escorpiões," *Memórias do Instituto Oswaldo Cruz*, vol. 17, no. 1, pp. 5-26, 1924.

[161] M. A. Bravo-Becherelle and N. Arizmendi, "Valoración de la Protección Mecánica de las Casas Contra la Entrada de lo Alcaranes, " *Época*, vol. 9, pp. 209-211, 1967.

[162] J. M. Ramsey, L. Salgado, A. Cruz-Celis, R. Lopez, A. L. Alvear and L. Espinosa,"Domestic Scorpion Control With Pyrethroid Insecticides in Mexico," *Medical and Veterinary Entomology*, vol.16, no 4, pp. 356-363, 2002.

[163] Norma Oficial Mexicana NOM-033-SSA2-2002, Para la Vigilancia, Prevención y Control de la Intoxicación por Picadura de Alacrán (2003). [Online] Available: http://www.salud.gob.mx/unidades/cdi/nom/033ssa202.html

[164] A. Alagón, C. Carrillo, A. Chávez-Haro, C. De la Mora-Zerpa, C. Larralde, N. Lamas, E. Martín, N. Osnaya-Romero, Y. Pérez, L. Possani and L. Romero-Zamora, "Alacranismo (T63.2, X22)," *Práctica Médica Efectiva, vol. 5, no.1, 1-4,* 2003.

[165] V. R. D. von Eickstedt, L. A. Ribeiro , D. M. Candido , M. J. Albuquerque and M. T. Jorge, "Evolution of Scorpionism by *Tityus bahiensis* (Perty) and *Tityus serrulatus* Lutz and Mello and Geographical Distribution of the Two Species in the State of São Paulo – Brazil," *Journal of Venomous Animals and Toxins*, vol.2, no.2, pp. 92-105, 1996.

[166] R. L. M. Silva, M. Andrea and T. K. Amorim, "Envenomation by *Tityus stigmurus* (Scorpiones: Buthidae) in Bahia, Brazil," *Revista da Sociedade Brasileira de Medicina Tropical*, vol. 33, no. 3, 239-245, 2000.

[167] M. R. M. Soares, C. S. Azevedo and M. De Maria, "Escorpionismo em Belo Horizonte, MG: um Estudo Retrospectivo," *Revista da Sociedade Brasileira de Medicina Tropical*, vol. 35, pp. 359-363, 2002.

[168] W. R. Lourenço, J. L. Cloudsley-Thompson, O. Cuellar, V. R. D. Von Eickstedt, B. Barraviera and M. B. Knox, "The Evolution of Scorpionism in Brazil in Recent Years," *Journal of Venomous Animals and Toxins*, vol. 2, no. 2, pp. 121–134, 1996.

[169] F. A. Matthiensen, "The Breeding of *T. serrulatus* Lutz & Mello, 1927 in Captivity (Scorpiones, Buthidae)," *Revista Brasileira de Pesquisas Médicas e Biológicas,* vol. 4, pp. 299-300, 1971.

[170] K. Hoshino, A. T. V. Moura and H. M. G. DePaula, "Selection of Environmental Temperature by the Yellow Scorpion *Tityus serrulatus* Lutz & Mello, 1922 (Scorpiones, Buthidae)," *Journal of Venomous Animals and Toxins Including Tropical Diseases*, vol. 12, no. 1, pp. 59-66, 2006.

[171] W. R. Lourenço, *Scorpions of Brazil*. Paris: Les Éditions l'If, 2002.

[172] O. Magalhães, "O Combate ao Escorpionismo", *Memórias do Instituto Oswaldo Cruz*, vol. 49, no. 3, pp. 425-439, 1946.

[173] W. Bücherl, "Escorpiões e Escorpionismo no Brasil III: Substâncias Escorpionicidas e Outras Medidas de Combate aos Escorpiões," *Memórias do Instituto Butantan*, vol. 27, pp. 107-120, 1956.

[174] W. Bücherl, "Escorpiões e Escorpionismo no Brasil VIII: Combate a *Tityus serrulatus* em S. José dos Campos," *Memórias do Instituto Butantan*, vol. 29, pp. 243-254, 1958.

[175] P. Cupo, M. M. Azevedo-Marques, S. E. Hering, "Escorpionismo". In *Animais Peçonhentos do Brasil: Biologia, Clínica e Terapêutica dos Acidentes*, São Paulo, Savier/Fundação de Amparo à Pesquisa do Estado de São Paulo, 2003, pp.198-207.

[176] Secretaria de Estado da Saúde de São Paulo, *Manual de Diretrizes para Atividades de Controle de Escorpiões*. São Paulo: SESP, 1994.

[177] W. H. Stutz, O. Bendeck, J. C. C. Camargo, E. M. Macedo; F. S. Oliveira and R. F. Bonito, "Bioensaio Visando Controle de Escorpionídeos (*Tityus Serrulatus*), através do uso de Bendiocarb, Deltamethrina e Lambdacyhalothrin," *Boletim ABRASCO*, v. 70, p. 27, 1998.

[178] C. Carvalho-Neto, P. G. Alves-Filho and C. R. W. Yassuda, "Ensaio de Campo no Controle de Escorpiões (*Tityus serrulatus*) Empregando Diazinon Microencapsulado, na Cidade de Aparecida, SP," presented at the *XXX* Congresso da Sociedade Brasileira de Medicina Tropical, Salvador, Mar. 6-11, 1994.

[179] C. M. R. Albuquerque, M. O. B. and L. Iannuzzi, "*Tityus stigmurus* (Thorell, 1876) (Scorpiones; Buthidae): Response to Chemical Control and Understanding of Scorpionism Among the Population," *Revista da Sociedade Brasileira de Medicina Tropical*, vol. 42, no. 3, pp. 255-259, 2009.

[180] C. S. Nunes, P. D. Bevilacqua and C. C. G Jardim,."Demographic and Spatial Aspects of Scorpionic Accidents in the Northwest Region of Belo Horizonte City, Minas

Gerais, 1993-1996," *Cadernos de Saúde Pública, Rio de Janeiro*, vol. 16, no. 1, pp. 213-223, 2000.

[181] M. C. Carvalho, M. A. Luna,"Avaliação Laboratorial do Efeito do Diazion Micorencapsulado em Escorpiões Amarelos *Tityus serrulatus*," presented at the XXXI Congresso da Sociedade Brasileira de Medicina Tropical, São Paulo, Mar., 1995

[182] E. F. S. Spirandelli-Cruz, C. R. W. Yassuda and J. J. Barraviera, "Programa de Controle de Surto de Escorpião *Tityus serrulatus*, Lutz e Mello 1922, no Município de Aparecida, São Paulo," *Revista da Sociedade Brasileira de Medicina Tropical*, vol. 28, pp. 123-128, 1995.

[183] C. M. R. Albuquerque, M. O. Barbosa and L. Iannuzzi, "*Tityus stigmurus* (Thorell, 1876) (Scorpiones; Buthidae): Response to Chemical Control and Understanding of Scorpionism Among the Population," *Revista da Sociedade Brasileira de Medicina Tropical*, vol. 42, no. 3, pp. 255-259, 2009.

[185] E. Marques-da-Silva, "Escorpionismo em Bandeirantes, Paraná: Ações Integradas na Análise da Situação e Controle do Escorpião Amarelo - *Tityus serrulatus*," presented at 8ª Mostra de Experiências Bem-Sucedidas em Epidemiologia, Prevenção e Controle de Doenças, Brasília, Nov. 5-7, 2008.

[186] S. Papini, J. L. Oliveira, A. Mazzoni, M. I. O. Andrade and L. C. Luchini, "Abundância e Impacto do Controle de Pragas Urbanas na Região de uma Subprefeitura do Município de São Paulo," *Hygeia*, vol. 5, no. 9, pp. 32 - 41, Dez. 2009.

[187] E. T. Natwick. (Aug. 2003) *Pest Notes – Scorpions - Integrated Pest Management in and Around the Home - Publication 74110*. University of California, Agriculture and Natural Resources. [Online]. Available: http://www.ipm.ucdavis.edu/PDF/PESTNOTES/pnscorpions.pdf

[188] D. H. Gouge, K. A. Smith, C. Olson and P. Baker. *Scorpions*. Cooperative Extension, College of Agriculture & Life Sciences, The University of Arizona. [Online]. Available: http://cals.arizona.edu/pubs/insects/az1223/

[189] S. Daar, T. Drlik, H. Olkowski and W. Olkowski, *IPM for Schools: a How-To Manual.* Berkley: Bio-Integral Resource Center, 1997.

[190] J. Campos and C. F. S. Andrade, "Susceptibilidade Larval de Duas Populações de *Aedes aegypti* a Inseticidas Químicos." *Revista de Saúde Pública*, vol. 35, no 3, pp. 232-236, 2001.

[191] E. P. Russell,"Speaking of Annihilation: Mobilizing for War against Human and Insect Enemies, 1914-1945," *The Journal of American History*, vol. 82, no. 4, pp. 1505-1529, March 1996.

[192] N. E. McIntyre, "Ecology of Urban Arthropods: a Review and a Call to Action," *Annals of the Entomological Society of America*, vol. 93, pp. 825–835, 2000.

[193] C. A Caramori and B. Barraviera, "Universities and Neglected Diseases – it is not Enough to Have the Knowledge, it Must Be Applied," *The Journal of Venomous Animals and Toxins including Tropical Diseases*, vol. 17, no.1, pp.1-3, 2011

[194] D. R. Price and J. A. Gatehouse, "RNAi-Mediated Crop Protection Against Insects," *Trends in Biotechnology,* vol. 26, pp. 393–400, 2008.

[195] T. Mito, T. Nakamura, T. Bando, H. Ohuchi and S. Noji, "The Advent of RNA Interference in Entomology," *Entomological Science,* vol. 14, pp. 1-8, 2011.

[196] S. Schulz, "Semiochemistry of Spiders," In *Advances in Insect Chemical Ecology*, Cambridge, Cambridge University Press, 2004, pp. 110–150.

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Pesticide-Derived Aromatic Amines and Their Biotransformation

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1. Introduction

Aromatic amines (AA) are an important and diversified class of pollutants, including industrial products, drugs, combustion products, pesticides or dyes. Some AA, in particular pesticide-derived AA, are persistent in soils and ground waters and exhibit potential toxicity. These compounds may accumulate in the environment, where they have noxious effects. This is especially true for 3,4 dichloroaniline (3,4-DCA), a by-product of aniline-derived herbicides such as diuron. In treated soil, diuron is transformed into 3,4-DCA and then acetylated by soil bacteria and fungi into the less toxic acetyl-DCA. Microorganisms are thus good candidates to be used for decontamination of AA-polluted soils, a process known as bioremediation. Recent data indicate that filamentous fungi represent promising candidates for bioremediation of AA-contaminated soils. However, fundamental research is still needed to better understand the potential of fungi in metabolizing these pollutants.

2. Aromatic amines, an important and diversified class of pollutants

AA constitute an important and diversified class of pollutants. Many AA are toxic to most living organisms due to their genotoxic or cytotoxic properties (Kim & Guengerich, 2005). AA account for 12% of the 415 chemicals known or strongly suspected to be carcinogenic in humans (USA National Toxicology Program, 2005). AA are common by-products of chemical manufacturing (pesticides, dyestuffs, rubbers or pharmaceuticals), coal and gasoline combustion or pyrolysis reactions (Palmiotto et al., 2001). The presence of AA in ground waters or soil samples subject to industrial, agricultural or urban pollution is an increasing concern, particularly for persistent toxic AA contaminants, such as pesticide-derived anilines (Gan et al., 2004).

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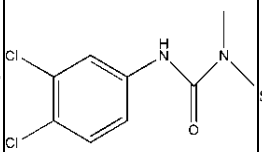
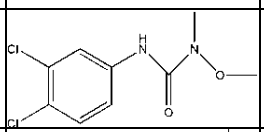
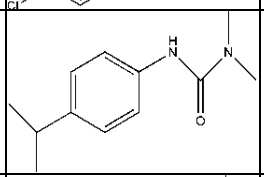
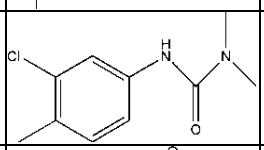
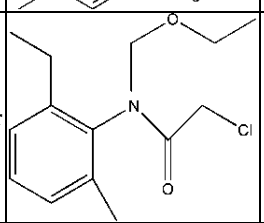
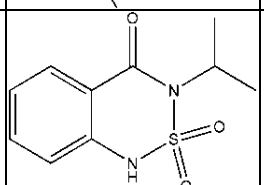
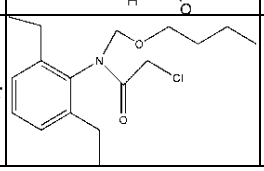
Major reports on pesticide-derived AA contaminations are listed in Table 1, including the chemical sources of contaminants. 3,4-dichloroaniline (3,4-DCA), a highly toxic pesticide-derived AA, is persistent in soil, surface and ground waters. This compound is the major breakdown product of the phenylurea herbicides diuron and linuron, and of the anilide propanil. At the cellular level, 3,4-DCA is known to activate the Ahr/Cyp1a1 pathway (Voelker et al., 2008; Ito et al., 2010). In the same way, the metabolite 3,5-dichloroaniline (3,5-DCA) is more toxic and persistent than its parent vinclozolin. In particular, toxicity of 3,5-DCA has been shown to be related to anti-androgenic activity (Lee et al., 2008).

Potential pollution source	Use	Structure	Reported sites of pollution	Aromatic amine derivative contaminants	References
diuron	herbicide (no longer used)		soils, waste waters, sewage sludges, surface waters	3,4-dichloroaniline	(Vroumsia et al., 1996; Ghanem et al., 2007; Albers et al., 2008; Badawi et al., 2009; Vercraene-Eairmal et al., 2010)
linuron	herbicide		soils	3,4-dichloroaniline	(Kolpin et al., 1998; Badawi et al., 2009; Heidler & Halden, 2009; Woudneh et al., 2009)
isoproturon	herbicide		soils, shallow ground waters	4-isopropylaniline	(Vroumsia et al., 1996; Spliid & Koppen, 1998; Muller et al., 2002; Hussain et al., 2009; Mosleh, 2009)
chlorotoluron	herbicide		soils, surface waters	3-chloro-4-methylaniline	(Vroumsia et al., 1996; Muller et al., 2002; Badawi et al., 2009)
acetochlor	herbicide (no longer used)		soils, ground waters	2-methyl-6-ethylaniline 2,6-diethylaniline	(Kolpin et al., 1998; Barbash et al., 2001; Osano et al., 2002; Dictor et al., 2008; Vercraene-Eairmal et al., 2010)
bentazon	herbicide		soils	2-amino N-isopropylbenzamide	(Kim et al., 1997)
butachlor	herbicide (no longer used)		soils	2,6-diethylaniline	(Ye et al., 2002)

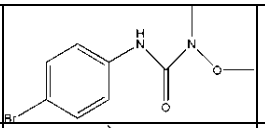
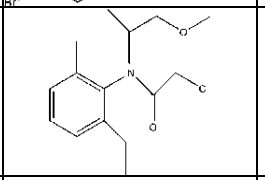
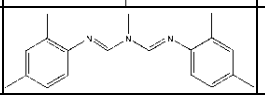
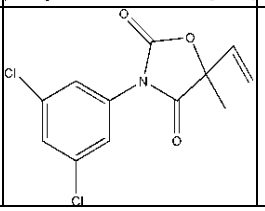
metobromuron	herbicide		soils	4-bromoaniline	(Van Eerd et al., 2003)
metolachlor	herbicide (no longer used)		ground and surface waters	2-ethyl-6-methylaniline	(Osano et al., 2002; Focazio et al., 2008; Huang et al., 2008; Vercraene-Eairmal et al., 2010)
amitraz	pesticide (no longer used)		soils, surface waters	2,4-dimethylaniline	(Osano et al., 2002)
vinclozolin	fongicide (no longer used)		soils	3,5-dichloroaniline	(Lee et al., 2008)

Table 1. Pesticide contaminants and their AA derivatives.

3. The fate of pesticide-derived aromatic amines in the soil environment

In the environment, organic pollutants can be degraded by different biological (Van Eerd et al., 2003), chemical (Sarmah & Sabadie, 2002) or photochemical (Katagi, 2004; Nelieu et al., 2009) processes. Furthermore, in soils, the occurrence and kinetics of degradation processes are modulated by retention through reversible or irreversible adsorption on soil components. Soils present a variety of complex and heterogeneous characteristics; among them, clay and organic contents as well as pH are known as the main properties driving its reactivity and thus the fate of chemicals (Calvet, 1989).

In addition, soils are complex ecosystems of many species, including plants, fauna and microorganisms (Spaink, 2002). Living organisms use several enzymatic pathways, including a variety of xenobiotic-metabolizing enzymes (XME) to protect themselves against the potentially toxic effects of the natural products and xenobiotics present in their environment. Overall, the metabolic fate of xenobiotics involves a two phase metabolism including cytochrome P450-mediated phase I reactions and transferase-mediated conjugation reactions (Phase II). Plant XME include cytochromes P450 (Morant et al., 2003) and glutathione *S*-transferases (Pflugmacher et al., 2000). XME are also expressed in soil microorganisms. Presently, most studies focus on bacterial XME (Van Eerd et al., 2003).

By-products of aniline-derived herbicides (such as 3,4-DCA) are among the most toxic chemicals found in soil (Dearfield et al., 1999; Harvey et al., 2002). The XME-dependent N-acetylation of these chemicals has been shown to detoxify them (Tweedy et al., 1970; Tixier et al., 2002) and has been reported to occur in soil bacteria such as *Pseudomonas sp.* (Vol'nova et al., 1980; Travkin et al., 2003; Westwood et al., 2005) and *Mesorhizobium loti* (Rodrigues-Lima et al., 2006). This pathway has also been described in filamentous fungi such as *Podospora anserina* (Martins et al., 2009).

Soil bacteria	Filamentous fungi
Pseudomonas aeruginosa (Westwood et al., 2005)	*Aspergillus niger, Beauveria bassiana, Cunninghamellae chinulata, Mortierella isabellina* (Tixier et al., 2002)
Mesorhizobium loti (Rodrigues-Lima et al., 2006)	*Podospora anserina* (Martins et al., 2009)

Table 2. Examples of species involved in microbial conversion of 3,4-DCA into 3,4-AcDCA.

The herbicide metobromuron is metabolized to 4-bromoaniline (4-BA), which is detoxified by certain soil microbes to acetyl-4-bromoaniline (Tweedy et al., 1970; Van Eerd et al., 2003).

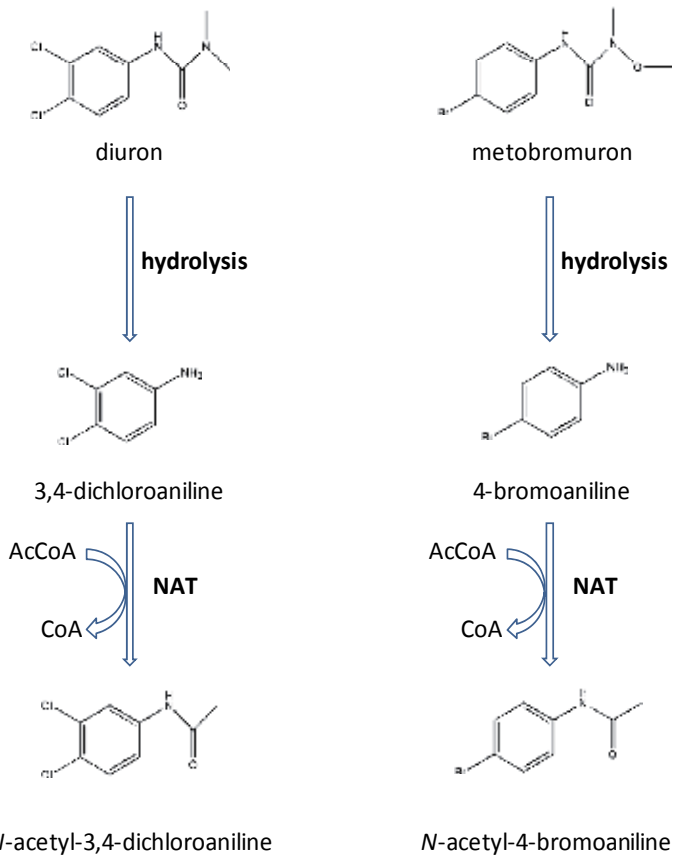


Fig. 1. Biotransformation of diuron and metobromuron by microorganisms.

4. Strategies for bioremediation of aromatic amine-contaminated soils

Soil contamination with pesticides and their derivatives is a major concern in industrialized countries. Exploiting the metabolic capability of microorganisms to transform pollutants is a

promising approach for bioremediation (degradation of pollutants using living beings) of contaminated soils.

Bioremediation is the use of living organisms for *in situ* treatment of contaminated soils, sediments or waters (Schnoor, 1997). Although bacteria are the most common group of organisms used for bioremediation, the use of plants (phytoremediation) and algae (phycoremediation) have been reported, whereas animals (zooremediation) are much more rarely considered (Gifford et al., 2007). The use of filamentous fungi (mycoremediation) may offer promising alternatives (Mougin, 2002; D'Annibale et al., 2006; Harms et al., 2011).

Phytoremediation has successfully been applied for remediation of soils contaminated with heavy metals or other xenobiotics. A form of phytoremediation is rhizoremediation or phytoremediation of the rhizosphere. Indeed, root exudates, soil bacteria and mycorrhizal fungi participate in degradation of organic chemicals in close proximity to the roots. Although the importance of the rhizosphere community for degradation of anthropic xenobiotics has been recognized, very little is known about the exact composition of the degrading populations (Kuiper et al., 2004).

Although the use of combined action between plants and microorganism is a promising strategy for bioremediation, other approaches may be proposed. Whereas it is known that the rhizosphere contains a great proportion of gram-negative bacteria such as *Pseudomonas sp.* (Kuiper et al., 2004), soil microbiological characteristics are generally more complex. Especially, it must be pointed out that the fungal/bacterial ratio in soil has shown the predominant contribution of fungi (Gilbertson, 1980; Durrieu, 1993; Boer et al., 2005). Due to the low substrate specificity of their degradative enzyme machinery (e.g. laccase, lignine peroxidase...), fungi are able to perform the breakdown of a wide range of organic pollutants in contaminated soils (Mougin et al., 2003, Tortella et al., 2005; D'Annibale et al., 2006). Thus, use of soil fungi may be a promising step for setting up new bioremediation development strategies.

However, the field of mycoremediation remains still to be explored (Harms et al., 2011). The best species suitable for such task are not well characterized and presently research focus on a few species, like *Fusarium* (many of them may belong to plant pathogenic species) or white-rots (i.e., basidiomycetes fungi that efficiently degrade lignin) (Mougin et al., 2002, Tortella et al., 2005; Chulalaksananukul et al., 2006; McErlean et al., 2006; Mougin et al., 2009).

Although a still limited number of data is available, bioremediation appears to induce reduced cost in comparison to conventional process such as incineration (Wood, 2008; Vangronsveld et al., 2009). *In situ* bioremediation techniques are in general less expensive since they avoid soil excavation and transport. Finally, soils treated by bioremediation may be re-used, at least for industrial crops, if acceptable target pollutant levels are reached (Mougin et al., 2009). However, current strategies generally categorized into *ex situ* and *in situ* bioremediation have not yet yielded the expected efficiency (Pandey et al., 2009). Fundamental research is needed to reduce cost and to increase performance of bioremediation techniques. A promising strategy is to explore the metabolic diversity of microorganisms and their ability to degrade pollutants. In the eyes of EU's regulation on genetic modified organisms (GMO) (European Commission DG ENV, 2010), the use of non-GMO with no pathogenic potential will be of great interest for future bioremediation approaches.

Exploiting the ability of microorganisms to transform AA pollutants is a promising approach for bioremediation of contaminated soils. Most studies focused on conversion of 3,4-DCA into its acetylated form. First, it has been shown that acetylated 3,4-DCA is less

toxic than 3,4-DCA; second, some soil bacteria and fungi strains are able to acetylate 3,4-DCA (Tixier et al., 2002; Westwood et al., 2005). Although aniline derivatives undergo complex transformations in soils, including mineralization (biodegradation), co-metabolic and condensation reactions, binding to humus complexes (Albers et al., 2008) and mineralization (Mougin et al., 2009), studies opened up new possibilities in bioremediation (Rodrigues-Lima et al., 2006; Martins et al., 2009, Mougin et al., 2009), as detailed below.

The major detoxification pathway of AA depends on the activity of arylamine *N*-acetyltransferase enzymes (NAT) (Dupret & Rodrigues-Lima, 2005). NAT catalyze the acetyl-CoA-dependent *N*- and/or *O*- acetylation of AA and their N-hydroxylated metabolites. The *N*-acetylation reaction leads to the detoxification of AA through the production of arylacetamides that are chemically stable. In humans, variations of NAT activity have long been associated with susceptibility to different cancers in relation with exposure to certain AA (Hein, 2002).

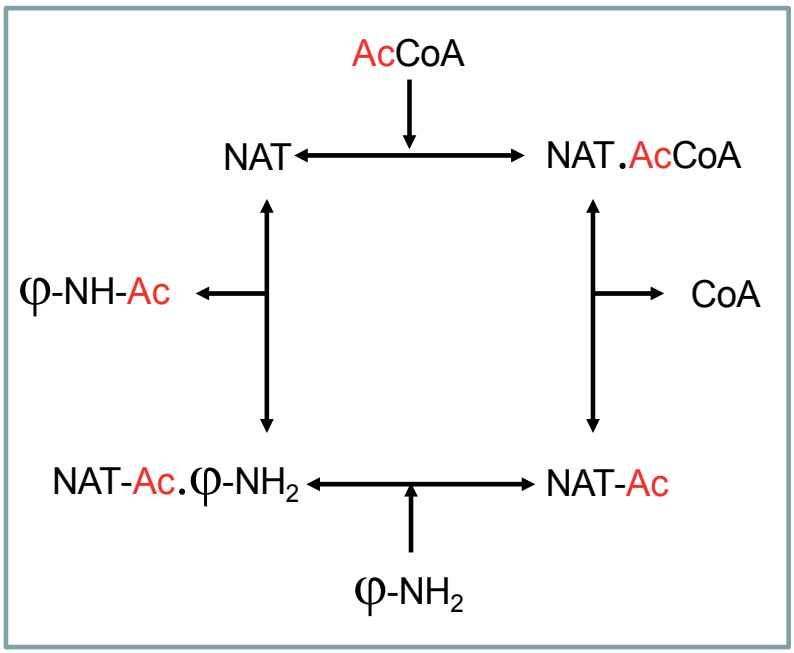


Fig. 2. Reaction mechanism of NAT enzymes

Using the potential detoxifying properties of NAT enzymes expressed in soil microorganisms, putative AA bioremediation pathways were explored using soil bacteria or filamentous fungi.

Legume plants and nitrogen-fixing roots represent a promising plant-bacterial rhizoremediation system, which turned the attention of biotechnologists in recent years. Two NAT enzymes were characterized in Mesorhizobium loti, a rhizobial nitrogen-fixing bacterium that lives in symbiosis with several species of the genus Lotus, including Lotus

corniculatus and Lotus japonicus. These two enzymes were shown to catalyze the N-acetylation of several known NAT substrates including aniline-derived pesticide residues (3,4-DCA, 4-BA and 4-iodoaniline). The existence of a functional NAT-dependent acetylation pathway in the root nodules of Lotus japonicus inoculated with M. loti was also demonstrated. M. loti was the first non-eukaryotic organism shown to express two catalytically active NAT isoforms. This work also provided the first evidence for acquisition of a xenobiotic detoxification pathway by a plant through symbiosis with a soil microbe (Rodrigues-Lima et al., 2006). As many aromatic pollutants are detoxified by the NAT-dependent pathway, these results opened up new possibilities in the field of rhizoremediation for aromatic soil pollutants. Indeed, leguminous plants grow in a wide variety of environments; moreover, root systems penetrate from 50 cm to a few meters into the soil. However, technical constraints are important and fungal remediation represents a promising alternative for AA bioremediation.

To date the potential of fungi in bioremediation is not well understood although these organisms form the large majority of the soil microorganisms (Harms et al., 2011). Indeed, it has been estimated that in a typical soil 80% of the biomass, with the exception of the plant roots, is of fungal origin (Gilbertson, 1980; Durrieu, 1993) and fungi out compete bacteria in many degradation processes or synergize their activity (Boer et al., 2005). Fungi efficiently secrete in the medium a large array of enzymes involved in cellulose/lignin degradation, which have been shown to display xenobiotic-metabolizing enzyme (XME) activities (Tortella et al., 2005; McErlean et al., 2006). They also produce and encounter many secondary metabolites (Misiek & Hoffmeister, 2007), including harmful polyketides, cyclic peptides, alkaloids and others, for which they must have detoxifying enzymes with XME activities (Glenn & Bacon, 2009).

Filamentous fungi are able to degrade aromatic ring containing compounds. The most efficient species in term of known enzymatic capacity are white-rot fungi such as *Pleurotus*, *Trametes* or *Phanerochaetes*. Lignin modifying enzymes from white rots, such as lignin peroxidases (LiP), manganese-dependent peroxidases (MnP) and laccases play key roles in degradation reactions. So far, fungal remediation studies using white rots have been performed on polycyclic aromatic hydrocarbons, nitro-aromatic compounds, or endocrine-disrupting phenolic chemicals (Mougin et al., 2009). However, white rots are not very competitive in soils. Therefore, the use of a cosmopolitan fungus to bioremediate AA-contaminated soils is a promising alternative.

Four well known fungal species (*Fusarium graminearum*, *Phycomyces blakesleeanus*, *Podospora anserina*, and *Rhizopus oryzae*) from different ecosystems (natural habitats of fungi are wheat (*F. graminearum*), soil (*R. oryzae*; *P. blakesleeanus*), dung (*P. anserina*) or soil/dung (*C. globosum*)), for which complete genome sequences are available, were screened for radial growth in the presence of three toxic AA: 3,4-DCA (pesticide residue), 2-aminofluorene (2-AF, carcinogen), and 4-butoxyaniline (4-BOA, chemical intermediate) (Martins et al., 2009). The growth of *R. oryzae* and *P. blakesleeanus* was almost completely abolished by these three AA at concentrations of 100–250 μM in standard minimal growth medium. In the same conditions, little effect on growth was observed in the other two studied species (*P. anserina* and *F. graminearum*), suggesting that they have mechanisms of tolerance to AA. Genomic DNA sequence analysis indicated that *P. anserina* had two putative NAT enzymes and that *F. graminearum* had three such enzymes, whereas the two sensitive fungi had no genes encoding NAT enzymes.

The bioremediation potential of *Podospora anserina* was explored. *P. anserina* only reproduces by sexual means, it is a cosmopolitan non-pathogenic species and its spread is easy to control. In addition, its genome has been sequenced and powerful genetic tools permit to construct single and double deletion mutants. Two NAT enzymes, PaNAT1 and PaNAT2, were characterized in *P. anserina*. PaNAT2 was systematically more active than PaNAT1. Targeted gene disruption experiments revealed that PaNAT2 was required for the growth and survival of the fungus in the presence of toxic AA.

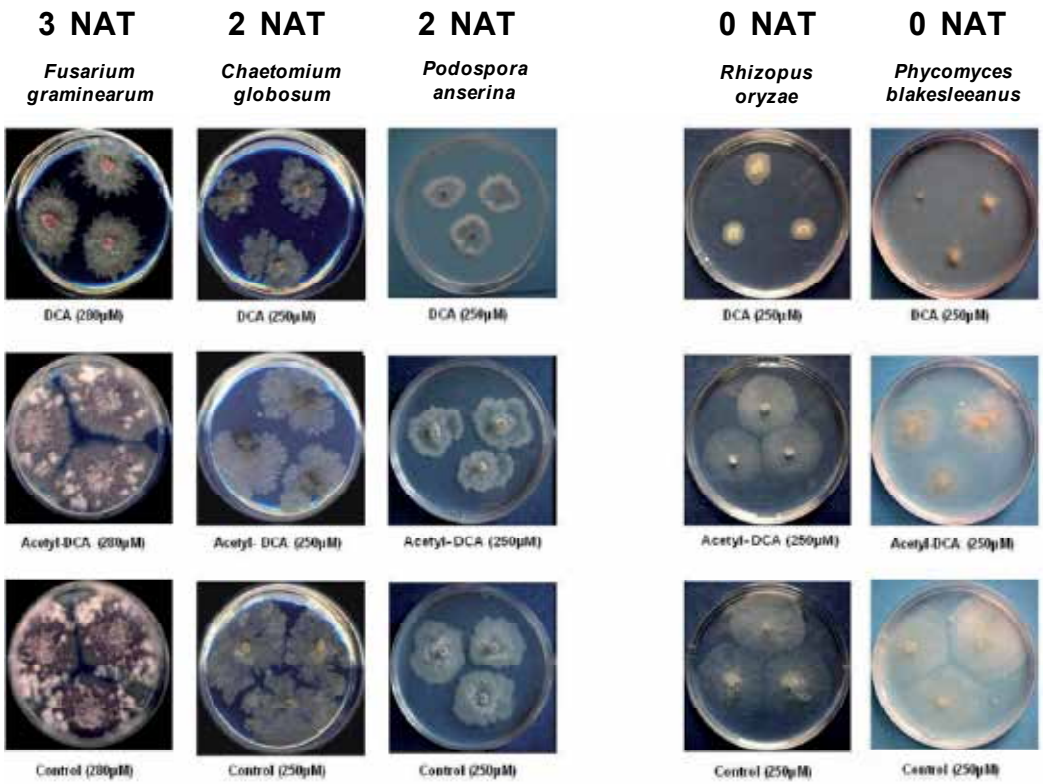


Fig. 3. NAT genes in fungi and tolerance to 3,4-DCA in five species.
The figure shows that three common fungal species, Fusarium graminearum, Chaetomium globosum and Podospora anserina tolerate 3,4-DCA at concentrations of 250 µM. By contrast, two other species, Rhizopus oryzae and Phycomyces blakesleeanus, are much less tolerant to this compound. In all cases, acetylated 3,4-DCA is less toxic than 3,4-DCA. Finally, the presence, in species of the left columns, of NAT genes appears to be associated with tolerance to 3,4-DCA.

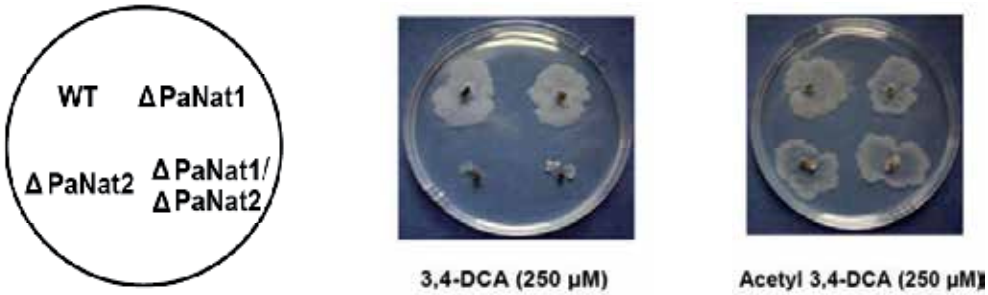


Fig. 4. PaNAT2 plays a key role in P. anserina tolerance to 3,4-DCA.
The figure shows that the radial growth of strains lacking PaNAT2 was strongly impaired in the presence of 3,4-dichloroaniline (3,4-DCA). Conversely, acetylated 3,4-DCA was much less toxic. Tested mutants lacked either one (ΔPaNat1 or ΔPaNat2) or both (ΔPaNat1/2) NAT genes. Petri dish contained the indicated strains grown on M2-agar medium with 3,4-DCA or its acetylated form at the indicated final concentration. Photographs were taken after 3 days of growth at 27°C.

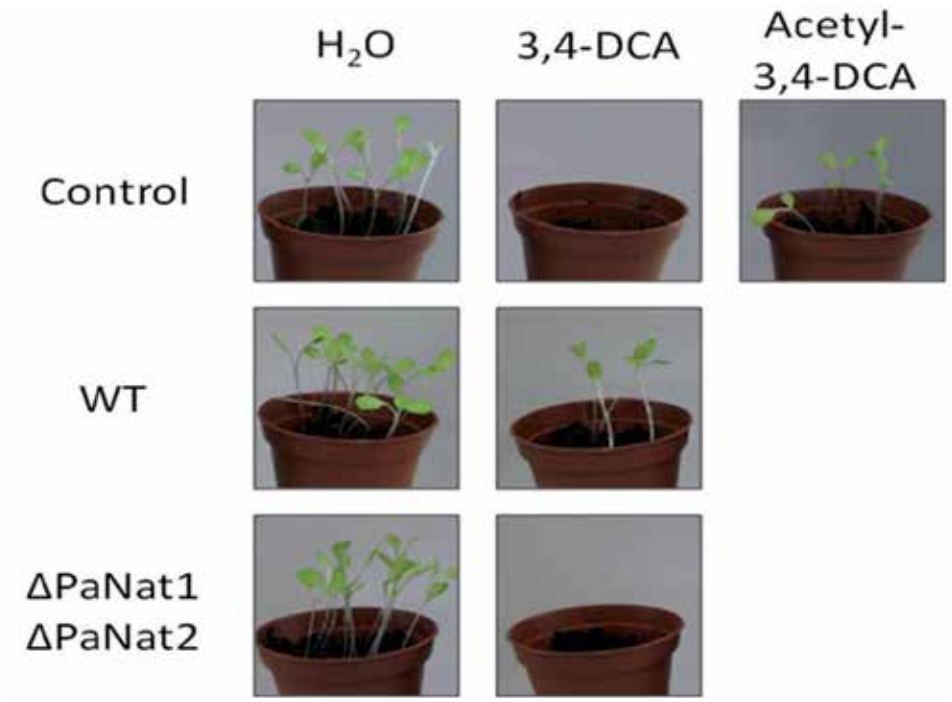


Fig. 5. Remediation of soil samples contaminated with 3,4-DCA.
3,4-DCA-contaminated soils (20 g/pot, 80 mg of 3,4-DCA/kg of soil) were inoculated every 24 hours, over a period of three days, with 0.5 g of wild-type Podospora anserina or with NAT-deleted strains (ΔPaNat 1/2 strains) and incubated for 72h at 25°C. Lactuca sativa seeds were sown and allowed to germinate and grown for 8 days at 25°C. Controls were set up with acetyl-3,4-DCA (80 mg/kg of soil) and water. Germination and growth were abolished in soils contaminated with 3,4-DCA. Conversely, in contaminated soils treated with P. anserina, seed germination and growth were restored.

Finally, proof-of-concept remediation experiments were performed where *P. anserina*, through its PaNAT2 enzyme, was able to detoxify 3,4-DCA in experimentally contaminated soil samples. 3,4-DCA concentration (80 mg/kg of soils) used were higher than concentrations observed in contaminated soils (around 0.02 to 5 mg/kg of dry soils). These findings provided a basis for new systems based on fungi for the bioremediation of contaminated soils (Martins et al., 2009).
Overall, given the detoxifying activity of NAT enzymes, the presence of NAT genes in many fungi (Martins et al., 2009; Martins et al., 2010) and the fungal biomass in fungi-bearing soils, these studies show that fungal bioremediation of pesticide-derived AA represent a promising perspective.

5. Conclusion

What are the remediation potential of bacteria and fungi for pesticide-derived AA? This question is still open. Whereas bacteria are abundant, fungi represent the largest biomass in soils. Moreover, the NAT activity of fungi seems to be higher than that of bacteria. So our preliminary studies suggest that fungi represent better candidates for bioremediation of AA-contaminated soils. Given the fungal biomass in fungi-bearing soils, these reports keep with promising perspectives in the field of bioremediation for a variety of toxic AA. Such perspectives should now be assessed in field studies.

6. References

Albers, C. N., G. T. Banta, P. E. Hansen & O. S. Jacobsen (2008). Effect of different humic substances on the fate of diuron and its main metabolite 3,4-dichloroaniline in soil. *Environ Sci Technol*, 4223, 8687-8691.

Badawi, N., S. Ronhede, S. Olsson, B. B. Kragelund, A. H. Johnsen, O. S. Jacobsen & J. Aamand (2009). Metabolites of the phenylurea herbicides chlorotoluron, diuron, isoproturon and linuron produced by the soil fungus *Mortierella sp*. *Environ Pollut*, 15710, 2806-2812.

Barbash, J. E., G. P. Thelin, D. W. Kolpin & R. J. Gilliom (2001). Major herbicides in ground water: results from the National Water-Quality Assessment. *J Environ Qual*, 303, 831-845.

Boer, W., L. B. Folman, R. C. Summerbell & L. Boddy (2005). Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS Microbiol Rev*, 294, 795-811.

Calvet, R. (1989). Adsorption of organic chemicals in soils. *Environ Health Perspect*, 83, 145-177.

Chulalaksananukul, S., G. M. Gadd, P. Sangvanich, P. Sihanonth, J. Piapukiew & A. S. Vangnai (2006). Biodegradation of benzo(a)pyrene by a newly isolated *Fusarium sp*. *FEMS Microbiol Lett*, 2621, 99-106.

D'Annibale, A., F. Rosetto, V. Leonardi, F. Federici & M. Petruccioli (2006). Role of autochthonous filamentous fungi in bioremediation of a soil historically contaminated with aromatic hydrocarbons. *Appl Environ Microbiol*, 721, 28-36.

Dearfield, K. L., N. E. McCarroll, A. Protzel, H. F. Stack, M. A. Jackson & M. D. Waters (1999). A survey of EPA/OPP and open literature on selected pesticide chemicals. II. Mutagenicity and carcinogenicity of selected chloroacetanilides and related compounds. *Mutat Res*, 4431-2, 183-221.

Dictor, M. C., N. Baran, A. Gautier & C. Mouvet (2008). Acetochlor mineralization and fate of its two major metabolites in two soils under laboratory conditions. *Chemosphere*, 714, 663-670.

Dupret, J. M. & F. Rodrigues-Lima (2005). Structure and regulation of the drug-metabolizing enzymes arylamine N-acetyltransferases. *Curr Med Chem*, 123, 311-318.

Durrieu, G. (1993). Ecologie des champignons.

Focazio, M. J., D. W. Kolpin, K. K. Barnes, E. T. Furlong, M. T. Meyer, S. D. Zaugg, L. B. Barber & M. E. Thurman (2008). A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States--II) untreated drinking water sources. *Sci Total Environ*, 4022-3, 201-216.

Gan, J., P. L. Skipper, M. Gago-Dominguez, K. Arakawa, R. K. Ross, M. C. Yu & S. R. Tannenbaum (2004). Alkylaniline-hemoglobin adducts and risk of non-smoking-related bladder cancer. *J Natl Cancer Inst*, 9619, 1425-1431.

Ghanem, A., P. Bados, A. R. Estaun, L. F. de Alencastro, S. Taibi, J. Einhorn & C. Mougin (2007). Concentrations and specific loads of glyphosate, diuron, atrazine, nonylphenol and metabolites thereof in French urban sewage sludge. *Chemosphere*, 699, 1368-1373.

Gifford, S., R. H. Dunstan, W. O'Connor, C. E. Koller & G. R. MacFarlane (2007). Aquatic zooremediation: deploying animals to remediate contaminated aquatic environments. *Trends Biotechnol*, 252, 60-65.

Gilbertson, R. I. (1980). Wood-rotting fungi of north america. *Mycologia*, 72, 1-49.

Glenn, A. E. & C. W. Bacon (2009). FDB2 encodes a member of the arylamine *N*-acetyltransferase family and is necessary for biotransformation of benzoxazolinones by *Fusarium verticillioides*. *J Appl Microbiol*, 1072, 657-671.

Harms, H., D. Schlosser & L. Y. Wick (2011). Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. *Nat Rev Microbiol*, 93, 177-192.

Harvey, P. J., B. F. Campanella, P. M. Castro, H. Harms, E. Lichtfouse, A. R. Schaffner, S. Smrcek & D. Werck-Reichhart (2002). Phytoremediation of polyaromatic hydrocarbons, anilines and phenols. *Environ Sci Pollut Res Int*, 91, 29-47.

Heidler, J. & R. U. Halden (2009). Fate of organohalogens in US wastewater treatment plants and estimated chemical releases to soils nationwide from biosolids recycling. *J Environ Monit*, 1112, 2207-2215.

Hein, D. W. (2002). Molecular genetics and function of NAT1 and NAT2: role in aromatic amine metabolism and carcinogenesis. *Mutat Res*, 506-507, 65-77.

Huang, S. B., T. J. Mayer & R. A. Yokley (2008). Direct aqueous injection LC-ESI/MS/MS analysis of water for 11 chloro- and thiomethyltriazines and metolachlor and its ethanesulfonic and oxanilic acid degradates. *J Agric Food Chem*, 568, 2595-2602.

Hussain, S., S. R. Sorensen, M. Devers-Lamrani, T. El-Sebai & F. Martin-Laurent (2009). Characterization of an isoproturon mineralizing bacterial culture enriched from a French agricultural soil. *Chemosphere*, 778, 1052-1059.

Ito, Y., Y. Matsuda & T. Suzuki (2010). Effects of 3,4-dichloroaniline on expression of ahr2 and cyp1a1 in zebrafish adults and embryos. *Comp Biochem Physiol C Toxicol Pharmacol*, 1522, 189-194.

Katagi, T. (2004). Photodegradation of pesticides on plant and soil surfaces. *Rev Environ Contam Toxicol*, 182, 1-189.

Kim, D. & F. P. Guengerich (2005). Cytochrome P450 activation of arylamines and heterocyclic amines. *Annu Rev Pharmacol Toxicol*, 45, 27-49.

Kim, J. E., C. J. Wang & J. M. Bollag (1997). Interaction of reactive and inert chemicals in the presence of oxidoreductases: reaction of the herbicide bentazon and its metabolites with humic monomers. *Biodegradation*, 86, 387-392.

Kolpin, D. W., E. M. Thurman & S. M. Linhart (1998). The environmental occurrence of herbicides: the importance of degradates in ground water. *Arch Environ Contam Toxicol*, 353, 385-390.

Kuiper, I., E. L. Lagendijk, G. V. Bloemberg & B. J. Lugtenberg (2004). Rhizoremediation: a beneficial plant-microbe interaction. *Mol Plant Microbe Interact*, 171, 6-15.

Lee, J. B., H. Y. Sohn, K. S. Shin, J. S. Kim, M. S. Jo, C. P. Jeon, J. O. Jang, J. E. Kim & G. S. Kwon (2008). Microbial biodegradation and toxicity of vinclozolin and its toxic metabolite 3,5-dichloroaniline. *J Microbiol Biotechnol*, 182, 343-349.

Martins, M., J. Dairou, F. Rodrigues-Lima, J. M. Dupret & P. Silar (2010). Insights into the phylogeny or arylamine *N*-acetyltransferases in fungi. *J Mol Evol*, 712, 141-152.

Martins, M., F. Rodrigues-Lima, J. Dairou, A. Lamouri, F. Malagnac, P. Silar & J. M. Dupret (2009). An acetyltransferase conferring tolerance to toxic aromatic amine chemicals: molecular and functional studies. *J Biol Chem*, 28428, 18726-18733.

McErlean, C., R. Marchant & I. M. Banat (2006). An evaluation of soil colonisation potential of selected fungi and their production of ligninolytic enzymes for use in soil bioremediation applications. *Antonie Van Leeuwenhoek*, 902, 147-158.

Misiek, M. & D. Hoffmeister (2007). Fungal genetics, genomics, and secondary metabolites in pharmaceutical sciences. *Planta Med*, 732, 103-115.

Morant, M., S. Bak, B. L. Moller & D. Werck-Reichhart (2003). Plant cytochromes P450: tools for pharmacology, plant protection and phytoremediation. *Curr Opin Biotechnol*, 142, 151-162.

Mosleh, Y. Y. (2009). Assessing the toxicity of herbicide isoproturon on *Aporrectodea caliginosa* (Oligochaeta) and its fate in soil ecosystem. *Environ Toxicol*, 244, 396-403.

Mougin, C. (2002). Bioremediation and Phytoremediation of Industrial PAH-Polluted Soils. *Polycyclic Aromatic Compounds*, 225, 1011-1043.

Mougin, C., C. Jolivalt, P. Briozo & C. Madzak (2003). Fungal laccases: from structure-activity studies to environmental applications *Environmental Chemistry Letters*, Vol 1, Number 2; 145-148.

Mougin, C., H. Boukcim & C. Jolivalt (2009). Soil Bioremediation Strategies Based on the Use of Fungal Enzymes *Advances in Applied Bioremediation* 17123-149.

Muller, K., M. Bach, H. Hartmann, M. Spiteller & H. G. Frede (2002). Point- and nonpoint-source pesticide contamination in the Zwester Ohm catchment, Germany. *J Environ Qual*, 311, 309-318.

Nelieu, S., F. Perreau, F. Bonnemoy, M. Ollitrault, D. Azam, L. Lagadic, J. Bohatier & J. Einhorn (2009). Sunlight nitrate-induced photodegradation of chlorotoluron: evidence of the process in aquatic mesocosms. *Environ Sci Technol*, 439, 3148-3154.

Osano, O., A. A. Oladimeji, M. H. Kraak & W. Admiraal (2002). Teratogenic effects of amitraz, 2,4-dimethylaniline, and paraquat on developing frog (Xenopus) embryos. *Arch Environ Contam Toxicol*, 431, 42-49.

Palmiotto, G., G. Pieraccini, G. Moneti & P. Dolara (2001). Determination of the levels of aromatic amines in indoor and outdoor air in Italy. *Chemosphere*, 433, 355-361.

Pandey, J., A. Chauhan & R. K. Jain (2009). Integrative approaches for assessing the ecological sustainability of in situ bioremediation. *FEMS Microbiol Rev*, 332, 324-375.

Pflugmacher, S., P. Schroder & H. Sandermann, Jr. (2000). Taxonomic distribution of plant glutathione S-transferases acting on xenobiotics. *Phytochemistry*, 543, 267-273.

Rodrigues-Lima, F., J. Dairou, C. L. Diaz, M. C. Rubio, E. Sim, H. P. Spaink & J. M. Dupret (2006). Cloning, functional expression and characterization of *Mesorhizobium loti* arylamine *N*-acetyltransferases: rhizobial symbiosis supplies leguminous plants with the xenobiotic N-acetylation pathway. *Mol Microbiol*, 602, 505-512.

Sarmah, A. K. & J. Sabadie (2002). Hydrolysis of sulfonylurea herbicides in soils and aqueous solutions: a review. *J Agric Food Chem*, 5022, 6253-6265.

Schnoor, J. L. (1997). Phytoremediation. Iowa City, Ground-Water Remediation Technologies Analysis Center.

Spaink, H. P. (2002). Plant-microbe interactions: a receptor in symbiotic dialogue. *Nature*, 4176892, 910-911.

Spliid, N. H. & B. Koppen (1998). Occurrence of pesticides in Danish shallow ground water. *Chemosphere*, 377, 1307-1316.

Tixier, C., M. Sancelme, S. Ait-Aissa, P. Widehem, F. Bonnemoy, A. Cuer, N. Truffaut & H. Veschambre (2002). Biotransformation of phenylurea herbicides by a soil bacterial strain, *Arthrobacter sp*. N2: structure, ecotoxicity and fate of diuron metabolite with soil fungi. *Chemosphere*, 464, 519-526.

Tortella, G. R., M. C. Diez & N. Duran (2005). Fungal diversity and use in decomposition of environmental pollutants. *Crit Rev Microbiol*, 314, 197-212.

Travkin, V. M., I. P. Solyanikova, I. M. Rietjens, J. Vervoort, W. J. van Berkel & L. A. Golovleva (2003). Degradation of 3,4-dichloro- and 3,4-difluoroaniline by *Pseudomonas fluorescens* 26-K. *J Environ Sci Health B*, 382, 121-132.

Tweedy, B. G., C. Loeppky & J. A. Ross (1970). Metobromuron: acetylation of the aniline moiety as a detoxification mechanism. *Science*, 168930, 482-483.

USA National Toxicology Program (2005). 11th Report on Carcinogens, U.S. Department of Health and Human Services.

Van Eerd, L. L., R. E. Hoagland, R. M. Zablotowicz & J. C. Hall (2003). Pesticide metabolism in plants and microorganisms. Weed Science. *Weed Science*, 51, 472-495.

Vangronsveld, J., R. Herzig, N. Weyens, J. Boulet, K. Adriaensen, A. Ruttens, T. Thewys, A. Vassilev, E. Meers, E. Nehnevajova, D. van der Lelie & M. Mench (2009). Phytoremediation of contaminated soils and groundwater: lessons from the field. *Environ Sci Pollut Res Int*, 167, 765-794.

Vercraene-Eairmal, M., B. Lauga, S. Saint Laurent, N. Mazzella, S. Boutry, M. Simon, S. Karama, F. Delmas & R. Duran (2010). Diuron biotransformation and its effects on biofilm bacterial community structure. *Chemosphere*, 817, 837-843.

Voelker, D., N. Stetefeld, K. Schirmer & S. Scholz (2008). The role of cyp1a and heme oxygenase 1 gene expression for the toxicity of 3,4-dichloroaniline in zebrafish (*Danio rerio*) embryos. *Aquat Toxicol*, 861, 112-120.

Vol'nova, A. I., E. G. Surovtseva & G. K. Vasil'eva (1980). [Acetylation of 3,4-dichloroaniline by representatives of the genus Pseudomonas]. *Mikrobiologiia*, 491, 167-170.

Vroumsia, T., R. Steiman, F. Seigle-Murandi, J. L. Benoit-Guyod & A. Khadrani (1996). Biodegradation of three substituted phenylurea herbicides (chlortoluron, diuron, and isoproturon) by soil fungi. A comparative study. *Chemosphere*, 3310, 2045-2056.

Westwood, I. M., S. J. Holton, F. Rodrigues-Lima, J. M. Dupret, S. Bhakta, M. E. Noble & E. Sim (2005). Expression, purification, characterization and structure of *Pseudomonas aeruginosa* arylamine *N*-acetyltransferase. *Biochem J*, 385Pt 2, 605-612.

Wood, T. K. (2008). Molecular approaches in bioremediation. *Curr Opin Biotechnol*, 196, 572-578.

Woudneh, M. B., Z. Ou, M. Sekela, T. Tuominen & M. Gledhill (2009). Pesticide multiresidues in waters of the Lower Fraser Valley, British Columbia, Canada. Part I. Surface water. *J Environ Qual*, 383, 940-947.

Ye, C. M., X. J. Wang & H. H. Zheng (2002). Biodegradation of acetanilide herbicides acetochlor and butachlor in soil. *J Environ Sci (China)*, 144, 524-529.

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The present book is a collection of selected original research articles and reviews providing adequate and up-to-date information related to pesticides control, assessment, and toxicity. The first section covers a large spectrum of issues associated with the ecological, molecular, and biotechnological approaches to the understanding of the biological control, the mechanism of the biocontrol agents action, and the related effects. Second section provides recent information on biomarkers currently used to evaluate pesticide exposure, effects, and genetic susceptibility of a number of organisms. Some antioxidant enzymes and vitamins as biochemical markers for pesticide toxicity are examined. The inhibition of the cholinesterases as a specific biomarker for organophosphate and carbamate pesticides is commented, too. The third book section addresses to a variety of pesticides toxic effects and related issues including: the molecular mechanisms involved in pesticides-induced toxicity, fish histopathological, physiological, and DNA changes provoked by pesticides exposure, anticoagulant rodenticides mode of action, the potential of the cholinesterase inhibiting organophosphorus and carbamate pesticides, the effects of pesticides on bumblebee, spiders and scorpions, the metabolic fate of the pesticide-derived aromatic amines, etc.

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