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Insecticides

Basic and Other Applications

Edited by Sonia Soloneski and Marcelo Larramendy

INSECTICIDES – BASIC AND OTHER APPLICATIONS

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Meet the editors

Sonia Soloneski holds a PhD in Natural Sciences and is an Assistant Professor of Molecular Cell Biology at the Faculty of Natural Sciences and Museum of La Plata, National University of La Plata, Argentina. Her graduate studies in Finland helped her in developing her doctoral thesis at the Department of Medical Genetics, University of Finland, Finland. She is a member of the National Scientific and Technical Research Council (CONICET) of Argentina, the Latin American Association of Environmental Mutagenesis, Carcinogenesis and Teratogenesis (ALAMCTA), the Argentinean Society of Toxicology (ATA) and the Argentine Society of Genetics (SAG). Dr Soloneski has co-authored more than 40 scientific publications in reviewed scientific journals, and 100 abstracts of research papers as a first author. She is a regular lecturer at the International A. Hollaender courses, held by the International Association of Environmental Mutagen Societies (IAEMS), and is a referent for subjects related to genetic toxicology and the ecotoxicology field.

Marcelo L. Larramendy, PhD, is a Professor of Molecular Cell Biology at La Plata National University (UNLP), Argentina, and has been a researcher at the National Scientific and Technical Research Council (CONICET) of Argentina since 1981. He is also a member of the Executive Committee of the Latin American Association of Environmental Mutagenesis, Carcinogenesis and Teratogenesis (ALAMCTA). Dr Larramendy has written more than 365 scientific papers in the field and has received several national and international awards. He is a Regular Lecturer at the International A. Hollaender Courses, held by the International Association of Environmental Mutagen Societies (IAEMS), and among others, a former guest scientist at NIH, Bethesda, USA, and University of Helsinki, Finland. Dr Larramendy is an expert in Molecular Cytogenetics and Genetic Toxicology.

Contents

Preface

Following *Herbicides, Theory and Applications* (InTech, 2011), this new addition aims to shed more light on matters of scientific interest in pesticide and crop management.

Insects have played a role in shaping the history of mankind since the dawn of time. Reference to them can be found in ancient books, for example in three (or four) of the ten plagues of Egypt, to persuade Pharaoh to release the people of Israel from slavery.

The purpose of pest control is to be able to produce more and better quality food as well as decrease costs. Traditional undernourished nations now export foodstuffs, but also suffer from the epidemics of obesity and diabetes among others. The benefits of insecticides speak for themselves. The pitfalls and dangers of their excessive use to animal and human health, as well as the environment, do not.

We cannot disregard the interplay that exists between science, national governments, international agencies and economy, to name but a few. While many agents have very strict or forbidden indications of use in many countries, the truth is that these criteria are not seriously enforced or, even worse, are simply disregarded in some. A growing trend is for countries to rent their lands for cultivation to others that are less favored by geographical conditions and who have an urgent need to feed those populations. These "host" countries are usually in need of cash and, in some cases, without rule of law.

Standardization of agricultural practices is another matter that should not be disregarded in the overall equation. There is a general aversion to rely less on the old farmer's eye and apply the same rule(s), whether they are appropriate or not. It has been estimated that an apple tree receives no less than 26 "treatments". While minimum levels of insecticides are set, the equation sometimes does not consider the overall sum of those minimum values, which reaches alarming proportions in some cases.

Scientists have the right and obligation to raise their voice and air concerns about double standards. Active principles that are forbidden to be used in most European and North American countries are still being produced for sale in far away destinations. In other cases, local production is achieved by means of subsidiaries, sale of licenses and local reformulations. Two of the BRIC countries are the main world

producers of agrochemicals today. There are many reasons for this, and the list would be too extensive and not inclusive but, among other things, we have to consider the means governments have at their disposal to sanction proper legislation, enforce bans, lobbying groups, struggle against smuggling or parallel import, higher cost of alternatives, attractiveness to foreign investments and the price these commodities reach in international markets. As a director of corporate communications of one of the largest agrochemical companies once mentioned in an interview, "his company does not have to guarantee the safety of biotech food (goes without saying that other products by this conglomerate fall into this category by default). Assuring safety is FDA´s job"(Food and Drug Administration of USA). Companies change names and use green colored logos with plants or flowers to convey an image that cannot be further from the truth.

Few people do not recognize that this is actually a boomerang. A large portion of these crops will be shipped to far away destinations, thus finding a way (directly or indirectly) to the consumer's tables or to be used as animal feed. The "green credentials" of foodstuffs and their packaging are another misleading factor. Several international food crises that have taken place in the last decades have demonstrated this. Usually, appropriate measures are taken after a serious incident has taken place but, in general terms, too late for the victims involved. Scientists should also bear responsibilities vis à vis consumers and not only governments and companies in order to avoid repeating the mistakes from the past. Not only human beings, but also biomes, land and riparian organisms (fauna and flora) have to be considered. We all share one land, one air; political or legal borders are totally meaningless in this question.

It is our hope that this book will be of interest and use not only to scientists, but also to the food-producing industry, governments, politicians and consumers as well. If we are able to stimulate this interest, albeit in a small way, we have achieved our goal.

Dr Guillermo Eli Liwszyc,
Physician, Specialist in Internal Medicine,
former Guest Scientist at the University of Helsinki,
Finland

Part 1

Basic and Alternative Control of Insect Pests

1

Insecticide Thiamethoxam: A Bioactive Action on Carrot Seeds (*Daucus carota* L.)

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1. Introduction

Explorations of the substances potential that can act as promoters of growth-inducing activities are in substantial contribution of research to agriculture. Many of these activities are related to the activation capacity of the plants resistance mechanisms, thus enabling seek control through integrated management.

With the modernization of agriculture, marked advances in farming techniques have been obtained, allowing mitigate the limiting factors with weather conditions such diseases, pests, among others. The plant physiology has promoted great advances in recent years with the advent of modern techniques such as the production of plants by tissue culture, genetic engineering and biotechnology. Among these modern techniques, the use of bioactive, capable of increasing the productive potential of plants, is an increasing use in the practice of modern agriculture and widespread in countries highly technical.

In Brazil, the use of bioactive beginning to be explored and the results of several studies have shown that these substances provide significant increases in productivity and, in quality, as observed, for example, significant increases in the amount of larger fruits. Bioactivators are natural substances of plant origin that have actions similar to the main plant growth regulators, aimed at growth and development of the plant. Provide better physiological balance, favoring closer ties to the genetic potential of culture.

Moreover, they are complex organic substances, not bioregulators, growth modifiers, capable of working in the plant transcription factors and gene expression in membrane proteins by altering the ion transport. They also act in metabolic enzymes could affect the secondary metabolism and may alter the mineral nutrition, induce the production of precursors of plant hormones, leading to hormone synthesis and more intense response to nutrients and plant hormones.

Applied to plants, bioactivators cause modification or alteration of specific metabolic and physiological processes, such as increasing the division and cell elongation, stimulation of chlorophyll synthesis and photosynthesis, flower bud differentiation, increasing the life of plants, softening the effects of adverse weather conditions and increasing the absorption of nutrients and setting their roots.

The bioactivator acts in the expression of genes responsible for synthesis and activation of metabolic enzymes related to plant growth by altering the production of amino acid precursors of plant hormones. With the increased production of hormones, plant expressed greater vigor, germination and root development. With a greater number of roots, increases the absorption and resistance of plant stomata to water loss, which benefits the metabolism and increases resistance to stress.

Thiamethoxam, 3-(2 – chloro – thiazole - 5 - ylmethyl) - 5 - methyl (1,3,5) oxadiazinan - 4 - ylidene-N- nitroamine, whose chemical structure is shown in Figure 1, is a systemic insecticide neonicotinoid group, family nitroguanidine, which acts on acetylcholine nicotinic receptor in membrane of insects, damaging the nervous system and causing them to death. It is used successfully in pest control in beginning cycle from different cultures. Due to numerous reports of field observations describing increases in vigor development and productivity, even in the absence of pests, has been considered a product that has phytotonic effect.

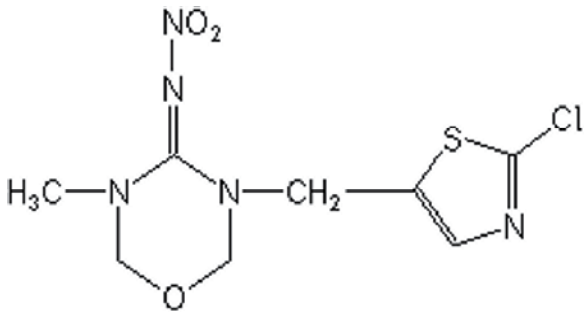


Fig. 1. Chemical structure of thiamethoxam. Source: Oliveira, et al., (2009).

With reference to the mechanism of action of thiamethoxam, the molecule has the ability to induce physiological changes in plants. Due to results obtained, it is concluded that this insecticide acts as bioactive two ways. The first, activating carrier proteins of cell membranes allowing greater ion transport by enhancing plant mineral nutrition. This increase in the availability of minerals promotes positive responses in the development and plant productivity. The second is related to increased enzyme activation caused by thiamethoxam at both the seed and the plant, thereby increasing both primary and secondary metabolism. It also increases the synthesis of amino acids, precursors of new proteins and endogenous synthesis of plant hormones. The responses of plants to these proteins and hormone biosynthesis may be related to significant increases in production.

Results of research related with soybean (*Glycine max*) (Castro et al., 2008; Cataneo, 2008), rice (*Oryza sativa*) (Clavijo, 2008; Almeida et al.; 2010), cotton (*Gossypium hirsutum*) (Lauxen et al., 2010), bean (*Phaseolus vulgaris*) (Almeida et al., 2010) e vegetables like, lettuce (*Lactuca sativa*), tomato (*Lycopersicon esculentum*), pumpkin (*Cucurbita pepo* L.) and carrot (*Daucus carota*) indicate benefit effect of thiamethoxam uses.

2. Bioactivator in physiological performance of carrot seeds

Carrot (*Daucus carota* L.) is the most economical expression of vegetables among those whose edible portion is the root, and to highlight the nutritional value, as a major vegetable

sources of provitamin A (Spinola et al, 1998). Careful selection of cultivars allows sowing of carrots over the years in many regions (Filgueira, 2000).
The success of horticulture generally depends on the establishment of suitable stand for each crop; otherwise reductions may occur in the quantity and quality of final product (Silva and Vieira, 2006).
Under field conditions, carrot seed germination may have low, slow and irregular, resulting in uneven emergence and a heterogeneous population of plants (Corbineau et al., 1994).
With increasing mechanization in vegetable production, establish rapid and uniform culture becomes increasingly important, and it is desirable that the evaluation of seed quality to provide information on their performance on the field.
Establishment of appropriate stand depends on the use of seeds with high physiological potential, able to germinate rapidly and uniformly under a large variation of the environment. Speed and timing are very important because they allow reducing the degree of exposure of seeds and plants to adverse factors (Marcos Filho, 2005).
Reduced or uneven emergence can lead to developmental delays, problems with weed control, non-uniformity of culture in different phenological stages, interference on product quality and characteristics related to the efficiency of harvesting (Marcos Filho, 2005).
In vegetables, backwardness and uneven development may be reflected in product quality and reducing the commercial value, such as lettuce, cabbage, carrots, cauliflower, eggplant and onion (Kikuta and Marcos Filho, 2007).
A survey on physiological quality of seeds of different kinds of vegetables such as carrots, peas, beets, tomato and watermelon industry, marketed and / or used by farmers, it was observed that germination of these seeds do not always fit the minimum standard of marketing required for each species (Nascimento, 1994).
Therefore, failure to stand and seedling vigor at low field level are frequent, with the need for appropriate and sensitive methods to detect these differences in seed quality.
In this context, considering the lack of information concerning the effect of thiamethoxam and the potential benefits that treatment can provide, the present study was to evaluate the influence of thiamethoxam in physiological performance of carrot seeds.

3. Methodology

This work was conducted at the Laboratory of Seed Analysis Textbook and greenhouse, Faculty of Agronomy Eliseu Maciel, Universidade Federal de Pelotas (UFPel), Pelotas / Brazil.
Seed lots of carrot cultivar Brasilia represented by four lots.
To establishment concentrations of thiamethoxam, it was used the following concentrations: 0.0, 0.05, 0.1, 0.2, 0.4, 0.8 mL / L, based on germination test three were selected. Tests conducted to evaluate the quality of seeds were performed with and without water stress.
The seeds were treated in a plastic bag containing distilled water at 0.1 mL of water to 0.05 mL and 0.4 mL of product thiamethoxam to 3g of carrot seeds. The product was applied directly to the bottom of the plastic bag before putting the seeds. Then the seeds were placed in plastic bag and mixed until uniform coating of the seeds. It was used a volume of mixture (product + water) sufficient to promote a more even distribution of product on the seeds. For measurement of product and distilled water were used micropipettes.
Water stress was achieved by the water potential of -0.4 MPa, using aqueous solutions of polyethylene glycol (PEG 6000). The calculation of the solute quantities was performed

according to Villela et al. (1991). Polyethylene glycol solutions, thus obtained were applied to the paper substrate, in an amount equivalent to 2.5 times its dry weight in all parameters evaluated in the laboratory involving the germination test.
To evaluate the physiological quality of seeds were conducted the following tests:
Germination: it was used four replications of 50 seeds of each batch distributed in transparent plastic boxes (gerbox) on two sheets of white blotter paper, moistened 2.5 times the weight of paper, placed in a germination chamber set to maintain the temperature constant 25 ^{o} C. Counts were made in the seventh and fourteenth days after sowing, and assessments, carried out according to ISTA (2010) by computing the percentages of normal seedlings.
Accelerated aging: conducted with 4.0 g of seeds distributed in wire screen suspended and placed inside plastic boxes, type gerbox (mini-camera). Inside the germination boxes were placed 40 mL of water and then the boxes were taken to an incubator set at a constant temperature of 41° C for 48 hours and subsequently subjected to germination tests, as described above. The evaluation was performed seven days after sowing, by computing the percentage of normal seedlings.
Root length: four replicates of 50 seeds were sown on a line drawn in the upper third part of paper substrate. The rolls containing the seeds remained at 25 ° C for seven days, after being evaluated, the root length of normal seedlings, with the aid of a millimeter ruler. The root length was obtained by adding the measurements of each replicate and dividing by the number of seedlings, with results expressed in centimeters.
Speed of germination: performed according to the methodology of the germination test, determined by daily counts to stabilize the number of seedlings in the test and the speed calculation made according to Maguire (1962).
Emergence of seedlings in the greenhouse: four replications of 50 seeds were distributed in individual cells of polystyrene trays (Styrofoam), containing commercial substrate Plantimax ®. The trays were kept in the greenhouse and evaluations were performed at 16 days after sowing, counting seedlings in length and more than 1.0 cm. The results were expressed as a percentage of emergence.
Statistical procedure: completely randomized factorial 4x3 (four lots and three concentrations of the product) separately in the evaluation with and without water stress, with three replications. For comparison of means between control and concentrations, it was used Dunnet test, probability level of 5%.

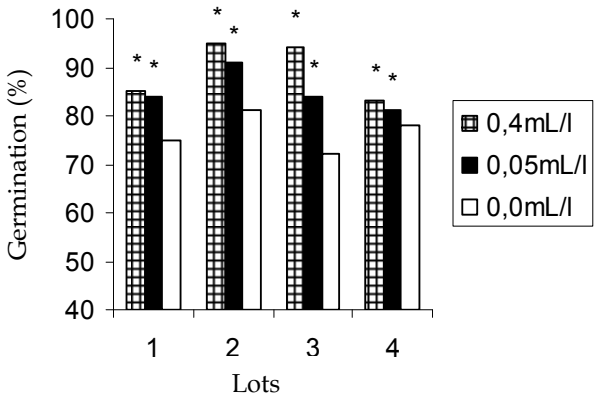
4. Results establishment of concentrations

The concentrations selected based on the result of germination test of treated seeds with different concentrations of the product thiamethoxam, beyond control were 0.05 and 0.4 mL.
Germination of treated seeds in accordance with product concentrations were 70% (zero), 75% (0.05 mL / L), 72% (0.1 mL / L), 72% (0.2 mL / L), 75% (0.4 mL / L) and 70% (0.8 mL / L) without stress. The choice of the concentration of 0.05 mL / L was based on the fact that seeds showed germination similar to other concentrations and spend less of the product. On the other hand, the concentration of 0.4 mL / L was selected because germination test were seedlings showed well developed, open cotyledons and normal roots. At the concentration 0.8 mL / L was found that the seedlings were developed, but their roots had necrosis.
Statistical analysis performed by Dunnet's test showed significant results for comparison of means between control and concentrations of all parameters.

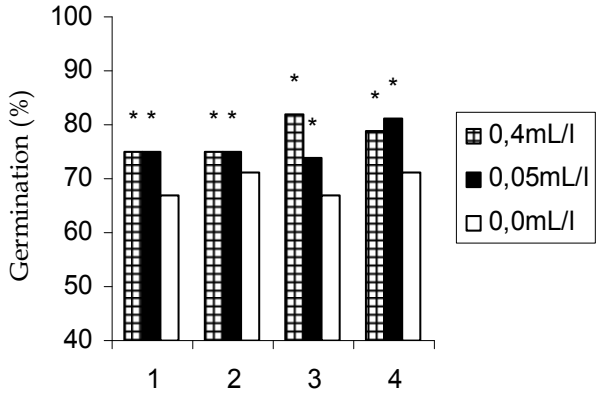
It is noted in Figure 1 that germination of four seed lots, without (Figure 1A) and with (Figure 1B) water stress, treated with thiamethoxam showed significant difference compared to control. Increases in germination were marked and varied according to the lots from 5 to 23 percentage points if the seeds have not been subjected to water stress and 4 to 15 to be subjected to stress.

In Figure 1B it appears that water stress reduced the percentage germination of seed lots. Lots 1 and 3 not treated, after the water stress reached below the standard of marketing, however, treatment stimulated germination of seeds and lots reached the minimum germination (70%) of the standard marketing. In soybean seeds was also observed that thiamethoxam accelerates germination, induces more growth of the embryonic axis and minimize the negative effects in situations of presence of aluminum, salinity and water deficiency (Cataneo et al., 2006).

There is a trend of germination of treated lots with different concentrations of the product showed similar results, with the exception of Lot 3, in which the concentration of 0.4 mL / L was more efficient.



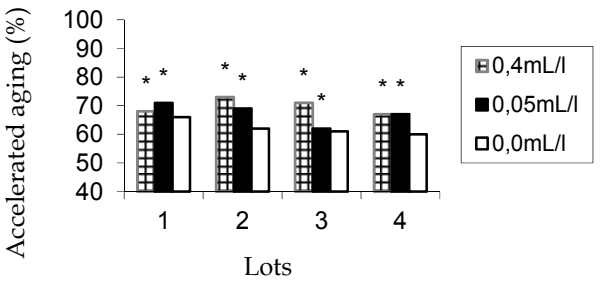
(A)



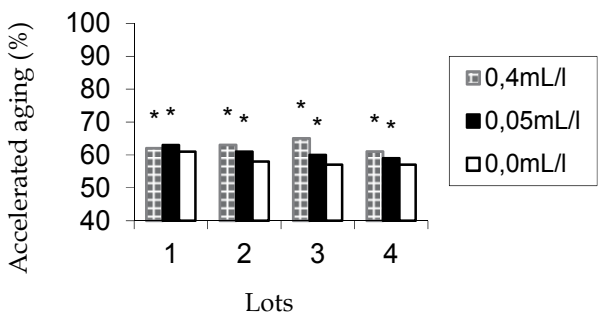
(B)

Fig. 1. Germination (%) of four seed lots of carrot, cultivar Brasilia, submitted (A) or not (B) to water stress. * Different from the control by Dunnet test at probability level of 5%.

According to Figure 2, germination after accelerated aging of treated seeds without (Figure 2A) and with (Figure 2B) water stress showed significant differences related to control. Positive difference varied according of lots, 2 to 11 percentage points in seed not submitted to stress and 2 to 9 in submitted to water stress. This superiority resistance occurs because thiamethoxam move through plant cells and actives several physiological reactions, such as functional protein expression related with plant defense mechanism avoid stress factors like drought, high temperatures, toxic effects, among others, improving productivity, leaf and radicular area, as found in soybean seed (Tavares e Castro, 2005).
Concentrations showed positive results in situations with and without water stress, but the concentration of 0.4 mL / L performed better for lots 2 and 3, without stress and 2, 3 and 4 with stress.



(A)



(B)

Fig. 2. Accelerated aging (%) of four seed lots of carrot, cultivar Brasilia, without (A) and with (B) water stress.* It differs from the control by Dunnet test at probability level of 5%.

According to data presented in Figures 3 and 4, treated seeds showed marked differences in root length compared to untreated, on average 4 cm, in both cases without (Figure 3 and 4 A) and with (Figures 3 and 4 B) water stress. This effect of thiamethoxam of supporting the growth of the root system, confirming the effect of rooting observed by Pereira et al. (2007) in sugar cane and potatoes; and also by Tavares et al. (2007) in soybean. It is believed that the thiamethoxam increase water uptake and stomatal resistance, improving the water

balance of the plant, tolerating water deficit better (Castro, 2006). As observed in soybean root development increases the absorption of nutrients, increases the expression of leaf area and plant vigor (Tavares and Castro, 2005).

The data speed of germination, without (Figure 5A) and with (Figure 5B) stress show that the treated seeds had a higher rate compared to control. The concentrations used had similar results. Treated seeds germinated on average one day soon if they have not been subjected to water stress and two days are subject to stress. This effect is very promising because carrot seeds in field conditions have poor germination, slow and irregular resulting in uneven emergence (Corbineau et al., 1994). This increased speed of germination is caused by physiological changes that occur in the plant indirectly stimulating the production of hormones, resulting in increased vigor, root growth, water absorption and primary and secondary metabolism, as observed in the sugarcane crop (Castro, 2007).

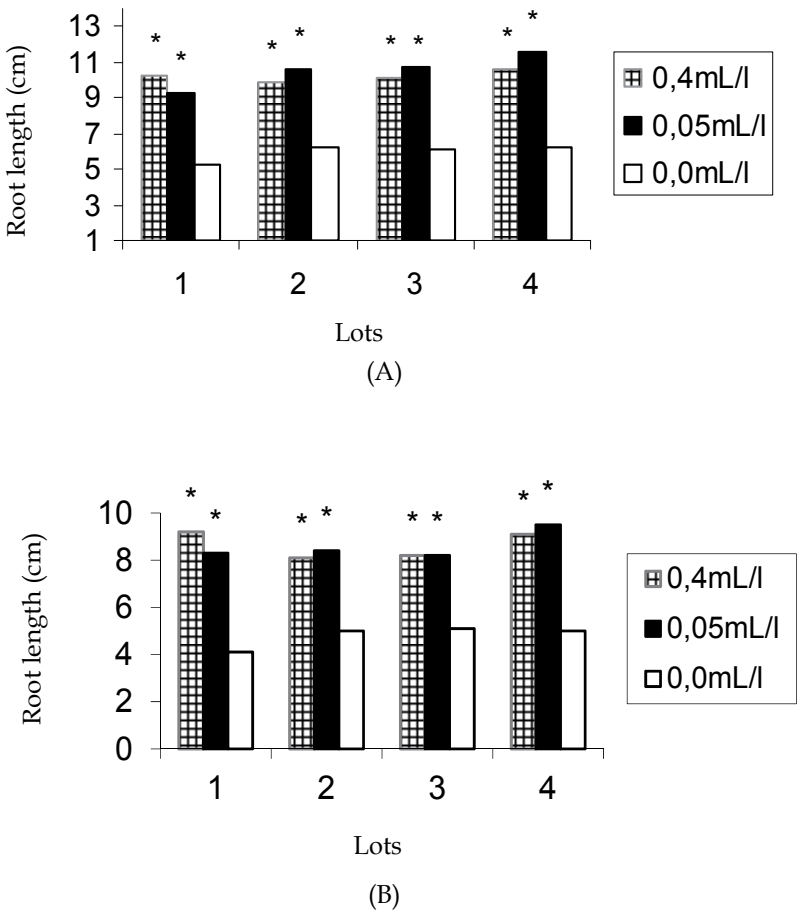
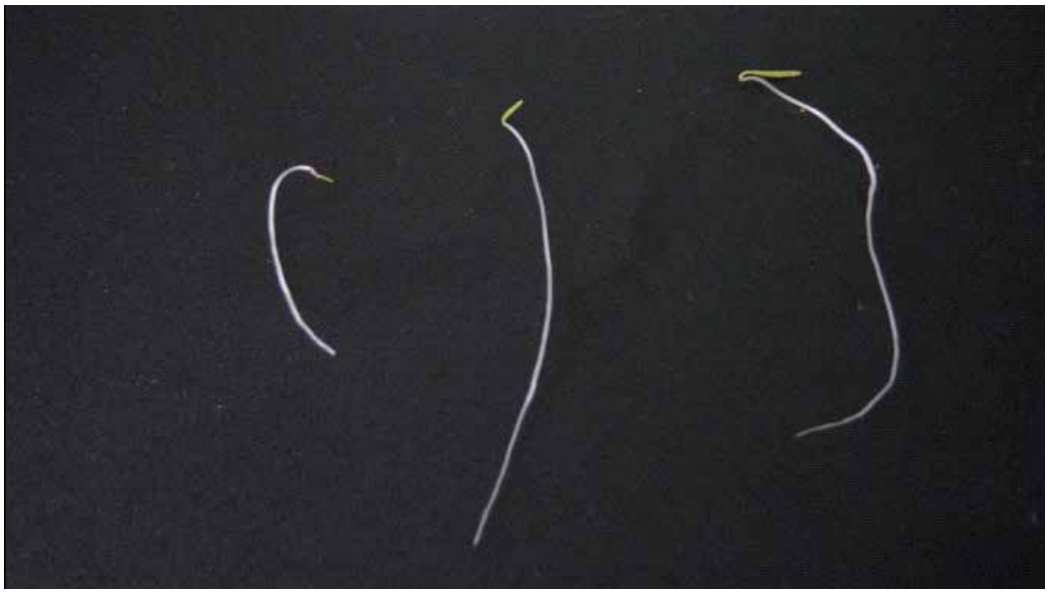


Fig. 3. Root length (cm) of seedlings of four seed lots of carrot, cultivar Brasilia, without (A) and with (B) water stress. * Different from the control by Dunnet test at probability level of 5%.



(A)

mL of product/ 3g of seed

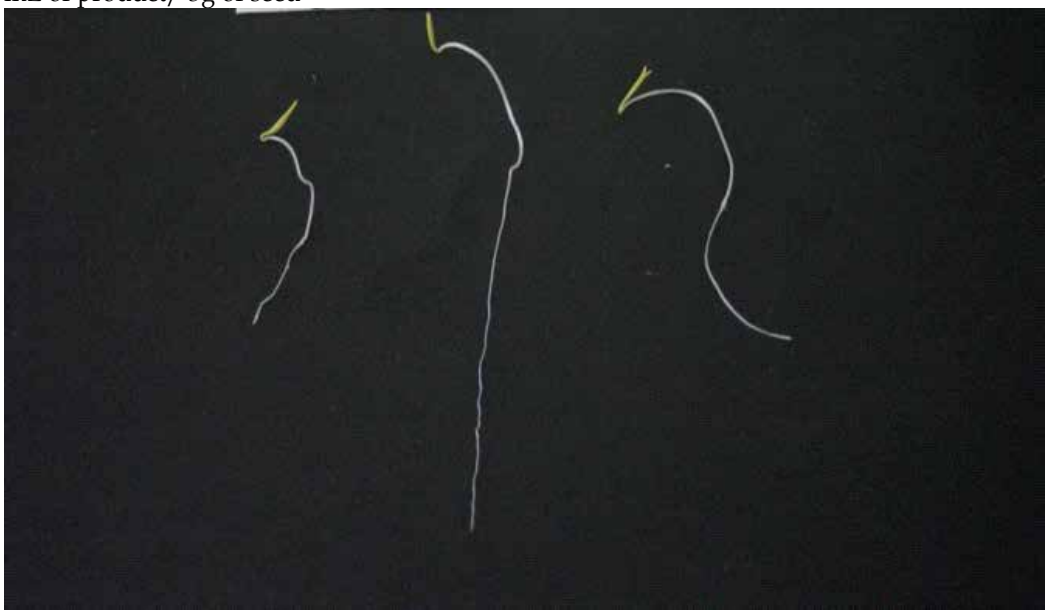
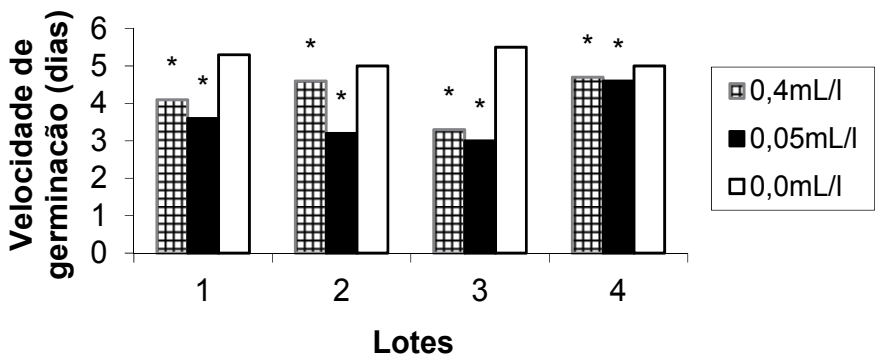
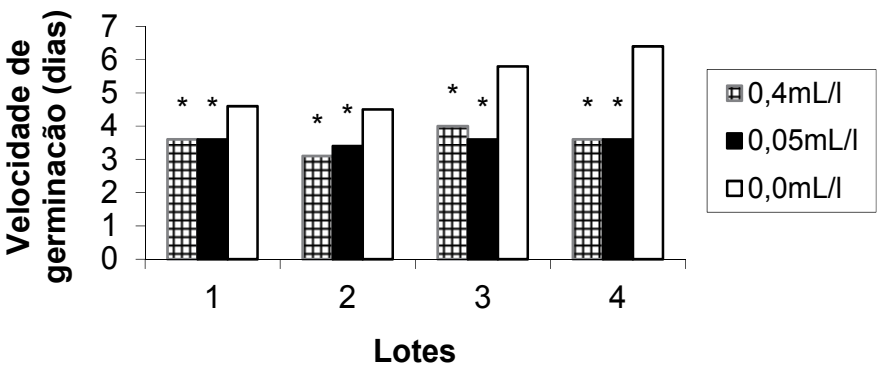


Fig. 4. Root length (cm) of seedlings of four seed lots of carrot, cultivar Brasilia, without (A) and with (B) water stress.

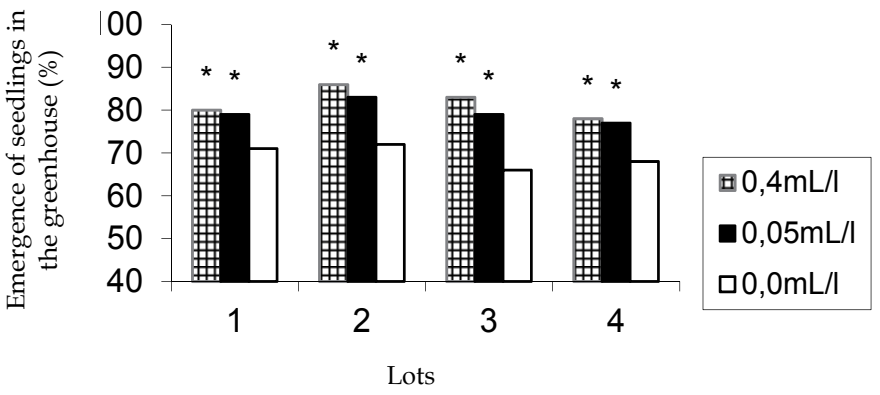


(A)



(B)

Fig. 5. Speed of germination (days) of four seed lots of carrot cultivar Brasilia, without (A) and with (B) water stress.* It differs from the control by Dunnet test at probability level of 5%.



(A)

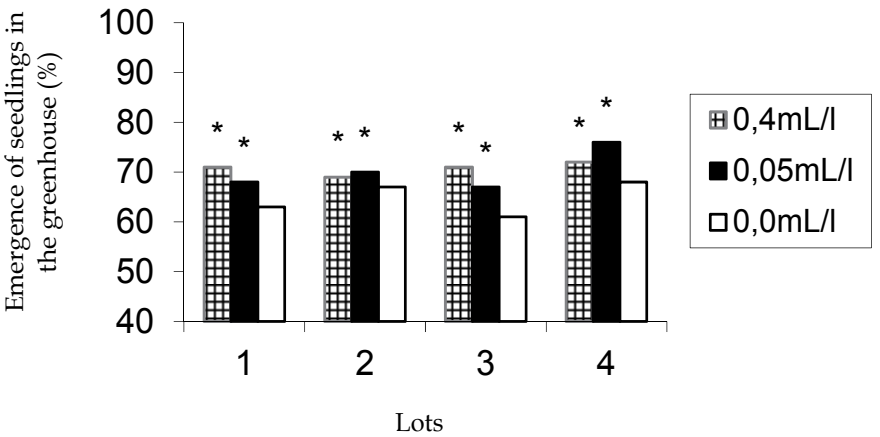


Fig. 6. Emergence of seedlings in the greenhouse for four seed lots of carrot, cultivar Brasilia without (A) and with (B) water stress. * Different from the control by Dunnet test at probability level of 5%.

In Figure 6, without (Figure 6A) and with (Figure 6B) water stress, it was observed that the emergence of seedlings in the greenhouse was stimulated, and the seeds treated with thiamethoxam showed significant differences compared to control. The positive differences compared to control vary according to lots, 9 to 17 percentage points if the seeds have not been subjected to water stress and 20 to 10 percentage points when subjected to stress. The two concentrations showed similar responses. These results confirm those found in soybean, to be seen increase in the root system and the percentage of seedling emergence also in water deficit conditions (Castro et al., 2006). According to the literature, soybean seeds treated with thiamethoxam have higher levels of amino acids, enzyme activity and synthesis of plant hormones that increase the plant responses to these proteins and these events provide significant increases in production and reducing the time of establishment of culture in the field, making it more tolerant to stress factors (Castro, 2006).

The results obtained can be described that the product stimulated the performance of carrot seeds in all parameters evaluated, both in seeds subjected to water stress or not. Carrot seeds treated with the product thiamethoxam showed significant increases in germination and vigor for all lots. Among the aspects of vigor, the product stimulated the growth of the root length, which is of great importance to the culture of carrots and this result was obtained in the laboratory confirmed in the greenhouse.

The product was more effective in stimulating the quality of seeds not subjected to water stress, with the exception of root length which positive change was similar for seeds subjected to stress or not. In all parameters evaluated, increases in the quality varied according to the lot. Concentrations of the product for most tests evaluated did not differ, however there was a trend of higher concentration to the higher values.

The application of thiamethoxam has strong interest for the culture of carrot, whose edible portion is the root and, moreover, by presenting, in field conditions, poor germination, slow, irregular with uneven emergence, the product acts as an enhancer, by allowing the expression of seed germination potential, accelerate the growth of roots and increase the absorption of nutrients by the plant. These features of thiamethoxam combined with the use of genetics and physiological high-quality seed powers the productive capacity of the culture.

5. Conclusions

Thiamethoxam product stimulates the physiological performance of carrot seeds subjected to water stress or not, with variable intensity according to lot.

Concentrations of 0.05 and 0.4 mL of the product is effective, however there is a tendency of higher concentration to the higher increases in quality.

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2

The Pyrethroid Knockdown Resistance

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1. Introduction

New promising insect control efforts are now being evaluated such as biological alternatives or even transgenic insects and *Wolbachia* based strategies. Although it is increasingly clear that successful approaches must involve integrated actions, chemical insecticides unfortunately still play a central role in pest and vector control (Raghavendra et al., 2011). Development of new safe and effective compounds in conjunction with preservation of those currently being utilized are important measures to insure insecticide availability and efficiency for arthropod control. In this sense, understanding the interaction of insecticides with the insect organism (at physiological and molecular levels), the selected resistance mechanisms and their dynamics in and among natural populations is obligatory.

Pyrethroids are synthetic compounds derived from pyrethrum, present in *Chrysanthemum* flowers. Currently, pyrethroids are the most used insecticides against arthropod plagues in agriculture and livestock as well as in the control of vectors of veterinary and human health importance. They are chemically distinguished as type I (such as permethrin, compounds that lack an alpha-ciano group) and type II (with an alpha-ciano group, like deltamethrin) (T. G. Davies et al., 2007b). Pyrethroid insecticides have been largely adopted against vector mosquitoes through indoor, perifocal or ultra-low volume (ULV) applications. As of yet pyrethroids are the only class of insecticides approved for insecticide treated nets (ITNs), an important tool under expansion against malaria, mainly in the African continent (Ranson et al., 2011). The consequence of intense and uncontrolled pyrethroid use is the extremely rapid selection of resistant populations throughout the world.

Just like DDT, pyrethroids act very fast in the central nervous system of the insects, leading to convulsions, paralysis and eventually death, an effect known as *knockdown*. However, unlike DDT, pyrethroids are not claimed to cause severe risks to the environment or to animal or human health, hence its widespread use. The main pyrethroid resistance mechanism (the knockdown resistance phenotype, *kdr*) occurs due to a point mutation in the voltage gated sodium channel in the central nervous system, the target of pyrethroids and DDT.

Herein we aim to discuss the main mechanism of pyrethroid resistance, the knockdown resistance (*kdr*) mutation, its effect and its particularities among arthropods. The most common methods presently employed to detect the *kdr* mutation are also discussed. Some aspects regarding the other main pyrethroid resistance mechanisms, like alterations in behaviour, cuticle and detoxifying enzymes will be only briefly addressed. The proposal of this chapter is to review knockdown resistance to pyrethroids, nowadays the preferred insecticide class worldwide. This topic discusses aspects of general biology, physiology,

biochemistry, genetics and evolution, with focus on disease vector mosquitoes. It is expected that the amount and diversity of material available on this subject may well illustrate insecticide resistance in a broader context.

2. Insecticide resistance mechanisms

Besides the resistance to chemical insecticides caused by modifications in the target site (also called phenotypic resistance), other mechanisms commonly associated are: metabolic resistance, behavioral modification and alterations in the integument. In the first case, endogenous detoxifying enzymes become more efficient in metabolizing the insecticide, preventing it from reaching its target in the nervous system. This occurs due to 1) increase in the number of available molecules (by gene amplification or expression activation) or 2) mutation in the enzyme coding portion of the gene, so that its product metabolizes the insecticide more efficiently. These processes can be very complex and involve three major enzyme superfamilies: Esterases, Multi function Oxidases P450 and Glutathion-S-Transferases (Hemingway & Ranson, 2000; Montella et al., 2007). In contrast, there are few examples in literature regarding insect behavioral changes and tegument alterations.

Resistance to insecticides may be functionally defined as the ability of an insect population to survive exposure to dosages of a given compound that are lethal to the majority of individuals of a susceptible lineage of the same species (Beaty & Marquardt, 1996). Resistance is based on the genetic variability of natural populations. Under insecticide selection pressure, specific phenotypes are selected and consequently increase in frequency. Resistance can result from the selection of one or more mechanisms. In order to elucidate the molecular nature of resistance, many studies report laboratory controlled selection of different species (Chang et al., 2009; Kumar et al., 2002; Paeoporn et al., 2003; Rodriguez et al., 2003; Saavedra-Rodriguez et al., 2007). With selected lineages, it becomes easier to separate the role of each distinct mechanism. In a more direct approach, the current availability of a series of molecular tools enables detection of expression of altered molecules in model organisms so that the effect of the insecticide can be evaluated under specific and controlled circumstances (Smith et al., 1997).

Regardless of the mono or multi-factorial character of resistance, this phenomenon may be didactically divided into four categories: behavioral, cuticular, metabolic and phenotypic resistance. In the first case the insect simply avoids contact with the insecticide through behavioral adaptations, which are presumably related to genetic inheritance (Sparks et al., 1989). Among arthropods, mosquitoes are by far the group most intensely investigated in relation to behavioral resistance (Lockwood et al., 1984). For instance, *Anopheles* malaria vector mosquitoes from the Amazon Region had the habit of resting in the walls after a blood meal. There are registers that some populations changed their behavior after a period of indoor residual application of DDT to the dwelling walls (Roberts & Alecrim, 1991). Behavioral changes that minimize contact between insect and insecticide may cause a severe impact in the insecticide application efficacy, especially if resistance is selected by physiological features (Ranson et al., 2011).

Certain alterations in the insect cuticle may reduce insecticide penetration. However, these effects are unspecific, leading to resistance to a series of xenobiotic compounds. This mechanism is known as reduced penetration or cuticle resistance. It is probably not related to high levels of resistance by itself, but it can interact synergistically with other mechanisms. The physiological processes or molecular pathways which describe this type of

resistance remain to be elucidated. With respect to pyrethroid resistance, recent evidences point to an increase in the levels of expression of two cuticle genes in populations of two *Anopheles* species (Awolola et al., 2009; Vontas et al., 2007).

The increased ability to detoxify insecticides is one of the main types of resistance, commonly referred to as metabolic resistance. It takes place when the activity of naturally detoxifying enzymes is enhanced, impeding the insecticide to reach its target. Among these enzymes, Multi function Oxidases (or Monoxigenases P450), Esterases and Glutathion-S-Transferases (GST) (ffrench-Constant et al., 2004; Hemingway & Ranson, 2000) are the major representative families. Although the molecular basis of metabolic resistance has been extensively studied, only few reports have investigated the specific metabolic pathways involved or their location in the insect organism. Many different mutations may be attributed to metabolic resistance, such as those leading to production of more enzymes, via gene duplication events or either increases in gene transcription rates, alterations in the normal tissue/time specificity of expression, point mutations leading to a gain of function or changes in the substrate specificity (ffrench-Constant et al., 2004; Hemingway et al., 2004; Perry et al., 2011). Detoxifying enzymes belong to superfamilies composed of numerous genes (Ranson et al., 2002), and it is not unusual for different enzymes to produce the same metabolites. Additionally, an alteration in one type of enzyme may lead to cross-resistance among different classes of insecticides (Ranson et al., 2011). However, population genetic markers that make feasible a complete diagnostic of the resistance mechanisms or their distribution are not yet available. Current studies are generally based on biochemical assays (Valle et al., 2006) and, to a lesser extent, on *microarray detox chips* (David et al., 2005; Vontas et al., 2007). Due to technical limitations, the most common reports are hence oriented to single gene responses, such as punctual mutations that increase the ability of a specific enzyme in detoxifying an insecticide (Lumjuan et al., 2011; Morin et al., 2008).

Multi function P450 Oxidases are the enzymes most commonly associated to metabolic resistance to pyrethroids. However, despite much indirect evidence of P450 total activity increase or even detection of higher expression of some related genes (*cyp*), little is known about their metabolic activity. For instance, 111 genes code for P450 in *Anopheles gambiae*, but only two (*cyp6p3* and *cyp6m2*) were described to be involved in pyrethroid metabolism (Muller et al., 2008). Surprisingly, metabolic resistance can still vary during the course of the day. This is the case of an *Ae. aegypti* population whose resistance to the pyrethroid permethrin is mediated by the *cyp9M9* gene. Expression of this gene is regulated by transcriptional factors enrolled in the circadian rhythm of the insect, varying along the day (Y. Y. Yang et al., 2010).

Finally, phenotypic or target site resistance is designated by modification of the insect molecule where the insecticide binds, inhibiting its effects. Neurotoxic insecticides have as their ultimate target different molecules from the insect central nervous system: the enzyme Acetylcholinesterase (for organophosphates and carbamates), the gama-aminobutiric acid receptor (for ciclodienes), the nicotinic acetylcholine receptors (for spinosyns and neonicotinoids) and the voltage gated sodium channel (for DDT and pyrethroids). Although the mutated target molecule decreases or even abolishes its affinity for the insecticide, it is essential that this alteration does not result in loss of function regarding the insect physiological processes. Since the classical target molecules are much conserved among animals, few mutations are permissive to guarantee the viability of their carriers (ffrench-Constant et al., 1998; Raymond et al., 2001).

The voltage gated sodium channel (Na_V) is the effective target for a number of neurotoxins produced by plants and animals, as components of their predation or defense strategies. Knowledge that mutations in the Na_V gene can endow resistance to both the most popular insecticides of the past (DDT) and nowadays (pyrethroids) is leading to significant progress in the understanding of the physiology, pharmacology and evolution of this channel (ffrench-Constant et al., 1998; O'Reilly et al., 2006).

3. The role of the voltage gated sodium channel (Na_V) in the nerve impulse propagation in insects

The membrane of all excitable cells (neurons, myocites, endocrinous and egg cells) have voltage gated ion channels responsible for the generation of action potential. These cells react to changes in the electric potential of the membrane, modifying their permeability status (Alberts et al., 2002; Randall et al., 2001). Voltage gated sodium channels (Na_V) are transmembrane proteins responsible for the initial action potential in excitable cells (Catterall, 2000). They are members of the protein superfamily which also includes voltage gated calcium (Ca_V) and potassium (K_V) channels (Jan & Jan, 1992). Both Na_V and Ca_V channels are constituted of four homologous domains whilst K_V is a tetramer with only one domain. A proposed evolution pathway assumes that Ca_V have evolved from K_v by gene duplication during the evolution of multicelular eukaryotes. Na_V channels are supposed to have evolved from an ancestral Ca_V family (family Ca_V3) (Spafford et al., 1999). Accordingly, the four Na_V domains are more similar to their Ca_V counterparts than among themselves (Strong et al., 1993). The sodium channel is completely functional by itself, unless the kinetics of opening and closure of the voltage gated channel can be modified by other proteins, sometimes referred to as complementary subunits (beta subunit in mammals and TipE in *Drosophila*) (Catterall et al., 2003).

Cell action potential starts with the depolarization of the membrane, with the internal side attaining a more positive state (compare Figure 1, pannels A and B). A stimulus that causes the depolarization in a given region of the cell membrane promotes activation (opening) of the Na_V in the vicinity. This process results in the influx of Na^+ to the cell, enhancing depolarization of the membrane. The action potential works in a positive feedback, that is, once started there is no need of additional stimuli to progress. However, one millisecond after the channel has been activated, the surrounding membrane reaches the Na^+ equilibrium potential, and the channel is deactivated. In this state, the pore is still open, but it assumes a conformation that halts the ion influx into the cell (Figure 1, C). After some further milliseconds, the membrane is repolarized and the channel closes, finally returning to its resting configuration (Figure 1, D). This whole process occurs in consonance with other channels and pumps, such as K_V and sodium/ potassium pumps that restore the original electric potential of the cell (Catterall et al., 2003; Randall et al., 2001). The correct operation of sodium channels is essential for nerve impulse propagation. Hence, if the regular propagation of an impulse is altered, as due to the interaction with an insecticide, the organism suffers paralysis and can eventually die.

The structure of Na_V is organized in four homologous domains (I-IV), each containing six hydrophobic segments (S1-S6) and a *P-loop* between S5 and S6 (Figure 2). The segments S1-S4 work as a voltage sensitive module. Since S4 segments are positively charged and sensitive to voltage changes, they move across the membrane in order to initiate the channel activation in response to membrane depolarization (schematically represented in Figure 1,

compare relative position of the Na_v blue domains in the different pannels). The pore forming module is composed of the S5-S6 segments and the loop between them, the latter acting as an ion selective filter in the extracellular entrance of the pore (Catterall et al., 2003; Goldin, 2003; Narahashi, 1992). Additionally, the *P-loop* residues D, E, K and A, respectively from domains I, II, III and IV, are critical for the Na^+ sensitivity (Zhou et al., 2004).

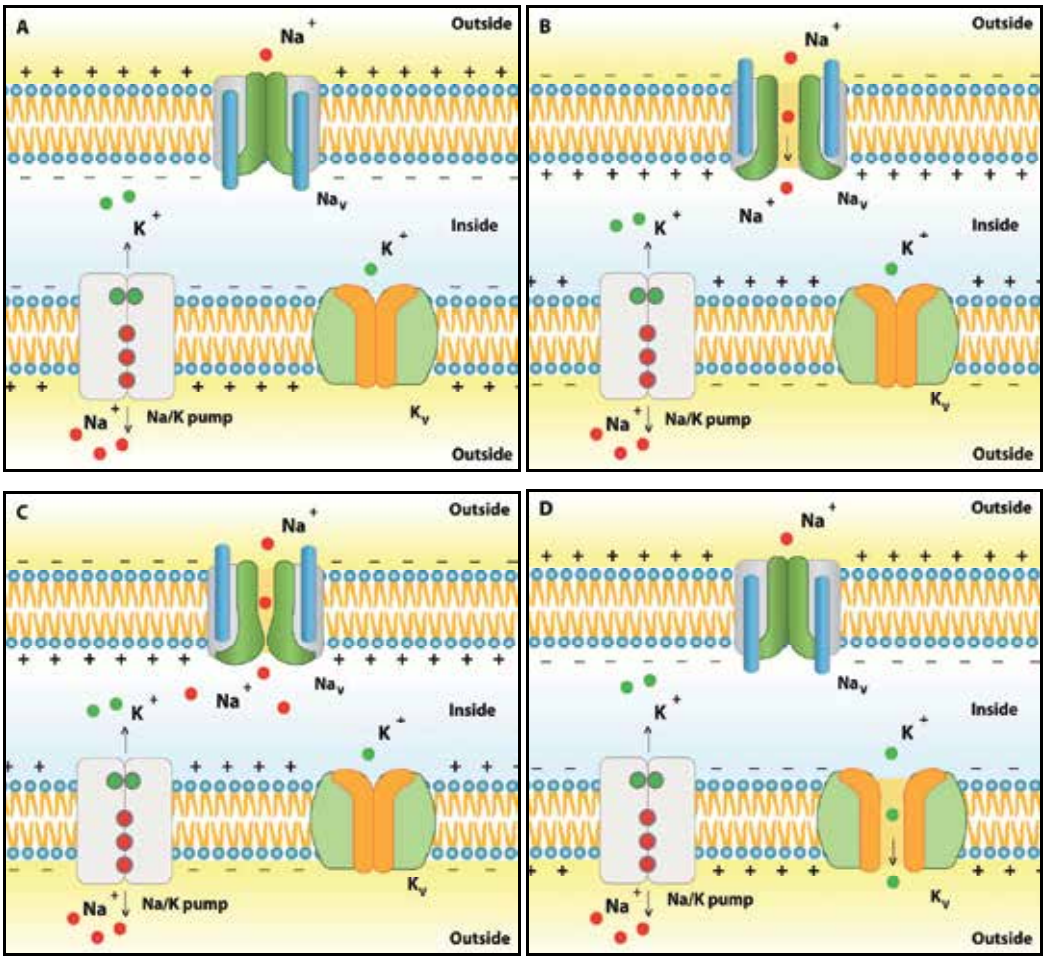

Fig. 1. Propagation of the action potential through a neuronal axon - In the resting potential stage (A) the axon cytoplasm has Na^+ and K^+ respectively in low and high concentrations compared to the surrounding extracellular fluid. The Na/K pump is constantly expelling three Na+ from the cell for every two K+ it transfers in, which confers a positive charge to the outer part of the membrane. When there is a nervous stimulus, the Na_V opens and the membrane becomes permeable affording the influx of Na^+, depolarizing the membrane charge (B). This is the rising phase of the action potential. Soon (~1 millisecond), the Na_V is deactivated, precluding further Na+ entrance to the cell (C), whilst K^+ exits the cell through K_V which is now opened, characterizing the falling phase of the action potential (D). The Na/K pump helps to reestablish the initial membrane potential. The action potential generates a wave of sequential depolarization along the axon. Figure based on T. G. Davies et al. (2007b).

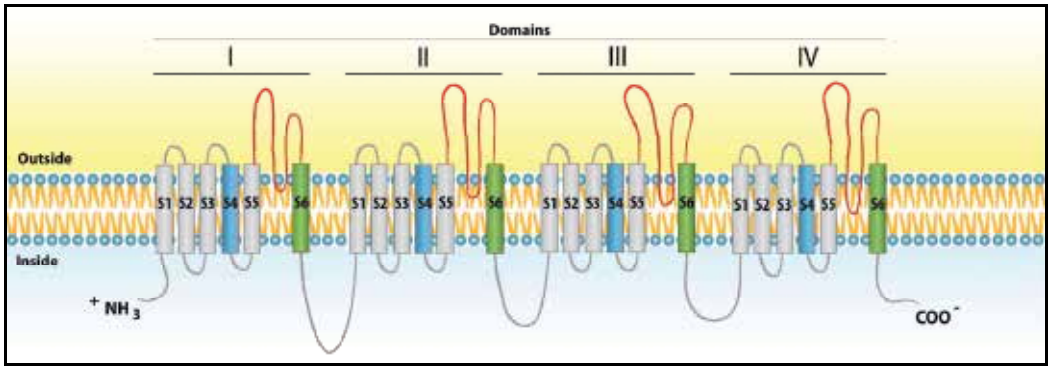

Fig. 2. The voltage gated sodium channel - Scheme representative of the Na_V inserted in a cell membrane, showing its four homologous domains (I-IV), each with six hydrophobic segments (S1-S6). In blue, the voltage sensor segments (S4); in green, the S6 segments, which form the channel pore together with the S5 segments and the link (*P-loop*, in red) between them. Figure adapted from Nelson & Cox (2000).

In the closed state, the putative insecticide contact sites are blocked, corroborating the assumption that pyrethroids and DDT have more affinity to the Na_v channel in its open state when the insecticide stabilizes the open conformation (O'Reilly et al., 2006). These insecticides, therefore, inhibit the channel transition to the non-conducting and deactivated states (T. E. Davies et al., 2008). By interacting with the channel, they form a sort of wedge between segments IIS5 and IIIS6 that restricts displacement of the pore forming helices S5 and S6, preventing closure of the channel. Consequently, the influx of Na^+ is prolonged, and the cell is led to work at an abnormal state of hyper-excitability. The amplitude of the Na^+ current will not decrease unless the cell's level of hyper-excitability is overcome by its ability to keep the sodium-potassium pump under operation. This process is responsible for the pyrethroid sublethal effect in insects, known as *knockdown* effect, which may lead to paralysis and death if prolonged (T. E. Davies et al., 2008; T. G. Davies et al., 2007b).
Predictive models suggest that DDT and pyrethroids interact with a long and narrow cavity delimited by the IIS4-S5 linker and the IIS5 and IIIS6 helices, accessible to lipophilic insecticides. Moreover, some of the aminoacids belonging to the helices engaged in contact with these insecticides are not conserved among arthropods and other animals, and this could be responsible for the selectivity of pyrethroid effects against insects (O'Reilly et al., 2006). The crystal structure of a Na_V has been recently published (Payandeh et al., 2011), pointing to a better understanding of the channel function and to its interaction with targeted compounds in a near future.
Besides pyrethroids and DDT, other insecticides act on the voltage gated sodium channel, like the sodium channel blocker insecticides (SCBIs) and N-alkylamide inseticides (like BTG 502). There are few reports about these compounds. However, it is known that SCBIs, such as indoxicarb, act by blocking the impulse conduction, an effect opposite to that of DDT and pyrethroids (Du et al., 2011).

4. The knockdown effect and the *kdr* phenotype

In the early 1950s, no sooner had DDT been introduced as an insecticide than resistant strains of houseflies were described. When exposed to DDT, these insects in general did not

suffer paralysis followed by death (*knockdown*) but, at most, presented a momentary paralysis followed by complete locomotion recovery, this phenotype being named *kdr* (*knockdown* resistance) (Busvine, 1951; Harrison, 1951; Milani, 1954). Since the introduction of pyrethroids, plenty of insect species exhibiting the *kdr* phenotype have been observed, attributed to previous DDT selection pressure, characterizing cross-resistance between both insecticides (Hemingway & Ranson, 2000). *Kdr* resistance results in 10-20 fold decrease in the sensitivity to the insecticide. However, *kdr* lineages of some species can exhibit up to 100 X increased pyrethroid resistance, an effect denominated *super-kdr*. *Kdr* and *super-kdr* alleles act as recessive traits and hence may persist at low levels in the population in heterozygous individuals (T. G. Davies et al., 2007a).

Over three decades after the description of the *kdr* effect, electrophysiological studies based on neuronal cells and tissues suggested that Na_V had to be the target site for pyrethroids. These reports also indicated that cross-resistance between pyrethroids and DDT must be related to that channel (Pauron et al., 1989). Concomitantly, the gene *paralytic* (*para)* from *Drosophila melanogaster* was cloned and sequenced. This gene is placed in the *locus* related to behavioral changes and paralysis after exposure to high temperatures, similar to the *knockdown* effect produced by DDT and pyrethroids (Loughney et al., 1989). Comparisons within vertebrate nucleotide sequences revealed that *para* is homologous to the voltage gated sodium channel gene (Na_V) (Loughney & Ganetzky, 1989). It was then shown, with a DDT resistant housefly lineage, that the *locus* homologous to *para* was in strong linkage with the *kdr* phenotype (Williamson et al., 1993). This evidence was soon extended to other insect species plagues or vectors, such as the tobacco budworm *Heliothis virescens* (Taylor et al., 1993), the cockroach *Blatella germanica* (Dong & Scott, 1994) and the mosquito *Aedes aegypti* (Severson et al., 1997).

Hitherto, Na_V is the only molecule incriminated as the target site for DDT and pyrethroids. Although it has been implied that type II pyrethroids can interact with the GABA receptor (which is the target, for instance, of the insecticide dieldrin), this interaction has not been considered toxically important (Soderlund & Bloomquist, 1989). Research on the molecular interaction between pyrethroids and their target site presently guides a series of approaches towards the development of a great variety of natural and synthetic neurotoxicants acting on the Na_V (Soderlund, 2010).

5. Molecular biology of the insect Na_V and the *kdr* mutation

A great variety of sodium channels have been identified by electrophysiological assays, purification and cloning (Goldin, 2001). In mammals, nine Na_V genes are known, with distinct electrophysiological properties as well as different expression profiles in the tissues and throughout development (Goldin, 2002; Yu & Catterall, 2003), phylogenetic analyses revealing that all are members of only one unique family, deriving from relatively recent gene duplications and chromosome rearrangements. On the other hand, Ca_V and Ka_V have little protein sequence identity and present diverse functions, indicative of more ancient segregation of their coding genes (Catterall et al., 2003).

The Na_V orthologous genes and cDNAs from *D. melanogaster* and *An. gambiae* share, respectively, 56-62% and 82% of nucleotide identity, evidencing a high degree of conservation between these species. The Na_V C-terminal is the most variable region, but as in all dipterans, it is mainly composed of aminoacids of short (Gly, Ala, Ser, Pro) or negative (Asp, Glu) side chains, suggesting a conserved function in this domain (T. G. Davies et al.,

2007a). Concerning size, the voltage gated sodium channel of *Ae. aegypti* (*AaNa$_V$*), for instance, presents 293 Kb of genomic DNA, with 33 exons. Its longer observed transcript has an ORF of 6.4 Kb, coding for 2,147 aminoacids for a protein estimated in 241 KDa (Chang et al., 2009).

The existence of two Na$_V$ evolutionary lines in invertebrates, represented by the genes *para* and *DSC1* in *D. melanogaster*, has been suggested (Spafford et al., 1999). These lines do not correspond to the different genes observed among vertebrates, and they are supposed to have arisen after vertebrate and invertebrate splitting (Goldin, 2002). DSC1 plays a role in the olfactory system (Kulkarni et al., 2002) as it has been found in the peripheral nervous system and also at high density in the synaptic regions. DSC1 is sensitive to tetradotoxin, a specific Na$_V$ blocker (Zhang et al., 2011), while BSC1, its homologous in *B. germanica*, has also been identified as a putative sodium channel, being expressed in the cockroach nerve cord, muscle, gut, fat body and ovary (Liu et al., 2001). Neither *DSC1* nor *BSC1*, however, mapped with any *locus* related to insecticide resistance (Loughney et al., 1989; Salkoff et al., 1987). Actually, these channels probably represent prototypes of a new Ca$_V$ family, highly related to the known Na$_V$ and Ca$_V$ (Zhang et al., 2011; Zhou et al., 2004). On the other hand, in invertebrates, the *D. melanogaster para* gene (or *DmNa$_V$*) and its equivalent in other species actually code for sodium channels and are related to pyrethroid/DDT resistance and to behavioral changes, as aforementioned.

In his review, Goldin (2002) suggested that two to four genes coding for sodium channels should exist in insects and that differences among them would not result from distinct genes but from pos-transcriptional regulation. Accordingly, even after publication of many insect genome sequences, there has been no mention whatsoever of Na$_V$ gene duplication. Furthermore, recent reports attribute the diversity in Na$_V$ sequences to alternative splicing and RNA editing. These modifications seem to be tissue and stage specific and might also have some influence on pyrethroid resistance (Liu et al., 2004; Song et al., 2004; Sonoda et al., 2008).

5.1 Alternative mRNA splicing in the Na$_V$

Briefly, alternative splicing is a post-transcriptional regulated event characterized when certain exons are removed together with introns. This is a common mechanism of gene expression regulation and increment of protein diversity in eukaryotes. The process may occur in different ways: complete exons can be included or excluded (optional exons), splicing sites can be altered and introns can be retained in the mature mRNA. There are also mutually exclusive pairs of exons, when two exons never unite in the same transcript. Alternative mRNA splicing introduces variability in both sequence and size of the Na$_V$ intracellular region, which by itself should have an impact on its operation (T. G. Davies et al., 2007a).

The regulation for excision of an exon, in detriment of others, may be tissue and development specific. In the context of pyrethroid resistance, it is important to know to what extent alternative splicing events compromise the interaction between the insecticide and the channel. It is also necessary to investigate the amount of alternative transcripts in the course of development and their distribution in the different tissues of the insect. The sodium channel genes have alternative exons that potentially synthesize a great number of different mRNAs (Figure 3). There are also mutually exclusive exons that occur in the transmembrane regions of domains II and III (T. G. Davies et al., 2007a). In *D. melanogaster*, many alternative splicing sites have been identified, with seven optional regions and two

pairs of mutually exclusive exons (Figure 3) (Olson et al., 2008). These sites are conserved in *M. domestica* (Lee et al., 2002) generating, in both species, 512 potential Na_V transcripts by alternative splicing. However, they are not all necessarily expressed as less than 10 were actually observed in mRNA pools (Soderlund, 2010).

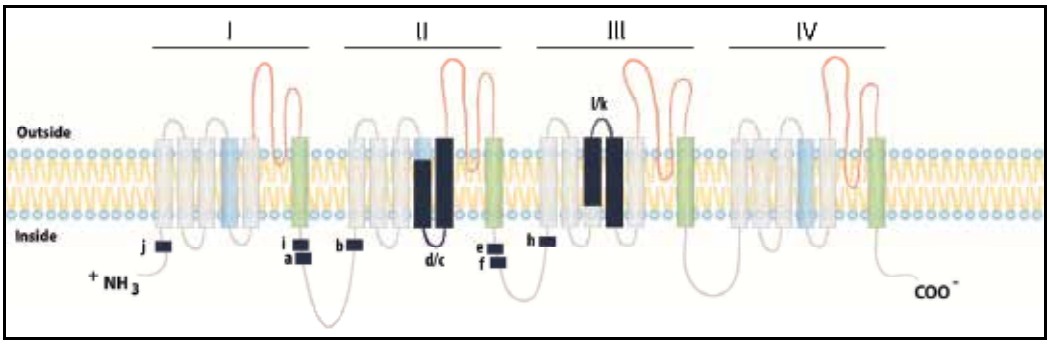

Fig. 3. Alternative splice in the insect voltage gated sodium channel gene. Scheme of Na_V with the sites of alternative exons of $DmNa_V$ indicated in dark color. Exons *a*, *b*, *i*, *j*, *e* and *f* are optional, while *d*/*c* and *l*/*k* are mutually exclusive. Figure adapted from Oslon et al. (2008).

The aminoacid sequences translated from optional exons are conserved and generally consist of intracellular domains of the channel, suggesting functional relevance to these events. Na_V transcript diversity derived from alternative splicing has been investigated in insects of many orders, revealing a high level of conservation, as shown in the cockroach *B. germanica* (Liu et al., 2001; Song et al., 2004), the silk worm *Bombyx mori* (Shao et al., 2009), the moth *Plutella xylostella* (Sonoda et al., 2008) and the mosquitoes *An. gambiae* (T. G. Davies et al., 2007a) and *Ae. aegypti* (Chang et al., 2009). However, in some species not all exons were observed nor their expression detected (see Davies et al., 2007a).

There are two mutually exclusive exons (called c/d) that code for a region between IIS4 and IIS5 segments (Figure 3). The absence of one of these exons might be important for pyrethroid resistance, since the *super-kdr* mutation (Met918Thr) is located in this region, as will be discussed further. In the cockroach *B. germanica*, the mutually exclusive exon pair *k*/*l* codes for the voltage sensitive region at domain III. The two varieties $BgNa_V1.1a$ and $BgNa_V1.1b$[1], which contain the exons *l* and *k* respectively, exhibit distinct electrophysiological properties. Furthermore, $BgNa_V1.1b$ is 100X more resistant to the pyrethroid deltamethrin than $BgNa_V1.1a$ (Du et al., 2006).

5.2 Sodium channel RNA editing

RNA editing has an important role in the regulation of gene expression and protein diversity. Recent studies implicate RNA editing in the removal of exons in alternative splicing sites, in the antagonism of interference RNA process (iRNA), in the modulation of mRNA processing and in the generation of new exons (for a review see Y. Yang et al., 2008). The basic mechanism of diversity generated by RNA editing includes nucleoside modifications such as C to U or A to I deaminations. Besides, it is possible that non-

[1] The genes annotation is in accordance with the nomenclature suggested by Goldin (2000).

templated nucleotides can be inserted in the edited mRNA. This process alters the protein aminoacid constitution so that it differs from the predicted genomic DNA sequence (Brennicke et al., 1999).

Liu et al. (2004) claimed that RNA editing should be the main regulatory mechanism to modulate the insect Na_V function. For instance, no correlation was found between a variety of *DmNa*$_V$ originated by alternative splicing and the observed changes in gating properties. Therefore it was implied that RNA editing might play a primary role in determining the voltage dependence of activation and deactivation of *DmNa*$_V$ variants (Olson et al., 2008). At least 10 A/I RNA editing substitutions were observed in the *DmNa*$_V$ in different points of the Drosophila life cycle indicating developmental regulation (Palladino et al., 2000). These sites are highly conserved in various organisms. Type U/C editing, which is more usual in mitochondria and plastids from higher plants, was also observed in *DmNa*$_V$ and *BgNa*$_V$, with electrophysiological alterations in both cases (Liu et al., 2004). Hence, RNA editing should play an important role in the generation of channels with distinct affinities to insecticides. Thus, it seems reasonable to infer that insecticide pressure selects for an adaptive mechanism which might spatially and temporally modulate Na_V mRNA editing. Still, in *Cx. quinquefasciatus* mosquitoes, diversity based on U/A editing in the sodium channel mRNA was shown to be related to pyrethroid resistance (Xu et al., 2006). In *Ae. aegypti*, however, recent analysis of *AaNa*$_V$ transcripts from a pyrethroid resistant lineage did not identify any sign of RNA editing (Chang et al., 2009).

5.3 The *kdr* mutation

The very first mutation identified as responsible for the *kdr* trait was a leucine to phenylalanine substitution (Leu1014Phe)[2] in the Na_V IIS6 segment of *M. domestica* (Ingles et al., 1996). Since then, the genomic sequence spanning the region coding for the IIS6 segment has been explored in a vast number of insects, in most of which, the same substitution being found at homologous sites (1014). Besides Phe, Ser is also encountered replacing Leu at the 1014 site in *An. gambiae*. They were initially observed respectively in western and eastern African regions, being commonly referred to as *w-kdr* and *e-kdr* mutations (Pinto et al., 2006). However, nowadays it is known that none of these alleles is restricted to either part of the continent (Ranson et al., 2011). A different substitution in the same 1014 site, Leu1014His, was also associated to pyrethroid resistance in the tobacco budworm *Heliothis virescens* (Park et al., 1999). Many studies identified at least 20 additional substitutions in the Na_V sequence, the majority being placed between segments S4 and S5, or internally to segments S5 or S6 of domain II. However, for most of them, the relationship with pyrethroid resistance is only speculative. Good compilations have recently been presented (T. G. Davies et al., 2007a; Dong, 2007; Du et al., 2009).

It is noteworthy that many of these mutations are not in the precise domain of interaction between insecticide and Na_V (O'Reilly et al., 2006). On the other hand, it is likely that substitutions in these points of interaction could result in the *super-kdr* trait, which has a more pronounced resistance effect (T. G. Davies et al., 2007b). This phenotype was also first described in *M. domestica* (Williamson et al., 1996) and *Haematobia irritans* (Guerrero et al., 1997). In both species, beyond the Leu1014Phe substitution, a Met918Thr mutation (in the IIS4-S5 linker) was disclosed in flies with very high resistant ratios to pyrethroids, referred

[2] Number refers conventionally to the position in the voltage gated sodium channel primary sequence of *M. domestica Vssc1*, according to Soderlund & Knipple 2003.

to as the *super-kdr* mutation (Jamroz et al., 1998). However, since it occurs only in association with the Leu1014Phe mutation, its isolated effects are as yet unknown. Although no *super-kdr* mutation has so far been identified in mosquitoes, it was suggested that Leu932Phe, in association with Ile936Val (both also in the IIS4-S5 linker), in *Culex* might play this role, being the first example of *super-kdr* in this group (T. G. Davies et al., 2007a). Accordingly, these sites have proved to be important for the interaction between Na_V, in the *D. melanogaster* sodium channel and pyrethroids or DDT (Usherwood et al., 2007).

Substitutions in site 929 are also involved in enhanced pyrethroid resistance, as is the case with the Lepidoptera *Plutella xylostella* mutation Thr929Ile, detected in association with Leu1014Phe (Schuler et al., 1998). However, in the maize weevil *Sitophilus zeamais*, the Thr929Ile was found alone (Araujo et al., 2011). In the louse *Pediculus capitis*, in turn, the Thr929Ile mutation was together with Leu932Phe (Lee et al., 2000). There were other substitutions in the same site: Thr/Cys and/or Thr/Val in the diamondblack moth *Frankliniella occidentalis* (Forcioli et al., 2002) and in the cat flea *Ctenocephalides felis* (Bass et al., 2004).

Ae. aegypti mosquitoes do not present any substitution in the classic 1014 *kdr* site, unlike many other insects or even mosquitoes from other genera, such as *Anopheles* and *Culex*, very likely because the 1014 site of *Ae. aegypti* Na_V is coded by a CTA, in place of the TTA codon present in the majority of other insects. For this reason, two simultaneous nucleotide substitutions would be necessary in order to change from Leu (CTA) to Phe (TTT) or Ser (TCA) (Martins et al., 2009a; Saavedra-Rodriguez et al., 2007). Instead, mutations in different positions have been observed in *Ae. aegypti* populations from Latin America and Southeast Asia, but at least two sites seem to be indeed related to pyrethroid resistance: 1016 (Val to Ile or Gly) and 1534 (Phe to Cys), respectively in the IIS6 and IIIS6 segments (Brengues et al., 2003; Harris et al., 2010; Martins et al., 2009a, b; Saavedra-Rodriguez et al., 2007). Mutations in the vicinity of this site in the IIIS6 segment were also encountered in the southern cattle tick *Rhipicephalus microplus* (He et al., 1999) and in the two-spotted spider mite *Tetranychus urticae* (Tsagkarakou et al., 2009).

Although different Na_V site mutations are known to confer resistance to pyrethroids, their number is quite restricted; additionally, far related taxa present alterations in the same homologous sites. For instance, the Leu1014Phe *kdr* mutation must have arisen at least on four independent occasions in *An. gambiae* (Pinto et al., 2007). Alterations that do not interfere with the endogenous physiological functions of the Na_v must be rare as it is much conserved among animals (ffrench-Constant et al., 1998). As a matter of fact, most of the species studied so far have the *kdr* mutation in the 1014 site, few species proving otherwise due to codon constraints, like *Ae. aegypti* and some anopheline species.

6. Molecular assays for monitoring frequency of *kdr* mutation in insect natural populations

Currently, there are many PCR based diagnostic methods for *kdr* mutation available with elevated sensitivity and specificity. For technique choice, one must consider mainly the laboratory resources, facilities and training of technical personnel, which is as important as establishing an defining localities and frequency of sampling. There is neither consensus nor strict rules suitable for all insect species or even for different populations of the same species. Resistance is a very dynamic process depending upon a series of external factors. Therefore, resistance level as well as the selected mechanisms may fluctuate in a short

period of time and space (Kelly-Hope et al., 2008). Moreover, one must be aware about the patterns of distribution and structure of the evaluated populations in order to determine an adequate frequency and sampling size (Ranson et al., 2011).

Allele-specific PCR assays (AS-PCR), as the name suggests, consists of amplification and detection of a specific allele from the DNA of an individual, who is further classified as hetero or homozygous for that allele. Many methodologies based on this strategy have been well succeeded in high-throughput individual diagnostic of *kdr* mutations. Herein, we highlight some PCR based amplifications by allele-specific primers and TaqMan genotyping.

There is ample variation for PCR methods based on allele specific primers. As a first example, one can use two primers (forward and reverse) common for both alleles that amplify a region spanning the mutation site. In this case, additional specific primers, bearing the SNP (single nucleotide polymorphism) at the 3'-end, have opposite orientations in relation to each other (Figure 2-A). The common primers will pair themselves giving rise to a bigger product (that can also be assumed as the positive control reaction) and shorter ones, the consequence of pairing with each allele-specific primer of contrary orientation. The common primers must anneal at sites that result in differently sized products when paring with the specific ones. If both alleles are present (cases when the individual is heterozygous) three products with distinct sizes will be produced (Chen et al., 2010; Harris et al., 2010).

Instead of amplifying a common region for both alleles, it is possible to directly obtain only the specific products (Figure 2-B). This can be accomplished by using only one common primer in one orientation and the two allele specific primers in the opposite sense. However, since the specific primers are at the same orientation and their specificity continues lying upon the 3'-end, something should be incremented in order to obtain distinguishable products. Germer & Higuchi, (1999), later improved by Wang et al. (2005), proposed attaching a GC-tail of different sizes to the 5'-end of the specific primers in a way that the products could be distinguishable by their Tm in a melting curve analysis. In this case the mix reaction contains a fluorescent dye, which lights up when bounded to double strand DNA, carried out in a fluorescence-detecting thermocycler ("Real time PCR"). Additionally, a different mismatch (pirimidine for purine or vice–versa) is added to the third site before the 3'-end of each allele specific primer, in order to strengthen their specificity (Okimoto & Dodgson, 1996). Alternatively, the products can also be distinguishable in a gel electrophoresis.

The second group of techniques is based on the amplification of a region spanning the *kdr* mutation site followed by the detection of the different alleles by specific hybridization with minor groove binding (MGB) DNA fluorescent probes, also known as TaqMan assay (Figure 2-C). Different alleles can be detected in the same reaction, since each probe is attached to a distinct fluorophore. The probe is constituted of an oligonucleotide specific for the SNP with a reporter fluorescent dye in the 5'-end and a non fluorescent quencher in the 3'-end (Araujo et al., 2011; Morgan et al., 2009; Yanola et al., 2011). Bass et al., (2007) concluded that TaqMan probes were the most accurate for *kdr* genotyping among six different evaluated methods.

Other techniques have also been applied. The *Hola* (Heated Oligonucleotide Ligation Assay, see details in Black et al., 2006) revealed high specificity in detecting different Na_V alleles in the 1011 (Ile, Met and Val) and 1016 (Val, Ile and Gly) sites from Thai *Ae. aegypti* populations (Rajatileka et al., 2008) and in the 1014 site of *Cx. quinquefasciatus* from Sri Lanka (Wondji et

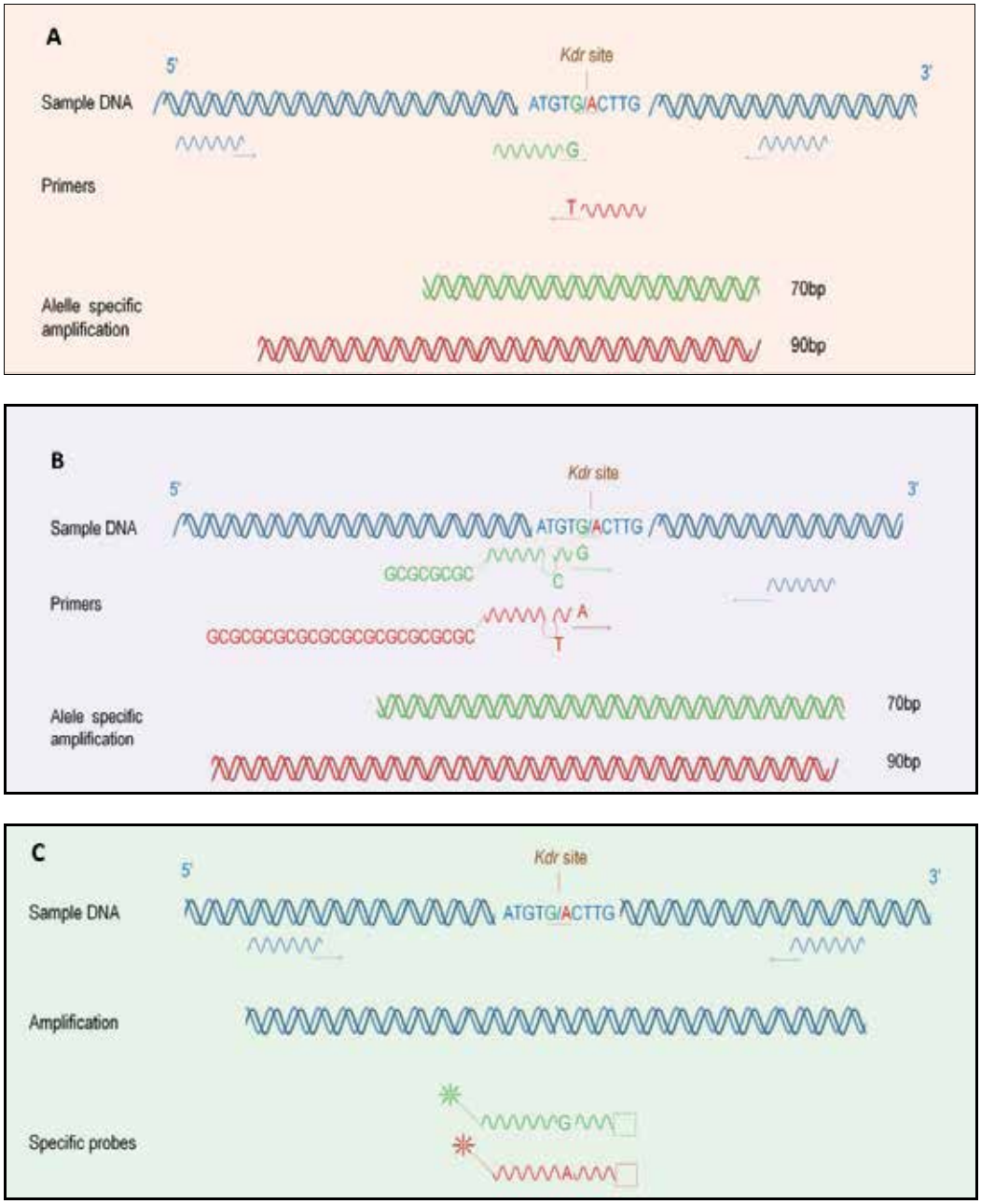

Fig. 4. Examples for kdr genotyping based on PCR methods. A – Allelic specific PCR with specific primers in different orientations; B – Allelic specific PCR with specific primers in the same orientation but with additional and differently sized $[GC]_n$ tails, in addition to a mismatch in the 3rd base before the 3'-end; C – TaqMan assay based on specific probes with a different luminescence for each allele. Figure adapted from Yanola et al. (2011).

al., 2008). However, comparison between *HOLA* and pyrosequencing revealed more specificity for this latter method in the diagnostic of the *kdr* mutation Leu1014Phe in *Cx. quinquefasciatus* (Wondji et al., 2008). Sequencing of regions that encompass the SNP allows a direct visualization of the nucleotide allele sequences, eliminating the problem of unspecific amplification or hybridization of PCR based protocols. Moreover, it enables visualizing potential novel variations that would never be identified by PCR diagnostic SNP techniques. However, sequencing in large scale is much more expensive than the aforementioned genotyping tools. It is also mandatory that the eletropherograms generated have a clean profile, so that the heterozygous individuals can be undoubtedly discriminated.

7. Conclusions

New strategies for arthropod control based on the release of laboratory manipulated insects that would suppress or substitute natural populations are being tested in the field with great prospect. The release of transgenic insects carrying a dominant lethal gene (RIDL) (Black et al., 2011) or of mosquitoes with the intracellular *Wolbachia*, that lead to refractoriness to other parasites (Werren et al., 2008) are currently the most discussed strategies. However, the laboratory handling process has to consider specific and sometimes complex aspects for each insect species, and it may take many years until field control based on this kind of approach can be effectively accomplished. Moreover, field studies that guarantee the environmental safety of releasing manipulated insects may take even longer. Hence, even if these strategies prove to be efficient to reduce, extinguish, or substitute a target insect population, the use of insecticides may still indeed play an essential role for many years to come, especially during periods of high insect or disease incidence.

Pyrethroids are largely the most adopted insecticide class in agriculture and for public health purposes. Their use tends to increase, since pyrethroids are the only safe compound to impregnate insecticide treated nets (ITNs), a strategy under expansion against mosquitoes. Advances regarding knowledge of its target, the voltage gated sodium channel, can contribute to the design of new compounds as well as the rapid identification of resistance related mutations. The continuous monitoring of insecticide resistance status, and its mechanisms, in natural populations has proven to be an important tool in the preservation of these compounds.

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3

Photoremediation of Carbamate Residues in Water

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1. Introduction

Pesticides are extensively world wide used for agriculture and for non-agricultural purposes. The major environmental concern of used pesticides is their ability to leach down to subsoil and contaminate the ground water, or, if they immobile, they could persist on the top soil and become harmful to microorganisms, plants, animal and people (Jha & Mishra 2005; Radivojević et al., 2008). Harmfull pesticide residues can contaminate the environment and accumulate in ecosystems than entering the human food chain (Đurović et al., 2010; Gašić et al., 2002a; Gevao et al., 2000). Pesticides have various characteristics that determine how act once in soil where it could accumulate to toxic level. Generally, soil and groundwater pollution are the major consequences environmental effects of pesticides application. Pesticides can reach water through surface runoff from treated plants and soil. Pesticide sprays usually directly hit non-target vegetation or can drift or volatilize from the treated areas that contaminate air, soil, and non-target plants. Finally, using of pesticides has resulted in acute and chronic ecological damage either by direct injury such as birds and fish or by indirect.

Carbamates are large group of pesticides which have been extensively used in almost sixty years. In this chapter an attempt is made to give the available data of the carbamates used as pesticides, their physico-chemical and toxicological characteristics, behaviour and fate in the environment, types of formulations which exist on the market as well as photochemical degradation for the certain members. Owing to widespread use in agriculture and relatively good solubility in water carbamate compounds can contaminate surface and ground waters and therefore carries a risk to various consumers, as well as the environment.

In this chapter we will also discuss some very important photocatalytic methods for remediation of water containing carbamate residues: direct photodegradation (photolysis), photosensitized degradation and photocatalytic degradation (including heterogeneous TiO_2 and ZnO processes and photo-Fenton and Fenton-like processes).

2. Carbamates

Carbamates were developed into commercial pesticides in the 1950s. It is a very huge family which members are effective as insecticides, herbicides, and fungicides, but they are most commonly used as insecticides. More than 50 carbamates are known. The most often used

members of carbamate group are: aldicarb, asulam, bendiocarb, carbaryl, carbetamid, carbofuran, carbosulfan, chlorpropham, desmedipham, ethiofencarb, formetanate, furatiocarb, fenoxycarb, isoprocarb, methiocarb, methomyl, oxamyl, phenmedipham, pirimicarb, promecarb, propamocarb and propoxur.
Carbamates are N-substituited esters of carbamic acid. Their general formula is:

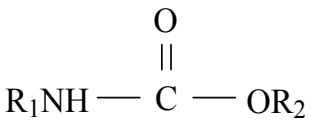


Fig. 1. General carbamate structure, where R_2 is an aromatic or aliphatic moiety, if R_1 is a methyl group it is carbamate insecticide, if R_1 is an aromatic moiety it is carbamate herbicide and if R_1 is a benzimidazole moiety it is carbamate fungicide (WHO, 1986).

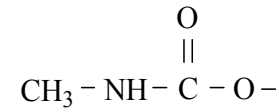
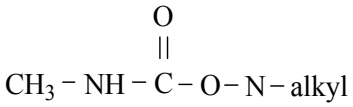
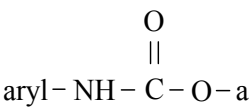
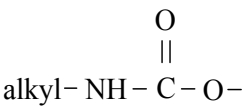
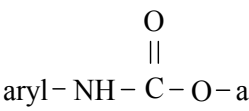
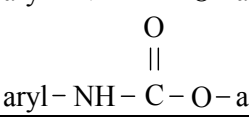
Pesticide activity	Chemical structure	Common or other names
Insecticide		aldoxycarb, allyxycarb, aminocarb, BPMC, bendiocarb, bufencarb, butacarb, carbanolate, carbaryl, carbofuran, cloethocarb, dimetilan, dioxacarb, ethiofencarb, formetanate, hoppcide, isoprocarb, trimethacarb, MPMC, methiocarb, metolcarb, mexacarbate, pirimicab, promacyl, promecarb, propoxur, MTMC, XMC, xylylcarb
		aldicarb, methomyl, oxamyl, thiofanox, thiodicarb
Herbicide		asulam, barban, carbetamide, chlorbufam, desmedipham, phenmedipham, swep
		dichlormate, karbutilate, terbucarb
Herbicide and sprout inhibitors		propham, chlorpropham
Fungicide		benomyl, carbendazim, thiophanate-methyl, thiophanate-ethyl

Table 1. Relationship of chemical structure and pesticide activity of carbamates (WHO, 1986).

2.1 Carbamates physical and chemical properties

It is known that esters or N-substituted derivates of carbamic acid are unstable compounds, especially under alkaline conditions. Decomposition under this conditions takes place and the compounds as alcohol, phenol, ammonia, amine and carbon dioxide are formed.
Derivates of carbamic acid as salts or esters are more stable than carbamic acid. This enhanced stability is the basis for synthesis of many derivates that are biologically active pesticides.
Carbamate ester derivates are crystalline solids of low vapor pressure with variable, but usually low water solubility. They are moderately soluble in solvents such as benzene, toluene, xylene, chloroform, dichloromethane and 1,2-dichloromethane. Generally, they are poorly soluble in nonpolar organic solvents such as petroleum hydrocarbons but highly soluble in polar organic solvents such as methanol, ethanol, acetone, dimethylformamide, etc (WHO, 1986).

2.2 Carbamates mode of action and toxicity

Most carbamates are active inhibitors of acetylholinesteraze (AChE), but some carbamates as benzimidazole have no acetylcholinesterase activity. Carbamates toxicity to insects, nematodes, and mammals is based on inhibition of acetylcholinesterase, which is the enzyme responsible for the hydrolysis of acetycholine into choline and acetic acid. Acetylcholine (ACh) is a substance that transmits a nerve impulse from a nerve cell to a specific receptor such as another nerve cell or a muscle cell. Acetylcholine, in essence, acts as a chemical switch. When it is present (produced by nerve cell) it turns the nerve impulse on. When it is absent, the nerve impulse is discontinued. The nerve transmission ends when the enzyme aceylcholinesterase breaks down the acetylcholine into choline and acetic acid. Without the action of this enzyme acetylcholine builds up at the junction of nerve cell and the receptor site, and the nerve impulse continues. Carbamate insecticides block (or inhibit) the ability of this enzyme, acetylcholinesterase, to break down the acetylcholine and the nerve impulse (Kamrin, 1997; Machemer & Pickel, 1994).
In mammals, cholinesterase inhibition caused by carbamates is labile, reversible process. Estimates of the recovery time in humans range from immediate up to four days, depending on the dose, the specific pesticide and the method of exposure. The breakdown of carbamate compounds within an organisms is a complex process and is depended on the specific pesticide structure. The rapid degradation of carbamates *in vivo* by mammals occurs by hydrolysis, oxidation and conjugation. The end products include amines, alcohols or phenol derivates. The urinary route is the main excretory route (Machemer & Pickel, 1994).
Inhibition of acetylholinesteraze (AChE) by carbamates causes toxic effects in animals and human beings that result in variety of poisoning symptoms. Carbamates acute toxicity and poisoning are dose related. Acute poisoning occurs rapidly after exposure. Ingestion of carbamate insecticides at low doses can cause excessive salivation and an increase in the rate of breathing within 30 min. At higher doses this is followed by excessive tearing, urination, no control defecation, nausea and vomiting. At the highest doses, symptoms can include those listed above along with violent intestinal movements, muscle spasm and convulsions. Death has occurred in a few instances, usually due to respiratory failure resulting from paralysis of the respiratory muscles (Kamrin, 1997; WHO, 1986).
While the insecticidal carbamate produce the typical symptoms of cholinesterase inhibition, they don't appear to induce a delayed neurotoxic reaction similar to that seen with some organophosphourus compound. Chronic exposure to carbamate compounds may cause

adverse effects on organs or acetylcholinesterase levels. These effects are unlikely to occur in humans at expected exposure levels (Kamrin, 1997).

The acute toxicity of different member of carbamates ranges from highly toxic to only slightly toxic. The LD_{50} for the rats ranges from less than 1 mg/kg to over 5000 mg/kg body weight. The acute dermal toxicity of carbamates is generally low to moderate except aldicarb which is very toxic. The carbamates in short term and long term toxicity studies showed different toxicity. Some carbamates are very toxic and others less. Carbamate pesticides are transformed metabolically by variety of chemical reactions in more water soluble molecules which can be excreted via the urine. Rats eliminate carbamate compounds rapidly in that way. Most metabolites are excreted within 24 h of exposure and therefore carbamate residues don't accumulate in animals (Kamrin, 1997; WHO, 1986). In the study of carbofuran toxicity on rats during subhronic exposure the histopathological changes in liver and kidneys were observed but there was cell regeneration in all test groups as well (Brkić et al., 2008).

Aldicarb is the most toxic among the carbamates and establish acceptable daily intake (ADI) for humans is 0.001 mg/kg/body weight. The other carbamates have ADIs values in range of 0.001-0.1 mg/kg/ body weight (WHO, 1986). According to the European Food Safety Authority (2009), the lowest ADI has carbofuran 0.00015 mg/kg/body weight (EU Pesticide Database, 2011).

Many carbamates have been studied for reproductive, teratogenic, mutagenic and carcinogenic effects and the results of this is that a few members of this family has been banned by the regulatory bodies worldwide.

2.3 Environmental fate

Generally, carbamates remain active for a few hours to a few month in soils and crop, but they may leave residues in agricultural products (Takino et al., 2004). The rate of degradation in soil depends on soil type, soil moisture, adsorption, pH, soil temperature, concentration of pesticide, microbial activity and photodecomposition. The higher the organic content, the greater the binding to soil and thus the greater the persistence. Also, the higher the soil acidity, the longer it takes for carbamates to be degraded. Carbamate insecticides are mainly applied on the plants, but can reach the soil, while carbamate nematocides and herbicides are applied directly to the soil. Generally, in soil carbamates degraded by chemical hydrolysis and microbial processes. Microorganisms that have capability to degrade carbamate pesticides play a significant role in the break dawn and elimination of them from environment. Because the different carbamates have different properties, it is clear that each of them should be evaluated on its own merits, and no extrapolation of results can be made from one carbamate to another. One carbamate may be easily decomposed, while another may be strongly adsorbed on soil. Some leach out easily and may reach groundwater. In these processes, the soil type and water solubility are of great importance. Furthermore, it should be recognized that this not only concerns the parent compound but also the breakdown products or metabolites (Kamrin, 1997; WHO, 1986).

Persistence of carbamate herbicides is increased by application to dry soil surface or by soil incorporation. Environmental factors which increasing microbial activity in soil generally decrease the persistence of carbamate herbicides. In most of degradation reactions the initial cleavage of the molecule occurs at ester linkage. Enzymatic hydrolysis of some carbamates can be correlated to soil acidity, and rate differences explained by consideration of certain

steric and electronic properties of the carbamates. The carbamate derivates with herbicidal action are substantially more stable to alkaline hydrolysis than the methyl carbamate derivatives, which have an insecticidal action (Kaufman, 1967).
Carbamate compounds degrade through chemical hydrolysis and this is the first step in the metabolic degradation. The hydrolysis products will be further metabolized in soil and plant. Chemical degradation does not appear to have much influence in the total degradation of pesticides in soil. Carbamate compounds are adsorbed and translocated through plants and treated crops. In most cases, carbamates will break down quickly in plants and the residues in plants will last not very long .
Finally carbamates are metabolized by microorganisms, plants and animals or broken down in water or soil. In water carbamates degraded by chemical hydrolysis, but photodegradation and aquatic microbes may also contribute degradation. Generally, in alkaline water and under sunlight carbamate compounds will decompose more rapidly (WHO, 1986).

2.4 Formulations

Carbamate products come in variety of solid and liquid formulations on the market. They contain beside carbamate compounds inert ingredients which could be toxic, flammable or reactive. Examples of inert ingredients are wetting agents, spreaders, dispersing agents, solvents, solubilizers, carriers, ticker, surfactants and so on. A surfactant is a substance that reduced surface tension of a system, allowing oil-based and water-based substances to mix more readily. A common groups of non-ionic surfactants are the alkylphenol polyethoxylates or alcohol ethoxylates which may be used in pesticide formulations. Nonyl phenols, one of the members of above mention alkylphenol surfactant has been linked to endocrine-disrupting effects in aquatic animals and should be substituted by less hazardous alternatives. Commonly used formulation types include liquid and dry formulations as emulsifiable concentrates (EC), soluble concentrates (SL), suspension concentrates (SC), than wettable powders (WP), water dispersible granules (WG), granules (GR), etc, and they are signed by international coding system (CropLife, 2008).
Pesticides are very often formulated as emulsifiable concentrates (EC) which produce emulsions when dissolved in water. The first problem in defining this formulation is the selection of an adequate surfactants (emulsifiers) for the intended purposes (Gašić et al., 1998a, 1998b, 2002b; Shinoda & Friberg, 1986). Recently there is increasing interest in the effect of emulsifiers on toxicity to mammals and fish. These effects can be due to inherent toxicity of the surfactant itself or to the enhancing effect that the emulsifiers may have on toxicity of active ingredient. So, the formulation type can have implications for product efficacy and exposure to humans and other non-target organisms (Knowles, 2005, 2006; Sher, 1984).
While the toxicity of the active ingredient of a pesticide is property which can not be changed, the acute toxicity effects of the formulation are strongly influenced by the way in which the active ingredient is formulated. While pesticide formulations are influenced by both the physical and chemical properties of the active ingredient and the economic pressures of the marketplace, there are formulation choices which will increase the safety of pesticide formulations (Mollet & Grubenmann, 2001).
The type of pesticide formulations and in, some cases, the choice of product of the same formulation type can significantly affect the results obtained in practical use. Safety, efficacy, residual life, cost, availability and ease of use must all be considered in selecting

formulation. The ways in which pesticides are formulated considerably influence their persistence. Formulations in order of increasing persistence on plants are prepared in the way that more readily adsorbed on the soil fractions and not appreciably degradated (Edwards, 1975).

3. Photodegradation processes for carbamates wastewater treatments

3.1 Photolysis

Photolysis (direct photodegradation reaction) is photodegradation process without any catalysts and use light only for degradation of different organic molecules, including pesticides and related compounds. Direct irradiation will lead to the promotion of the pesticides to their excited singlet states and such excited states can then undergo among homolysis, heterolysis or photoionization processes (Burrows et al., 2002). Direct photodegradation by solar light is limited and various lamps have been used for irradiation of contaminated water solutions. The photolysis of contaminants (including pesticides) in aqueos solution depends on the different reaction parameters such as type of light, lamp distance, temperature, initial concentration of pesticides, type of water, pH, the presence of humic and fulvic acids, the presence of O_2, O_3, O_2/O_3 and H_2O_2, the presence of inorganic ions and organic matter dissolved in water (Burrows et al., 2002; Tomaševic at al., 2010a).

3.2 Photosensitized degradation

The photosensitized reaction is based on the absorption of light by a molecule of the sensitizer and includes an energy transfer from molecul excited state to the pesticides. The most famous sensitizers are aceton, rose Bengal, methylene blue and humic and fulvic acids (Burrows et al., 2002).

3.3 Advanced oxidation processes

Advanced Oxidation Processes (AOP_s) include catalytic and photochemical methods and have H_2O_2, O_3 or O_2 as oxidant. The principal active species in this system is the hydroxyl radical $^{\bullet}OH$, which is an extremely reactive and non-selective oxidant for organic contaminants (Legrini at al., 1993; Sun & Pignatello, 1993). The main advantage of these processes is a complete mineralization of many organic pollutants (Andreozzi at al., 1999; Neyens & Baeyens, 2003). Several of AOP_s are currently employed for the elimination of pesticides from water: heterogeneous photocatalytic reactions with semiconductor oxides TiO_2 (Malato et al., 2002a, 2002b; Tomaševic at al., 2010a) or ZnO (Tomaševic at al., 2010a) as photocatalysts, photo-Fenton (Malato et al., 2002a; Tamimi et al, 2008; Tomaševic at al., 2010b) and photo-assisted Fenton processes (Huston & Pignatello, 1999). Electro-photo-Fenton (Kesraoui Abdessalem et al., 2010) and electrochemical oxidation processes (Tomašević et al., 2009a) have been seldom studied.

Heterogeneous photocatalysis is combination of semiconductor particles (TiO_2, ZnO, Fe_2O_3, CdS, ZnS), UV/solar light and different oxidants (H_2O_2, $K_2S_2O_8$, KIO_4, $KBrO_3$). The main equations of the heterogeneous photocatalysis are (Andreozzi et al., 1999; Daneshvar et al., 2003; Karkmaz et al., 2004; Legrini at al., 1993):

$$C + h\nu \rightarrow C\,(e^- + h^+) \quad (1)$$

$$h^+ + H_2O \rightarrow {}^{\bullet}OH + H^+ \quad (2)$$

$$e^- + O_2 \rightarrow O_2^{\bullet -} \quad (3)$$

Among AOP_s, heterogeneous photocatalysis using TiO_2 as photocatalyst appears as the most emerging destructive technology. The following mechanism of the TiO_2 photocatalysis has been proposed (Daneshvar et al., 2003; Gomes da Silva & Faria, 2003; Karkmaz et al., 2004; Tomaševic at al., 2010a):

a) absorption of efficient photons by titania ($h\nu \geq Eg=3.2$ eV):

$$TiO_2 + h\nu\,(UV) \rightarrow e_{CB}^- + h_{VB}^+ \quad (4)$$

b) oxygen ionosorption:

$$(O_2)_{ads} + e_{CB}^- \rightarrow O_2^{\bullet -} \quad (5)$$

c) neutralization of OH^- groups into $^\bullet OH$ by photoholes:

$$(H_2O \leftrightarrow H^+ + OH^-)_{ads} + h_{VB}^+ \rightarrow H^+ + {}^\bullet OH \quad (6)$$

d) oxidation of the organic reactant via successive attacks by $^\bullet OH$ radicals:

$$R + {}^\bullet OH \rightarrow R'^\bullet + H_2O \quad (7)$$

e) or by direct reaction with holes:

$$R + h^+ \rightarrow R^{\bullet +} \rightarrow \text{degradation products} \quad (8)$$

ZnO is also frequently used as a catalyst in heterogeneous photocatalytic reactions. The biggest advantage of ZnO in comparison to TiO_2 is that it absorbs over a larger fraction of the UV spectrum and the corresponding threshold wavelength of ZnO is 387 nm. Upon irradiation, valence band electrons are promoted to the conduction band leaving a hole behind. These electron-hole pairs can either recombine or interact separately with other molecules. The holes at the ZnO valence band can oxidize adsorbed water or hydroxide ions to produce hydroxyl radicals. Electron in the conduction band at the catalyst surface can reduce molecular oxygen to superoxide anion. This radical may form organic peroxides or hydrogen peroxide in the presence of organic scavengers. The hydroxyl radical attacks organic compounds (R) and intermediates (Int) are formed. These intermediates react with hydroxyl radicals to produce the final products (P). The mechanism of heterogeneous photocatalysis in the presence of ZnO can be given by the following reactions (Behnajady et al., 2006; Daneshvar et al., 2004, 2007; Pera-Titus at al,. 2004; Tomaševic at al., 2010a):

$$ZnO + h\nu\,(UV) \rightarrow e_{CB}^- + h_{VB}^+ \quad (9)$$

$$e_{CB}^- + h_{VB}^+ \rightarrow \text{heat} \quad (10)$$

$$h_{VB}^+ + H_2O_{ads} \rightarrow H^+ + {}^\bullet OH_{ads} \quad (11)$$

$$h_{VB}^+ + {}^-OH_{ads} \rightarrow {}^\bullet OH_{ads} \quad (12)$$

$$e_{CB}^- + O_2 \rightarrow O_2^{\bullet -} \quad (13)$$

$$O_2^{\bullet -} + HO_2^\bullet + H^+ \rightarrow H_2O_2 + O_2 \quad (14)$$

$$O_2^{\bullet-} + R \rightarrow R\text{-}OO^{\bullet} \quad (15)$$

$$^{\bullet}OH_{ads} + R \rightarrow Int. \rightarrow P \quad (16)$$

Fenton's processes belong to AOP_s and utilize H_2O_2 activation by iron salts. The classic Fenton's reagent is a mixture of ferrous ion and H_2O_2 in acidic solution or suspension (Neyens & Baeyens, 2003; Tamimi at al.,2008):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {\bullet}OH \quad (17)$$

Equation (17) presents the most important steps of a Fenton reaction and involves electron transfer between H_2O_2 and Fe(II) with oxidation of Fe(II) to Fe(III) and the resulting production of highly reactive hydroxyl radical •OH and potentially reactive ferryl species. The degradation of pesticides by Fenton's reagent can be strongly accelerated upon UV or UV-visible light. This process is the photo-Fenton reaction (Malato et al., 2002a, 2002b; Tamimi et al, 2008; Tomaševic at al., 2010b). Equation (17) is the key of photo-Fenton processes. The obtained Fe^{3+} ion or its $Fe(OH)^{2+}$ complexes act as light absorbing species, that produce another hydroxyl radical, while the initial Fe^{2+} ion is regained:

$$Fe(OH)^{2+} + h\nu \rightarrow Fe^{2+} + {\bullet}OH \quad (18)$$

The main advantage of the photo-Fenton process is light sensitivity up to a wavelength of 600 nm (Malato et al., 2002a).

4. Photodegradation of carbamate pesticides

4.1 Aldicarb

Aldicarb (IUPAC name: 2-methyl-2-(methylthio)propionaldehyde O-methylcarbamoy-oxime) is a systemic oxime carbamate pesticide, effective against a variety of insects, mites, and nematodes. It is sold commercially only in granular form (GR). Aldicarb is applied on a variety of crops, including cotton, sugar beet, sugarcane, citrus fruits, potatoes, sweet potatoes, peanuts, beans (dried beans), soybeans, pecans, and ornamental plants. Home and garden use is not permitted in many countries. The current regulation status of this active ingredient under directive 91/414/EEC is not included in Annex 1 (EU Pesticide Database, 2011; Tomlin, 2009).

The complete conversion of 38 mg/L of aldicarb and 62% reduction in TOC content using the photo-Fenton reaction ($Fe(III)/H_2O_2/UV$) within 120 min in acidic aqueous solution (pH 2.8) at 25 °C with fluorescent blacklight irradiation (300-400 nm) has been considered (Huston & Pignatello, 1999). They also observed the formation of sulfate and nitrate ions during the photo-Fenton process.

4.2 Asulam

Asulam (IUPAC name: methyl sulanylcarbamate) is selective systemic herbicide, which is used for control of annual and perennial grasses and broad-leaved weeds in spinach, oilseed poppies, alfalfa, some ornamentals, sugar cane, bananas, coffee, tea, cocoa, coconuts, rubber, fruit trees and bushes, and forestry. It could be found only as soluble concentrate (SL) on the market. The current regulation status of this active ingredient under directive 91/414/EEC is not included in Annex 1 (EU Pesticide Database, 2011; Tomlin, 2009).

The degradation of asulam was studied in homogeneous aqueous solution in the presence of molecular oxygen at pH 3.0-3.4, by irradiation at 365 nm and by solar irradiation (Catastini et al., 2002a). When the iron(III) aquacomplexes was photoreduced to iron(II) ions and hydroxyl radicals the degradation of asulam in the presence of oxygen continud to completion. The Fe^{2+} ions are oxidized back to Fe^{3+} ions through various pathways such as photooxidation and oxidation by H_2O_2 generated within the system, where another $^{\bullet}OH$ forms. Their experimental results indicate that the presence of Fe^{3+}, Fe^{2+} and molecular oxygen accelerate the mineralization of asulam. Also, less than 10% conversion of asulam was observed when the irradiation was performeds in the presence of 0.01 M 2-propanol, used as hydroxyl radical scavenger. Complete conversion and nearly complete TOC reduction of 23 mg/L of asulam was achieved with 16.7 mg/L of Fe^{3+} ions, within 17 h (at 365 nm) and 28-30 h (under solar light). In this process intermediates or degradation by-products of asulam were not identified. The photodegradation of the herbicide asulam in aqueous solution (1.0×10^{-4} M or 23 mg/L) has been investigated with and without Fe(III) (Catastini et al., 2002b).The asulam disappearance were monitored by photolysis at 254 nm as a functuion of pH and oxygen concentration and no complete transformation of organic carbon into CO_2 was observed. In the presence of Fe(III) at 365 nm the complete mineralization of asulam has been achieved.

4.3 Bendiocarb

Bendiocarb (IUPAC name: 2,3-isopropyldenedioxyphenyl methylcarbamate, 2,2-dimethyl-1,3-benzodioxol-4-yl methylcarbamate) is systemic insecticide with contact and stomach action. It is active against many public health, industrial and storage pest. This active ingredient is especially useful inside buildings, due to its low odor and lack of corrosive and staining properties. It comes in variety formulations type as DP, FS, GR, SC, WP on the market. The current regulation status of this active ingredient under directive 91/414/EEC is not included in Annex 1 (EU Pesticide Database, 2011; Tomlin, 2009).

Evaluation of different pathway (photolysis, photo-Fenton, H_2O_2/UV and electro-Fenton) of bendiocarb (112-188 mg/L) photodegradation have been proposed (Aaron & Oturan, 2001). The conversion of insecticide was apparently much faster in the H_2O_2/UV and photo-Fenton proces (λ = 254 nm, 68 mg/L of H_2O_2 and 55.8 mg/L of Fe^{3+}) than in the other processes. Also, the degradation mechanism of bendiocarb has been proposed. The photolysis of aqueous bendiocarb (3.3×10^{-3} M, 4 h, room temperature, 125 W medium-pressure mercury lamp) has been examined by GC-MS (Climent & Miranda, 1996). Upon irradiation the only one photo-product (corresponding phenol) was detected and 30% conversion of bendiocarb was achieved.

4.4 Carbaryl

Carbaryl (IUPAC name: 1-naphthyl methylcarbamate) is insecticide with contact and stomach action and has slight systemic properties. It is used for control of chewing and sucking insects on more than 120 different crops, including vegetables, tree fruit (including citrus), mangoes, bananas, strawberries, nuts, vines, olives, okra, cucurbits, peanuts, soya beans, cotton, rice, tobacco, cereals, beet, maize, sorghum, alfalfa, potatoes, ornamentals, forestry, etc, than for control earthworms in turf and as a growth regulator for fruit thinning of apples. Also it is used against an animal ectoparasiticide. Carbaryl can be found formulated as DP, GR, OF, RB, SC, TK and WP. The current regulation status of this active

ingredient under directive 91/414/EEC is not included in Annex 1 (EU Pesticide Database, 2011; Tomlin, 2009).
The degradation of carbaryl under UV light using a continuous flow of TiO_2 slurry shown that the degradation proceeds through a multi-step process involving the attack of the substrate by •OH radicals (Peris at al., 1993). The studies on the degradation of carbaryl under simulated solar light in aqueous TiO_2 dispersions showed that the reaction follows pseudo-first-order kinetics and the complete mineralization (to CO_2, nitrate and ammonium ions) is achieved in less 30 min (Pramauro et al., 1997). The effect of ionic and non-ionic aliphatic surfactants (constitute an important ingredient of pesticide formulations and can influence the degradation of pesticide) on the degradation of aqueous carbaryl solutions (20 mg/L) containing 500 mg/L of TiO_2 (anatase) in the presence of simulated solar light (1500 W Xenon lamp with 340 nm cut-off filter) was investigated (Bianco Prevot at al., 1999). Depending on the surfactant and on the initial pH of the solution, an inhibition ot the photodegradation rate was observed. Also, mineralization of the carbaryl to CO_2, nitrate and ammonium ions was evidence in the presence of added surfactants, suggesting the feasibility of photocatalytic treatment of aqueous pesticide wastes.

4.5 Carbetamid

Carbetamid (IUPAC name: (R)-1-(ethylcarbamoyl)ethyl carbamilate) is selective herbicide, absorbed by the roots, and also by the leaves. It is used for control of annualgrasses and some broad-leaved weeds, alfalfa, sainfoin, brassicas, field beans, peas, lentils, sugar beast, oilseed rape, chicory, endive, sunflowers, caraway, strawberries, wines, and fruit orchards. Formulations types for this active ingredient are EC and WP. The current regulation status of this active ingredient under directive 91/414/EEC is included in Annex 1, expiration of inclusion: 31/05/2021 (EU Pesticide Database, 2011; Tomlin, 2009).
Photodegradation of herbicide carbetamide with ultraviolet light ($\lambda > 290$ nm) in the presence of TiO_2, H_2O_2 and ozone was studied in the aqueous solutions (Mansour et al., 1992). Using spectrometric methods several photoproducts were isolated and identified, suggesting that photodegradation pathways of carbetamide in the presence of TiO_2 and H_2O_2 are hydroxylations of the aromatic ring. Also, UV-ozonation rapidly oxydized carbetamide to water, ammonia and CO_2. The kinetics of photodegradation of carbetamide in water in the presence of TiO_2 (Degussa P 25 grade, surface area 50.0 m^2/g) or ZnO (surface area 9.5 m^2/g) were examined upon $\lambda \geq 310$ nm (Percherancier et al., 1995). The effects of various parameters, such as the kind of semiconductor, mass of TiO_2, initial concentration of pesticide, radiation flux and quantum yield were studied. The degradation with ZnO is faster than that with TiO_2 in spite of the lager surface area of the later catalyst. Also, the mechanism of the carbetamide photocatalytic degradation has been proposed.

4.6 Carbofuran

Carbofuran (IUPAC name: 2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is systemic insecticide with predominantly contact and stomach action. It is used for control of soil-dwelling and foliar-feeding insects and nematodes in vegetables, ornamentals, beet, maize, sorghum, sunflowers, oilseed rape, potatoes, alfalfa, peanuts, soya beans, sugar cane, rice, cotton, coffee, cucurbits, tobacco, lavender, citrus, wines, strawberries, bananas, mushrooms and other crops. This active ingredient is prepared as FS, GR, SC and WP formulation. The current regulation status of this active ingredient under directive 91/414/EEC is not included in Annex 1 (EU Pesticide Database, 2011; Tomlin, 2009).

Various carbofuran photodegradation processes (by ozon, UV photolysis, Fenton, O_3 + UV, UV + H_2O_2 and photo-Fenton) upon polychromatic UV irradiation were evaluated (Benitez et al., 2002). For all these reactions, the apparent pseudo-first–order rate constants are evaluated in order to compare the efficiency of each process. The most effective process in removing carbofuran from water was the photo-Fenton system (UV + Fe^{2+} + H_2O_2) with rate constants k from 17.2 x 10^{-4}/s to >200.0 x 10^{-4}/s. The degradation of pure carbofuran and commercial product Furadan 4F in acidic aqueous solution upon polychromatic light (300-400 nm) by photo-assisted Fenton process has been studied (Huston & Pignatello, 1999). The complete conversion of 2.0 x 10^{-4} M of pure carbofuran and more than 90% TOC reduction in the water solution within 120 min has been achieved. Nitrate and oxalate ions were detected as organic ionic species after the treatment. Also, the results show that the adjuvants in Furadan 4F have little or no influence on degradation of carbofuran nor of TOC mineralization. Two different Advanced oxidation processes (photo- and electro-Fenton) have been used for photodegradation of carbofuran in water (Kesraoui Abdessalem et al., 2010). For the photo-Fenton process TOC removal ratio was influenced by the initial concentration of the pesticides and the amout of Fe^{3+} and H_2O_2. The TOC measurement indicate an efficient mineralization of 93 and 94% respectively, for photo- and electro-Fenton processes after 480 min of treatment. Carbofuran could not be mineralized on AlFe-PILC and Fe-ZSM-5 zeolite catalysts in the heterogeneous photo-Fenton reactions at 575.6 nm, even in the catalytic reaction promoted at high temperature (Tomašević et al., 2007a, 2007b).

4.7 Ethiofenocarb

Ethiofencarb (IUPAC name: ∝-ethythio-∝-tolyl methylcarbamate) is systemic insecticide with contact and stomach action. It is applied for control of aphids on pome fruit , stone fruit and soft fruit, than vegetables, ornamentals and sugar beet. Formulations types which can be found on the market are: emulsifieble concentrate (EC), emulsions oil in water (EW) and granules (GR). The current regulation status of this active ingredient under directive 91/414/EEC is not included in Annex 1 (EU Pesticide Database, 2011; Tomlin, 2009).

Solar photodegradation of ethiofencarb was examined in pure water, natural water and in the pure water containing 10mg/L of humic acids (Vialaton & Richard, 2002). Photosensitized reactions are main degradation pathway of pesticide in natural water and in the presence of humic acids. Photosensitized transformations were shown to be largely due to photoreactants other than singlet oxygen and hydroxyl radicals. A comparative photolysis reactions of ethiofencarb in water and non-water media were performed in the presence of simulated solar light (Sanz-Asensio et al., 1999). The studies showed that the photolysis reaction follows pseudo-first-order kinetics and that the degradation kinetics depend on the solvent polarity. In the water media the reaction of pesticide degradation was completed for 30 h. Also, the photoproducts are dependent on the solvent and the main photoproduct in water was 2-(methyl)phenyl-N-methylcarbamate. The photolysis of aqueous ethiofencarb (3.3 x 10^{-3} M, 4 h, room temperature, 125 W medium-pressure mercury lamp) has been examined by GC-MS (Climent & Miranda, 1996). Upon irradiation three photoproducts were detected and 66% conversion of ethiofencarb was achieved. The main product was 2-methylphenyl methylcarbamate, and two corresponding phenols also were registered.

4.8 Formetanate

Formetanate (IUPAC name: 3-dimethylaminomethyleneaminophenyl methylcarbamate) is acaricide and insecticide with contact and stomach action. It is used for control of spider mites and some insects on ornamentals, pome fruit, stone fruit, citrus fruit, vegetables and alfalfa. It is sold commercially only as soluble powder (SP). The current regulation status of this active ingredient under directive 91/414/EEC is included in Annex 1, expiration of inclusion: 30/09/2017 (EU Pesticide Database, 2011; Tomlin, 2009).

The solar driven photo-Fenton process using pilot-scale compound parabolic collector was applied to the degradation of formetanate in the form of AgrEvo formulated product Dicorzol (Fallman et al., 1999). The results shown that a good conversion of formetanate was achieved (about 25 min was a TOC half-life and about 70 min was the time necessary for degradation of 80% of TOC). The heterogeneous photocatalysis with TiO_2 (200 mg/L) and homogeneous photocatalysis by photo-Fenton (0.05 mM of $FeSO_4$ x $7H_2O$) of 50 mg/L of formetanate have been studied (Malato et al., 2002b). In the presence of 2.8 mg/L of Fe^{2+} complete conversion of formetanate and more than 90% TOC reduction was demonstrated in pilot-scale solar reactor. The kinetics of formetanate degradation by the TiO_2 solar photocatalysis and by the solar photo-Fenton process were also investigated (Malato et al., 2002b, 2003).

4.9 Methomyl

Methomyl (IUPAC name: S-methyl N-(methylcarbamoyloxy)thioacetimidate) is systemic insecticide and acaricide with contact and stomach action. It is used for control of a wide range of insects and spider mites in fruit, vines, olives, hops, vegetables, ornamentals, field crops, cucurbits, flax, cotton, tobacco, soya beans, etc. Also it can be used for control of flies in animal and poultry houses and dairies. Formulations types for this active ingredient are SL, SP, WP. The current regulation status of this active ingredient under directive 91/414/EEC is included in Annex 1 expiration of inclusion: 31/08/2019 (EU Pesticide Database, 2011; Tomlin, 2009).

The solar driven homogeneous photo-Fenton and heterogeneous TiO_2 processes for methomyl detoxification in water have been evaluated (Malato et al., 2002b, 2003). According to TOC removal, the photo-Fenton process was more efficient in degrading 50 mg/L of methomyl than was the TiO_2 process. The both processes were capable of mineralizing more than 90% of the insecticide (Malato et al., 2002b). The photodegradation of methomyl by Fenton and photo-Fenton reactions were investigated (Tamimi et al., 2008). The degradation rate and the effect of reaction parameters (initial concentration of pesticide, pH, ferrous and H_2O_2 dosage, etc) were monitored. The photo-Fenton was more efficient than Fenton, both for methomyl degradation and TOC removal. The catalytic wet peroxide oxidation of methomyl at 575.6 nm (photo-Fenton reaction) with two types of heterogeneous iron catalysts (Fe-ZSM-5 zeolite and AlFe-pillared montmorillonite) were performed (Lazar et al., 2009; Tomašević et al., 2007c, 2009c, 2010a, 2010b; Tomašević, 2011). The effect of catalyst type on the reaction is shown in Fig. 2. The photolysis of 16.22 mg/L of methomyl in different types of water (deionized, disstiled and sea water) at 254 nm was performed (Tomašević et al., 2009c, 2010a; Tomašević, 2011) and the influence of reaction parameters to degradation of pesticide were investigated. The studies showed that the photolysis reactions depend on the lamp distance (Fig. 3), water type (Fig. 4), reaction temperature and pH. The photocatalytic removal of the methomyl from aqueous solutions upon UV/Vis (366 and 300-400 nm) and natural solar light in the presence of TiO_2 and ZnO has been examined

(Tomašević et al., 2009b, 2010a; Tomašević, 2011) and the influence of reaction conditions (initial concentration of methomyl, catalysts type and concentration, pH, presence of Cl^- ions) were studied. The results (Table 2) showed that the degradation of methomyl was much faster with ZnO than with TiO_2. The IC results confirmed that mineralization of methomyl led to the formation of sulfate, nitrate, and ammonium ions during the all investigated processes (Tomašević et al., 2010a, 2010b; Tomašević, 2011).

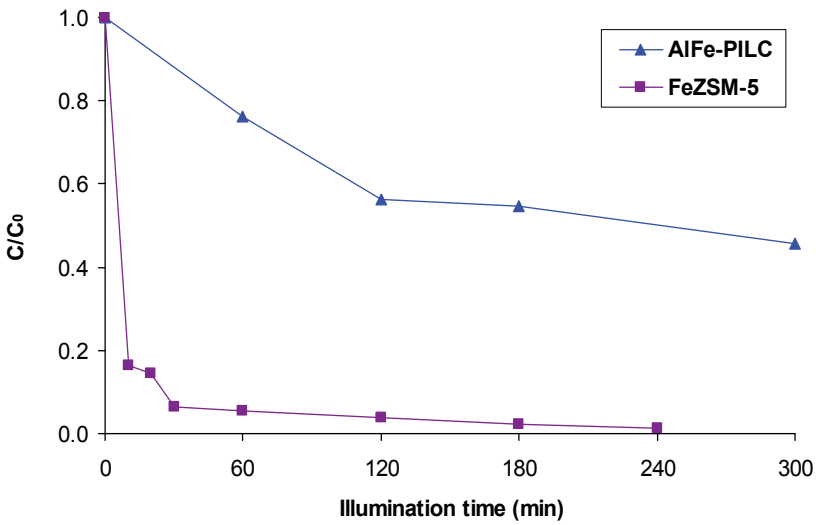


Fig. 2. Photodegradation of methomyl with 5 g/L of catalysts (Tomašević, 2011).

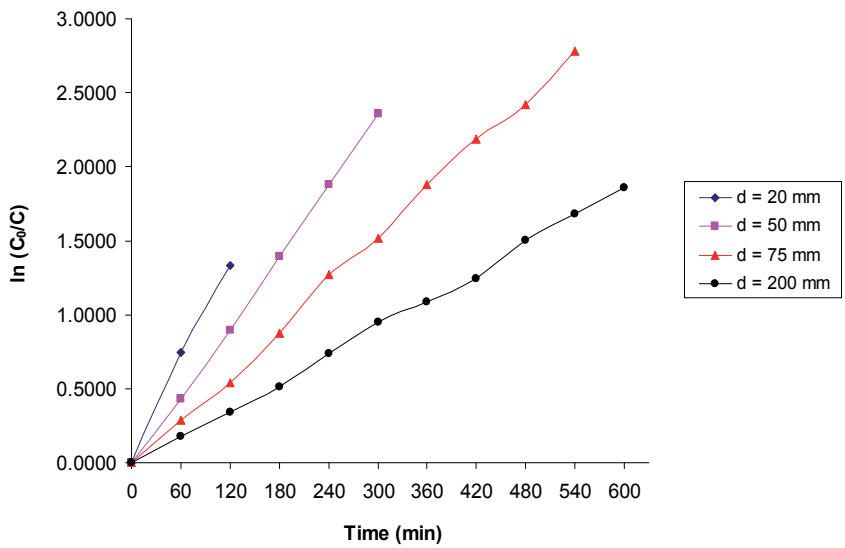


Fig. 3. The effect of lamp distance on the photolysis rate of methomyl (Tomašević, 2011).

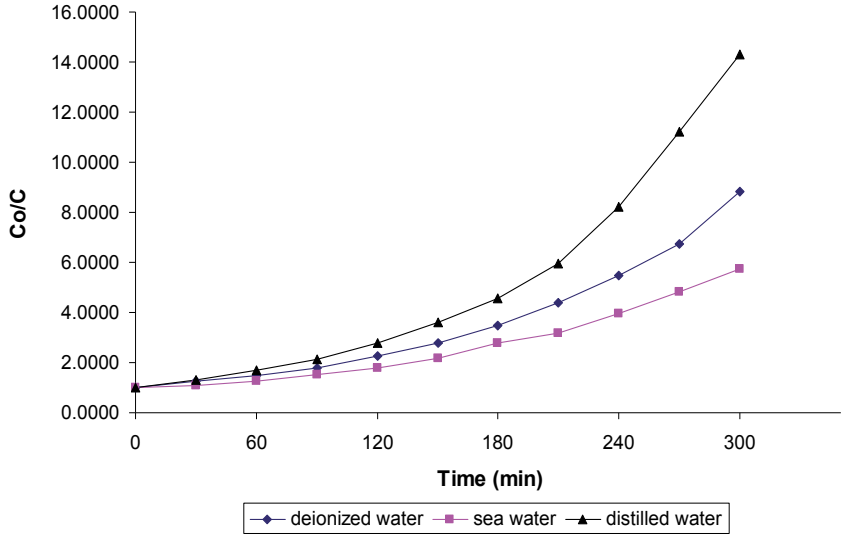


Fig. 4. The effect of the type of water on the photolysis rate of methomyl (Tomašević, 2011).

Technical methomyl	Parameters	Water type Deionized
With 2.0 g/L of TiO_2	k (min^{-1})	0.0058
	R	0.9880
	$t_{1/2}$ (min)	119.51
With 2.0 g/ L of ZnO	k (min^{-1})	0.0120
	R	0.9915
	$t_{1/2}$ (min)	57.76

Table 2. Kinetics of methomyl photodegradation at 366 nm (Tomašević, 2011).

4.10 Oxamyl

Oxamyl (IUPAC name: N,N-dimethyl-2-methylcarbamoyoxyimino-2-(methylthio) acetamide) is contact and systemic insecticide, acaricide and nematocide. It is used for control of chewing and sucking insects, spider mites and nematodes in ornamentals, frut trees, vegetables, cucurbits, beet, bananas, pineapples, peanuts, cotton, soya beans, tobacco, potatoes, and other crops. It could be found only as soluble concentrate (SL) on the market. The current regulation status of this active ingredient under directive 91/414/EEC is included in Annex 1, expiration of inclusion: 31/07/2016 (EU Pesticide Database, 2011; Tomlin, 2009).

An pre-industrial solar treatmen is used to prevent pollution of waters with commercial pesticide Vydate L, containing 24% oxamyl (Malato et al., 2000). Oxamyl is completely photodegraded, but mineralization is slow with illuminated TiO_2 only. The use of additional oxidants such as peroxydisulphate enhanced the degradation rate by a factor of 7 compared to TiO_2 alone. Solar photodegradation in aqueous solution of oxamyl in the presence of two photocatalysts TiO_2 and sodium decatungstate $Na_4W_{10}O_{32}$ is reported (Texier et al., 1999).

For pure compounds TiO_2 was a better catalyst than $Na_4W_{10}O_{32}$, concerning the rate of photodegradation and mineralization. When the pesticide is used as formulation product, the decatungstate anion becomes as efficien or even more efficient than TiO_2. This difference of reactivity is accounted for by the different nature of the active species during both photodegradation processes. The solar driven photo-Fenton process was applied to the degradation of oxamyl in the form of DuPont formulated product Vydate (Fallman et al., 1999). The obtained results shown that oxamyl was relatively recalcitrant (about 100 min was a TOC half-life and about 160 min was the time necessary for degradation of 80% of TOC).

4.11 Pirimicarb

Pirimicarb (IUPAC name: 2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate) is selective systemic insecticide with contact , stomach, and respiratory action. It is used as a selective aphicide for control a wide range of crops, including cereals, oil seeds, potatoes and other vegetables, ornamentals, and other non-food uses. Formulations types for this active ingredient are AE, DP, EC, FU, WG and WP. The current regulation status of this active ingredient under directive 91/414/EEC is included in Annex 1, expiration of inclusion: 31/07/2017 (EU Pesticide Database, 2011; Tomlin, 2009).

Photolysis of pirimicarb upon simulated solar light in natural water and in different aqueous solutions was investigated (Taboada et al., 1995). Aceton strongly increased degradation of pesticide, while methanol did not have any significant effect. The rate of pesticide degradation in the presence of river water was 4.5 times slower than in distilled water, and the half-life of pirimicarb in presence of dissolved humic and fulvic acids was 2-10 times longer than in distilled water. In all studied solutions the degradation reaction followed a first-order kinetics. The solar light and simulated sunlight were used for the photolysis of pirimicarb in water (Romero et al., 1994). The photodegradation mechanism seemed to be similar under both conditions, but the half-life of pirimicarb was found to be about three times longer under natural than under simulated conditions. Also, four main products were isolated and identified by spectroscopic methods. The photolysis of aqueous pirimicarb (3.3×10^{-3} M, 4 h, room temperature) has been examined by GC-MS (Climent & Miranda, 1996). Upon irradiation with 125 W medium-pressure mercury lamp three main photoproducts were detected.

4.12 Promecarb

Promecarb (IUPAC name: 3-methyl-5-methylphenyl methylcarbamate) is an obsolete carbamate insecticide once used to combat foliage and fruit eating insects. It is systemic insecticide. Promecarb is highly toxic by ingestion and is adsorbed through the skin. Formulations type is EC. The current regulation status of this active ingredient under directive 91/414/EEC is not included in Annex 1 (EU Pesticide Database, 2011; Tomlin, 2009).

The photolysis of promecarb in water solution (3.3×10^{-3} M, 4 h, room temperature, 125 W medium-pressure mercury lamp) has been examined by GC-MS (Climent & Miranda, 1996). Upon irradiation, 24% conversion of promecarb was achieved and photolysis of promecarb led to the phenol derivative (22%) as major product. Also, minor amounts of two compounds (isomers arising from photo-Fries rearrangement) were also obtained.

4.13 Propamocarb

Propamocarb (IUPAC name: propyl 3-(dimethylamino)propylcarbamate) is systemic fungicide with protective action. It is used for specific control of Phycomycetes. Also it is used against of wide variety of pest on tomatoes and potatoes, lettuce, cucumber, cabbages, ornamentals, fruit, vegetables, and vegetable seedbeds. Formulations types on the market are SC and SL. The current regulation status of this active ingredient under directive 91/414/EEC is included in Annex 1 expiration of inclusion: 30/09/2017 (EU Pesticide Database, 2011; Tomlin, 2009).

The application of solar photo-Fenton process for degradation of DuPont commercial product Previcur (Fallman et al., 1999) confirmed that propamocarb was one of the hardest pesticides to degrade by process (106 min was a TOC half-life and more than 200 min was the time necessary for degradation of 80% of TOC).

4.14 Propoxur

Propoxur (IUPAC name: 2-isopropoxyphenyl methylcarbamate) is non-systemic insecticide with contact and stomach action. It is used for control of cockroaches, flies, fleas, mosquitoes, bugs, ants, millipedes and other insect pests in food storage areas, houses, animal houses, etc. Also it is used for control of sucking and chewing insects (including aphids) in fruit, vegetables, ornamentals, vines, maize, alfalfa, soya beans, cotton, sugar cane, rice, cocoa, forestry, etc, and against migratory locusts and grasshoppers. There are a lot of different formulations with this active ingredient as AE, DP, EC, FU, GR, RB, SL, UL, WP and Oil spray. The current regulation status of this active ingredient under directive 91/414/EEC is not included in Annex 1 (EU Pesticide Database, 2011; Tomlin, 2009).

An study of the photodegradation of aerated aqueous propoxur solution is given very interesting data (Sanjuan et al., 2000). Photolysis of 1.0×10^{-3} M solution (pH 6.8) with 125 W medium-pressure mercury lamp leads to an almost complete degradation of pesticide and the formation of photo-Fries rearrangement products, but with a relatively minor degree of mineralization. Photocatalyzed degradations in the presence of TiO_2 (40 mg) or with 150 mg of triphenylpyrylium-Zeolite Y (TPY) were shown the same degree of propoxur mineralization. Laser flash photolysis (266 nm) has shown that the degradation could be initiated by a single electron transfer between excited 2,4,6-triphenylpyrylium cation and propoxur to form the corresponding 2,4,6-triphenylpyrylium radical and propoxur radical cation.

5. Conclusion

The reviewed literature reflects that in case of carbamate pesticides the most of the studies have been reported using photo-Fenton processes, photolysis and heterogeneous catalysis with TiO_2 as a catalyst. This photodegradation processes have been proposed as an effective and attractive techniques for degradation of carbamate residues in water. The kinetics of all photodegradation processes depend on several main parameters such as the nature of pesticides, type of light, initial concentration of pesticides (and catalysts), pH of solution, temperature, and presence of oxidant. The AOP_s provide an excellent opportunity to use solar light as an energy source. Photocatalytic processes can lead to the mineralization of toxic and hazardous carbamate pesticides into carbon dioxide, water and inorganic mineral salts.

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4

Tree Injection as an Alternative Method of Insecticide Application

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1. Introduction

Injection directly into the conductive tissues of trees was a method first investigated systematically by Leonardo da Vinci, but some of the most early tree injection experiments were not recorded until early in the 20th century (Roach, 1939, May, 1941, Costonis, 1981). Dutch elm disease, a destructive vascular wilt disease of elm renewed interest in tree injection in the 1970s (Jones and Gregory, 1971; McWain and Gregory, 1971; Jones et al., 1973; Gregory et al., 1973; Gregory and Jones, 1975; Shigo and Campana, 1979; Kielbaso et al. 1979; Shigo et al., 1980), when more common fungicide applications proved ineffective. During this time, several injection methods, including trunk infusion (Schreiber 1969), and pressurized trunk injections (Filer 1973; Helburg et al. 1973; Reil and Beutel 1976, Sachs et al., 1977; Kondo, 1978, Darvas et al., 1984, Navarro et al., 1992), were developed. Tree injection was also used for treatment of other tree pathogens (Guest et al., 1994; Fernández-Escobar et al.1994, 1999), insects, and physiological disorders (i.e., interveinal chlorosis) in the EU (Fernández-Escobar et al. 1993). Interest in tree injection technologies (McClure, 1992, Doccola et al., 2007; Smitley et al., 2010) in the US has also increased, with the introduction of several tree killing insects such as hemlock woolly adelgid (*Adelges tsugae*), Asian longhorned beetle (*Anoplophora glabripennis*) and emerald ash borer (*Agrilus planipennis*). In addition to new injection technology, formulations are being designed for injecting into trees that improve plant safety and reduce application time. Examples of the new technologies are the TREE I.V. micro-infusion system and Air/Hydraulic micro-injector (Arborjet, Inc. Woburn, MA, USA) and the Eco-ject® Microinjection System (Bioforest Technologies, Canada). Today, tree injection is an alternative method of chemical application with certain advantages: (1) efficient use of chemicals, (2) reduced potential environmental exposure, and (3) useful when soil and foliar applications are either ineffective or difficult to apply (Stipes, 1988; Sanchez-Zamora and Fernandez-Escobar, 2004). Tree injection into roots, trunks or limbs requires wounding of the tree, which has implications to the tree's health. The question often asked is, does the benefit gained by tree injection outweigh the risk of the wound caused by treatment? This question of cost-benefit is certainly valid. However, this concern must also be weighed against environmental (and off target) exposures when trees are sprayed or insecticides are applied to the soil. An underlying assumption is that the value of the tree and its treatment is greater than sustaining tree loss. Key factors weigh in to wound responses in trees that likewise demand consideration. These include (1) the tree species, (2) tree health, (3) the attributes of the

chemistry applied and (4) the frequency that applications are made. Such issues present a broader and more complex paradigm and carry over into tree injection practices. In order to apply tree injections effectively, one needs a basic understanding of the (1) method of application, (2) the chemistry applied, and (3) tree condition. The aim of this paper is to recommend tree injection as an alternative application method for systemic insecticides to (1) protect trees against destructive insects, (2) to minimize potential environmental exposures, and (3) to manage tree wound responses.

2. Tree anatomy and physiology

The introduction and movement of liquid insecticides by injection is dependent upon tree vasculature. Anatomically, trees are highly connected systems (Shigo, 1989, 1991). Fibrous, non-woody roots absorb water and solutes (i.e., minerals in dissolved form) from the rhizosphere (root-soil environment). Hydraulic movement upward in the xylem is dependent upon transpiration from stomates, driven by the moisture lost from leaf surface to the ambient atmosphere (Greulach, 1973). Upward translocation of systemic insecticides also depends upon the rise of sap in trees.

Although movement of sap in the stem is generally upward (i.e., straight sectorial ascent), there is considerable variation in the path of water movement across species (Zanne et al., 2006). The ascent of water in trees follows two basic patterns, that of, spiral and vertical ascents. Systemic chemicals move upward in tree stems along the path of their respective ascents. Crown distribution of water is the most complete by spiral ascent (e.g., red oak), the least effective, by vertical ascent (e.g., white oak) (Rudinski and Vité, 1959). Spiral ascent occurs in a number of species, including conifer xylem (Kozlowski and Winget 1963, Kozlowski et al., 1967).

The size, pattern and distribution of vessels vary in trees. Hardwoods may be grouped as ring- or diffuse- porous; conifers are considered non-porous species (Chaney, 1988). Angiosperm trees have large, wide vessels associated with comparatively high flow rates, while gymnosperms rely solely on very small diameter tracheids to move water. The rate of water flow differs with tree species. Hagen-Poiseulle law describes the rate of flow as a function of the xylem radius to the 4^{th} power (Kramer et al., 1996). Therefore hardwoods (e.g., oaks, elms) move injected liquid at a faster rate than conifers (e.g., pines, hemlocks). In feet per hour, ring porous hardwoods (red oak, ash, elm) move water at 92, 85 and 20; diffuse porous hardwoods (black walnut, maple, beech) move water at 13, 8 and 4; while conifers (pine, hemlock) move water at 6 and 3 (Coder, 1999). Conifers and diffuse porous hardwoods tend to use a larger proportion of sapwood than the ring porous hardwoods for water movement. Drilling more deeply (i.e., 30 rather than 15 mm) in these species serves to access a larger area of sapwood for the injection of systemic chemicals. Sinclair and Larsen investigated wood characteristics that correlated with ease of injection for deciduous trees and suggested the formula, relative frequency of vessels divided by specific gravity (1981).

3. Sapwood composition

Xylem (sapwood) is the conductive tissue of plants, made up of cellulose, lignin and other substances. Cellulose $(C_6H_{10}O_5)_n$ is an organic polymer made up of glucose molecules linked together in long chains (Raven, Evert & Curtis, 1981). Lignin is a complex organic polymer that functions to strengthen wood. Cellulose makes up the cell wall of plants, and

is 44.4% carbon (Heukelekian, H. and S.A. Waksman. 1925). When mature, the xylem protoplast dies, leaving only cell wall. It is through the remaining lumen that water conduction occurs. The lumen simultaneously functions as a continuous and extensive conductive and adsorptive structure.

4. Soil and trunk spray applications compared to tree injection

Water soluble insecticides are differentially absorbed by tree roots comparative to insoluble chemistries such as the avermectins (Wislocki, 1989). Imidacloprid and acephate are labeled in the US for soil application, but restricted in areas of ground water concern (for example, Long Island, N.Y., U.S.). In coarse textured, sandy soils and in areas with high precipitation, there is the potential for insecticide leaching. The insecticidal treatment of eastern hemlock (*Tsuga canadensis*) for hemlock woolly adelgid (*Adelges tsugae*) is an example. Eastern hemlock is a riparian species, which grows in moist soils, and near streams and rivers. In these environments, the use of trunk sprays increases the potential for exposure to off target organisms (e.g., aquatic invertebrates, fish). Tree injection of insecticides is an alternative method of application where these conditions exist. Tree injected imidacloprid applied directly to the vascular tissues is conducted upward within those tissues; the procedure reduces the potential for unintended exposures.

5. Pros of tree injection

Canopy sprays are used to control defoliating insects, but drift and limited reach are issues in very tall (>15 meters) trees, where coverage from hydraulic sprayers is inadequate. Employing tree injections resolves these issues; the chemistries move within the vascular system into the canopy for systemic activity. Systemic injections are used to effectively control borers that feed under the bark, where active ingredients sprayed onto the surface of trees may not penetrate in biologically active concentrations. Soil applications are also used, but have a number of limitations. For example, they may be slower acting, require higher amounts of product or repeated applications, may migrate off-target, and be subject to microbial degradation. Finally, tree injections may be more economical to use. Although hydrolysis occurs within the plant, systemically injected chemistries may provide greater residual activity compared to other methods, (i.e., spray, drench) which are subject to drift, leaching, photolysis or microbial degradation. Repeated spray applications each season are necessary for adequate insect control. Aqueous photolysis and mean aerobic soil half-life of selected chemistries appear in Table 1. Soil applications of systemic insecticides are often made at significantly higher volumes (e.g., 5 to 10x) compared to tree injection in order to compensate for leaching, binding to soil particles, microbial degradation or the vagaries of pH and soil moisture. If there are good reasons to utilize tree injection, why are they not employed more often? The objection most often cited is that the application requires drilling into trees. This concern includes the physical wound, and the tree defenses triggered by the introduced formulation. Wounding in trees needs to be placed within context of other types of wounding against which trees evolved effective survival strategies. Trees are wounded in nature when insects bore into the bark and sapwood and when woodpeckers peck and bore into trees after them. People also create wounds in trees for specific purposes.

Half-lives (days)				
Insecticide	**Water Sol (g/L)**	**$K_{o/c}$***	**Aqueous Photolysis**	**Soil+**
Acephate	700 (Worthing, 1987)	0.48 (Montgomery, 1993)	stable (Chevron, 1972d)	0.5 (Chevron, 1972g)
Imidacloprid	0.514 (Yen & Wendt, 1993)	300-400 (Cox et al., 1997)	$3.98x10^{-2}$ (Anderson, 1991)	38.9 (Yoshida, 1990)
Emamectin	0.024 (Tomlin, 2004)	>25000 (Mushtaq et al. 1996)	3.6-10.9 (Mushtaq et al., 1998)	193.4 (Chukwudebe et al., 1997a)

*organic carbon adsorption coefficient
+mean aerobic

Table 1. Water solubility's, organic carbon adsorptions and half-lives of three chemistries systemically injected into trees.

6. Wood boring insects

Insect borers include species of Lepidoptera, Hymenoptera and Coleoptera. Borers may be further categorized as wood or cambium borers. Most native insects are opportunistic, attacking stressed and declining trees. When conditions favor epidemiology, trees are attacked and killed. Exotic insects are comparatively more aggressive and attack and kill healthy trees.

Lepidoptera: Clear-winged borers (Sessidae) include some serious pests including the ash borer (*Podesia syringae*). *Dioryctria* borers (Pyralidae) attack pines causing large masses of sap to exude. The Zimmerman pine moth (*Dioryctria zimmermani*) is a pest of Austrian and Scotch pines (*Pinus nigra, P. sylvestris*) in ornamental landscapes (Cranshaw & Leatherman, 2006).

Hymenoptera: Horntails (Siricidae) are sawflies that develop in damaged or stressed trees. A recent introduction in the US, the Sirex woodwasp (*Sirex noctilio*), a native of Europe, Asia and northern Africa has the potential to cause significant mortality in native pine stands (Haugen & Hoebeke, 2005).

Coleoptera: Several families of beetles bore into trees, which include the Scolytidae (bark beetles), Cerambycidae (Longhorned beetles or roundheaded borers), and Buprestidae (flat-headed borers). Some species vector spores of destructive pathogens.

Scolytidae: In Lodgepole pine (*Pinus contorta*) a native scolytid mountain pine beetle (*Dendroctonus ponderosae*) vectors *Ophiostoma clavigerum*, a blue staining fungus (Solheim and Krokene, 1998). MPB also infests ponderosa (*P. ponderosa*), sugar (*P. lambertiana*) and white (*P. monticola*) pines (Amman et al., 2002). An epidemic can cause widespread tree mortality. The Smaller European Elm bark beetle (*Scolytus multistriatus*) vectors spores of the bluestain fungus (*Ophiostoma novo-ulmi*) that cause Dutch elm disease, a vascular wilt disease that has devastated the American elm (*Ulmus americana*) in the United States.

Cerambycidae: Locust borer (*Megacyllene robiniae*) is a native that attacks, and can severely damage or kill stressed and healthy black locust *(Robinia pseudoacacia*) (Galford, 1984). The Asian longhorned beetle (*Anoplophora glabripennis*) was introduced from Asia (China) and identified in Brooklyn, New York in 1996. ALB has a broad host range in the US but preferentially infests maple (*Acer*), and birch (*Betula*) trees (Sawyer, 2010).

Buprestidae: Emerald Ash Borer (*Agrilus planipennis*), an exotic introduced from Asia (China) was identified in Detroit, MI in 2002 (McCullough and Siegert, 2007; Anulewicz et al., 2008.). EAB attacks native ash (*Fraxinus*) species, preferentially Green (*F. pennsylvanica*) and Black (*F. nigra*), but also White (*F. americana*) and Blue (*F. quadrangulata*) ashes. EAB mines the phloem, cambium and scores the xylem as an actively developing larva. The vascular disruption reduces water movement upward into the canopy, and photosynthate transport through the phloem; unchecked infestations result in tree death. Unlike maple and birch attacked by ALB, ash trees do not bleed and EAB larvae do not remove frass from their galleries, so there are no visible signs of early infestation. Infestations often go undetected for several years, and symptoms in ash (epicormic sprouts, bark cracks, woodpecker flecks) and signs (d-shaped exit holes) do not occur until the damage has occurred. Goldspotted Oak Borer (*Agrilus coxalis*) is native to Southeastern Arizona, detected in San Diego County, California in 2004. It attacks coast live oak (*Quercus agrifolia*), canyon live oak (*Q. chrysolepis*) and California black oak (*Q. kelloggii*). Regarded as an invasive species in California, larval feeding kills phloem and cambium, which results in crown dieback and tree mortality (Coleman & Seybold, 2008). Other Buprestid borers include the two-lined chestnut borer (*A. bilineatus*) and the bronze birch borer (*A. anxius*). Adult two-lined chestnut borers attack stressed or declining oak trees. The bronze birch borer preferentially attacks European cutleaf birches such as *Betula jacquemontii, B. pendula and B. pendula* 'Youngii' (Dirr, 2009).

7. Birds that drill into trees

The yellow-bellied sapsucker (*Sphyrapicus varius*) bores into the bark of trees to obtain sap. More than 250 species of woody plants are known to be attacked, but birch (*Betula* spp.), maple (*Acer* spp.) and hemlock (*Tsuga* spp.) are preferentially attacked (Ostry & Nicholls, 1978). Sapsucker damage is characterized by many closely spaced holes on the tree. The tree responds by proliferating new tissues at the wound sites. Woodpeckers feed primarily on wood boring insects. The Northern flicker (*Colaptes auratus*), Red-bellied woodpecker (*Melanerpes carolinus*), Downy woodpecker (*Picoides pubescens*), Hairy woodpecker (*Picoides* villosus) and Red-headed woodpecker (*Melanerpes erthrocephalus*) drill holes into trees to extract insects or sap (Barnes, 1989). These woodpecker behaviors are generally not regarded as detrimental to trees.

8. People drill into trees

People drill into trees for sap extraction and to apply treatments, including injection. In the northeastern US and Canada, Sugar maples (*Acer saccharum*) are tapped annually for maple syrup production. Healthy trees that are tapped according to established guidelines do not suffer adverse health effects and remain productive (Davenport & Staats, 1998), some for over 100 years. Arborists drill into trees to install cabling and lightning protection (ANSI A300 Part 3, 2006; ANSI A300 Part 4, 2008). Tree care specialists treat by injection to protect trees against destructive pests. In the US, destructive, exotic insects such as hemlock woolly adelgid (USDA/FS 2003), Asian long-horned beetle (USDA/FS 2008) and emerald ash borer (USDA/FS 2008a) have recently renewed interest in tree injection technology as an alternative method of insecticide application (McClure, 1992, Doccola et al., 2007; Smitley et al., 2010). To apply tree

injections effectively, one needs a basic understanding of the (1) method of application, (2) the chemistry applied, and (3) tree condition.

9. Tree injection methodology

Systemic tree injections effectively treat destructive insect pests of trees. Examples of the new technologies are the TREE I.V. micro-infusion system and the Air/Hydraulic micro-injector (Arborjet, Inc. Woburn, MA, USA) and the Eco-ject® Micro-injection System (Bioforest Technologies, Inc., Canada). The TREE I.V. micro-infusion system and Air/Hydraulic micro-injector deliver 0.50 and 2.0 liters at injection pressures of 172 to 1379 kPa, respectively. These methods require the insertion of an interface into the sapwood (Arborplug™) to inject a systemic insecticide. The Arborplug has an internal rubber septum which is pierced by an injector needle for liquid delivery. The Arborplug is 15 mm in length and has a diameter of either 7 or 9 mm. Drilling 15 mm deep provides a volumetric capacity of 0.6 to 1.1 cm^3, respectively. The Eco-ject Micro-injection System loads re-usable micro-injection capsules, but does not use a plug. Using such devices, one may deliver a number of systemic chemistries by tree injection. Here we discuss three insecticides which are, (1) acephate, (2) imidacloprid and (3) emamectin benzoate.

9.1 Acephate

Acephate (O,S-dimethyl acetylphosphoramidothioate) is water soluble (700 g/L) and readily absorbed by tree roots for systemic activity (Worthing, 1987; Kidd & James, 1991). It has a low $K_{o/c}$ (organic carbon adsorption coefficient) of 0.48 (Montgomery, 1993); it is only weakly adsorbed in the soil. Acephate is an organo-phosphate insecticide designed for insecticidal activity and quick degradation. Acephate's stability is affected by pH. It has a comparatively shorter half-life (of 16-d, pH 9) in alkaline environments (Chevron, unpublished 1972b). Acephate is particularly mobile in coarse textured soils and has the potential to leach (Yen et al., 2000), but it is quickly degraded by microbial activity. In plants, acephate's half-life is approximately 5 to 10-d. Approximately 5 to 10% of acephate is degraded to methamidophos (which has insecticidal activity), the remainder to salts (of N, P and S) (Chevron, unpublished 1973). Acephate has both translaminar and systemic activity in plants. Acephate is a broad spectrum systemic, used for control of aphids, leaf miners, Lepidopterous larvae, sawflies, and thrips. 97.4% acephate is a soluble granular offered as an implant (Ace-Cap, Creative Sales, Fremont Nebraska) or tree injection formulation (ACE-jet, Arborjet, Inc.).

9.2 Imidacloprid

Imidacloprid (1-[(6-chloropyridin-3-yl) methyl]-N-nitro-4, 5-dihydroimidazol-2-amine) is a chloronicotinyl (neonicotinoid) chemistry with a water solubility of 0.51 g/L (Yen and Wendt, 1993). Imidacloprid has moderate binding activity ($K_{o/c}$ of 300 to 400) to clay and organic matter (Cox et al., 1997), however there is potential for the compound to move through porous, coarse textured soils (Jenkins, 1994). Imidacloprid has translaminar and systemic activity in plants (Buchholz and Nauen, 2002). Imidacloprid controls sucking insects such as adelgids, aphids, thrips, whiteflies, and some beetles, including Cerambycids. Examples of tree injection formulations of imidacloprid are Imicide (JJ Mauget, Arcadia, CA), Xytect (Rainbow Treecare Scientific Advancements, Minnetonka, MN) and IMA-jet (Arborjet, Inc.).

9.3 Emamectin benzoate

Emamectin benzoate is a semi-synthetic compound derived from the fermentation by-product of a soil actinomycete, *Streptomyces avermitilis* (Jansson et al., 1996). Emamectin benzoate is a mixture of the benzoic acid salt of two structurally complex heterocyclic (glycoside) compounds. It occurs as a mixture of ≥90% benzoic acid salts of 4'-epi-methylamino-4'-19 deoxyavermectin B1a and ≤10% 4'-epi-methylamino-4'-deoxyavermectin B1b (Wood, 2010). Emamectin benzoate is poorly (0.024 g/L) soluble in water (Tomlin, 2004). It has a $K_{o/c}$ of >25,000 and is immobile in soils (Mushtaq et al. 1996). Emamectin benzoate has translaminar activity, but limited plant systemic activity when applied to the foliage (Copping, 2004). A novel micro-emulsion formulation (TREE-äge, Syngenta Crop Protection, LLC, Greensboro, NC) used for systemic tree injection is registered for use in the US against specific Coleoptera and Lepidoptera pests.

10. Behaviors of injected chemistries

Injected chemistries differ in their rate of movement in the vascular system, and in their residual activity. In Avocado (*Persea americana*), Acephate peaked in foliage 2 weeks following tree injection, whereas peak imidacloprid residues were not observed for 7-9 weeks following application (Morse et al., 2008). The slow upward movement of imidacloprid may be explained by its comparatively higher carbon adsorption, and may play a role in the extended activity observed in field studies (Doccola et al., 2007; Morse et al., 2008). Studies in green ash (*Fraxinus pennsylvanica* Marsh) and white ash (*F. americana* L.) have demonstrated that imidacloprid accumulates in the canopy, but tree injection could also provide a reservoir for continued systemic activity (Cregg et al., 2005; Tanis et al., 2006, 2007, 2009). Takai et al. (2003), reported 3 years of protection in pine trees against pine wilt nematode after injecting a liquid formulation of emamectin benzoate. In the US, emamectin benzoate was reported to provide 2 or more years of protection against Lepidopterous and Coleoptera pests, including Pine cone worm (*Dioryctria*), Southern pine beetle (*Dendroctonus frontalis*) and Emerald ash borer (*Agrilus planipennis*) (Grosman et al., 2002, Grosman et al., 2009; Smitley et al., 2010).

Injection into plant tissues protects the chemistry from phytolysis and microbial degradation, mechanisms that breakdown the chemistry in the environment relatively quickly. Although hydrolysis occurs within the plant, some of the metabolites have insecticidal activity (for example, olefinic-, dihydroxy- and hydroxy-imidacloprid breakdown products of imidacloprid) (Sangha & Machemer, 1992; Suchail et al., 2001). Residual activity is based on the half-life of the chemistry, but carbon adsorption may also play a role in the activity observed in perennial tissues (such as in twig, branch and stem) over time. Injected formulations that provide multiple years of activity must move (spatially) from the original injection site in the xylem tissue into new vascular tissue in order to be effective against insects that perennially attack and feed in the lateral cambium. Residual activity of an injected insecticide provides protection against insect pests that have extended emergence periods, multiple generations per year, or are epidemic (i.e., increase exponentially over time).

11. When to treat trees

Apply treatments before damage (defoliation, vascular mining) occurs for optimum results. Oak trees defoliated by gypsy moth must use stored carbohydrates for recovery

(Shigo, 1989; Shigo, 1991). Furthermore, native insects are opportunistic: oaks that have been defoliated by insects such as gypsy moth (*Lymantria dispar*) are predisposed to attack by the two-lined chestnut borer (Haack & Acciavatti, 1992). Minimizing defoliation in trees is a sound practice to protect tree health. Rather than resorting to "rescue" treatments to save trees at risk of wood and bark infesting insects, treat them when they still appear visibly healthy. Late insecticide treatments (e.g., >33% canopy dieback, epicormic sprouting, bark cracks, woodpecker flecks, exit holes) are contra-indicated. This approach minimizes negative outcomes, such as canopy dieback, delayed recovery or tree mortality.

As discussed earlier, the upward movement of an injected chemistry is dependent upon plant evapo-transpiration. Therefore, tree injections are most efficiently applied when trees are transpiring. Transpiration is dependent on a number of factors, such as soil moisture, soil and ambient temperature, the relative humidity and time of day. For optimal uptake, apply when the soil is moist, soil temperatures are above 7.2°C (45˚F), and during the 24 hour period when transpiration is greatest.

When using insecticides with short-residual activity (an example is acephate), make the application when the pest is active. Application of chemistries with greater residual activity are somewhat less dependent upon insect feeding activity (e.g., imidacloprid, emamectin), but are typically applied 30-d or more of expected pest activity. Fall applications may be applied in some instances. For example, imidacloprid applications in evergreen trees may be applied late in the season. Imidacloprid applications for HWA applications are made in the autumn to coordinate with resumption of sistens nymphal activity following summer aestivation. Imidacloprid activity is retained in hemlock (leaves of 3-6 age classes persist in trees) for extended residual activity (Doccola et al., in press). In addition, systemic insecticides with high adsorption coefficients (>5000) may be applied in the fall (at leaf senescence) for activity in the next growing season. TREE-äge (emamectin benzoate) is an example of a fall application used to protect ash trees against EAB (Smitley et al., 2010).

12. Tree defense responses

When trees are wounded, whether by an insect boring into the tree or by a mechanical drill bit, tree defense mechanisms come into play. These defense reactions and responses were systematically described by Shigo and Marx (1977). Dujesiefken and Liese have elaborated on the (CODIT) model taking into account the role of air exposure and embolism formation in the process of walling the damage in trees (2008). Individual trees may vary considerably in the strength of their response to similar types of wounds depending on genetics or tree health (Shigo, 1999). A discussion of tree wound responses must consider basic tree anatomy, in particular the secondary vascular tissues. Of most interest is the lateral meristem (cambium). This secondary cambium is only a few cells thick and occurs between the sapwood (xylem) and inner bark (phloem). This tissue is embryonic in nature. Periclinal divisions form xylem cells inward and phloem cells outward. The cambium is not transport tissue. Sapwood consists of living (symplast) and non-living (apoplast) cells. The living cells within the sapwood are non-differentiated parenchyma. The parenchyma cells store starch, oils and ergastic substances (Esau, 1977). Parenchyma occurs both as radial and axial tissues. Radial parenchyma extends into the phloem. The conductive xylem is functional when it matures and dies. The side walls of the xylem are pitted. Parenchyma cells sometimes balloon into the lumen of the xylem through the sidewall pits to form a tylose, or a physical

barrier. Tyloses may be formed in older wood naturally (e.g., white oak, *Quercus alba*, forms tyloses in second year wood), or are a consequence of trauma (e.g., red oak, *Q. rubra*, forms tyloses in response to wounding) (Shigo, 1999). When a tree is physically injured, both biochemical and structural changes occur. The biochemical reactions (changes of stored carbohydrates to phenolic and terpene defense chemicals) are observed in tree sections in three dimensions. These were named reaction zones (or boundary walls) 1 – 3. Reaction zone 1 occurs in the axial direction (i.e., with the stem axis) and is the least limiting boundary. Reaction zone 2 occurs in the radial direction (i.e., with the tree radius, inward toward the pith), and reaction zone 3 occurs in the tangential direction (i.e., with the tree's circumference), and is the strongest limiting boundary of the three reaction zones. The fourth wall, referred to as the barrier zone occurs after injury, and is the strongest limiting boundary. Meristematic cells (cambium) divide to form callus tissue, which later differentiates into new woundwood (new xylem, cambium and phloem). Native insect attacks to healthy trees are fended off by the biochemistry and by the subsequent physical responses. Emerald ash borer attacks to Asian species of ash (*Fraxinus chinensis*, *F. manchurica*) do not result in tree mortality: plant defense responses effectively isolate the larva in early stages of attack and limit its progression. In *F. pennsylvanica* (a native), the larvae are compartmentalized via physical boundaries (wall 4), but the biochemistry (phenols, terpene chemistries) does not effectively stop the insect's development. Injection of an insecticidal chemistry to compensate for insufficient tree response is the basis of successful tree protection. EAB research has demonstrated that this strategy is very effective (Smitley et al., 2010).

Tree wound responses are dependent upon a number of intrinsic and extrinsic variables such as tree species, tree health, method of treatment and chemistry applied. Tree wound response is under genetic control (Santamour, 1979). For example, birch (*Betula* spp.) poplar (*Populus* spp.) and willow (*Salix* spp.) are considered weak compartmentalizers, whereas oak (*Quercus* spp.), sycamore (*Platanus* spp.) and linden (*Tilia* spp.) are considered strong compartmentalizers (Dujesiefken and Liese, 2008). Santamour (1986) described fourteen cultivars of maple (*Acer*), ash (*Fraxinus*), oak (*Quercus*) and linden (*Tilia*) that were strong wall 2 compartmentalizers. As a group, trees have evolved to resist assaults and are successful, long-lived perennial plants. Tree health is another variable with numerous contributing factors. These include the age of the tree, soil conditions (texture, structure, moisture, pH, minerals and drainage), and exposure (sun, shade). Trees require light, water and minerals for essential life functions (including defense). Photosynthesis is the basis of carbohydrate synthesis. Woundwood responses utilize energy (carbohydrate, lipid) stores. When injections are made to trees in relatively good health (preventative-early therapeutic treatments) tree woundwood development readily proceeds to close wounds. However, the prognosis for recovery is comparatively lower, when making late therapeutic (rescue) applications, because energy stores are reduced. Optimal wound responses are observed when applications are made early, relative to infestation (Doccola et al., 2011). To further manage wounds in trees, make the fewest number of injection sites to apply the dose, and whenever possible, avoid drilling in the valleys between roots (Shigo and Campana, 1977).

The Wedgle Direct-Inject (ArborSystems, LLC, Omaha, NE) is a method of tree injection that does not require drilling into the sapwood. The system relies on forcing the de-lamination (slippage) of the bark from the sapwood to apply a small amount of a formulation. This method directly exposes the lateral cambium to concentrated solvents. A consequence is phytotoxicity (e.g., hypersensitive reactions, necroses) to the tissues of the lateral meristem

(the initials for woundwood development). The small doses and exposures to the lateral cambium by this method offers no clear advantage over drilling into trees for injection. Protection of the lateral cambium is of greater consequence to tree wound response compared to drilling into the sapwood. Further, wound closure rates of trees are positively correlated with trunk growth, and greater callus is produced around larger wounds than around smaller diameter wounds (Neely, 1988). Arborjet, Inc. employs a (7 or 9 mm) diameter drill hole to efficiently deliver higher volumes of insecticides into trees. The larger diameter hole is strongly limited by boundary wall 3 (this strong boundary reduces the likelihood of girdling and is an advantage to tree survival). With this system, a plastic Arborplug is inserted into the drilled hole, which creates the injection interface. The Arborplug from a tree wound defense perspective, reduces exposure of the lateral cambium to the solvent carriers in the injection formulation and minimizes wood exposure to air. Placing backflow preventers into the bark do not function in the same manner. Further, when the Arborplug is set correctly (at the sapwood-bark plane), it provides a flat surface for callus and woundwood development and wound closure. This encapsulation is the survival strategy of trees following injury (Dujesiefken and Liese, 2008).

13. Multiple-year activity

It is possible to make applications that are effective against a persistent and destructive tree pest and not require an annual treatment. The residual activity of tree injected imidacloprid may be due to protection against photolysis and microbial degradation. Foliar half-life of imidacloprid is ~9.8-d (Linn, 1992d, unpublished). Plants metabolize imidacloprid via hydrolysis, but some of the metabolites have insecticidal activity. The predominant metabolites associated with toxicity in insects are olefinic-, dihydroxy- and hydroxy-imidacloprid (Sangha & Machemer, 1992; Suchail et al., 2001). In studies of large (50 cm) diameter hemlock infested with HWA, both soil and tree injections with imidacloprid were made (Doccola et al., in press). Two methods of tree injections were employed, one using low volume micro-injection (QUIK-jet, Arborjet, Inc.) and the second using high volume micro-infusion (TREE I.V., Arborjet, Inc.). The soil applications were made using the Kioritz injector (Kioritz Corporation, 7-2, Suehirocho 1 –Chome, Ohme, Tokyo, 198 Japan). Tree injection administered 0.15 g imidacloprid per 2.5 cm dbh, micro-infusion applied 0.3 g per 2.5 cm dbh whereas soil injection applied 1.45 g per 2.5 cm dbh. In that study, data was collected on HWA infestation, tree growth and imidacloprid residues in the foliage over a three year period. Tree foliage responses were greater in the tree injection treatments. Imidacloprid residues taken annually from 70 to 1165-d were above the LC_{50} value of 0.30 µg/g for HWA (Cowles et al., 2006) for all the imidacloprid treatments. At 1165-d, foliage residues (of 1.35 µg/g) in the lowest dose injections continued to protect trees. This residual activity of imidacloprid was attributed to both the perennial nature (of 3-6 years) of the foliage, and to the slow, upward movement of imidacloprid. Green ash trees treated with emamectin benzoate tree injections were protected from EAB for up to four years (Smitley et al., 2010). A recently completed 3 year study using low dose injections of emamectin benzoate protected trees for three years (Deb McCullough, personal communication). These studies point to efficacy and duration of tree injection methods. The TREE-äge label is approved (by US EPA) for up to two years of control against listed arthropods, including EAB. Injection is a very efficient use of insecticidal chemistry to protect trees.

14. Tree injection as an alternative

Today, tree injection is an alternative method of chemical application with definite advantages: (1) efficient use of chemicals, (2) reduced potential environmental exposure, and (3) useful when soil and foliar applications are either ineffective or difficult to apply (Stipes, 1988; Sanchez-Zamora and Fernandez-Escobar, 2004). Tree injection is used when trees are at risk from attack from destructive or persistent pests. It may be put to good use in tall trees. They are administered in trees growing in environmentally sensitive locations (e.g., near water, in sandy soils). Tree injection does create wounds, however the benefit of the introduced chemistry to protect trees often outweigh the drilling wound. The new paradigm weighs the potential of off target consequences of application to the consequences of the drilled wound made by tree injection. Unintended off target exposures include toxicity to earthworms, fish, aquatic arthropods, pollinators and applicator. Insecticides are by design, toxic, albeit useful, substances. Tree injection is a method to deliver specific toxicants to the injurious pest and to minimize non-intended exposures. In this chapter, three specific insecticides used in tree injection were considered, each with unique attributes for specific applications in trees. Tree injection is an alternative methodology to apply systemic insecticides for tree protection.

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5

Development of a Prophylactic Butyrylcholinesterase Bioscavenger to Protect Against Insecticide Toxicity Using a Homologous Macaque Model

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1. Introduction

Organophosphorus (OP) and carbamate pesticides are extensively used to control agricultural, household and structural pests. Each year approximately 5.6 billion pounds of pesticides are used worldwide potentially exposing ~1.8 billion people who use pesticides to protect the food and commercial products that they produce (Alavanja, 2009). Although unintentional occupational poisonings represent only a small number, estimated to be ~10% (Litchfield, 2005) or 25 million agricultural workers globally (Jeyaratnam, 1990), large scale exposure of both civilian and military personnel has become an ever increasing threat, as a result of deliberate insecticide contamination of the environment and critical water supplies by terrorists. In this context, pesticide use is one of only two exposures consistently identified by Gulf War epidemiologic studies to be significantly associated with the multisymptom illness profiles described as Gulf War illness (Cao et al., 2011). Pesticide use has also been associated with neurocognitive deficits and neuroendocrine alterations in Gulf War veterans in clinical studies conducted following the end of the war.

While OP nerve agents and WHO Class I and Class II OP pesticides constitute a diverse group of chemical structures, all potentially exhibit a common mechanism of toxicity, that is, active site phosphorylation of acetylcholine (AChE) resulting in AChE inhibition and accumulation of acetylcholine, overstimulation of cholinergic receptors, and consequent clinical signs of cholinergic toxicity such as seizures, brain damage and cognitive and behavioural defects (Millard et al., 1999; Rosenberry et al., 1999; Colosio et al., 2009). The relationship between AChE inhibition and symptoms showed that prevalence ratios were significantly >1 for respiratory, eye and central nervous system symptoms for workers with >30% inhibition (Ohayo-Mitoko et al., 2000). More recent studies indicate that insecticide exposure to DFP (diisopropyl fluorophosphate) causes a prolonged increased in hippocampal neuronal Ca++ plateau which may underlie morbidity and mortality (Deshpande et al., 2010). These findings are consistent with those indicating persistent changes in locus coeruleus noradrenergic neuronal activity and lasting changes in this brain area after removal of the insecticide chlorpyrifos oxon; reminiscent of the lasting cognitive

symptoms of Gulf War illness in soldiers exposed to these compounds (US DOD, Pesticides-Final Report, 2003).
Currently, the standard (approved) treatment for acute OP pesticide poisoning involves administration of intravenous (iv) atropine and an oxime e.g. obidoxime, pralidoxime to reactivate inhibited AChE (Worek et al., 2010). However, the effectiveness and safety of oxime administration in acute OP pesticide-poisoned patients has been challenged and a recent clinical trial showed no clinical benefits and a trend towards harm in all sub-groups, despite clear evidence that these doses reactivated AChE in the blood (Buckley et al., 2011).
An efficacious prophylactic therapeutic treatment for preventing insecticide poisoning that can bind and scavenge the OP before it reaches and targets AChE in neuromuscular junctions is therefore a high priority. The leading candidate of this type is native (plasma) butyrylcholinesterase (BChE) whose potent OP bioscavenging ability has been demonstrated in many animal models and against varied OP neurotoxins (Doctor et al., 2001; Lenz et al., 2001). While several new catalytic and other stoichiometric enzymes also exhibit this ability (Lenz et al., 2007), based on availability, broad spectrum efficacy, stability and safety (Sun et al., 2005), BChE is the most advanced in terms of development of a human treatment. In Turkey, frozen plasma (BChE levels of 3,000 - 5,700 units) given as an alternative or adjunctive treatment with atropine and oximes, has been shown to prevent mortality and intermediate syndrome in acutely insecticide-exposed and hospitalized individuals (Güven et al., 2004). Currently, BChE also finds use as a treatment of cocaine overdose and for the alleviation of succinylcholine-induced apnea.
Structurally, BChE (also known as pseudocholinesterase or non-specific cholinesterase) is a serine esterase (MW=345,000) comprised of four identical subunits each containing 574 amino acids, held together by non-covalent bonds, with 36 carbohyrdrate chains (23.9% by weight) (Lockridge, 1990; Nachon et al., 2002). BChE is found in all species at levels of 1-20 ug/ml in plasma (Rosenberg, unp. data) and is also abundant in liver, intestine and lung. Recombinant (r) human butyryl-cholinesterase (HuBChE), like the native form, is also a potent bioscavenger of OP neurotoxins (Doctor et al., 2001; Lenz et al., 2001; Raveh et al., 1997) but its development as a human treatment for pesticide exposure has been disadvantaged by: (i) poor in vivo stability (bioavailability) of the unmodified forms and the presence of potentially immunogenic glycans using certain expression systems (ii) a 1:1 stoichiometry between the enzyme and OP (Raveh et al., 1997) and (iii) the high LD_{50} of insecticides (ug-mg/kg levels). This necessitates the delivery of large, costly, rBChE doses to detoxify exposed individuals which is problematic when intramuscular (im) or subcutaneous (sc) injections are the chosen routes of delivery. In this chapter, we shall describe our experience of how the chemistry, glycosylation, chemical modification, animal model and route of administration may reduce or enhance the potential of BChE bioscavengers as prophylactic therapeutic human antidotes for OP insecticide exposure.

2. Production of tetrameric and monomeric forms of rMaBChE and rHuBChE

Macaque (Ma) and human (Hu) BChE molecules are very similar molecules differing by only 22 amino acids and sharing ~96% DNA sequence identity, critical glycosylation sites, cysteines and disulfide bridging (Boeck et al., 2002; Rosenberg et al., 2010). Thus, most anti-BChE antisera react with both molecules. Native HuBChE and MaBChE in plasma are composed predominantly of tetramers (98%) with the tetramerization domain being located within the last 40 C-terminal residues of each monomeric subunit (534-574) (Blong et al.,

1997). In human serum, the association of lamellipodin proline rich peptides with the monomeric chains results in the formation of BChE tetramers (Li et al., 2008). Recombinant BChE produced in mammalian cells, in contrast, has only 10-20% tetrameric forms and therefore optimal tetramerization requires the addition of either poly(L-proline) to the culture medium or co-expression of the full length BChE monomers with the proline-rich attachment domain (PRAD) of ColQ gene (Altamirano & Lockridge, 1999).

To date, rHuBChE and rMaBChE molecules have been produced in transgenic mammalian cells (Chilukuri et al., 2008; Rosenberg et al, 2010), goat milk (Huang et al., 2007) and in plants (Geyer et al., 2010; Jiang, unpub. data). Our approach has been to utilize two expression systems for the production of rMaBChE and rHuBChE. Initially, Chinese hamster ovary cells (CHO) were used because of their human-like glycosylation. More recently, a transient plant expression platform was adopted to increase the yield and reduce the time and cost of producing rBChE. Although CHO cells and plants are able to produce significant levels of tetrameric BChE molecules (Li et al., 2008; Geyer et al., 2010), in the present studies, co-transfection of the BChE and PRAD genes has been shown to increase both levels of tetramerization and yields in each expression system. While the CHO cell expression of recombinant proteins is very well established, recent innovations in transient plant expression systems e.g. Bayer's Magnifection system (Gleba et al., 2005) and the Cow Pea Mosaic Virus Hyper-translatable Protein Expression System (PBL Technology) (Sainsbury et al., 2008) have been shown to be some of the most rapid, cost effective and productive expression systems in existence; capable of producing grams of recombinant proteins in weeks (Goodin et al., 2008).

CHO-derived (Stable Transfection)*				Plant-derived (Transient Transfection)*		
rMaBChE#+		rHuBChE		rMaBChE#		
				N. tobacum		*N. benthamiana*
Monomeric	Tetrameric	Monomeric	Tetrameric	Monomeric	Tetrameric	Tetrameric
8U/ml (9mg/L)	25U/ml (28mg/L)	16 U/ml (22.9mg/L)	45 U/ml (64.3 mg/L)	60 U/gm (66.6 mg/kg)	140 U/gm (155.5 mg/kg)	400 U/gm (444 mg/kg)

*All tobacco plants and leaves from *Nicotiana tobacum* and *N. benthamiana* were transfected using Agrobacterium-mediated infiltration

CHO supernatants and whole leaf extracts are prepared for purification.

+ BChE activity is determined spectrophotometrically (Grunwald at al., 1997), using butyrylthiocholine (BTC) (0.5 mM each) as substrate. One unit of enzyme activity is the amount required to hydrolyze 1 umol substrate/min. One mg MaBChE has 900 units of activity and one mg HuBChE has 700units.

Table 1. Expression levels of different forms of rBChE using CHO-and plant-based expression systems.

In addition to the tetrameric forms, a truncated monomeric form of rBChE (MW=~81KDa) that is incapable of oligomerization has also been produced by the insertion of a stop codon at G534 resulting in a monomeric form lacking 41 C-terminal residues (Blong et al., 1997). The smaller monomeric molecules may more rapidly gain access to the blood from muscle or lungs (depending on the route of delivery) with transiently higher bioavailablity in the plasma, which would be advantageous in emergency situations that require real time responses and rapid treatment or booster administrations.

3. In vitro biological properties of rMaBChE

To test the chemical properties of CHO- and tobacco-derived rMaBChE, inhibition and reactivation assays using diisopropyl fluorophosphate (DFP) and paraoxon (diethyl 4-nitrophenyl phosphate) have been performed with and without the oxime 2-PAM (pyridine-2-aldoxime methochloride)(Luo et al., 2008). DFP is an OP compound that has been used as an experimental insecticide agent in neuroscience because of its ability to inhibit cholinesterase and cause delayed peripheral neuropathy. Paraoxon is an insecticide and will be described in detail in a later section. Following purification of the CHO supernatant and the plant leaf extract using procainamide sepharose, rMaBChE molecules conjugated with polyetheleneglycol (PEG) using succinimidyl-propionate-activated methoxy-PEG-20K (SPA-PEG-20K; Nektar Inc., Birmingham, AL) or Sunbright ME-200HS 20K PEG (NOF, Tokyo, Japan) (Chilukuri et al., 2008a; Cohen et al., 2001) to test the effects of PEGylation on enzyme plasma stability. Initially, the biochemical properties of both the unmodified and PEGylated forms of both monomeric and tetrameric rMaBChE were examined using DFP inhibition; bimolecular rate constants (k_i ($\times$ 10^7) M^{-1} min^{-1}) for inhibition of all the recombinants forms ranging from 2.58 - 2.23 ($\times$ 10^7) M^{-1} min^{-1} which were indistinguishable from the well characterized native HuBChE (2.29 +/- 0.1) and native MaBChE (2.22 +/- 0.1) (data not shown).

3.1 Inhibition and reactivation of plant derived CHO-derived and plant-derived rBChE by paraoxon

The kinetics of inhibition of both plant-derived and CHO-derived rMaBChE by paraoxon were further examined as shown in Fig. 1A. At low paraoxon concentrations (0.01 and 0.02*uM*), the reciprocal value of $E_t/E_{t,0}$ was highly correlated with the reaction time; the reaction rate constant of plant-derived rMaBChE at 0.01*uM* paraoxon being slightly faster than that of CHO-derived MaBChE (0.035 $M^{-1}min^{-1}$ vs 0.022 $M^{-1}min^{-1}$ *respectively)*. These values follow the simple 2nd-order (reciprocal) model.

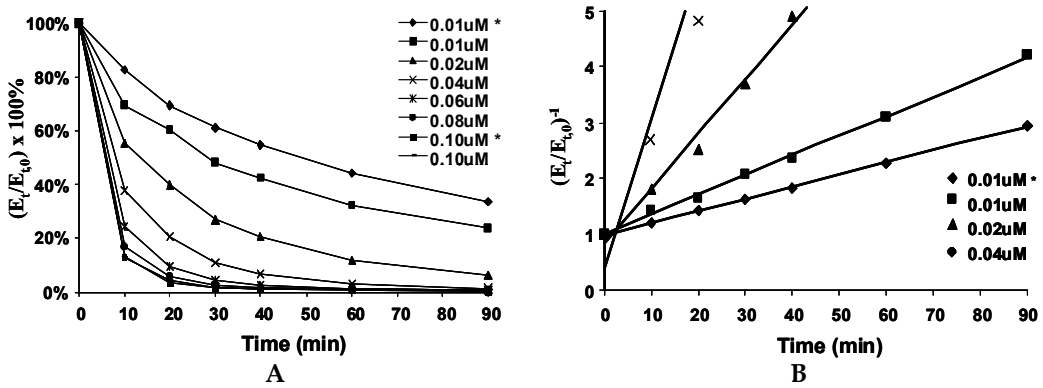

Fig. 1. Inhibition kinetics of plant- and CHO-derived* rMaBChE by different concentrations of paraoxon (0.01uM - 0.10uM) A: Percent inhibition of BChE by paraoxon. (Percent BChE activity was obtained by dividing the BChE activity with paraoxon at each time point with control BChE activity at the same time point. B: Reciprocal plot of BChE inhibition by paraoxon.

3.2 Reactivation of paraoxon-inhibited plant-derived rMaBChE by 2-PAM

Since a 1 hour incubation of 0.016 uM plant-derived MaBChE (1.2U/ml) with 0.02uM paraoxon resulted in 80-90% inhibition of the enyme (Fig. 1A), the same conditions (incubation of paraoxon with rMaBChE at a final enzyme concentration of 0.04-0.05uM), was used to prepare inhibited rMaBChE. Reactivation of inhibited rMaBChE was then initiated by adding different concentrations of 2-PAM (0.4mM-6.4mM) for various times (Fig.2). The kinetics of reactivation of paraoxon-inhibited CHO- and plant-derived rMaBChE were found to follow the simple first-order (mono-exponential) model.

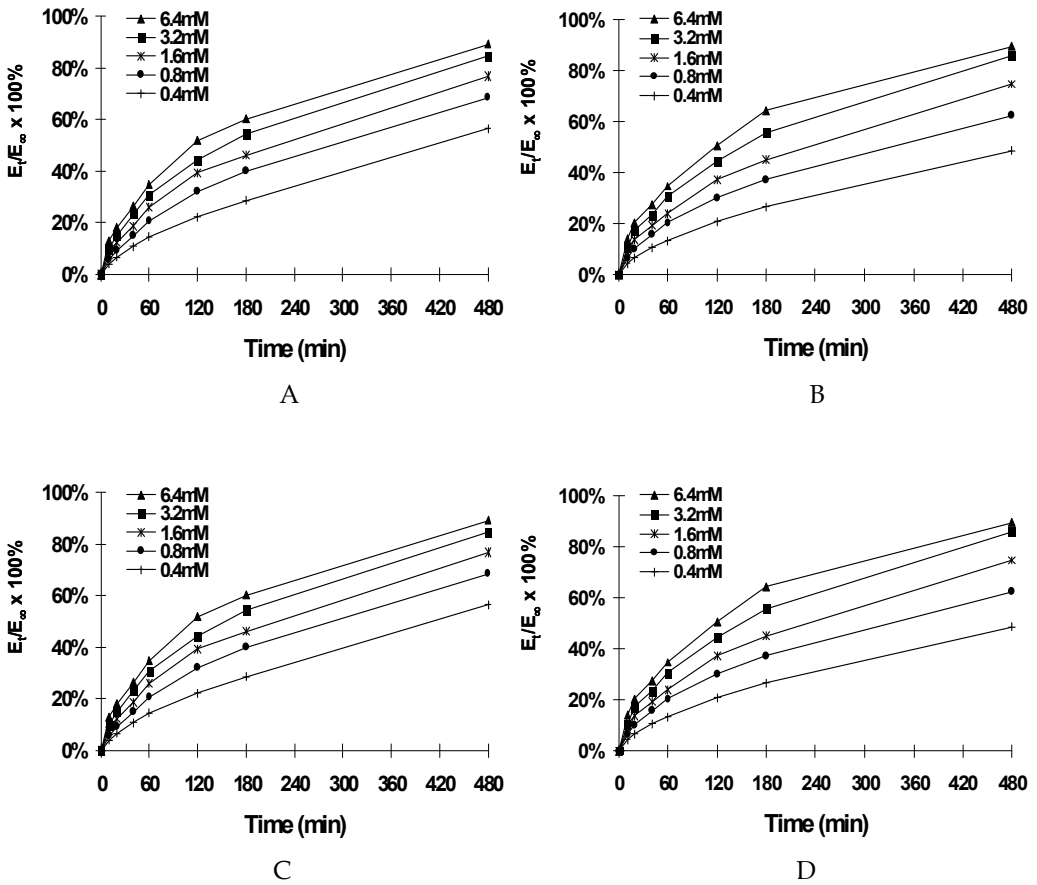

Fig. 2. Reactivation kinetics of paraoxon inhibited plant- and CHO-derived MaBChE by 2-PAM. A, C: Plant-derived MaBChE; B, D: CHO-derived MaBChE; A and B: Direct plot of the time course vs % reactivation; C and D: Semi-logarithmic plot of time course of reactivation. For inhibition controls, inhibited BChE was incubated with reaction buffer without 2-PAM. Triplicate BChE assays were performed at the times indicated.

The results indicate that both paraoxon-inhibited plant- and CHO-derived rMaBChE showed very similar patterns of reactivation by different concentrations of 2-PAM (Fig. 2A, 2B) with nearly 100 % reactivation of each rMaBChE form being achieved by 24 hours at >1.60 mM 2-PAM; the k_{app} values of CHO-rMaBChE ranging from 0.0014 to 0.004 min^{-1} and plant-rMaBChE from 0.0013 to 0.0051 min^{-1} (Fig. 2C, 2D). The reactivation k_{app}s at each 2-PAM concentration was linear when plotted against 2-PAM concentration (mM) expressed logrithmically.

4. In vivo testing of rBChE

In the area of insecticide exposure/contamination, there is a high likelihood that agricultural workers or military personnel will be exposed multiple times during their lives and thus multiple prophylactic treatments must be considered a possibly. This is often problematic since administration of heterologous HuBChE into macaques or other species eg mice has been shown to generate anti-BChE antibody responses and rapidly eliminate enzyme on repeated injections (Matzke et al., 1999; Chiluluri et al., 2008b; Sun et al., 2009). Thus, in vivo retention times of exogenously administered recombinant proteins can only be accurately assessed using homologous systems (rMaBChE → macaques and rHuBChE → humans) in which antibodies or other immune responses are not induced. In this context, homologous BChE enzyme has been shown to have a long half-life (8-12 days) with no adverse effects and no immunogenicity following either (i) transfusions of human plasma into humans (ii) daily administrations of partially purified native HuBChE into humans for several weeks (Jenkins et al., 1967; Cascio et al., 1988) or (iii) injection of purified native MaBChhE or PEG-rMaBChE into macaques (MRT= 200-300 h)(Rosenberg et al., 2002, 2010). These data are in contrast to exogenously administered heterologous HuBChE which displayed a rapid clearance in macaques (MRT = 33.7 h) (Raveh et al., 1989). While the choice of the animal model for PK, immunogenicity and efficacy testing is always important, the animal species utilized for the evaluation of an efficacious human cholinesterase bioscavenger is critical, since potential treatments against OP toxicity cannot be tested in humans and will require extensive testing in animal models and the Animal Rule (CFR 601.90 for biologics) for regulatory approval.

4.1 Pharmacokinetics of clearance in rodent and macaque models

Pharmacokinetic profiles following administration of biologics in many rodent and primate species are used to indicate the periods after administration that such biologics are likely to exhibit optimal benefit or protection. An efficacious therapeutic for preventing OP poisoning is a molecule that: (i) can bind and scavenge the OP before it reaches the targeted AChE in neuromuscular junctions and (ii) has the ability to remain at therapeutic levels in the blood for prolonged periods to counteract a known or impending OP exposure. The in vivo parameters generally used to assess PK performance after administration are mean retention time (MRT), maximal concentration (Cmax), time to reach maximal concentration (Tmax), elimination half life (T1/2) and area under the plasma concentration curve extrapolated to infinity (AUC).

Generally pharmacokinetics of recombinant molecules differs considerably depending on the structure, glycoslyation, size, route of administration, immunogenicity, and animal model utilized. Interestingly, despite protein sequence identity, rBChE proteins, similar to many other recombinant biologics, have been shown to be rapidly cleared following injection (Saxena et al., 1998; Cohen et al., 2006) in contrast to the good plasma stability of native BChE. Thus, rBChE molecules require post-translational modification to provide protection as therapeutic scavengers. A common means of increasing the radius of the target molecule permitting slower renal clearance and prolonging plasma retention is by PEG conjugation. This has been successfully used with proteins, peptides, oliogonucleotides and antibody fragments to improve pharmacokinetic and immunological profiles (Kang et al., 2009). Accordingly, both monomeric and tetrameric forms of rMaBChE have been conjugated with 20KD PEG (without interference of in vitro biological properties) and the pharmacokinetic profiles of the unmodified and PEG-conjugated rMaBChE forms compared in monkeys and mice (Rosenberg et al., 2010).

Figure 3 shows the PK profiles in 24 monkeys following iv injection of 1.2 -3 mg/kg of unmodified or PEG-rMaBChE and illustrates several aspects of BChE clearance: (i) PEG-rMaBChE exhibits good stability in the lower range of the native form; the hierarchy of clearance being native BChE ~ PEG-rMaBChE >>> unmodified monomeric rMaBChE > unmodified tetrameric rMaBChE. (ii) Surprisingly, five of the monkeys demonstrated unexpected dramatic decreases in BChE levels (shown in bold between days 150 and 230 days post injection). In each case, these decreases always occurred immediately after the weekend treatment of the grass surrounding the animal facility and presumably resulted from exposure of the housed monkeys to insecticide; highlighting the unintentional consequences of routine insecticide use on plasma BChE activity and (iii) despite very poor retention of the unmodified monomeric rBChE, administration of the PEGylated monomeric rMaBChE showed overlapping pharmacokinetic parameters with the larger PEG-rMaBChE tetrameric form despite lack of oligomerization.

Importantly, the extended circulatory retention afforded by PEG conjugation of rMaBChE in monkeys (injected iv) was not observed in mice (injected ip) where unmodified and modified monomeric and tetrameric rMaBChE all exhibited the same high MRT and T1/2 (Rosenberg et al., 2010). This indicates that, depending on the parameter measured, the mouse model does not accurately predict the outcome in monkeys with MRT and T1/2 values appearing to be less predictive indicators of circulatory stability in macaques than parameters such as AUC and Cmax. Similar differential pharmacokinetic behaviour was observed following the administration of recombinant rhesus (Rh) and HuAChE in mice and monkeys (Cohen et al., 2004).

These studies highlight the potential problems inherent in choosing an animal model to test human biologics. Notwithstanding the differences in pharmacokinetic behaviour of the same protein in different species and the high potential for immunogenicity in rodents due to the evolutionary distance between rodents and humans, other influences may also play a role in the circulatory stability of proteins following even the first injections into heterologous species. Table 2 shows the pharmacokinetic parameters (MRT, Cmax, Tmax, T1/2 and AUC) following injection of different forms of BChE into several different animal species determined from the time course curve of blood BChE concentrations and using a Windows-based program for non-compartmental analysis. Several conclusions can be made.

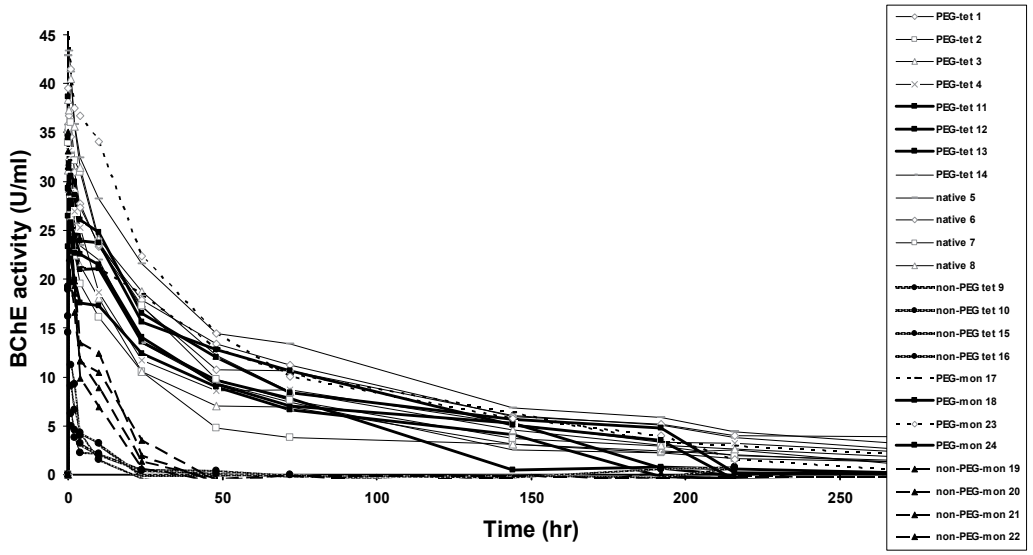

Fig. 3. Pharmacokinetics of clearance following iv injection of 1.2 - 3.0 mg/kg rMaBChE into 24 monkeys. Each line represents a single monkey. Different forms of rMaBChE were used except for 4 macaques receiving native BChE.

For example, while the Cmax following first injections appear to be similar in any animal model at comparable doses, the AUC, MRT and T1/2 are often significantly higher in homologous systems (e.g. PEG-rMaBChE into macaques and native mouse (Mo) BChE into mice) than heterologous injections (native HuBChE into monkeys or mice or PEG-rHuBChE into monkeys). This indicates that heterologous proteins, even when PEGylated and given at a time when anti-BChE titers are absent or low, appear to be eliminated faster than homologous proteins suggesting that pharmacokinetic parameters are less than optimal in all heterologous systems.

It should also be noted, that while PEG conjugation markedly improves the pharmacokinetic profile of therapeutic rMaBChE and other biologics, effects relating to immunogenicity have been mixed. Thus, reduced immunogenicity has been observed following PEGylation of enzymes, cytokines and hormones, while administration of PEGylated interferon-β1a to monkeys actually resulted in increased immunogenicity (Pepinsky et al., 2001). In the case of rHuBChE produced in HEK-293 cells, PEGylation failed to eliminate immunogenicity in mice as demonstrated by the rapid clearance of a repeat 100U injection of (heterologous) PEG-rHuBChE, coincident with induction of high levels of serum anti-BChE antibody (Sun et al., 2009). Likewise, when tested in a sandwich ELISA, the presence of 4–7 PEG molecules per rMaBChE monomer did not prevent the binding of BChE epitopes to either an anti-BChE MAb or a polyclonal rabbit anti-BChE antibody when antigen concentrations were increased to as little as 4–8 U/ml (Rosenberg et al., 2010) which, as mentioned above, is in the range of BChE in normal plasma. These studies raise the question whether chemical modification by PEG will be able to mask any "foreign" rBChE epitopes, such as non-human glycans, sufficient to prevent humoral immune responses and also highlights the importance of using homologous animal models to perform in vivo PK, immunogenicity and efficacy testing.

Human and Mouse BChE								
BChE	**Dose** [Units, mg, mg/kg]	**Animal**	**Route**	**MRT** (hr)	**AUC** (U/ml.h)	**Cmax** (U/ml)	**Tmax** (hr)	**T1/2** (hr)
natHuBChE (Raveh,1997)	11.5 mg (8,000 U)	Monkey	iv	33	710			
natHuBChE	11.5 mg (8,000 U)	Monkey	im		582	16.2	9.5	
natHuBChE (Lenz,2005)	5.25 mg/kg (12,000 U)	Monkey	im		2576	21	9.27	79.3
	8.75 mg/kg (20,000 U)	Monkey	im		3822	33	10.3	73.5
natHuBChE (Sun, 2005)	34 mg/kg (30,000 U)	Monkey	iv	73	16,538	222	0	37
natHuBChE (Sun, 2009)	100 U	Mouse	im	48	1,300	19		21
natMaBChE *	100 U	Mouse	im	73	2,500	25		24
Monkey BChE								
BChE	Dose [Units, mg, mg/kg]	Animal	Route	MRT (hr)	AUC (U/ml.h)	Cmax (U/ml)	Tmax (hr)	T1/2 (hr)
natMaBChE *	3 -5 mg/kg (7,000 U)	Monkey	iv	191				
(Rosenbberg, 2002)	1.3 - 1.65 mg/kg (3,000 U)	Monkey	iv	50				
natMaBChE (unpub)*	1.8 mg/kg	Monkey	iv	142	2950	27		
	1.8 mg/kg	Monkey	iv	142	4010	37		
natMaBChE*	2.9 mg/kg	Monkey	iv	224	4431	38		143
(Rosenberg, 2010)	2.9 mg/kg	Monkey	iv	307	4299	40		126
	1.9 mg/kg	Monkey	iv	200	2097	26		157
PEG-rMaBChE*	2.9 mg/kg	Monkey	iv	168	2141	33		112
(Rosenberg, 2010)	2.9 mg/kg	Monkey	iv	223	3312	39		85
	1.9 mg/kg	Monkey	iv	134	1724	24		97
PEG-rMaBChE (unpub)*	3.0 mg/kg	Monkey	iv		4359	51		
PEG-rHuBChE (unpub)*	3.0 mg/kg	Monkey	iv		1101	40		

MRT: mean retention time, Cmax: maximal concentration, Tmax: time to reach maximal concentration, T1/2: elimination half life, AUC: area under the plasma concentration curve extrapolated to infinity. nat: native, Mon: monomeric, Tet: tetramer.

Table 2. Pharmacokinetic parameters of different forms of BChE in homologous* and heterologous systems.

4.2 The role of glycosylation and oligomerization on pharmacokinetics

The BChE molecule is a soluble protein, protected from proteolysis by a heavy sugar coating from nine N-linked glycans (Li et al., 2008). N-glycosylation is one of the major post-translational modifications of proteins and can be critical to their bioavailability. Importantly, while the first steps in the *N*-glycosylation pathway, leading to the formation of oligomannosidic structures, are conserved in plants and animals, the final steps in the formation of complex N-glycans may differ with the expression system used. Thus, in contrast to native HuBChE molecules which have highly sialylated bi- and triantennary type glycans (Saxena et al., 1998; Kolarich et al. 2008) containing the N-acetyl neuraminic acid (NANA, NeuAc) form of sialic acid (Varki, 2001), rHuBChE molecules may exhibit under-sialyated or immunogenic non-human glycan structures that accelerate in vivo clearance

due to rapid uptake by asialoglycoprotein and mannose receptors in the liver or by antibody-mediated mechanisms (Park et al., 2005). For example, CHO cells produce recombinant proteins which contain human-like glycans that may be undersialyted, compared to those produced in livestock systems which append the non-human galactose-α-1,3-galactose and the N-glycolyl neuraminic (NGNA, NeuGc) form of sialic acid (Chung et al., 2008; Diaz et al., 2009) and those produced in plants which are non-sialylated and append the non-human β-1,2 xylose and α-1,3 fucose containing glycans (Altmann, 2007).

The relationship between sialic acid levels and oligomerization of recombinant molecules with their circulatory longevity has been extensively studied. For example, administration to mice of recombinant bovine and rhesus acetylcholinesterase (rBoAChE, rRhAChE) as well as plant-derived rHuBChE have supported the idea that pharmacokinetic behaviour is governed by hierarchical rules (Kronman et al., 2000); efficient enzyme tetramerization and high sialic acid occupancy both being required for optimal plasma retention. However, other data from monkey and mice studies do not closely obey these classical rules for circulatory retention. For example: (i) the requirement for tetramerization of rAChE molecules was less important when performed in macaques rather than mice (Cohen et al., 2004) (ii) CHO-derived monomeric PEG-rMaBChE resulted in high MRT when injected into in monkeys (Fig.3, Rosenberg et al., 2010) and (iii) the MRT and T1/2 of unmodified and PEG-modified monomeric rMaBChE were both unexpectedly high following injection into mice; PEG-conjugation offering no significant advantages.

While the short lived circulatory retention of asialylated BChE attests to the importance of sialylation in retention/clearance, the degree to which silaic acid occupancy is required does not always seem straight forward. Thus, although the rapid clearance of monomeric (13% non-silayted) and tetrameric (25% nonsialyted) rMaBChE in monkeys, compared to the native or PEGylated forms, has been thought to result from undersialylation, glycan analysis by MALDI-TOF of the highly stable native HuBChE and MaBChE proteins indicates that these also contain a significant percentage of nonsialyted or undersilayted proteins. For example, native HuBChE contains 23% monosialyted glycans (99.9% NANA) and a significant percentage of non-sialyted glycans (Kolarich et al., 2008) while native MaBChE is comprised of 21.3% non-sialayted glycans and 21.8% monosialylated glycans (99.9% NGNA) (Rosenberg, unp. data). This means that heterologous animal models invariably involve the administration of native or CHO-derived human proteins containing NANA into animals containing the NGNA form of sialic acid (monkeys, rodents). These findings showing either high percentages of undersialylated glycans in the stable native proteins and those showing lower pharmacokinetic parameters following heterologous injections, raise the interesting question as to whether the type of sialic acid type as well as the degree of sialic acid occupancy may determine the rate of clearance of recombinant glycoproteins.

It is also important to note that recent engineering of different expression systems is now permitting the production of glycoproteins with human-like glycans. For example, while the inability to perform appropriate *N*-glycosylation has been a major limitation of plants as expression systems, these are being overcome by new approaches involving the generation of knockout or knockdown plants that: (i) completely lack xylosyl transferase (XylT) and fucosyl transferase (FucT) activity (Strasser et al., 2004) and accumulate high amounts of human-like N-glycan structures that contain no 1,2-xylose or core a1,3-fucose (ii) lack complex N-glycans resulting from the inactivity of N-acetlyglucosaminyltransferase 1 (GnT1) (Strasser et al, 2005; Wenderoth & von Schaewen, 2000) and (iii) contain glycans

terminating in sialic acid (Paccalet et al., 2007; Castilho et al., 2010). In addition, different glycoforms of plant derived proteins can be generated by protein targeting to different compartments (i) cytosol (aglycosylated) (ii) ER (high mannose) or (iii) secreted into the apoplast (complex) (Stoger et al., 2005)

4.3 Effects of the route of administration on pharmacokinetics

As mentioned, delivery of PEG-rBChE as a pre-exposure modality is disadvantaged by its large size and a 1:1 stoichiometry between the enzyme and OP requiring high doses due to the high LD_{50} of many insecticides (ug-mg/kg levels). The route of systemic delivery of high doses of native BChE (MW~350KDa) and tetrameric PEG-rMaBChE (MW>800KDa) will determine the pharmacokinetics (PK) of clearance and is critical to efficacy and safety. Currently very little monkey data exists on the delivery of a stoichiometrically equivalent dose of PEG-rBChE calculated to protect against a known LD_{50} of a toxic OP insecticide. Although immediate release requiring intravenous (iv) injection may be necessary in certain high threat situations, these are usually impractical in the field. Needleless cutaneous delivery via the dermis and epidermis (chemical mediators, electroporation) appear quite promising, but are unlikely to deliver high doses. Thus, self-administered transdermal injections through the skin either by subcutaneous (sc) or intramuscular (im) routes have been the approaches most commonly used; virtually all human vaccines currently on the market being administered via these routes. Traditionally, autoinjectors, devices for im delivery of a self administered single dose of a drug are used in the military to protect personnel from chemical warfare agents and are currently used to deliver morphine for pain and atropine, diazepam and 2-PAM-Cl for first-aid against nerve agents. For this reason, most animal protection studies with OP bioscavengers have routinely been delivered im to rodents (Lenz et al., 2005; Mumford et al, 2010; Saxena, et al., 2011).

Despite all the pharmacokinetics data generated using im and sc routes of delivery of many drugs and biologics, little is known about the factors that govern the rate and extent of protein absorption from the injection site and the role of the lymphatic system in the transport of large molecules to the systemic circulation. With smaller molecules, the time to maximal concentration is usually shorter following im injections compared to sc injections where absorption is slow and prolonged and accounts for the lag in entering the blood. However with larger therapeutic molecules (MW>16KDa), the lymphatics are thought by some groups to be the primary route of absorption from sc (and im) injection sites. Large molecules are thought to exit the interstitium via cleft like openings into the lymph and enter the systemic circulation via the thoracic duct (Supersaxo et al., 1990; Porter et al., 2001; McLennan et L., 2006). To assess the effects of different routes of delivery, pharmacokinetic behaviour using different doses of PEG-rMaBChE tetrameric molecules was compared in monkeys following im and sc injections.

4.3.1 Intramuscular delivery of PEG-rMaBChE

Four monkeys each received an im injection of either 2.5 or 3 mg/kg of PEG-rMaBChE. As shown in Fig. 4, unlike the delivery of the smaller native HuBChE which appear to behave uniformly following im injection (Lenz et al., 2005), the much larger PEG-conjugated form exhibits very variable results when delivered into the muscle with Tmax values in the 4 macaques having values of 8, 24, 48 and 48 hr respectively; the 8-hour peak looking more like an iv injection than an im injection. It is not clear whether this more rapid exit from the

muscle injection site into the blood reflects a more vascularised muscle or whether im delivery has more potential to damage blood vessels and promote faster draining. It is clear however that delivery of large doses of a therapeutic such as PEG-rHuBChE will require many im injections to achieve required peak values and will increase the likelihood of targeting a blood vessel. The stoichiometric dose of BChE required to protect humans against 2 LD_{50} of soman has been considered to be 3 mg/kgm (200 mg/70 kg); the antidotal efficacy of BChE being contingent upon both the rate of OP detoxification and its levels in blood (Raveh, 1997; Ashani & Pistinner, 2004). It would be unlikely that Cmax values (20 and 23 U/ml at 3 mg/kg and 17 and 10 U/kg at 2.5 mg/kg) following im administration would be sufficient for protection. In addition, the variable times of peak enzyme make it difficult to choose a time for prophylactic dosing.

4.3.2 Subcutaneous delivery of PEG-rMaBChE

Extensive pharmacokinetics have been performed on many well known biologics in monkeys and humans, either PEGylated or unmodified, using the sc routes of delivery (Boelaert et al., 1989; Ramakrishnan et al., 2003; Heatherington et al., 2001; Radwanski et al., 1987; Mager et al., 2005), although extrapolation from these studies may be problematic because all used considerably smaller molecules than native or PEG-rBChE. Generally, sc injections have been the delivery route of choice for compounds with limited oral bioavailability, as a means of modifying or extending the release profiles of these molecules, or as a means of delivering drugs that require large quantities (Yang, 2003) since larger volumes may be injected. In one case, a highly concentrated form of a therapeutic requiring large doses for its effects has be prepared as a crystalline and successfully delivered sc in a small volume (Yang et al. 2003).

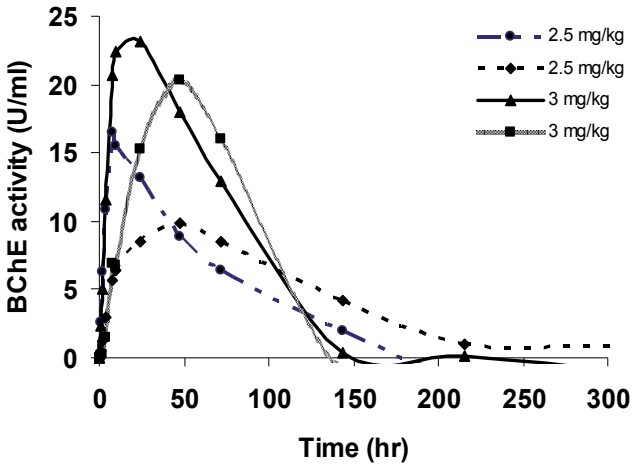

Fig. 4. Pharmacokinetic profiles of PEG-rMaBChE delivered by im injection. Four monkeys were injected into the thigh muscles using a 1-ml syringe.

Figure 5 shows the pharmacokinetic profiles following sc delivery of the tetrameric PEG-rMaBChE at 2.5, 3 and 5 mg/kg. Tmax values were all consistently ~48 hrs, regardless of the

dose. However, while Cmax was generally associated with dose, there was a good deal of overlap between the 3 mg/kg and 5mg/kg doses; the larger doses being retained at higher levels in the blood for many days. This once again raises the question as to whether a high dose of very large molecules can leave the site of the sc injection and enter the blood at levels required for protection. By contrast 3 mg/kg delivered iv reaches a peak of >50 U/ml. It is important to note that despite the apparent low bioavailability of sc administered proteins compared to those given intravenously (17-65%), sc delivery often produces equivalent efficacy to iv administration and is assumed to be due to prolonged absorption leading to reduced receptor saturation.

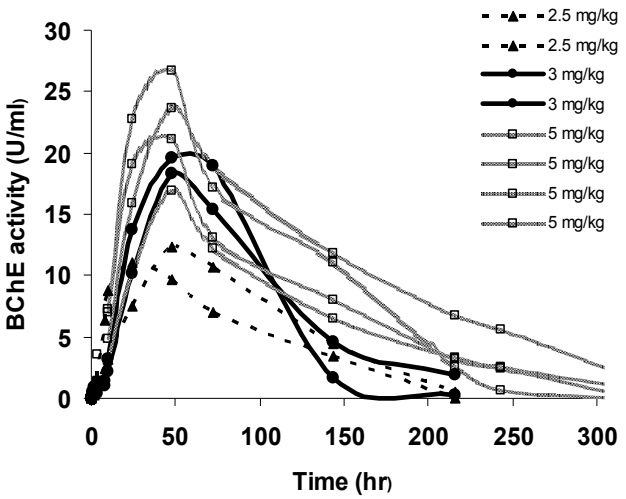

Fig. 5. Pharmacokinetics of PEG-rMaBChE delivered by sc injection. Eight monkeys were injected with the doses indicated in 2-4 ml sc between the shoulder blades.

A direct comparison of the pharmacokinetic parameters following im versus sc injections of 4 monkeys at does of 2.5 mg/kg and 3 mg/kg is shown in Table 3 and indicates that the im and sc values are quite similar. Overall, the results indicate that for a very high MW protein such as PEG-rMaBChE or PEG-rHuBChE, neither im or sc administrarion are optimal to achieve good plasma retention with high PK parameters. For this reason, a different non-parenteral route of delivery via the lung, where the high MW becomes an advantage, is now the choice route of delivery.

Parameters	Subcutaneous injection				Intramuscular injection			
	Four individual monkeys				Four individual monkeys			
MRT (h)	62.23	90.12	110.2	73.4	49.37	60.99	58.6	108.0
T1/2 (h)	25.2	42.3	77.8	37.8	23.3	19.4	24.0	58.7
Cmax (U/ml)	19.6	18.3	12.3	11.0	23.1	20.3	16.5	9.8
AUC (U/ml·h)	1706	1856	1489	1128	1762	1675	1089	1367

Table 3. Comparison of the pharmacokinetics parameters four following sc and im injections performed in parallel.

4.4 Protection studies with PEG-rMaBChE

Many studies have demonstrated efficacy of native HuBChE, both pre-and post–exposure, in rodents and monkeys to protect against OP nerve agents delivered by sc injection, iv injection or vapour. (Lenz et al., 2005; Sun et al., 2008; Saxena et al., 2011; Mumford et al, 2010). Protection has also been shown in mice and guinea pigs using PEG-rBChE produced in goat and plants (Huang et al. 2007, Geyer et al., 2010). However, very few studies have utilized the non-human primate monkey model for assessing insecticide toxicity and none have used respiratory exposure.

Two types of protection studies using different routes of delivery are currently being performed to assess the ability of BChE to protect against toxicity resulting from exposure to the insecticide paraoxon.

1. Aerosolized PEG-rMaBChE 1 hr prior to aerosolized paraoxon exposure.
2. Intravenous delivery of PEG-rMaBChE 1 hr prior to sc delivery of paraoxon.

4.4.1 Paraoxon

The majority of OP insecticides are lipophilic, not ionised, and are absorbed rapidly following inhalation or ingestion (Vale, 1998). Dermal absorption is slower and can be prevented by removing clothes and bathing, but severe poisoning may still ensue if exposure is prolonged. Respiratory pesticide exposure by inhalation of powders, airborne droplets or vapours is particularly hazardous because pesticide particles can quickly enter the bloodstream via the lungs and cause serious damage. Under low pressure, droplet size is too large to remain airborne**.** However, when high pressure, ultra low volume application (ULV) or fogging equipment is used for agricultural purposes, respiratory exposure is increased due to the production of mist- or fog-size particles, which can be carried on air currents for a considerable distance (Armed Forces Pest Management Board Technical Guide No. 13). Small children are highly vulnerable because they breathe in greater volumes of air, relative to their body weight, than adults.

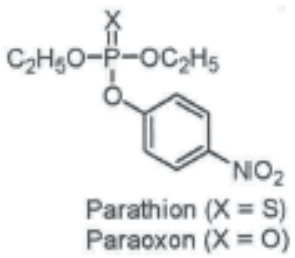

Fig. 6. Chemical structure of parathion and paraoxon.

Paraoxon is the active metabolite of the inactive parathion (Fig. 6) produced by a sulfur-for-oxygen substitution carried out predominantly in the liver by the mixed-function oxidases (Dauterman, 1971). It was chosen for these studies because it inhibits AChE, BChE and carboxylesterase (Levine, 2006), it has a relatively low LD_{50}, and low volatility and stability in aqueous solution. Parathion has probably been responsible for more cases of accidental

poisoning and death than any other OP insecticide (Lotti & Moretto, 2005) and was recently phased out of use in the US. In humans, parathion is absorbed via skin, mucous membranes, and orally and is rapidly metabolized to paraoxon which can result in headaches, convulsions, poor vision, vomiting, abdominal pain, severe diarrhea, unconsciousness, tremor, dyspnea and finally lung-edema as well as respiratory arrest. Symptoms of severe poisoning are known to last for extended periods of time, sometimes months. Additionally, peripheral neuropathy including paralysis is noticed as late sequelae after recovery from acute intoxication (http://extoxnet.orst.edu/pips/parathio.htm). Parathion has been extensively used for committing suicide and potentially for the deliberate killing of people.

4.4.2 Aerosolized PEG-rMaBChE protection against aerosolized paraoxon exposure

As an alternative to delivering high doses of a large molecule into the systemic circulation by sc or im routes, studies are currently being performed using aerosol therapy for delivering rBChE directly to the lung in order to create an effective "pulmonary bioshield" that will detoxify incoming inhaled insecticide in situ and prevent or reduce respiratory toxicity. This takes advantage of the large size of the molecule which will be retained in the lung due to its inability to pass through the lung endothelium into the blood. In this context, inhalation serves as a major means of intoxication because of rapid accesses of the OP to the blood. An efficient pre-exposure pulmonary therapeutic in the form of aerosolized PEG-rBChE could be delivered before a known use/release of insecticides and prevent the lung damage and delayed neuropathy often associated with exposure, while reducing the need for post-exposure atropine and oximes.

Maxwell et al. (2006) have recently shown that for OP compounds (including the insecticides paraoxon, DFP and dichlorvos) the primary mechanism of in vivo toxicity is the inhibition of AChE and the residual unexplained variation in OP toxicity represents <10% of the total variation in toxicity. Almost all of the variation in the LD_{50} of OP compounds in rats was explained by the variation in their in vitro rate constants for inhibition of AChE. Thus, to develop a paraoxon/monkey animal model for aerosolized insecticide exposure and to avoid unnecessary stressing and killing of monkeys in developing the model, the dose of aerosolized paraoxon required to achieve a ~50% inhibition of RBC AChE and serum BChE has been used initially as a readout for toxicity and a basis from which to analyse protection by CHO-derived rMaBChE. Thus, paraoxon which is not neutralized in the lung will enter the blood and can be measured by the inhibition of AChE and BChE activity in lysed whole blood using using a modified assay (Ellman et al, 1961) with 5,5'-dithiobis(2-nitrobenzoic acid), the substrate acetyl-thiocholine (ATC) and 20uM etho-propazine to inhibit BChE activity.

Initially, the dose of aerosolized paraoxon required to produce ~50% inhibition of red blood cell (RBC) AChE and BChE in the circulation was first determined in mice prior to the macaque studies. The LD_{50} of paraoxon in rodents has been established using oral, percutaneous (pc) and subcutaneous (sc) routes (mice: 760 ug/kg orally; 270 - 800 ug/kg sc and for rats: 1800 ug/kg orally and 200 - 430 sc (reviewed in Levine, 2006; Villa et al., 2007). Milatovic et al. (1996) showed that a single acute injection of 0.09, 0.12, or 0.19 mg/kg paraoxon in rats, representing 40% LD_{50}, 52% LD_{50} and 83% LD_{50} respectively, did not produce signs of cholinergic hyperactively. In the present study, the effective dose of aerosolized paraoxon resulting in 50% inhibition in mice was found to be 150-180 ug/kg which is less toxic than the parenteral route. In addition, aerosolized BChE given 24 hr prior

to the paraoxon significantly reduced the AChE inhibition (our unpub. data). Rodents contain a high endogenous levels of CaE, another stoichiometric OP scavenger (Dirnhuber et al. 1979) and are known to be ~10-fold less sensitive to soman than non-human primates (Maxwell et al., 2006). Accordingly, a dose of 15 ug/kg of aerosolized paraoxon has been shown to result in 50-60% RBC AChE inhibition and preliminary data indicate that PEG-rMaBChE , delivered as a pre-exposure aerosol one hour prior to exposure, can totally reduce this inhibition in a dose –dependent manner.

4.4.3 Intravenous PEG-rMaBChE protection against subcutaneous paraoxon exposure

These studies are being formed to compare routes of delivery with efficacy of protection and indicate that while paraoxon delivered sc is also more toxic than as an aerosol, complete protection can be achieved by PEG-rMaBChE pretreatment.

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6

Adverse Effect of Insecticides on Various Aspects of Fish's Biology and Physiology

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1. Introduction

Today, water quality management faces greater problems than at any time in its history. In addition to natural pollutants, varied contaminants exist in surface waters including multiple chemical compounds and different products of industrial and agricultural revolution. The insecticides constitute one group of these pollutants, both synthetic and natural, which contribute to the environmental problems. At present, it seems that the problem is more conspicuous in developing countries, where lately there has been an increase in the use of insecticides as a means of increasing agricultural productivity, without much concern to the consequences of indiscriminate application. There are many pathways by which insecticides leave their sites of application and distribute throughout the environment and enter the aquatic ecosystem. The major route of insecticides to water ecosystems in urban areas is through rainfall runoff and atmospheric deposition. Another source of water contamination by insecticides is from municipal and industrial dischargers. Most insecticides ultimately find their way into rivers, lakes and ponds (Tarahi Tabrizi, 2001; Honarpajouh, 2003; Bagheri, 2007; Shayeghi *et al*., 2007; Vryzas *et al*., 2009; Werimo *et al*., 2009; Arjmandi *et al*., 2010) and have been found to be highly toxic to non-target organisms that inhabit natural environments close to agricultural fields. The contamination of surface waters by insecticides is known to have ill effects on the growth, survival and reproduction of aquatic animals. In the past few years, the increase of mortality among the fish in various streams, lakes and ponds of around the world has drawn scholars' attention to the problems caused by insecticides and pesticides runoff associated with intense agricultural practices. Different concentrations of insecticides are present in many types of wastewater and numerous studies have found them to be toxic to aquatic organisms especially fish species (Talebi, 1998; Üner *et al*., 2006; Banaee *et al*., 2008). Fishes are particularly sensitive to the environmental contamination of water. Hence, pollutants such as insecticides may significantly damage certain physiological and biochemical processes when they enter into the organs of fishes (John, 2007; Banaee *et al*., 2011). Authors found out that different kinds of insecticides can cause serious impairment to physiological and health status of fishes (Begum, 2004; Monteiro *et al*., 2006; Siang *et al*., 2007; Banaee *et al*., 2009). Since fishes are important sources of proteins and lipids for humans and domestic animals, so health of fishes is very important for human beings. Recently, many studies have been conducted to determine the mechanisms of insecticides in fishes, with the ultimate goal of

monitoring, controlling and possibly intervening in xenobiotics exposure and its effects on the aquatic ecosystem. This chapter presents further information concerning the toxic effects of different concentrations of insecticides on various aspects of fish's biology and physiology. In other words, this chapter depicts the effects of insecticides on the survival chance, blood biochemical parameters, tissues and organs, reproduction, development and growth, nervous system, behavior, genetic and immune system of fish. The information given in this part facilitates the evaluation of potential toxic hazard resulting from exposure to different levels of these compounds.

2. Biokinetics and bioteransformation

After exposure to difference concentrations of insecticides in water, the fish absorbs them in its gills, skin or gastrointestinal tract. In the other words, Due to their lipophilicity, most insecticides easily permeate the biological membranes and it increases the sensitivity of fish to aqueous insecticides. Then, these compounds are rapidly metabolized and extracted, and may be bio-concentrated in various tissues of fish. In other words, bio-accumulation occurs if the insecticides is slowly metabolized or excreted from the body. As the amount of insecticide increases, it becomes more harmful to the consumer or animal. Accumulated insecticide can cause death or long-term damage. Ballesteros *et al.* (2011) showed that during the initial 24 h of exposure, insecticides may be transformed in various tissues of fish. However, some differences exist among tissues relating to metabolism rates, leading to different distribution models of the original compounds and their metabolites. For example, the low biodegradation and the high lipid solubility of some insecticides such as organochlorine insecticides have led to problems with the bio-concentrations of these compounds in different tissues of fish. In addition, since some fish are lower on the food chain, bioaccumulation of insecticides may increase in tissues of their predators and consumers, such as humans and thus affecting their health and survival. So, the bioaccumulation of these contaminants in fish and the potential biomagnification in humans are perceived as threats (Favari *et al*., 2002). Bioaccumulation rate of insecticides in fish depends on the species, life stages, the amount of fat reservation in different tissues and diet of fish, chemical and physical properties of insecticides and the rate of water pollution.

In order to facilitate the elimination and detoxification of toxic compounds, fishes have developed partly complex detoxification mechanisms including the release of several enzymes collectively termed xenobiotic metabolizing enzymes. Enzymatic biotransformation of insecticides can potentially alter their activity and toxicity. Enzymes participating in the biotransformation of insecticides are classified into phase I and phase II enzymes. The phase I enzymes, cytochrome P450 enzymes including CYP1A and CYP3A, are generally involved in the biotransformation of exogenous and endogenous compounds; thereby creating a more polar and water soluble compound. A great diversity of cytochrome P450 enzymes in fish has been recognized (Stegeman and Hahn, 1994), and CYP1A, CYP2B, CYP2E1, CYP2K1 and CYP3A have been recently identified in liver of some freshwater fish (Nabb *et al*., 2006) which play an important role in the detoxification of organophosphate and carbamate insecticides (Ferrari *et al*., 2007). The common pathways of biotransformation of different kinds of insecticides include three cytochrome P450 (CYP) mediated reactions: *O*-dealkylation, hydroxylation, and epoxidation of insecticides (Soldano *et al*., 1992; Keizer *et al*., 1995; Kitamura *et al*., 2000; Straus *et al*., 2000; Behrens & Segner, 2001; Nebbia, 2001). In phase II reactions, metabolites produced in phase I detoxification often conjugate with

glutathione, uridyl-diphosphate glucose (UDPG), uridyl-diphosphate-glucuronic acid (UDPGA), amino acid derivatives and sulfate derivatives and can readily excrete from the fish body (Iannelli *et al*., 1994; Keizer *et al*., 1995; Kitamura *et al*., 2000; Straus *et al*., 2000; Behrens & Segner, 2001; Nebbia, 2001). In other words, final metabolites may also be excreted from the body of fish through the skin, gills, genital products, urine as sulphated and glucuronidated metabolites and stool as glutathione conjugated metabolites (Kitamura *et al*., 2000; Straus *et al*., 2000; Behrens & Segner, 2001; McKim & Lein, 2001; Nebbia, 2001).
Since metabolites produced during detoxification process may be more dangerous than parental compounds, these metabolites can cause serious damage in fish. Furthermore, the production of reactive oxygen species (ROS) during detoxification process can induce oxidative damage and may be a mechanism of toxicity for aquatic organisms living in environments receiving insecticides (Monteiro *et al*., 2009). ROS can indiscriminately attack and react with susceptible vital macromolecules -lipids, proteins and DNA- in living cells, inducing cytotoxicity and can result in serious disturbances in physiological cell processes (Dogan *et al*., 2011; Jin *et al*., 2011). Lipid peroxidation, the major contributor to the loss of cell function, DNA damage, enzyme inactivation, and hormone oxidation are bio-indicators of oxidative cell damage and examples of toxic mechanisms of insecticide induced ROS being involved in pathological processes and in the etiology of many fish diseases (Üner *et al*., 2006; Dogan *et al*., 2011).

3. Acute toxicity

In acute toxicity, sudden and intense mortality may be observed in a fish population exposed to toxic materials. The most apparent symptoms of insecticides' acute poisoning in fishes include lethargy, forward extension fins, pallor or blur parts of body, severe reaction to external stimuli and muscle spasms and sudden fast swimming in circles. The main clinical internal sings that can lead to death of fishes include neurological disorder and disruption of nerve functions, respiratory dysfunction and suffocation (Banaee *et al*., 2011).
Acute toxicity testing is widely used in order to identify the dose or exposure concentration and the time associated with death of 50 percent of the fish exposed to toxic materials which is expressed as LC_{50} in parts per million (ppm) or milligrams per liter (mg/L). In addition, we can use the LC_{50} value in the classification of insecticides based on potential toxicity for fishes. Furthermore, the relative acute toxicity of chemicals to fish can be categorized as follows:

Toxicity rating	**96 hr LC_{50}**
Slightly toxic	10-100 ppm
Moderately toxic	1-10 ppm
Highly toxic	0.1-1.0 ppm
Extremely toxic	Less than 0.1 ppm

Our literature reviews demonstrate that different fish species, even from the same family, show differences in the sensitivity to high concentrations of insecticides in water. Acute toxicity of different insecticides is influenced by the age, sex, genetic properties and body size of fish, water quality and its physicochemical parameters, and purity and formulation of insecticides.
The eight tables, which give relative acute toxicity of some insecticides to fishes, can be used to determine the potential toxicity to fish of using these compounds in farms around surface waters and to select products which less likely to cause problems. The data are derived from

laboratory studies and are given only as a guideline and not absolute data of the acute toxicity of the insecticides to different species of fish (Table 1-11).

Insecticide	species	Range of 96h LC_{50}	Reference
Akton	Channel catfish, Bluegill, Rainbow trout, Fathead minnow	0.17-1370 ppb	Jonson & Finley, 1980
Aldicarb	Fathead minnow	1.3-2.4 ppm	Pant *et al*., 1987
Aldrin	Chinook salmon, Rainbow trout, Fathead minnow, Black bullhead, Bluegill, Largemouth bass	2.3-53 ppb	Jonson & Finley, 1980
Allethrin	Rainbow trout, Bluegill	19-56 ppb	Jonson & Finley, 1980
Aminocrab	Cutthroat trout, Rainbow trout, Bluegill, Atlantic salmon, Fathead minnow, Channel catfish, Largemouth bass, Yellow perch, Brook trout	3.1-31 ppm	Jonson & Finley, 1980
Azinphos ethyl	Rainbow trout, Bluegill	1.1-20 ppb	Jonson & Finley, 1980
Azinphos methyl	Gilthead seabream, Coho salmon, Rainbow trout, Bluegill, Atlantic salmon, Brown trout, pike, Goldfish, Carp, Fathead minnow, Black bullhead, Channel catfish, Green sunfish, Largemouth bass, Black crappie, Yellow perch	2.1-4270 ppb	Arufe *et al*., 2007; Jonson & Finley, 1980
Azodrin	Rainbow trout, Bluegill, Channel catfish. Fathead minnow	4.9-50 ppm	Jonson & Finley, 1980

Table 1. Summary of acute toxicity.

Insecticide	species	Range of 96h LC_{50}	Reference
Bensulide	Rainbow trout, Bluegill, Black bullhead, Common eel, Guppy, Sheephead minnow, Crucian carp	0.1-30 ppm	McAllister *et al*., 1986a, b;
Benzene hexachloride	Rainbow trout, Bluegill, Cutthroat trout, Goldfish, fathead minnow, Channel catfish, Largemouth bass	9-348 ppb	Jonson & Finley, 1980
Carbaryl	Coho salmon, Chinook salmon, Cutthroat trout, Atlantic salmon, Brown trout, Brook trout, Lake trout, Carp, Channel Catfish, Fathead Minnow, Rainbow Trout, Bluegill, Goldfish, Black bullhead, Green sunfish, Largemouth bass, Black crappie, Yellow perch	0.9-39 ppm	Jonson & Finley, 1980
Carbofuran	Walked catfish, *Poecilia reticulate*, Chubs	0.22-23 ppm	Dobšíková, 2003; Begum, 2004
Carbophenthion	Channel catfish, Bluegill, Green sunfish	0.02-6 ppm	Jonson & Finley, 1980
Carbosulfan	Bluegill, Cutthroat trout, Rainbow trout, Lake trout, Channel catfish	2.4-280 ppb	Yi *et al*., 2006; Jonson & Finley, 1980

Table 2. Summary of acute toxicity.

Insecticide	species	Range of 96h LC_{50}	Reference
Chlordane	Coho salmon, Cutthroat trout, Rainbow trout, Brown trout, Fathead minnow, Channel catfish, Bluegill, Largemouth bass	3-115 ppb	Jonson & Finley, 1980
Chlorethoxyphos	Cutthroat trout, Rainbow trout, Fathead minnow, Channel catfish, Bluegill, Largemouth bass	0.72-11.9 ppm	Jonson & Finley, 1980
Chlorfenapyr	Bluegill, Rainbow trout, channel catfish	6.5-14.7 ppb	Rand, 2004
Chlorpyrifos	Mosquito fish, Bluegill, Fathead minnow, Rainbow trout, Nile tilapia, Goldfish	0.57-3270 ppb	Davey *et al*., 1976; Holcombe *et al*., 1982; Bowman, 1988a, b; Gül, 2005; Wang *et al*., 2009
Coumaphos	Cutthroat trout, Rainbow trout, Lake trout, Channel catfish, Bluegill, Largemouth bass	0.34-1.2 ppm	Jonson & Finley, 1980
Cryolite	Rainbow trout, Bluegill	47-400 ppm	Jonson & Finley, 1980

Cypermethrin	Sheepshead minnow, Rainbow trout, Bluegill, Freshwater catfish	0.39-0.95 ppb	Jaber & Hawk, 1981; Sousa, 1998; Mishra et al., 2005
DDD	Rainbow trout, Fathead minnow, Channel catfish, Largemouth bass, Walleye	14-4400 ppb	Jonson & Finley, 1980
DDE	Rainbow trout, Atlantic salmon, Bluegill	32-240 ppb	Jonson & Finley, 1980

Table 3. Summary of acute toxicity.

Insecticide	**species**	**Range of 96h LC_{50}**	**Reference**
DDT	Coho samon, Rainbow trout, Fathead minnow, Channel catfish, Bluegill, Largemouth bass, Black bullhead, Yellow perch	1.5-21.5 ppb	Jonson & Finley, 1980
Deltamethrin	Guppies, Channa punctatus,	1.5-5.13 ppb	Viran et al., 2003; Sayeed et al., 2003
Diazinon	Cutthroat trout, Rainbow trout, Lake trout, Fathead minnow, Carp, Bluegill	0.9-2.6 ppm	Calmbacher, 1978a, b; Banaee *et al*., 2011; Banaee *et al*., 2008; Jonson & Finley, 1980
Dichlorvos	Lake Trout, Sheephead minnow	0.18-7.5 ppm	Mayer & Ellersieck 1986; Jones & Davis, 1994
Dicrotophos	Bluegill, Rainbow trout, Channel catfish	6.3-24.2 ppm	Jonson & Finley, 1980
Dieldrin	Cutthroat trout, Rainbow trout, Goldfish, Fathead minnow, Channel catfish, Bluegill, Largemouth bass	1.2-19 ppb	Jonson & Finley, 1980
Diflubenzuron	Cutthroat trout, Rainbow trout, Brook trout, Fathead minnow, Channel catfish, Bluegill, Yellow perch	25-240 ppm	Jonson & Finley, 1980
Dimethoate	Rainbow trout, Bluegill	6-9.3 ppm	Jonson & Finley, 1980
Dimethrin	Fathead minnow, Channel catfish, Yellow perch, Bluegill,	28-1275 ppb	Jonson & Finley, 1980
Dinitrocresol	Rainbow trout, Bluegill	66-360 ppb	Jonson & Finley, 1980

Table 4. Summary of acute toxicity.

Insecticide	species	Range of 96h LC_{50}	Reference
Dioxathion	Cutthroat trout, Rainbow trout, Largemouth bass	22-110 ppb	Jonson & Finley, 1980
Disulfoton	Rainbow trout, Fatheah minnow, Channel catfish, Bluegill, Largemouth bass	60-4700 ppb	Jonson & Finley, 1980
d-Trans Allethrin	Coho salmon, Steelhead, Lake trout, pike, Fathead minnow, Channal catfish, Largemouth bass, Yellow perch	2.6-66 ppb	Jonson & Finley, 1980
Endosulfan	Striped bass, Bluegill, Rainbow trout, Fathead minnows, Asian swamp eel, Milk fish, Zebra fish	0.1-20 ppb	Mayer & Ellersieck, 1986; Siang *et al*., 2007; Capkin *et al*., 2006; Magesh & Kumaraguru, 2006, Velasco-Santamaría *et al*., 2011
Endrin	Rainbow trout, Goldfish, fathead minnow, black bullhead, Channel catfish, Mosquito fish, Bluegill, Largemouth bass, Yellow perch, carp	0.15-1.8 ppb	Jonson & Finley, 1980
EPN	Cutthroat trout, Rainbow trout, Channel catfish, Bluegill, Largemouth bass, Walleye	110-420 ppb	Jonson & Finley, 1980
Ethion	Cutthroat trout, Rainbow trout, Channel catfish, Bluegill, Largemouth bass, Fathead minnow	0.17-7.6 ppm	Jonson & Finley, 1980

Table 5. Summary of acute toxicity.

Insecticide	species	Range of 96h LC_{50}	Reference
Ethyl Parathion	Coho salmon, Cutthroat trout, Rainbow trout, Brown trout, Goldfish, Carp, Fathead minnow, Channel catfish, Bluegill, Black bullhead, Largemouth bass, Yellow perch	0.4-3.52 ppm	Jonson & Finley, 1980
Fenitrothion	Coho salmon, Cutthroat trout, Rainbow trout, Brown trout, Brook trout, Atlantic salmon, Goldfish, Bluegill, Channel catfish, Fathead minnow, Carp	1.7-12 ppm	Johnson & Finley, 1980; Woodward & Mauck, 1980; Jonson & Finley, 1980
Fenthion	Coho salmon, , Rainbow trout, Brown trout, Brook trout, Atlantic salmon, Goldfish, Yellow perch, Bluegill, Channel catfish, Green sunfish, Fathead minnow, Largemouth bass, Carp	1.1-3.4 ppm	Jonson & Finley, 1980

Fenvalerate	Zebra fish	3.5-193 ppb	Ma *et al*., 2009
Heptachlor	Rainbow trout, Northern pike, Fathead minnow, Black bullhead, Channel catfish, Redear sunfish, Bluegill, Largemouth bass,	5.3-63 ppb	Jonson & Finley, 1980
Isoprocarb	Goldfish	4.61 ppm	Wang *et al*., 2009

Table 6. Summary of acute toxicity.

Insecticide	**species**	**Range of 96h LC_{50}**	**Reference**
Kepone	Rainbow trout, Channel catfish, Bluegill, redear sunfish	30-225 ppb	Jonson & Finley, 1980
Leptophos	Rainbow trout, Lake trout, Fathead minnow, Bulegill	0.03-30 ppm	Jonson & Finley, 1980
Lindane	Eel, Tilapia, African Catfish, Coho salmon, Rainbow trout, Brown trout, Goldfish, Carp, Fathead minnow, Black bullhead, Green sunfish, Largemouth bass, Yellow perch	0.03-1.29 ppm	Ferrando *et al*., 1988, Feltz, 1971; Lawson *et al*., 2011; Jonson & Finley, 1980
Linuron	Bluegill, Rainbow trout	3-16.2 ppm	Wetzel, 1986; Mayer & Ellersieck,1986
Malathion	Coho salmon, Cutthroat trout, Rainbow trout, Brown trout, Lake trout, Goldfish, Carp, Fathead minnow, Black bullhead, Bluegill, Green sunfish, Largemouth bass, Yellow perch, Redear sunfish	4-12900 ppb	Mayer & Ellersieck,1986; Jonson & Finley, 1980
Methamidophos	Rainbow trout, Fathead minnow, Channel catfish, Bluegill	1.6-100 ppm	Jonson & Finley, 1980
Methomyl	Cutthroat trout, Rainbow trout, Atlantic salmon, Brook trout, Fathead minnow, Channel catfish, Bluegill, Largemouth bass	0.3-6.8 ppm	Jonson & Finley, 1980; Yi et al., 2006

Table 7. Summary of acute toxicity.

Insecticide	species	Range of 96h LC_{50}	Reference
Methoxychlor	Rainbow trout, Atlantic salmon, Cutthroat trout, Brook trout, Lake trout, pike, Goldfish, Largemouth bass, Bluegill, Yellow perch, Fathead minnow, Channel catfish	15-64 ppb	Jonson & Finley, 1980
Methyl Parathion	Freshwater characid fish, Coho salmon, Cutthroat trout, Rainbow trout, Brown trout, Lake trout, Goldfish, Carp, Fathead minnow, Channel catfish, Bluegill, Black bullhead, Green sunfish, Largemouth bass, Yellow perch	0.25-9 ppm	Mayer & Ellersieck,1986; Monteiro et al., 2006; Jonson & Finley, 1980
Methyl Trithion	Cutthroat trout, Rainbow trout, Channel catfish, Bluegill, Largemouth bass	0.76-2.8 ppm	Jonson & Finley, 1980
Mexacarbate	Coho salmon, Cutthroat trout, Rainbow trout, Atlantic salmon, Lake trout, Carp, Fathead minnow, Channel catfish, Bluegill, Black bullhead, Largemouth bass, Yellow perch	0.32-23 ppm	Jonson & Finley, 1980
Mirex	Rainbow trout, Brown trout, Fathead minnow, Channel catfish, Bluegill, Largemouth bass, Yellow perch, Walleye	100 < ppm	Jonson & Finley, 1980

Table 8. Summary of acute toxicity.

Insecticide	species	Range of 96h LC_{50}	Reference
Monocrotophos	Tilapia, Mosquito fish	11.5-20.5 ppm	Rao, 2006; Kavitha & Rao, 2007
Naled	Cutthroat trout, Rainbow trout, Lake trout, Fathead minnow, Channel catfish, Bluegill, Largemouth bass	0.13-3.3 ppm	Jonson & Finley, 1980
Oxydemeton-methyl	Rainbow trout, Channel catfish, Bulegill, Largemouth bass, Walleye	13-31.5 ppm	Jonson & Finley, 1980
Permethrin	Brook trout	1.4-7.9 ppb	Jonson & Finley, 1980
Phorate	Cutthroat trout, Rainbow trout, Northern pike, Largemouth bass, Channel catfish, Bluegill	2-110 ppb	Jonson & Finley, 1980

Phosmet	Coho salmon, Rainbow trout, Fathead minnow, Channel catfish, bluegill, Smallmouth bass, Largemouth bass	0.15-10.6 ppm	Jonson & Finley, 1980
Phosphamidon	Rainbow trout, Fathead minnow, Channel catfish, Bluegill	3.4-100 ppm	Jonson & Finley, 1980
Phoxim	Coho salmon, Atlantic salmon, Rainbow trout, Brown trout, Brook trout, Northern pike, Fathead minnow, Channel catfish, bluegill	0.11-2.9 ppm	Jonson & Finley, 1980
Propoxur	Goldfish, Rainbow trout, Fathead minnow, Bluegill	4.8-36.2 ppm	Wang *et al*., 2009; Jonson & Finley, 1980

Table 9. Summary of acute toxicity.

Insecticide	**species**	**Range of 96h LC_{50}**	**Reference**
Pyrethrum	Coho salmon, Atlantic salmon, Brown trout, Lake trout, Channel catfish, bluegill	13-65 ppb	Jonson & Finley, 1980
Resmethrin	Cho salmon, Lake trout, Fathead minnow, Channel catfish, bluegill	1.7-9.9 ppb	Jonson & Finley, 1980
Ronnel	Rainbow trout, Channel catfish, bluegill, Cutthroat trout, Lake trout	0.6-1.6 ppm	Jonson & Finley, 1980
Rotenone	Rainbow trout, Channel catfish, bluegill	2.6-36 ppb	Jonson & Finley, 1980
RU-1169	Coho salmon, Atlantic salmon, Lake trout, fathead minnow, White sucker, Bluegill	0.3-28 ppb	Jonson & Finley, 1980
S-Bioallethrin	Fathead minnow, Channel catfish, Bluegill, Yellow perch	7.8-90 ppb	Jonson & Finley, 1980
SD-17250	Coho salmon, Rainbow trout, bluegill	1.5-5.7 ppm	Jonson & Finley, 1980
Strobane	Bluegill, Rainbow trout	8.7-12 ppb	Jonson & Finley, 1980
TEPP	Rainbow trout, Fathead minnow, Bluegill	240-980 ppb	Jonson & Finley, 1980

Table 10. Summary of acute toxicity.

Insecticide	species	Range of 96h LC50	Reference
Temephos	Cutthroat trout, Rainbow trout, Atlantic trout, Brook trout, Lake trout, Fathead minnow, Channel catfish, Bluegill, Largemouth bass	1.44-34 ppm	Jonson & Finley, 1980
Thanite	Rainbow trout, Channel catfish, Bluegill	1.2-3.7 ppm	Jonson & Finley, 1980
Thiodicarb	Bluegill, Rainbow trout	1.4-3.3 ppm	Yi *et al*., 2006
Toxaphene	Coho salmon, Rainbow trout, Brown trout, Goldfish, Carp, Fathead minnow, Black bullhead, Channel catfish, Bluegill, Largemouth bass, Yellow perch	2-18 ppb	Jonson & Finley, 1980
Trichlorfon	Eel, Rainbow trout, Cutthroat trout, Atlantic salmon, Brown trout, Brook trout, Lake trout, Fathead minnow, Channel catfish, Bluegill, Largemouth bass	0.36-9.2 ppm	Lopes *et al*., 2006; Jonson & Finley, 1980

Table 11. Summary of acute toxicity.

4. Sub-lethal toxicity

Sub-lethal toxicity testing was planned based on one tenth or more of LC_{50} dose in moderate periods. In sub-lethal toxicity, the organs or biological systems which may be affected at such exposure can be respiratory, hepatic, haematopoietic, nervous, cardiovascular, and reproductive and immune systems. Different biomarkers of fish exposed to insecticides are usually evaluated in these experiments. Insecticides may lead to changes in the blood biochemical parameters and haematological profile of fish which can be investigated as biomarker in pollution monitoring (Mushigeri & David, 2005; Banaee *et al*., 2008; Kavitha & Rao, 2009). In fact, these compounds may induce alterations in the activities or levels of a number of different enzyme systems, including those necessary for biochemical reactions in cells (Banaee *et al*., 2011). Decreased rate of growth, reproductive disorder, spinal deformities, histopathological changes (Benli & Özkul, 2010) in gills, liver, haematopoietic tissue such as spleen, head of kidney, and renal tubules, endocrine tissues as well as brain, neurological and behavioral disorder and genetic defects are other biological indicators of exposure to insecticides which are described in details in the following sections.

5. Chronic toxicity

Chronic toxicity tests commonly include the measurement of long term effects of low concentrations of insecticides on the survival, growth, reproduction, nervous system and other biological and physiological aspects of fishes. Type of injury to fish in chronic toxicity is similar to sub-lethal toxicity damage, but the frequency and intensity injury and lesion resulting from chronic toxicity may be more or even less than damage of sub-lethal toxicity. Therefore, this experiment is important in insecticides toxicology.

6. Side effect of insecticides on various aspects of fish's biology and physiology

6.1 Alterations in blood biochemical parameters

Insecticides can cause serious impairment to physiological and health status of fish. Therefore, biochemical tests are routine laboratory tests useful in recognizing acute or chronic toxicity of insecticides (Banaee *et al*., 2008; Al-Kahtani, 2011) and can be a practical tool to diagnose toxicity effects in target organs and to determine the physiological status in fish. Blood biochemistry test gives indicates what is happening in the body of fish exposed to insecticides. When different tissues are injured, the damaged cells release specific enzymes into plasma and we can recognize their abnormality levels in blood. Then it helps locate the lesions. Also, if certain organs are not eliminating certain waste products or not synthesizing certain important materials, this can tell us they are not functioning properly. In some cases due to the severity of the damage to tissues, particularly liver, synthesis of many biochemical parameters may reduce significantly in cells, which can decrease some biochemical factors in blood of fish exposed to insecticides. These changes were observed in *Channa punctatus* (Agrahari *et al*., 2007), *Oreochromis niloticus* (Velisek *et al*., 2004), *O.mossambicus* (Arockia and Mitton, 2006; Matos *et al*., 2007), *Heteropneustes fossilis* (Saha & Kaviraj, 2009), *Cirrhinus mrigala* (Prashanth & Neelagund, 2008) *Clarias batrachus* (Begum, 2005; Ptnaik, 2010), *Cyprinus carpio* (Banaee *et al*., 2008), *Oncorhynchus mykiss* (Banaee *et al*., 2011), *Colisa fasciatus* (Singh *et al*., 2004) which were exposed to monocrotophos, bifenthrin, carbaryl, dimethoate, cypermethrin, sevin, diazinon, and malathion respectively.

6.2 Tissue and organ damage

Histopathological investigations on different tissues of fish are valuable tools for toxicology studies and monitoring water pollutions. In histopathology, we can provide information about the health and functionality of organs. Tissues injuries and damages in organs can result in the reduced survival, growth and fitness, the low reproductive success or increase of susceptibility to pathological agents. Frequency and intensity of tissue lesions depend on the concentrations of insecticides and the length of the period fish are exposed to toxins. Nevertheless, many insecticides cause specific or non-specific histopathological damage (Fanta *et al*., 2003). For example, histopathological lesions in the liver tissue of freshwater fish (*Cirrhinus mrigala*) (Velmurugan *et al*., 2009) and common carp carp (*Cyprinus carpio*) (Banaee *et al*., In press) were observed after 10 and 30 days exposure to sublethal concentrations of dichlorvos and diazinon insecticides, respectively. Other researchers reported the same histopathological alterations in different tissues of fish treated with diazinon (Duttaa & Meijer, 2003; Banaee *et al*., 2011), deltamethrin (Cengiz, 2006; Cengiz & Unlu, 2006), fenitrothion (Benli & Özkul, 2010).

6.3 Reproductive dysfunction

Reproduction guarantees the survival of fish population. Any changes in environmental parameters or physiological conditions of fish can affect its reproductive success. Since fish may be exposed to environmental pollutants, including insecticides, herbicides, heavy metals and other xenobiotics, disorders may occur in their natural reproductive process. Recent researches showed the dysfunction in the reproductive systems of fishes exposed to insecticides. Insecticides' effects on reproductive biology of fishes are numerous, and include decreased fecundity, testicular and ovarian histological damage (Duttaa & Meijer,

2003; Banaee *et al*., 2009), vitellogenesis process impairment (Haider and Upadhyaya, 1985;), and disruption in steroidogenesis process (Zaheer Khan & Law, 2005), delay in gonads maturation (Skandhan *et al*., 2008), alter in reproductive and parental behavior (Jaensson *et al*., 2007), impairment in olfactory response and disorder in reproductive migrations (Scholz *et al*., 2000), as well as disruption in coordinating courtship behavior of male and female fish and time of spawning (Jaensson *et al*., 2007).

Some insecticides are known as endocrine disrupting chemicals (EDC) which can interfere with the normal functioning of endocrine system in fish. Adverse effects of insecticides on the hypothalamus-pituitary-gonads axis can also play a significant role in causing reproductive failures in fish. In fishes, chronic toxicity of insecticides can change sex steroid hormone levels in plasma. While the mechanism is not exactly known, it is possible that insecticides and their metabolites disrupt reproductive systems through activation or inhibition of key enzymes which participated in the steroid hormone biosynthesis in fishes. For example, DDT, endosulfan, methoxychlor and some other insecticides possess estrogenic properties and are probably capable of disrupting functions of endocrine system and causing disorder in the reproductive system of fish (Arukwe, 2001). These compounds may directly or indirectly interact with natural hormones, changing the hormone functions and thus altering physiological cellular response or mutate the natural patterns of hormone synthesis and metabolism. Impact of organophosphate insecticides such as malathion, diazinon and fenitrothion on the hypothalamus-pituitary-gonads axis and also disturbance in hormones associated with reproductive systems were studied by Kapur & Toor, (1978); Singh and Singh, (1987); Maxwell & Dutta, (2005); Skandhan *et al*. (2008).

Insecticides can also cause adverse effects on gonad histology, morphology and its growth. In addition, there are significant relationships between blood sex steroid hormone concentrations, sperm or oocytes quality, rate of fecundity and histopathological alterations in ovary and testis of fish exposed to different insecticides (Duttaa & Meijer, 2003; Maxwell & Dutta, 2005). Banaee *et al*., (2008) reported that diazinon inhibits steroidogenesis in testis of male carp by histopatological alterations. Research results showed that direct toxic effects of insecticides on seminiferous tubules or Leydig cells may be the most important parameter for the low quality of sperms in fish (Fadakar Masouleh *et al*., 2011). Similar results were reported in walking catfish (*Clarias batrachus*), freshwater eel (*Monopterus albus*), and Atlantic salmon (*Salmo salar*) that were exposed to different insecticides (Singh & Singh, 1987; Singh, 1989; Moore & Waring, 1996; 2001).

Exposure of fish eggs and milt to insecticides also reduced the level of fertilization, hatching rate and the larval survivability. Further studies on bluegills (*Lepomis macrochirus*), atlantic salmon (*Salmo salar* L.) demonstrated that the gametes and fertilized eggs were sensitive to the insecticides (Tanner & Knuth, 1996; Moore & Waring, 2001) suggesting a further toxic impact of these toxicants on the fish reproduction. In addition, the waste of energy in the fish exposed to insecticides reduces their reproductive ability.

6.4 Development disorders

Study of development disorders caused by insecticides is to emphasize the links between the concentrations of toxins and dysfunction in normal development from embryonic to puberty periods. So, impairment in the normal development and the growth may reduce the fish's survival chance.

Embryos and larvae may be directly exposed to insecticides, through the yolk or via parental transfer in viviparous fish (Viant *et al*., 2006). Spinal deformities, mostly scoliosis and lordosis, and morphological abnormalities were among the more adverse effects registered for insecticides toxicity. Other alterations in the embryo of fish exposed to insecticides also consist of yolk sac edema, pericardial edema and crooked body of larvae (Xu *et al*., 2010). Teratogenic effects of carbaryl insecticides on the embryo of fish have been proved (Todd and Leeuwen, 2002). Similar results were reported in silversides *Menidia beryllina* exposed to tebufos during embryogenesis (Middaugh *et al*., 1990; Hemmer *et al*., 1990).

The most important factors decreasing fish growth consist of disorder in feeding behaviors, decrease in feeding rate, dysfunction in metabolism process and waste of energy to overcome the stress caused by insecticide exposure (Tripathi *et al*., 2003). For example, disorder in the metabolism of carbohydrates, proteins and lipids in various tissues, particularly liver of fish exposed to insecticides, may reduce their growth rates. Begum (2004) found out that protein and carbohydrate metabolism in the liver and muscle tissue is disrupted on the exposure to a carbofuran insecticide. In addition, exposure during embryonic or larval stage can result in behavioral abnormalities, such as decreased ability to capture prey after hatching, functional deficiencies or slowing of growth and finally death (Kuster, 2005; Viant *et al*., 2006; Arufe *et al*., 2007). These changes were observed in larvae and embryo of zebra fish (*Danio rerio*) in contact with endosulfan (Velasco-Santamaria *et al*., 2011), beta-cyprmethrin (Xu *et al*., 2010); paraoxon-methyl (Küster, 2005) and sevin (carbaryl insecticide) (Todd and Leeuwen, 2002).

6.5 Neurotoxicity

The primary mechanism of organophosphate and carbamate insecticides toxicity is well known – they function as inhibitors of acetycholinestrase enzyme (AChE) or and butyrylcholinesterase (BChE), as well as disturbing the metabolism of other neurotransmitters such as γ-aminobutyrate (GABA). The synthetic pyrethroids change normal neuronal function by interfering in the function of ion channels in the nerve cell membrane, alterations in intracellular calcium ion concentrations and possibly by blocking GABA receptors. Organochlorine insecticides act primarily by changing the transport of ions across the nerve cell membranes, thus altering the ability of nerve to stimulate.

Fish exposure to these insecticides is frequently assessed by determining the alterations in AChE in brain, muscle, plasma and other tissues or probably GABA activity in brain (Banaee, 2010). AChE is an enzyme responsible for inactivating the neurotransmitter acetylcholine (Fulton & Key, 2001). AChE inactivation results in the accumulation of the neurotransmitter acetylcholine in cholinergic synapses space, leading to synaptic blockage and disruption of signal transmission (Ferrari *et al*., 2004; 2007a, b). Inhibition of AChE induces alteration in the swimming behavior, shaking palsy, spasms and other undesirable effects (Sharbide *et al*., 2011). Disturbances in AChE activity can also impair feeding, identification and avoidance and escaping from predators, spatial orientation of the species, and reproductive behavior (Bretaud *et al*., 2000). Thus, AChE inhibition is considered to be a specific biomarker of exposure to organophosphorus and carbamate insecticides like diazinon, chlorpyrifos, propoxur, isoprocarb, (Üner *et al*., 2006; Cong *et al*., 2008; 2009; Wang

et al., 2009; Banaee *et al*., 2011;). Similar results have been observed for pyrethroids insecticide toxicity (Koprucu *et al*., 2006). Disorder in γ-aminobutyrate (GABA) system in brain of rainbow trout exposed to sub-lethal lindane was reported by Aldegunde *et al*., (1999). GABA receptors inhibit the transmission of nerve impulses; thus disturbances in this receptor would also lead to an over stimulation of the nerves.

In addition, nervous tissue has weaker antioxidant defense system than other kinds of tissue. On the other hand, the brain as center of the nervous system in fish contains low levels of enzymatic and non-enzymatic antioxidant and higher levels of oxidizable unsaturated lipids and catecholamines. So, nerve tissue is very sensitive to oxidative stress damage induced insecticide toxicity compared with other tissues (Üner *et al*., 2006; Li *et al*., 2010). These results have been reported by other scientists (Senger *et al*., 2005).

6.6 Behavioral alterations

Behavioral changes are the most sensitive indicators of potential toxic effects. The behavioral and the swimming patterns of the fish exposed to different insecticides include changes in swimming behavior, feeding activities, predation, competition, reproduction and species-species social interactions such as aggression (Cong *et al*., 2008; 2009; Werner and Oram, 2008). Banaee *et al*. (2008; 2011) reported similar behavioral responses in common carp and rainbow trout exposed to sub-lethal levels of diazinon. In fact, most insecticides influence the behavior patterns of fish by interfering with the nervous systems and sensory receptors (Keizer *et al*., 1995; Pan & Dutta, 1998; Cong *et al*., 2008; 2009); and this incident may impair the identification of situation and development of appropriate response by the fish exposed to insecticide. The effect of certain insecticides on the activity of acetylcholinestrase may lead to a decreased mobility in fish (Bretaud *et al*., 2000). Researchers have reported the same alterations in *Oryzias latipes*, *Cyprinus carpio*, *Labeo rohita*, *Oncorhynchus tshawytscha*, *O.latipes*, *Cirrhinus mrigala*, *Oreochromis niloticus*, *Clarias gariepinus* treated with chlorpyrifos (Rice *et al*., 1997; Halappa & David, 2009), malathion (Patil & David, 2008), diazinon (Scholz *et al*., 2000), endosulfan (Gormley & Teather, 2003), Fenvalerate (Mushigeri & David, 2005), fenitrothion (Benli & Özkul, 2010), dimethoate (Auta *et al*., 2002), respectively.

6.7 Genotoxicity

In genetic-toxicology any heritable damage or DNA inactivation resulting from the animal's exposure to xenobiotics is studied. Genotoxic chemicals such as insecticides have common chemicals and physical properties that enable them interact with genetic materials (Campana *et al*., 1999; Çava & Ergene-Gözükara, 2003; Candioti *et al*., 2010; Dogan et al., 2011). The mutation that may result from an interaction between a chemical and the genetic material is a heritable change in the cell genotype, and thus the error may be transferred to the daughter cell or the next generation. Carcinogenesis and the formation of some tumors in different tissues of fish exposed to insecticides may also be caused by genotoxic properties of these xenobiotics. One of the ill effects of insecticides' arrival into surface waters may be an induction of chromosomal damage in eggs and larvae of fishes in different stages of development.

Some insecticides that behave as endocrine active compounds can change the expression of vital genes resulting in unusual concentrations of plasma steroid hormones and reproductive dysfunction or immuosuppression (Jin *et al*., 2010).

6.8 Immunosuppression

The immune system of fish is important for defense against a variety of pathogens. The system is very sensitive to homeostatic adjustments via endocrine regulation and is influenced by the biochemical status of the nervous system. Thus, any impairment in the nervous system and disturbance in the biochemical homeostasis can weaken the immune system of fish. On the other hand, insecticides may alter the function of the immune system and result in immunodepression, uncontrolled cell proliferation, and alterations of the host defense mechanisms including innate immunity and acquire immunity against pathogens.

Different insecticides at sub-lethal levels have been recognized as stressors causing immune-suppression in fish (Werner and Oram, 2008). In addition, some insecticides may exert immunotoxic effects by altering the transcription of important mediators of the fish's immune system (Eder *et al.*, 2009). Effects of insecticides like P,P'-DDE, lindane, cypermethrin, chlorpyrifos, diazinon on the immune factors of fish such as Interleukin-1β (IL-1β), IL-1β receptor (IL-1R1), Interferon gamma (IFN-γ2b), TNFα, MHCIα, MHCIIα, Mx, TLR9, IγML and C- reactive protein (CRP), TCRα in head- kidney leucocytes, Lysozyme activity, chemiluminuscence (CL) response and immunocompetent cells population size, IgM levels, value of white blood cells (WBC) and respiratory burst activity, head kidney phagocytes and peripheral blood leucocytes, etc., have been reported by scholars (Betoulle *et al*., 2000; Khoshbavar-Rostami *et al*., 2006; Banaee *et al*., 2008; Cuesta *et al*., 2008; Girón-Pérez *et al*., 2009; Shelley *et al*., 2009; Ahmadi *et al*., 2011; Jin *et al*., 2011, Wang *et al*., 2011). The exposure to sub-lethal concentrations of insecticides is what probably makes fish vulnerable to infectious diseases because of their immune-depressive effects (Zelikoff *et al*., 2000). For example, the susceptibility of juvenile chinoock salmon (*O.tshawytscha*) to infectious hematopoietic necrosis virus was significantly increased in fish exposed to sub-lethal concentrations of esfenvalerate (Clifford *et al*., 2005). Similar results were reported in goldfish and common carp that were exposed to carbaryl and lindane respectively (Shea, 1983; Shea & Berry, 1984; Cossarini-dunier & Hattenberger, 1988).

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Production of Insecticidal Baculoviruses in Insect Cell Cultures: Potential and Limitations

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1. Introduction

The potential of baculoviruses to be employed as insecticides is known since more than 75 years ago (Benz, 1986). To date, over 30 different baculoviruses are used to control several insect plagues in agriculture, horticulture and forestry (Moscardi, 1999). The use of baculovirus as insecticides is based on a set of useful properties, such as pathogenicity, specificity, narrow host range, environmental persistence, ability to act synergistically with other natural enemies of the pest and ability to induce artificial epizootics. Despite these advantages, very few baculoviruses have become widely used as insecticides, standing out as some successful examples the use of the *Anticarsia gemmatalis multiple nucleopolyhedrovirus* (AgMNPV) to control the velvetbean caterpillar in soybean crops in Latin America, *Cidia pommonella granulovirus* (CpGV) to fight the codling moth attacks in fruit orchards, and *Spodoptera exigua multiple nucleopolyhedrovirus* (SeMNPV) to control the armyworm in vegetable crops under cover in Europe (Moscardi, 1999). The causes of the limited acceptance of baculoviruses as insecticides are diverse, including slow speed of action, problems to register and market these biological insecticides and difficulties to produce them at an appropriate scale.

The technologies currently used to produce insecticidal baculoviruses are based on the infection of susceptible insect larvae (Black et al., 1997). However, the implementation of processes to produce baculovirus in insect larvae is hampered by several limitations: high labour requirements, lack of expertise in standardization and validation of such processes, difficulties in scaling production to levels consistent with the profitability of the process and difficulties to properly control both the process production and product quality. While several improvements in production systems in insect larvae have been described in the last years which could help overcome some of the problems described above (van Beek & Davis, 2007), it has been also proposed that the adoption of an alternative technology based on the viral propagation in insect cell cultures could enable the development of well standardized, controlled and scalable production processes for insecticidal baculoviruses (Szewczyk et al., 2006).

The purpose of this chapter is to review the current state of the art about insect cell culture technology and its application to the production of viral insecticides belonging to the family *Baculoviridae*. The several restrictions still existing to develop feasible processes as well as the prospects for overcoming these limitations will be also reviewed.

2. The baculoviruses

2.1 Structure and classification

The baculoviruses (family *Baculoviridae*) are a group of arthropod-specific viral pathogens that have a circular and supercoiled double-stranded DNA genome (Rohrmann, 2011). The size of the genome ranges from 80 to 180 kbp. The genome is contained in a rod-shaped nucleocapsid with helical symmetry. The baculoviruses generate two different progenies, called budded virus (BVs) and occluded virus (OVs) that share the same nucleocapsid. Both viral progenies play different roles in the natural cycle of these viruses. BVs, consisting of a single nucleocapsid surrounded by an envelope derived from the cellular plasmatic membrane, are responsible for the transmission of the infection from cell to cell in an infected animal. In OVs, on the other hand, one or more nucleocapsids are contained by an envelope synthesized *de novo* in the nucleus of infected cells. These virions are then included in a crystalline protein matrix, consisting mainly of a single polypeptide which is product of the hyperexpression of a very late viral gene, resulting in the so-called occlusion bodies (OBs). The OBs, whose polypeptide structure gives protection to the OVs contained therein, are responsible for the transmission of the infection between susceptible animals in nature and, in fact, constitute the viral progeny useful as insecticide. The structures of BVs, OVs and OBs are shown in Figure 1.

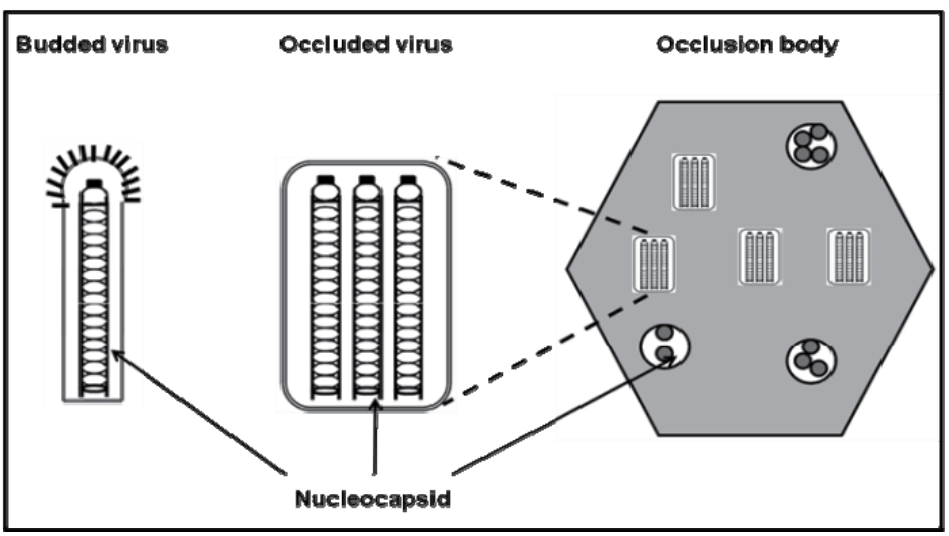

Fig. 1. schematic structures of budded virus, occluded virus and occlusion bodies of baculoviruses.

The members of the *Baculoviridae* family are classified into four different genera (Jehle et al., 2006). The viruses classified into the genus *a-baculovirus* are able to infect lepidopteran insects to produce nuclear polyhedrosis. The viruses belonging to the genus *β-baculovirus*

can also infect lepidopteran insects, but they produce granulosis. On the other hand, the viruses classified into the genus *γ-baculovirus* can infect hymenopteran insects to produce nuclear polyhedrosis, while the viruses of the genus *δ-baculovirus* are associated to the production of nuclear polyhedrosis in dipteran insects. As most baculoviruses used as insecticides so far belong to the genus *a-baculovirus*, the subject henceforth will be focused on lepidopteran nucleopolyhedroviruses.

2.2 Natural cycle and pathogenicity

The main route of infection of lepidopteran larvae with nucleopolyhedrovirus is the ingestion of food contaminated with viral OBs (Granados & Williams, 1986). Once ingested, OBs are transported to the larvae´s midgut, where they are dissolved to release the occluded virions, due to the combined action of the alkaline environment and the presence of alkaline proteases. The released OVs pass through the midgut peritrophic matrix and find the brush border membrane of the columnar midgut epithelial cells, which fuse with the viral envelope to enter the viral nucleocapsids within the cytoplasm. The ability of OVs to infect midgut epithelial cells is dependent on the expression of a set of genes whose products are denominated "*per os* infectivity factors" (PIFs) (Rohrmann, 2011). Most nucleocapsids are then transported to the nucleus through a process that is dependent on actin polymerization. Once the nucleocapsid has been entered into the cell nucleus, the viral DNA is naked and starts the transcriptional cascade that lead ultimately to the assembly of progeny nucleocapsids. A distinctive feature of the primary replication of nucleopolyhedroviruses in the midgut is that the nucleocapsids that were assembled in the nucleus are almost totally exported to the basal cytoplasmatic membrane, from where they finally egress as BVs. The budding of BVs occurs at regions of the plasmatic membrane that have been modified by insertion of the glycoproteins characteristic of the BV progeny, GP64 and/or F. Then, BVs would cross the basal lamina to begin the dissemination process that leads to secondary systemic infections. Alternatively, it has been proposed that baculoviruses can reach the main insect cavity through previous infection of tracheal cells (Pasarelli, 2011). Secondary infections, that affect almost all insect tissues, start when BVs penetrate into cells through a receptor-mediated endocytosis process. After the fusion of the viral envelope with the membrane of acidified endosomes, viral nucleocapsids are released into the cytoplasm and then transported to the nucleus, where the viral genome is naked. Differently from what occur in midgut epithelial cells, the transcriptional cascade in secondary infections drives the replication process to the production of nucleocapsids that, besides of feeding the budding of BVs, are assembled into the nucleus to form OVs. The assembly of OVs implies the retention of nucleocapsids inside the nucleus and the acquisition of an envelope synthesized *de novo* at the expense of the inner nuclear membrane. The OVs are finally occluded inside a crystalline matrix consisting mainly of the viral protein polyhedrin , whose gene is expressed at very high levels during the very late stage of the transcriptional cascade. The products of the occlusion process are the OBs, which are retained inside the nucleus until the death and lysis of the infected cell. At the end of the pathogenic process, the infected insect is full of OBs that, after its death and liquefaction of its tissues, are released into the environment to restart the cycle again.

The symptoms of the disease associated to baculovirus infection are not usually apparent during the first days post-infection (Granados & Williams, 1986). The change of color and the altered behavior of infected insects are often the earliest signs of infection with α-baculovirus. The lack of appetite, which after several days culminates in the total

interruption of feeding, is another sign of infection. The growth of infected larvae is delayed with respect to uninfected controls, and the death occurs after several days post-infection. The length of the interval of time between infection and death of the insect varies between 3 days and 3 weeks, and depends on many factors, including larval age, nutritional status, dose of virus and virulence of the viral strain, as well as on environmental factors. Anyway, nor the cessation of ingestion or death occur immediately to infection, facts that constitute strong constraints to the acceptance of baculoviruses as insecticides.

2.3 The transcriptional regulation of the baculovirus replication cycle and the production of viral progenies

The replication cycle of baculoviruses is mainly regulated at the transcriptional level. The baculovirus transcriptional program occurs in three stages, called early, late and very late, respectively, which are coordinated in a cascade (Table 1) (Blissard & Rohrmann, 1990; Rohrmann, 2011).

Time post-infection *	RNA polymerase	Transcriptional stage	DNA replication	BVs production	OVs production	OBs assembly
0 – 8 hs.	Cellular RNA polymerase II	Early	-	-	-	-
8 – 18 hs.	Viral RNA polymerase	Late	+++	+++	-	-
From 18 hs.	Viral RNA polymerase	Very late	-	+	+++	+++

Table 1. Cascade of transcriptional events during the replication of baculoviruses and temporal distribution of production of viral progenies. *AcMNPV replication.

Each transcriptional stage is characterized by the expression of a specific group of genes. Early transcription begins immediately after the parental viral DNA is naked into the nucleus. It is carried out by the cellular RNA polymerase II, and includes a set of genes whose products are trans-activators and enzymes that will then have a role in viral DNA replication. As an exception, in the early stage is also transcribed the *gp64* gene whose expression´s product is the principal glycoprotein of the group I nucleopolyhedrovirus´ envelope. The end of the early stage is determined by the onset of viral DNA synthesis, and involves a change in the pattern of transcription by which some of the genes initially transcribed are no longer expressed, and a new set of genes begins to be transcribed by a viral RNA polymerase, starting the late stage of transcription. The late stage involves the expression of genes whose products are proteins and glycoproteins that are part of the structure of the budded virions, which are assembled and released from the infected cell during this time. Finally, there occurs a further change in the pattern of transcription, whose most notable feature is the hyperexpression of genes whose products are proteins and glycoproteins involved in the assembly of occluded virions and occlusion bodies, such as polyhedrin and P10. The RNA polymerase involved in the very late transcription is the

same viral enzyme in charge of the late transcription. Although some BVs are produced during the very late stage, the hallmark of this period is the assembly of occlusion bodies, which extends up to the cell death.

3. Technological applications of baculoviruses

Baculoviruses are arthropod-specific pathogens, with a host range that is generally very narrow, and lack the ability to replicate and produce pathogenic effects in other animals and plants, properties that have promoted their use as safe insecticides with reduced environmental impact (Huber, 1986). In addition, the expression of very late genes in the baculovirus genome is under the control of regulatory elements with a very high promoter activity, a property that has allowed the development of one of the most widely used expression systems for the production of recombinant proteins, the baculovirus vector expression system (Luckow & Summers, 1988). Also, baculoviruses are able to penetrate into mammalian cells, although they can not replicate into them. This property permits the use of these viruses as vectors for gene delivery (Kost & Condreay, 2002). Baculoviruses also exhibit a potent immunostimulating activity in mammals, opening the possibility of their use as adjuvants in the formulation of novel vaccines (Abe & Matsuura, 2010). Some of these applications have yet to demonstrate its market potential, but others are a reality and products based on these viruses are used today in agriculture, veterinary medicine and human medicine, among others.

3.1 Baculoviruses as insecticides

Baculoviruses presents a number of advantages over traditional synthetic chemical insecticides (Moscardi, 1999). Their high specificity makes them safe for other insects, and thus helps to preserve and even enhance the natural mechanisms of plagues control. In addition, although baculoviruses can infect mammalian cells, including human cells, they can not replicate in them and therefore they lack of pathogenicity for the human being and other animals, making safe their use. The multiplication in their natural hosts, and their capacity to persist in the environment make them suitable for the inoculative control of plagues in forestry. In addition, the same properties coupled with the aforementioned preservation of natural enemies, permit the reduction of the number of the applications needed to keep the insect plague under control in annual crops, thus contributing to reduce the costs of protection. Finally, its use in replacement of synthetic insecticides helps to reduce the overall levels of chemical pollution.

The use of baculoviruses as insecticides also has limitations. Their high specificity is also a disadvantage to their widespread use, since they are only useful when the damage to the crop to be protected is produced predominantly by a single insect, and they are not effective in controlling pest complexes. The insecticidal effect of baculoviruses is not evident immediately after application, and the delay usually is accompanied by an increase of the level of crop damage. This defect can be counteracted by an earlier application of the virus, but it requires a close quantitative following of the insect population. Also, since baculovirus production processes are based on viral replication in living hosts, their yields can not match the high yields obtained at relatively low costs in the synthesis of chemical insecticides. Table 2 presents a list of selected baculoviruses belonging to the genus *a-baculovirus* registered for their use as insecticides. As can be

observed, most of them are rather specific for one lepidopteran specie, except AcMNPV that has a wider host range.

Virus	Insect target	Crops
AgMNPV	*Anticarsia gemmatalis*	Soybean
AcMNPV	*Autographa californica*, *Trichoplusia ni*, *Pseudoplusia includens*, etc.	Cotton, cabbage, tomato, broccoli
HzSNPV	*Helicoverpa zea*, *Heliothis virescens*	Cotton, corn, tomato, vegetables
HaNPV	*Helicoverpa armigera*	Cotton, tomato
LdMNPV	*Lymantria dispar*	Forest
MbMNPV	*Mamestra brassicae*	Cabbage
OpMNPV	*Orgya pseudotsugata*	Forest
SeMNPV	*Spodoptera exigua*	Vegetables
SlNPV	*Spodoptera littoralis*	Cotton
SpltMNPV	*Spodoptera litura*	Vegetables, cotton

Table 2. Examples of *a-baculovirus* registered for insecticidal use.

3.2 Genetically modified baculoviruses as improved insecticides

As explained above, the adoption of baculoviruses as insecticides is limited by some of its pathogenic properties. One of the strategies developed to overcome this limitation is the modification of the viral genome in order to improve the insecticidal capabilities of the modified virus. To this end, two alternative routes have been followed: the insertion of foreign genes whose expression gives the virus an increased virulence, and the deletion of viral genes responsible for the delay in the evolution of the viral pathogenic process (reviewed by Szewczyk et al., 2006). The genes corresponding to several specific insect toxins, hormones or enzymes have been cloned and expressed in different baculoviruses, resulting in most cases in increased virulence, decreased time to insect death, and decreased plant damage. Besides, the deletion of the viral gene codifying for the ecdysteroid glycosil transferase (*egt*) - involved in the metabolism of the hormone ecdysone - also resulted in reduction of food consumption and faster killing of infected larvae.

Although the genetic modification has demonstrated to be a promissory strategy to improve the insecticidal ability of these viruses, the public perception about the risks that would involve the field release of recombinant viruses has limited the interest in developing novel insectides based on genetically modified baculoviruses. In fact, although have elapsed 20 years since the first publications that described the development of genetically modified baculovirus with enhanced insecticidal activity, no one product based on these recombinant viruses has yet come to market, and companies that were involved initially in these developments have canceled the processes for obtaining approvals for its use.

4. Insect cell culture technology

4.1 A brief history

The first studies conducted *in vitro* on tissues of invertebrate animals were made by Goldschmidt in 1915 (Day & Grace, 1959). Thereafter, and for about 40 years, attempts to multiply insect cells and tissues *in vitro* have had limited success. After completion of the Second World War, and already having the air filtration technology that permitted the safe handling of animal cell cultures in sterile environments, the work of Wyatt et al. (1956) on the chemical composition of the insect hemolymph allowed the development of the first culture media specifically designed for the cultivation of lepidopteran cells (Grace, 1958). The establishment of the first insect cell lines obtained from tissues of lepidopteran insects was an achievement reached by Grace (1962). Since then until now, at least half thousand cell lines, from different insects and distinct tissues have been established. A milestone in this process was the establishment of the cell line IPLB-Sf21 (Vaughn et al., 1977). This cell line, used to plaque baculovirus for the first time, exhibited relevant technological properties, such as the ability to grow indistinctly in static cultures and in agitated suspension cultures. Also, IPLB-Sf21 was the insect cell line where the clone Sf9 was produced from. This clone was closely linked to the development of the baculovirus – insect cell expression system for recombinant proteins (Summers & Smith, 1983). At the same time, new culture media were developed, such as MM, TC-100, TNM-FH and IPL-41, and the insect haemolymph that was initially used to supplement them, was replaced by fetal calf serum. At the end of the 80's, two important developments opened the possibility of expanding the cultivation of insect cells to an industrial scale: first, the development of microemulsions of lipids and sterols allowed the formulation of serum-free media, and second, the demonstration of the protective effect of surfactant poli-alcohols on the integrity of insect cells in suspension cultures aerated by sparging permitted the scaling-up to large stirred tank reactors and airlift reactors (Maiorella et al, 1988). In recent years, the main contributions to the technology of cultivation of insect cells have come from the development of genetically modified cell lines, capable, for example, to produce proteins with humanized molecular structures (Shi & Jarvis, 2007).

4.2 Insect cell lines

In a process of baculovirus production in cell cultures is crucial to make a proper selection of the cell line to be used as substrate for virus multiplication. The selected cell line must be susceptible and permissive to the virus, which must replicate in abundance to produce high yields of both budded virus and occlusion bodies. Preferably, nutritional requirements and metabolism should be well characterized, and the cell line should show relevant technological properties such as adaptability to suspension cultures, capability to grow in a low-cost serum-free medium and ability to grow in industrial bioreactors. Furthermore, it should be genetically stable, and should not be a source of viral variability.

Currently there are hundreds of cell lines established from tissues and organs of lepidopteran insects (Lynn, 2007), but very few meet the requirements described above. Table 3 shows a list of the lepidopteran insect cell lines more used for producing wild-type and/or recombinant baculovirus. The IPLB-Sf21 cell line and its clone Sf9 have been used intensively and they are well characterized. They can grow in suspension cultures at high cell concentration in bioreactors and there are several serum-free media available for them. Both wild-type and recombinant AcMNPV replicate very well in Sf cell lines

(O´Reilly et al., 1994). In addition, these cell lines had shown to be susceptible and permissive to the replication of other baculoviruses (Claus et al, 1993). The cell line BTI-TN-5B1-4, known commercially as High Five®, is also being used widely to produce recombinant proteins due to its susceptibility to AcMNPV and elevated specific productivity (Chung & Shuler, 1993).

Cell line	Insect	Tissue of origin	Susceptibility to baculoviruses
IPLB-Sf21 / Sf9	*Spodoptera frugiperda*	Ovarioles	AcMNPV, AgMNPV, SfMNPV, SlNPV, TnSNPV
BTI-TN-5B1-4	*Trichoplusia ni*	Embryos	AcMNPV, AgMNPV, TnSNPV
BCIRL-HZ-AM1	*Heliothis zea*	Ovarioles	AcMNPV, HaNPV, HzSNPV
Bm5	*Bombyx mori*	Ovarioles	AcMNPV, BmNPV
IPLB-LdEIta	*Lymantria dispar*	Embryos	AcMNPV, LdMNPV
saUFL-AG-286	*Anticarsia gemmatalis*	Embryos	AcMNPV, AgMNPV

Table 3. Lepidopteran insect cell lines used frequently to produce wild-type and recombinant baculoviruses.

Besides the widely used cell lines that were mentioned in the preceding paragraph, other cell lines have been used more narrowly in processes involving the multiplication of other baculoviruses. In general, these lines were established from tissues of the natural host of the baculovirus to replicate, because viral yields tend to be higher in infected cultures of these homologous cell lines than in the heterologous ones. For instance, the cell lines saUFL-AG-286 (*Anticarsia gemmatalis*) (Sieburth & Maruniak, 1988), BM5 (*Bombyx mori*) (Grace, 1967), BCIRL-HZ-AM1 (*Heliothis zea*) (McIntosh & Ignoffo, 1981) and IPLB-LdEIta (*Lymantria dispar*) (Lynn et al, 1988) have been used to produce specifically the viruses AgMNPV, BmNPV, HaSNPV and LdMNPV, respectively. However, these cell lines are not as well characterized as the most widely used lines, and their technological properties (adaptation to suspension, ability to grow in serum-free culture media) are less remarkable or yet unknown.

4.3 Nutrition and metabolism of lepidopteran insect cells

Most existing data on nutrition and metabolism of lepidopteran insect cells in culture has been obtained from studies of a few cell lines, mostly Sf9 and BTI-TN-5B1-4. The comparative analysis of that information permits to conclude that each cell line is considerably flexible for satisfying their nutritional requirements. However, there are marked differences in metabolic behavior between different cell lines. Carbohydrates and amino acids are the most important nutrients, and the knowledge about their quantitative demands and metabolism will be briefly reviewed below. The information available about

the quantitative requirements of other important nutrients, such as lipids, sterols, vitamins and mineral salts, is much scarce.

Carbohydrates are essential components of all culture media for insect cells, due to their role as main sources of carbon and energy. Insect cells are capable to grow in culture media containing glucose as the unique carbohydrate, but insect cells can also consume other monosaccharides and disaccharides (Mitsuhashi, 1989). Sf9 cells consume glucose without production of lactate. This behavior has been attributed to the existence of an active tricarboxylic acid cycle, where 70 – 80% of the consumed glucose would be totally oxidized (Neermann & Wagner, 1996). However, these results are still controversial, because other studies have found that the percentage of glucose consumed that is derived to the tricarboxylic acid cycle is much lower (Benslimane *et al*., 2005). The production of other metabolites such as alanine, glycerol and ethanol, as well as fatty acid synthesis, could explain the fate of a significant fraction of carbon incorporated to Sf9 cells through glucose consumption (Drews et al., 2000; Bernal et al., 2009). BTI-TN-5B1-4 and BM5 cell lines have a different behavior: both cell lines produce lactate, displaying a behavior similar to that of mammalian cells (Rhiel et al., 1997; Stavroulakis et al., 1991). The flux of glucose was studied in BTI-TN-5B1-4 cells, resulting that the proportion oxidized in the tricarboxylic acids cycle was lower than in Sf9 cells (Benslimane et al., 2005). The influence of baculovirus infection on the metabolism of insect cells, and specifically on glucose metabolism, is a topic that has been scarcely addressed, and the information is contradictory (Bernal et al., 2009; Gioria et al., 2006).

According to Mitsuhashi (1989), 15 amino acids are essential to insect cells. Glutamine is the amino acid consumed faster and in a greater extension in cultures of most lepidopteran insect cell lines characterized to date. However, it has been demonstrated that glutamine is dispensable for the growth of Sf9 cells, providing that the cells have ammonium as nitrogen source (Öhman et al., 1996). Other amino acids that can be consumed in significant quantities are asparagine, aspartate, glutamate and serine, and precisely asparagine is consumed faster than glutamine in cultures of the BTI-TN-5B1-4 cell line (Rhiel et al., 1997). The demand of other amino acids is much lower. Besides serving as precursors for the synthesis of proteins and nucleic acids, amino acids that are consumed faster are used as sources of energy, such as glutamine and asparagine. The metabolism of glutamine has been studied in Sf9 cells, where the utilization pathways are depending on the availability of glucose (Drews et al., 2000). It has been proposed that, in glucose excess, the cytoplasmatic enzyme glutamate synthase transfers the amidic nitrogen of glutamine to the amine-position in glutamate, from where it is transaminated to alanine, the main product of glutamine metabolism. But when glucose is exhausted, glutamine is metabolized in mitochondria, where the amide-nitrogen and the amine-nitrogen of glutamine are sequentially released as ammonium ion, which accumulates as the main product of glutamine metabolism under glucose limitation in Sf9 cells. The metabolism of glutamine and other amino acids used as energy sources in other cell lines, such as BTI-TN-5B1-4 and saUFL-AG-286, is probably regulated differently, because they produce ammonia even in the presence of excess glucose (Gioria et al., 2006; Rhiel et al., 1997). The information about the influence of baculovirus infection on glutamine requirement and metabolism is scarce, but most results appear to indicate that insect cells tend to reduce the demand after infection (Gioria et al., 2006; Bernal et al., 2009).

4.4 Culture media for lepidopteran insect cells

Almost all media used for cultivation of insect cells have a chemical composition partially defined. They consist of a basal medium composed of chemically defined mixtures of carbohydrates, amino acids, vitamins, salts and, in some cases, organic acids. In addition, the media must be supplemented with compounds of undefined chemical composition that contribute to cell´s proliferation, such as fetal calf serum, microbial extracts and/or protein hydrolysates.

4.4.1 Basal culture media

TC-100, IPL-41, Grace and TNM-FH are the most commonly used basal media for *in vitro* culture of lepidopteran insect cells (Schlaeger, 1996).TC-100 and TNM-FH have almost the same amino acids composition as the Grace medium, from which they originated. IPL-41 has the same qualitative composition of amino acids, but with higher concentration for the ones consumed faster: glutamine, asparagine, glutamic acid, aspartic acid and cystine. In any case, it has been demonstrated for several different insect cell lines that the concentrations of most amino acids in basal culture media are oversized with respect to cellular requirements (Bédard et al., 1993; Ferrance et al., 1993; Lua & Reid, 2003) . TC-100 and TNM-FH contain also protein hydrolysates, and the last is additionally supplemented with yeast extract. With regard to carbohydrates, all media contain glucose, although at different concentrations, and IPL-41 contains also an additional monosaccharide, fructose. Grace, TNM-FH and IPL-41 media also contain disaccharides, all of them contain sucrose and the last also maltose. IPL-4 is richer in vitamins and also contains organic acids, not present in the original formulations of the media Grace, TNM-FH and TC-100. The composition of mineral salts of IPL-41 differs from the other three, and is also enriched with oligoelements. In spite of the differences in the chemical composition described above, the four basal media can support the growth of Sf9 and BTI-TN-5B1-4 cell cultures, provided they are supplemented with fetal calf serum. This fact highlights the nutritional plasticity of these cell lines. On the other hand, the richer medium IPL-41 has demonstrated to be better suited than the other three to formulate serum-free media (Ikonomou et al., 2001; Maiorella et al., 1988).

4.4.2 Fetal calf serum

Blood serum obtained from bovine fetuses is the most used undefined supplement for animal cell cultures, including both mammalian and insect cells. It concentrates, in a single component, several essential functions for cultured cells (Barnes & Sato, 1980). Serum provides, in a water-soluble vehicle, lipids and cholesterol, and it is also a rich source of growth factors, vitamins and mineral oligoelements. Its proteins´ transporters allow the supply of poorly soluble ions, such as Fe^{+++}. In addition, serum has detoxifying activity, and their proteins can contribute to the preservation of the structural integrity of cells when they are subjected to mechanical stress.

In spite of the advantages of fetal calf serum, they are accompanied by several disadvantages. Its composition is undefined and variable from batch to batch. It is also a possible vehicle for the introduction of chemical and biological contaminants, such as plaguicides, virus and prions, among others. The high concentration of serum proteins may involve interference with the extraction and purification of products. Besides, serum proteins are a source of foam in culture processes where aeration is made by sparging. The cost of using fetal bovine serum as a supplement of a basic medium is so high that it can

reach 90% of the final cost of complete culture medium, which can be unacceptable for the development of a production process for a viral bioinsecticide. Finally, the regulatory agencies are becoming increasingly restrictive in relation to the use of raw materials of animal origin.

4.4.2.1 Serum-free media for insect cell cultures

The problems about the use of serum as a supplement in animal cell cultures, described above, have driven the development of new culture media capable to support the growth of insect cell cultures in a serum-free environment. As the serum is a very complex substance, and its functions are diverse, it is necessary to use a mixture of various components to replace it. The supply of hydrophobic nutrients is replaced by adding microemulsions that contain a source of lipids and cholesterol (Maiorella et al, 1988; Ikonomou et al., 2001). The most used sources of lipids are the methyl esters of fatty acids isolated from the liver of marine fishes, but recently it has been described the use of cooking soybean oil, a cheaper and more abundant source of lipids (Micheloud et al., 2009). In addition, microemulsions also contain the surfactant Pluronic F68, whose presence protects the cells from the detrimental influence of bubbles in sparged bioreactors.

On the other hand, the contribution of growth factors that made the serum is replaced by mixtures of enzymatic hydrolysates of proteins and yeast extract (Schlaeger, 1996). Hydrolysates from milk proteins such as lactalbumin and casein, and meat proteins, are commonly used as cheap replacements of serum, but the peptides responsible for the growth factor activity have not been identified. The effects of the addition of yeast extract to culture media for insect cells are similar to that of fetal calf serum, modifying the specific growth rate and increasing the maximum cell density (Eriksson & Häggström, 2005).

There are currently several commercial serum-free media available that were specifically designed to cultivate either Sf9 or BTI-TN-5B1-4 cell lines, but eventually can also support the growth of other insect cell lines. The growth parameters of cultures in these media are remarkable, as well as the yields of baculovirus and recombinant proteins obtained in infected cultures. However, the cost of the commercial serum-free media is, at least, as high as the cost of complete medium supplemented with fetal calf serum, precluding its utilization for the economically feasible production of insecticidal baculoviruses.

New serum-free media were specifically designed in recent years for culturing a few cell lines of interest, due to their potential application to the development of production processes for insecticidal baculoviruses in insect cell cultures. A prototype low cost medium (LCM) was developed to both cultivate the cell line BCIRL-HZ-AM1 and to produce the baculovirus HaSNPV (Lua & Reid, 2003). Comparable maximum cell densities and growth rates were obtained in both the LCM and a commercial serum-free medium, but lower specific virus yields were reached in LCM. The composition of the LCM medium was not disclosed. On the other hand, the low-cost medium UNL-10 was developed to grow the saUFL-AG-286 cell line, useful to produce the baculovirus AgMNPV (Micheloud et al, 2009). The yields of occlusion bodies in suspension cultures, using optimized parameters of infection, were as high as 3×10^{11} OBs L^{-1}, with specific yields higher than 600 OBs/cell. The composition of the UNL-10 medium, that was optimized to improve the yield of OBs of AgMNPV, has glucose as the only source of carbohydrates, a lower concentration of most amino acids, an improved mixture of vitamins and a lipid emulsion made with cooking oil. The growth factor activity is exerted by an optimized mixture of an enzymatic hydrolysate of casein, tryptose broth and yeast extract.

4.5 Physicochemical conditions in insect cell cultures

The pH of the medium in insect cell cultures is determined by its chemical composition, depending mainly on the buffer activity of salts, although the mixture of amino acids can also play a role in pH regulation. The optimal pH for all lepidopteran insect cell cultures is acid, between 6.2 and 6.4 (Schlaeger, 1996). While the pH can be modified through the evolution of an insect cell culture, the changes tend to be limited and usually do not compromise the cellular physiology.

The optimum osmolarity can differ for distinct lepidopteran insect cell lines, which can react also differently according to the agents utilized to modify it. The osmolarity of most culture media for insect cells varies between 250 and 350 mOsm kg^{-1}, but the initial value is habitually modified through the evolution of the culture, usually without consequences on the cellular physiology (Kurtti & Munderloh, 1984).

Insect cells are cultivated in vitro at temperatures ranging between 25 and 30 ^{o}C. The optimum temperature for most lepidopteran cell lines is 28 ^{o}C (O´Reilly et al., 1994). Cells cultured in serum-free media are less tolerant of temperature changes that cells grown in media supplemented with serum (Mitsuhashi & Goodwin, 1989).

The dissolved oxygen concentration is a critical parameter in insect cell cultures due to the reduced solubility of oxygen in aqueous culture media (Palomares & Ramírez, 1996). Dissolved oxygen levels between 40 and 70% are usually appropriate to keep acceptable growth parameters in insect cell cultures. In addition, most of the available information indicates that it is also critical to keep a proper level of dissolved oxygen in baculovirus-infected cultures, because oxygen deprivation is a cause of low yield of virus or recombinant protein.

5. Bioreactors in insect cell cultures

Although the most used insect cell lines can grow in suspension or as static cultures indistinctly, the scaling-up of static cultures is not a feasible alternative for the production of insecticidal baculoviruses. Thus, in this section it will only be reviewed the information about insect cell suspension cultures, from agitated erlenmeyers and spinner-flasks for low-scale cultures up to stirred tank and airlift reactors at larger scale.

The suspension cell cultures at low scale in agitated erlenmeyers or spinner-flasks usually do not offer significant technological difficulties, being able to reach cell densities as high as 1 x 10^7 viable cells per milliliter, with doubling times from 18 to 30 hours during the exponential growth phase (Bédard et al., 1993; Benslimane et al., 2007; Gioria et al., 2006; Lua & Reid, 2003; Rhiel et al., 1997). This is true for most insect cell lines as long as the ratio between the areas of the gas phase –usually air- to the liquid phase –culture medium- is large enough to ensure that the superficial supply of oxygen is adequate. In practical terms this means that the volume of culture should never exceed 25% of the total volume of the container. In addition, the stirring speed should be adjusted to 60 - 80 rpm in spinner-flasks and 100 - 120 rpm in flasks with orbital shaking.

The large scale culture of animal cells in suspension requires an adequate mixing through agitation, either mechanical or pneumatic, in order to keep cells in suspension, as well as to ensure physicochemical homogeneity and adequate mass transference. But these requirements of scaling collide with certain morphological characteristics of the insect cells, as their large size and lack of cell wall, that make them fragile and sensitive to the effects of agitation and gas sparging (Trinh et al., 1994). A successful scaling-up of suspension

cultures of insect cells will be always the result of a compromise between the satisfaction of an adequate mixing and the preservation of the structural and functional cellular integrity. Stirred tank reactors, where agitation is performed mechanically through impellers, have demonstrated to be useful to cultivate insect cells at large scale (Maranga et al., 2004). The agitation rate should be carefully controlled in stirred reactors, especially when cultures are aerated by sparging, due to deleterious effects on cell functionality and viability that occurs when cultures are stirred at speeds over 300 rpm with Rushton turbines (Cruz et al., 1998). This is because the shear in the zone near to the impeller - where the energy of agitation is introduced into the reactor- is very high. Three different ways to aerate insect cell cultures in stirred tank reactors have been used: surface aeration (Kamen et al., 1991), bubble-free aeration (Chico & Jáger, 2000) and sparging (Cruz et al., 1998). Without a doubt, the aeration method that offers a better chance of escalation in stirred reactors is through direct gas bubbling. Two process parameters should be carefully considered in stirred tank reactors aerated through sparging in order to keep a proper cellular viability and functionality: the aeration rate and the bubble size. High aeration rates, as well as low bubble size are main causes of impaired growth and functionality in insect cell cultures (Trinh et al., 1994).

Airlift reactors have been much less used than stirred tank reactors, in spite they offer advantageous characteristics to cultivate large and fragile cells, as insect cells. One of the advantages of airlift reactors, when compared to classical stirred tank reactors is that they have the potential to provide high mass transfer rates with low and homogeneously distributed shear. Thus, adequate oxygen transfer rates from the gas to the liquid phase and from this one to the suspended cells can be obtained within a homogeneous environment and with a reduced exposure to sources of mechanical stress (Merchuk, 1991). On the other hand, these reactors improve their performance as it increases in size, provided that the optimal relations between the reactor geometrical parameters are preserved. In addition, due to its simplicity of design and construction, airlift reactors require less capital investment, and its operation and maintenance costs are also lower than in stirred tank reactors. While some articles have been published on the cultivation of insect cell lines in airlift reactor and its application to the production of baculovirus and recombinant proteins, there are few systematic studies for optimization of the geometrical parameters and operation in processes involving these reactors, nor models for their scaling-up (King et al., 1992; Maiorella et al., 1988; Visnovsky et al.; 2003).

In addition to the classical airlift and stirred tank reactors, other reactor designs were applied successfully to the cultivation of lepidopteran insect cells, such as the rotating-wall vessel (Cowger et al., 1999). However, given the level of production scale that should be achieved for the development of economically feasible processes, only stirred tank and airlift reactors appear to have the potential to be used for the large scale production of insecticidal baculoviruses in insect cell cultures. This is also because of the experience existing in the scale-up of cultures of microorganisms and animal cells in these reactors.

6. Baculovirus infection in lepidopteran insect cell cultures

Figure 3 outlines an infection process of infection with baculovirus in an insect cell culture. The preceding sections have addressed the characteristics of the cells, culture media and reactors. This section will deal with those aspects of the process that relate specifically to infection: the viral inoculums, the parameters of infection, the operation strategy and the product.

6.1 BVs: the viral inoculums in insect cell cultures

The baculovirus inoculum is composed by BVs that are added to the insect cell culture at the time of infection. The quality of the seed virus is a critical factor to determine the quality of the final product, the occlusion bodies, as well as the process productivity. First, the selected strain of virus should be virulent for the insect to be controlled. It should be also capable to replicate in the cell culture, yielding a high productivity of OBs. In addition, OBs produced from that inoculums should have a high biological activity. To meet these requisites, the viral inoculums should be free of genomic variants capable to reduce either the product yield or the biological activity of OBs. Two main types of genomic variants of baculovirus capable to reduce the OBs yield and biological activity have been described: the "few polyhedra" (FP) phenotype and the defective interfering particles (DIPs).

The mutations responsible for the FP phenotype, often associated with the inactivation of the gene 25k, are expressed through the following features: reduced yield of OBs, reduced content of OVs per occlusion body, reduced biological activity of OBs and increased yield of BVs (Beames & Summers, 1989; Harrison & Summers, 1995). The emergence of the FP phenotype is responsible for a sharp drop of the final yields of OBs, as well as its biological activity (Lua et al., 2002). Once emerged, the population of FP mutants tends to enrich through successive passages in insect cell cultures, due to its increased capability to produce BVs.

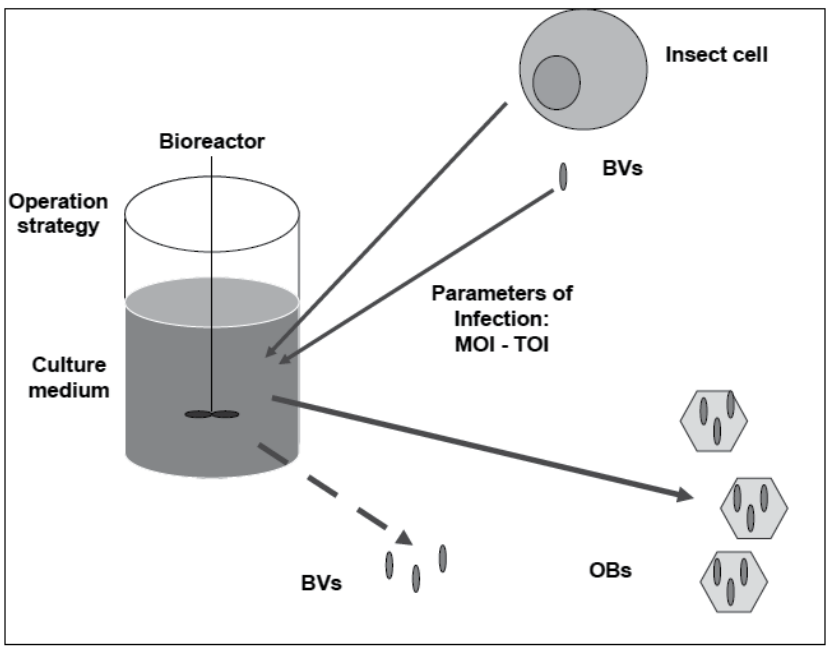

Fig. 3. Schematic representation of OBs insecticidal baculovirus production process in insect cell cultures. MOI: multiplicity of infection; TOI: time of infection.

The DIPs are generated as consequence of genomic deletions that originate shorter viral genomes (Kool et al., 1990). The DIP genomes can not replicate autonomously, but they can do it with the help of complete genomes. The replication of the DIPs competes and interferes with the replication of complete genomes, and leads to progressive enrichment of the

population of defective virus, at the expense of the population of wild-type virus (van Lier et al., 1990). The generation of DIPs in cell cultures infected with baculovirus is favored by conditions that increase the probability of homologous recombination, such as in infections at high multiplicity of infection. The proportion of DIPs in a viral population, as well as the proportion of FP mutants, increases with the number of passages in cell cultures, a phenomenon known as "passage effect"(Krell, 1996). This effect impairs the amplification of baculovirus stocks necessary to infect large scale cultures of insect cells.

The viral stock is the most expensive raw material in processes destined to the production of insecticidal OBs in insect cell cultures. Obtaining the stock for infecting the production reactor usually requires the amplification of the seed virus through successive rounds of infection in insect cell cultures at progressively larger scales (Rhodes, 1996). The optimization of the conditions of infection for the production of BVs would have a double beneficial effect on the whole production process: on the one side, it would allow to reduce the consumption of an expensive raw material and, on the other side, it would permit to reduce the steps of the seed virus amplification process, and therefore to reduce the probabilities to generate and propagate deleterious genomic variants. Despite its importance, the information available about the optimization of infection conditions for the production of the BV progeny of baculovirus is very scarce (Carinhas et al., 2009).

The quality of baculovirus stocks may also be affected by reduced infectivity in relation to the total amount of viral particles (Dee & Shuler, 1997). Although several causes have been proposed to explain this phenomenon, the inactivation of BVs could be an important detrimental factor, especially in stocks prepared in serum-free media (Jorio et al., 2006). An optimized management of the preservation of serum-free baculovirus stocks could have an important impact on the feasibility of the scaling-up process.

6.2 Parameters of infection

The fate of a baculovirus infection in an insect cell culture is strongly dependent on the selection of the multiplicity of the infection (MOI) and the time of infection (TOI) (Carinhas et al., 2009; Licari & Bailey, 1991; Micheloud et al., 2009). MOI is defined as the ratio between the number of infectious viral particles and the number of cells of the infected culture. The selection of the MOI determines the proportion of cells that become initially infected, as well as the distribution of infectious particles per cell. In cultures infected at high MOI (greater than 5 infectious units per cell), all the cells are infected synchronously. This prompts the viral replicative process to follow the same temporal pattern in all cells, with the emergence of a unique peak of viral progeny. On the other hand, when cultures are infected at lower values of MOI, only a proportion of the cellular population is infected initially. Thus, at least two cellular sub-populations will coexist in a culture infected at low MOI: infected and uninfected cells. Viral replication takes place immediately in initially infected cells, producing a viral progeny after one generation time. Uninfected cells, on the other hand, proliferate and will be infected later with the viral progeny of the initially infected cells. An important difference is that in cultures infected synchronously, viral replication occurs in cells that are in similar physiological state and subjected to similar environmental conditions, while in asynchronously infected ones viral replication takes place through successive rounds of infection, in cells under different physiological states and subjected to different environmental conditions. The importance of this difference is that the replicative capacity of insect cells varies, depending on both their physiological condition and the quality of the culture medium at the time that they are actually infected.

One factor that has contributed strongly to limiting the development of feasible production processes of insecticidal baculoviruses in insect cell cultures is the so called "cell density effect" (Wood et al., 1982). This phenomenon is characterized by the reduction of the intrinsic ability of insect cells to replicate baculovirus as the infection is delayed in time, and the cell density increases. The phenomenon has been observed also for recombinant proteins produced by genetically modified baculovirus. Several causes have been proposed to explain the "cell density effect", including nutrient limitation (Bédard et al., 1994), accumulation of toxic by-products (Taticek & Shuler, 1997), autocrine factors and cell cycle distribution (Braunagel et al., 1998; Calles et al., 2006), and inhibition of the central energy metabolism (Bernal et al., 2009), among others, but the causes remain to be identified. The cell density effect, that could be observed in several insect cell lines infected with different baculoviruses, both wild-type and recombinant, can be overcome, at least partially in Sf9 cell cultures by adopting alternative strategies for cell culture and viral infection, such as fed-batch or perfusion, as will be explained hereinafter.

6.3 Operation strategy

The typical strategy for the production of viruses in cultured animal cells is the infection of batch cultures. This strategy implies that a cell culture is cultivated in a proper medium up to reach the desired cellular concentration, when it is added the viral inoculums and the infection is allowed to progress without ulterior modification of the system until the harvest of the product is made. The batch production of insecticidal baculoviruses can be easily implemented, and it is possible to obtain high yields of occlusion bodies (Lua & Reid, 2003; Micheloud et al., 2009; Rodas et al., 2005). However, the cell density effect is a strong limitation to reach economically significant yields of virus, so that the MOI and the time of infection should be optimized to avoid an increase in cell density of infected cultures. This limitation could be partially overcome through the medium exchange before infection (Bédard et al., 2004). Although this strategy is feasible for working at low scale in shaken-flasks or spinner-flasks, it is difficult to implement at larger scale.

Fed-batch is an alternative operation strategy to overcome the cell density effect in cultures of the *Spodoptera frugiperda* cell lines. Concentrated solutions containing the more demanded nutrients (glucose, glutamine and other amino acids, yeast extract and lipids´ emulsion) were added to high density cell cultures for obtaining high yields of recombinant proteins and occlusion bodies (Bédard et al., 1994; Elias et al., 2000). The main advantage of this strategy resides in its technological simplicity, which makes it proper to be implemented in both low and large scale. However, the use of this kind of operation is limited to insect cell lines that do not accumulate toxic by-products, such as IPLB-Sf21 and Sf9. For other, cell lines, like BTI-TN-5B1-4 and saUFL-AG-286, fed-batch would not be a suitable strategy, since ammonia accumulation could be the cause of inhibition of viral replication.

The perfusion strategy, that implies the continuous removal of spent medium and its replacement by fresh medium with retention of the cell mass inside the cultivation device, has been also used. It has been employed to obtain high yields of recombinant proteins in insect cell cultures of high density, infected with genetically modified baculoviruses (Chico & Jäger, 2000). The advantage of this strategy with regard to fed-batch resides in the possibility to use it with cell lines that accumulate toxic by products, which are continuously removed with the spent medium. However, its implementation requires sophisticated devices to remove spent cell-free medium, making it unlikely use in the development of economically feasible processes for the production of insecticidal baculoviruses in large scale.

The production in culture systems that operate continuously appears to be an attractive option to reduce the operation costs in fermentation processes. Given its lytic nature, a continuous process for baculovirus production should involve the use of at least two cultivation devices, one for cellular propagation and a second one for virus infection and replication. The first reactor is continuously fed with fresh medium, and the viral product is continuously harvested from the second reactor, which is in turn fed with cell culture from the first reactor, also in a continuous way and at the same rate. Although it has been demonstrated that is possible to operate this kind of continuous process to produce baculovirus OBs by a limited time, the viral yield is affected rapidly by the passage effect, invalidating this strategy as an effective approach (van Lier et al, 1990).

6.4 Product yield and quality

The feasibility of a production process for an insecticidal baculovirus will finally reside on the yield and the quality of viral occlusion bodies. Rhodes (1996), through a detailed economic analysis of an *in vitro* process for production of an insecticidal baculovirus, has established that the limit yield to reach the economical feasibility should be at least 2×10^{11} $OBs.L^{-1}$. More recently, Nguyen et al. (2011) have argued that the minimum volumetric yield for an economically feasible process of production of HaSNPV in insect cell cultures should be 40 times higher. The OBs yields effectively reached for several baculoviruses in serum-free cultures of different cell lines, according to the available information, ranged from 1×10^9 to 3×10^{11} $OBsL^{-1}$, with cell specific yields ranging from 3 to 700 OBs per cell (Chakraborty et al., 1999; Gioria et al., 2006; Lua & Reid, 2003; McKenna et al., 1997; Micheloud et al., 2009; Rodas et al., 2005).

The insecticidal potency of baculovirus occlusion bodies that are produced in insect cell cultures is a controversial topic. Some papers have shown that occlusion bodies produced in infected insect cell cultures are less potent than polyhedra produced by infection of insect larvae (Chakraborty et al., 1999; McKenna et al., 1997). However, this reduced activity may not be an intrinsic characteristic of the occlusion bodies produced in cell cultures, but related to the extraction method used. Extraction with a solution of sodium dodecyl sulfate (SDS), a widely used procedure for releasing the occlusion bodies accumulated in the nuclei of infected cells, alters the structure of the polyhedron envelope and would reduce the content of occluded virus per polyhedron, and thus its insecticidal ability (Lua et al., 2003). Consequently, the extraction with SDS should be avoided for the purpose of preserving the quality of occlusion bodies. On the other hand, the occlusion bodies produced by infection in larvae contain an alkaline protease that is not codified in the viral genome, and that would be incorporated from the tissues of the infected insect. This protease, which is not present in the occlusion bodies produced in cell cultures, could be an additional factor of virulence, accelerating the dissolution of occlusion bodies in the insect midgut and thus contributing to increase its biological activity (Rohrmann, 2011). Finally, it has been also reported that the composition of the culture medium could affect the activity of occlusion bodies, but the causes are unknown (Pedrini et al, 2006).

7. Scaling-up: limitations and possibilities

Although the production of insecticidal baculoviruses in insect cell cultures has been proposed as an alternative to overcome the limitations of the processes *in vivo*, so far no process *in vitro* could be even implemented on an industrial scale, and occlusion bodies are

still produced in infected insect larvae. Some factors that 25 years ago have hindered the development of large-scale production processes for insecticidal baculoviruses in insect cell cultures, such as the sensitivity of insect cells to the stresses linked to the mechanical agitation in stirred tank reactors and to the bubble rupture in sparged bioreactors, have been resolved and several cell lines can be cultivated today in industrial bioreactors of large volume to produce occlusion bodies or recombinant proteins. However, other factors that still limit the development of feasible processes have not yet been satisfactorily resolved, and will be reviewed below.

Obtaining a cell line with relevant technological properties and with the ability to replicate the virus at a high yield of OBs, is a requirement to develop a feasible process for the production of an insecticidal baculovirus in insect cell cultures. Besides Sf9 and BTI-TN-5B1-4, there are few cell lines that fulfill these requisites. The cell line BCIRL-HZ-AM1, used to produce HaSNPV, is capable to grow in suspension cultures in a low-cost serum-free medium in stirred tank reactors. BCIRL-HZ-AM1 cells can produce high specific yields of HaSNPV OBs in infected cultures (Lua & Reid, 2003), but its ability to produce high yields of BVs, a property that is important for the scaling-up, is more limited (Pedrini et al., 2011). The cell line saUFL-AG-286, of election to produce AgMNPV, can generate high specific yields of OBs in serum-free suspension cultures, but the production of OBs is strongly inhibited at cell densities higher $8x10^5$ cells mL^{-1}, thus limiting the possibility to reach very high volumetric yields of OBs (Micheloud et al., 2009). As these cell lines are heterogeneous, the isolation of cell clones with improved ability to produce baculovirus OBs appears to be a reliable possibility to enhance the productivity of viral insecticides (Nguyen et al., 2011; Pasumarthy & Murhammer, 1994). For the production of other insecticidal baculoviruses will be necessary to establish new cell lines, obtained preferably from tissues of their respective target insects.

Another requisite that must be resolved before confronting the scaling-up of an insecticide baculovirus production process is the development of a low cost serum-free culture medium for the selected cell line. It has been indicated that the cost of the culture medium for an economically feasible process should not be higher than U$S 2.5 per liter (Rhodes et al., 1996), or it even should be lower than U$S 1 (Gong et al., 1997). Commercial serum-free media for Sf9 and BTI-TN-5B1-4 cells are sold at prices that are 30 times greater, and therefore are not useful for producing insecticidal baculoviruses at industrial scale. Besides, the cost of media specifically developed for producing insecticidal baculoviruses are yet above the acceptable limit for an economically feasible process. The rational approach to further reduce the cost of culture media for insect cell cultures is the simplification of the chemical composition, based on the deep knowledge of the nutritional demands and metabolism of insect cells, both uninfected and infected. However, most cell lines have not been sufficiently characterized as to progress towards a simplification of the composition of the culture medium. A more empirical approach to reduce the cost of the culture medium is the replacement of costly ingredients, such as amino acids and lipids, by optimized mixtures of raw materials of lower cost such as protein hydrolysates and cooking oil.

The usual strategy to produce baculovirus occlusion bodies in insect cell cultures has been the infection of batch cultures. However, the possibility to obtain high volumetric yields of viral OBs in batch cultures is impaired by the "cell density effect". Whenever possible, the adoption of alternative strategies of infection could be a way to overcome the cell density effect and thus improve the viral productivity. The fed-batch culture, which has proven to be a feasible alternative to increase the yield of recombinant proteins and BVs in Sf9 cell

cultures at high density, could also be an alternative strategy to increase the yield of occlusion bodies. A deeper understanding of the causes that lead to the manifestations of the cell density effect could help to design more rational feeding schedules than those used to date, and thus increase the viral productivity. However, the usefulness of the fed-batch strategy is restricted to cell lines that do not accumulate toxic by-products.

A large-scale process to produce insecticidal baculovirus OBs in insect cell cultures requires the completion of successive steps of viral amplification in growing scale (Rhodes, 1996). OBs are the final product of the whole process, but BVs are the product for each of the intermediate steps of scaling. Despite the importance to improve the yield of BVs, few studies have systematically explored the optimization of the production of this viral progeny (Carinhas *et al*, 2009). The optimization of BVs production could help to reduce the number of scaling steps necessary to get the number of virions needed to feed the OBs production reactor, and therefore reduce scaling cost. Furthermore, the reduction in the number of stages of scaling would contribute to limiting the probability of emergence of unproductive viral variants, such as FP mutants and DIPs. The approach patented by Lua and Reid (2005), using occluded virions extracted from occlusion bodies as seed, could alleviate the need for viral inoculum at the beginning of the scaling-up process, but does not prevent the need to improve the yields of BVs in the later stages. Additionally, the improvement of the ratio infectious particles/total particles, through better BVs preservation, could mean significant savings in the demand for seed virus, and therefore a step towards the feasible scaling-up of the viral insecticide production process in insect cell cultures.

8. Concluding remarks and perspectives

Baculoviruses are a group of arthropod-specific pathogens which have a significant potential to be used as safe and environmentally friendly insecticides in agriculture, horticulture and forestry. The replication of baculoviruses produces two viral progenies, budded and occluded viruses. The last are included into proteinaceous structures called occlusion bodies, which display insecticidal activity when ingested by susceptible insects. The current technology to produce insecticidal occlusion bodies is based on the viral infection of susceptible insects, but an alternative technology based on the viral replication in insect cell cultures could aid to overcome some of the limitations of the former. The insect cell line, the culture medium, the bioreactor, the virus, the infection parameters and the culture strategy are elements of the insect cell culture technology that must be optimized in order to develop i*n vitro* production processes for insecticidal baculoviruses. While it is now possible to grow insect cells in large-scale industrial reactors using serum-free media to produce high yields of occlusion bodies for several baculoviruses, the current technology is still insufficient to achieve economic feasibility. To do that, in the next future the efforts should be mainly orientated:

- to gain deep insight over the insect cell biology in order to identify the factors responsible of the cell density effect;
- to improve the composition and to reduce the cost of serum-free culture media for insect cell cultures;
- to increase, through the manipulation of the infection parameters in batch cultures or, whenever possible, through optimized fed-batch strategies, the volumetric yield of occlusion bodies;

- to optimize the production of budded virus in order to reduce the length and the cost of the viral inoculum scaling-up, and to minimize the risk of generating and selecting unproductive viral variants.

Only obtaining satisfactory solutions for these remaining problems will make possible to establish economically viable processes for the production of insecticidal baculoviruses in insect cell cultures on an industrial scale.

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8

Factors Affecting Performance of Soil Termiticides

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1. Introduction

Although baits have increased in popularity in recent years, the application of liquid termiticide to soil remains the most widely used method for protecting structures against subterranean termites (Anonymous, 2008). In addition to fast acting, repellent toxicants such as bifenthrin and other pyrethroids that act as barriers to termite movement, non-repellent, slower acting compounds including fipronil, imidacloprid and thiamethoxam are now among the preferred soil treatments. Delayed toxicity can provide opportunity for horizontal transfer of the active ingredient, potentially reducing termite activity (Remmen & Su, 2005; Shelton & Grace, 2003). While there is some evidence of colony suppression or elimination following perimeter treatments with imidacloprid (Parman & Vargo, 2010), other studies have shown that a reduction in activity occurs over only a small portion of a colony's foraging range, making it unlikely that soil treatments affect the overall termite population (Osbrink et al., 2005; Rust & Saran, 2006; Saran & Rust, 2007; Su, 2005). This limited potential for transfer emphasizes the importance of bioavailability of termiticides in soil over an extended period of time.

Failure of soil termiticide treatments is often related to factors other than the active ingredient (Su & Scheffrahn, 1990b). Efficacy and longevity of soil treatments varies greatly with application rate, soil properties (Gold et al., 1996; Su & Scheffrahn, 1990b), termite pressure (Jones, 1990), and application technique (Forschler, 1994; Su et al., 1995). Factors influencing the performance of soil termiticides can be grouped into those that determine toxicity, bioavailability, or persistence. Each of these factors is affected by properties of the termiticide and soil (Gold et al., 1996; Spomer et al., 2009; Wiltz, 2010). Although some generalizations can be made about relative toxicity of different termiticides, susceptibility differences occur among species and colonies (Beal & Smith, 1971; Osbrink & Lax, 2002). Termiticide rate and application technique influence both bioavailability and long-term persistence (Peterson, 2010). Termite population pressure and satellite nests can reduce availability of the toxicant. Finally, other environmental factors such as moisture, temperature, and microbial communities affect termiticide degradation (Baskaran et al. 1999, Saran & Kamble 2008).

2. Soil termiticides

Long-term studies evaluating chemicals as potential termiticides were initiated in the 1920's and 1930's (Randall & Doody, 1934), but it was not until after World War II that the

cyclodienes, a class of chemical compounds identified as highly effective termiticides, became commercially available (Ware, 2000). Pre-construction soil treatments with cyclodienes became the standard method of subterranean termite prevention from the late 1940s until 1988 (Lewis, 1980; Su & Scheffrahn, 1990b). The cyclodienes, particularly chlordane, were extremely efficacious and stable in soil, often protecting structures from subterranean termite infestation for several decades (Grace et al., 1993; Lenz et al., 1990; Su & Scheffrahn, 1990b).

Because of their residual longevity, questions were raised about the environmental impact of these chemicals (Lewis, 1980; Su & Scheffrahn, 1990a; Wood & Pierce, 1991). Chlordane and related chemicals were banned in most of the world in the 1970's and 1980's (Ware, 2000). However, they constitute a major environmental problem because of their high toxicity, persistence in the environment, and ability to bioaccumulate in the food chain and because they are still being used in certain countries for agricultural and public health purposes (Itawa et al., 1993; Ntow, 2005; Xue et al., 2006).

Following the loss of chlordane as a soil termiticide, the only termiticides available for use as soil barrier treatments were chlorpyrifos (an organophosphate) and several pyrethroids. The residual activity of chlorpyrifos was significantly shorter than that of the cyclodienes (Grace et al., 1993; Lenz et al., 1990). As a result of the Food Quality Protection Act of 1996, the U. S. Environmental Protection Agency (EPA) revised its risk assessment of chlorpyrifos and, in 2000, the use of chlorpyrifos as a soil termiticide was canceled (EPA, 2000).

With the loss of chlorpyrifos, pyrethroids were the primary weapon available for subterranean termite prevention. The pyrethroids are more persistent than chlorpyrifos, but less stable in the soil than the cyclodienes (Lenz et al., 1990; Pawson & Gold, 1996; Su & Scheffrahn, 1990b). Soil barriers composed of pyrethroids are more likely to fail than barriers composed of cyclodienes or chlorpyrifos (Forschler, 1994; Kard, 1999; Lenz et al., 1990; Su & Scheffrahn, 1990b; Su et al., 1993) because pyrethroids are repellant to subterranean termites (Rust & Smith, 1993; Su & Scheffrahn, 1990b; Su et al., 1993).

Beginning in 2000, several new nonrepellant soil termiticides appeared on the market: fipronil, a phenyl pyrazole (Aventis Corp., 2001), imidacloprid, a chloronicotinyl (Bayer Corp., 2000), and chlorfenapyr, a pyrrole (BASF Corp., 2001). Nonrepellant termiticides are an improvement over the pyrethroids because subterranean termites cannot detect gaps in the treatment and use them to gain access to structures (Potter & Hillery, 2001). Subterranean termites are unable to detect the termiticide and do not avoid soil that has been treated with them (Kuriachan & Gold, 1998). Chlorantraniliprole is a new termiticide belonging to the anthranilic diamide class of insecticides. It targets a unique receptor site, the ryanodine receptor, causing the release of stored calcium, resulting in loss of muscle control, cessation of feeding, and eventually death of the termite (Cordova et al., 2006). Unlike other soil termiticides, chlorantraniliprole has no known health effects to humans and no personal protective equipment is required for application (Dupont, 2010). Also being developed for subterranean termite control is indoxacarb, an oxadiazine proinsecticide that is metabolically activated after entering the insect (Spomer et al. 2009; Wing et al., 2000).

A large amount of the variability in effectiveness of different soil treatments can be attributed to the termiticide itself. In a study evaluating *Coptotermes formosanus* mortality on treated soils, bifenthrin performed better than fipronil or chlorfenapyr (Wiltz 2010). Bifenthrin was also found to have the highest activity against *Reticulitermes hesperus* when compared with other pyrethroids (Smith & Rust, 1990).

Saran and Rust (2007) found that *R. hesperus* tunneled through untreated sand and stopped near the interface of fipronil treated sand. There was little tunneling in the treated sand, but termites tunneled close enough to obtain a lethal dose of fipronil. To some extent, *C. formosanus* and *Reticulitermes flavipes* penetrated sand treated with 0 - 64ppm fipronil, indicating non-repellency, but complete penetration of the treated sand was prevented by high mortality (≥88% for *C. formosanus* and ≥89% for *R. flavipes* after 7 d) (Remmen & Su, 2005). While several studies conducted in small laboratory arenas have found high mortality in fipronil treatments, extended foraging arena assays demonstrated that fipronil barriers can split termite populations, with high mortality occurring close to the treatment site, but little mortality at distances >5 m (Su, 2005).

Although imidacloprid is slow to induce mortality, mobility impairment occurs within hours of exposure (Thorne & Breisch, 2001). Imidacloprid is non-repellent (Remmen & Su, 2005), but this combination of delayed mortality and rapid mobility impairment results in limited movement of termites into treated barriers and limited mortality after 7d in close proximity to imidacloprid-treated sand.

Several studies have demonstrated differences in degradation rates among insecticides. Baker and Bellamy (2006) found that of the termiticides tested, the organophosphate, chlorpyrifos, degraded the quickest, while chloronicotinyls and pyrethroids degraded at slower rates. Horwood (2007) measured termiticide residues in a weathered sand: loam mixture, finding that bifenthrin and chlorfenapyr were more persistent than chlorpyrifos, fipronil, and imidacloprid. Horwood (2007) found that after 15 months, chlorpyrifos and fipronil concentrations at lower depths were little changed from the time of treatment, but there was a major reduction in imidacloprid concentration at all depths.

3. Soil-termiticide interactions

Because soil consists of a heterogeneous mixture of mineral and organic particles, it is difficult to predict the influence of soil type on termiticides. When soil conditions fall outside an optimum range, termiticides can be immobilized or adsorbed by the soil or altered chemically to an inactive form.

Laboratory studies have found interactions between soil and termiticide properties. Effects of clay (Henderson et al. 1998; Smith & Rust 1993) and organic carbon (Felsot & Lew 1989; Forschler & Townsend, 1996; Gold et al., 1996; Spomer et al., 2009) content on bioavailability to termites differ with termiticide. Termiticide effectiveness diminishes over time, especially on soils that pose bioavailability problems (Gold et al., 1996; Su et al., 1993; Tamashiro et al., 1987).

Variation in soil properties, such as pH, clay and organic matter content, soil moisture, and electrolyte concentration, influence the adsorption and desorption characteristics of termiticides to soils. Of equal importance are the physical and chemical properties of the toxicant, including concentration, pH, and solubility.

3.1 Mobility

Mobility is one of the most important factors in determining bioavailability and efficacy of a soil treatment. If a pesticide is too mobile, it fails to protect the structure, while increasing risk of groundwater contamination. However, if the chemical is too tightly bound to soil particles, bioavailability is limited. Mobility is affected by the pesticide's sorption, water solubility, and vapor pressure and by external influences that include soil properties,

weather, topography, and vegetation. Sorption describes the attraction between a chemical and soil, vegetation, or other surfaces. However, the term most often refers to the binding of a chemical to soil particles. Sorption is defined as the attraction of an aqueous species to the surface of a solid (Alley, 1993). The sorbing species, usually an organic compound, is called the sorbate, and the solid, usually soil, to which the sorbate is attracted is known as the sorbent. This attraction results from some form of bonding between the chemical and adsorption receptor sites on the solid. Several mechanisms may operate in a particular situation, including ionic attraction, hydrophobic attraction, and hydrogen bonding. For pesticides that are weak acids or bases, sorption is influenced by soil pH.

Sorption is also influenced by soil moisture, organic matter content, and texture. Pesticides are more readily sorbed onto dry soil because water competes with pesticides for binding sites in moist soil. More sorption occurs in soils made largely of clay and organic matter. Organic matter and clay particles have small particle size, large surface area, and high surface charge. Sand particles provide less surface area for sorption, making pesticides more likely to move away from the point of application.

Several parameters are used to describe a pesticide's sorption behavior in soils. Table 1 contains sorption parameters for selected chemicals currently and previously used as soil termiticides.

Two related measures of a pesticide's sorption are the sorption coefficient (K_d) and the soil organic carbon coefficient (K_{OC}). These coefficients are ratios of adsorbed to dissolved pesticide for a specific soil (K_d) or for the organic carbon fraction of a soil (K_{OC}). These values are useful for broadly discriminating between leaching classes of pesticides, but actual adsorption depends on many factors, including soil moisture, temperature, soil pH, and type of organic matter (Wauchope et al., 2002).

Termiticide	K_{OC} (L/kg)	Log K_{ow}	H_2O Solubility (mg/L)	Henry's law constant ($atmm^3/mol$)	Reference
Bifenthrin	1.31×10^5 - 3.02×10^5	6.0	0.1 (25°C)	7.2×10^{-3}	Fecko (1999)
Chlordane	4.19 – 4.39	2.78	1.0×10^{-4}	1.3×10^{-3}	USEPA (1986)
Chlorantraniliprole	3.3×10^2 (average)	2.8	1.023 (20°C)	3.1×10^{-15}	USEPA (2008)
Cypermethrin	6.1×10^4 (average)	6.6	4×10^{-3} (20°C)	2.5×10^{-7}	Jones (1999)
Fipronil	3.8×10^3 – 1.2×10^4	4.01	2.4 (pH 5) 2.2 (pH 9)	3.7×10^{-5}	Connelly (2001)
Imidacloprid	1.3×10^2 - 3.1×10^2	0.57	514 (20°C, pH 7)	6.5×10^{-11}	Fossen (2006)
Indoxacarb	2.2×10^3 - 9.4×10^3	4.7	0.2 (25°C)	$< 6.1 \times 10^{-10}$	USEPA (2000)

Table 1. Soil sorption parameters of selected soil termiticides.

Because sorption coefficient values for the same pesticide vary widely with soil properties, reported values are not included in Table 1. However, K_d values are useful for comparing sorption of different chemicals to the same soil. The organic carbon sorption coefficient is a property of the pesticide and is independent of soil organic matter. The sorption coefficient and organic carbon sorption coefficient are related by the equation:

$$K_d = Koc\ (\%O.C.) \quad (1)$$

Where O.C. is the percentage organic carbon the soil contains. This relationship shows that as the organic fraction of soil increases, the distribution coefficient, $K_{d,}$ increases. For this relationship to hold true, the chemical must be non-ionic because soil pH affects sorption of ionic sorbates.

As Table 1 illustrates, K_{OC} values for a pesticide are not constant. Pesticide concentration affects adsorption (Kamble & Saran, 2005), but not to an extent that prevents comparison of relative mobility of different pesticides. For polar solutes, surfaces other than organic carbon can become important sorbents particularly when soils are low in organic matter (Cheung et al., 1979; Cox et al., 1998; Means et al., 1982).

Another useful measure of potential pesticide mobility is the octanol - water partition coefficient (K_{ow}). K_{ow} is a measure of the hydrophobicity of an organic compound. The more hydrophobic a compound, the less soluble it is, therefore the more likely it will adsorb to soil particles (Bedient et al, 1994). To evaluate hydrophobicity, the organic solvent octanol is used as a surrogate for organic matter. The octanol-water partition coefficient is the ratio of the concentration of a chemical in octanol and in water at equilibrium and at a specified temperature. K_{ow} is determined by adding a known amount of the pesticide to equal volumes of octanol and water. The coefficient is determined by calculating the concentration in the octanol phase compared to the concentration in the water phase. K_{ow} values vary by several orders of magnitude and may be reported as either K_{ow} or log K_{ow} values. The octanol-water partition coefficient is correlated with water solubility; therefore, the water solubility of a substance can be used to estimate its octanol-water partition coefficient.

Water solubility describes the amount of pesticide that will dissolve in a known amount of water. Highly soluble pesticides are more likely to be moved by runoff or leaching. As with sorption parameters, solubility values are useful as a means of comparison, but actual values will vary with field conditions. Solubility is affected by temperature, water pH, and the presence of other chemicals. The solubility of a compound tends to be inversely proportional to the amount of sorption that it can undergo.

In addition to being adsorbed to soil or transported by water, pesticides can be volatilized. Pesticide volatilization from moist soil is described by the Henry's law constant (K_h). K_h is defined as the concentration of pesticide in air divided by the concentration in water. K_h can be measured experimentally or estimated by dividing the saturation vapor pressure of the compound by its solubility (Suntio et al., 1988). Like other sorption parameters, K_h is temperature dependent, but values are useful for comparing volatility of different compounds. Pesticides with higher K_h are more likely to volatilize from moist soil. Because sorption affects the amount of pesticide in the soil water, the tendency to volatilize from moist soil depends on both the Henry's law constant and sorption coefficients.

3.2 Clay

Soil texture has a strong impact on termiticide performance, but effects differ with insecticide. In assays conducted with bifenthrin, chlorfenapyr, and fipronil, *C. formosanus* mortality was generally highest when clay content was low (Wiltz, 2010). Clay content of soil was significantly related to termite mortality across all termiticides, rates, and exposure times (Wiltz, 2010). Likewise, Osbrink and Lax (2002) found that *C. formosanus* workers experienced greater mortality in fipronil-treated sand than in treated potting soil or a mixture of soil and clay. Bobé et al. (1997) reported that for fipronil there was a significant decrease in adsorption coefficient as the soil clay content decreased, thereby increasing bioavailability. However, the opposite result occurs with some insecticides. Smith and Rust (1993) found that increased clay content increased the toxicity of certain pyrethroids, such as cypermethrin. The authors concluded that cypermethrin and clay apparently interacted creating a formulation similar to a wettable powder, which may have an increased affinity for the nonpolar termite integument. Gao et al. (1998) investigated the adsorption of seven pesticides and metabolites with different physiochemical properties, finding that adsorption was generally more effective on smaller and larger soil particles than on intermediate-sized particles.

3.3 pH

Effects of pH on adsorption and desorption vary with insecticide chemistry and interact with other soil properties. Low pH soils increase the adsorption of weakly acidic pesticides (Boivin et al., 2005; Carrizosa et al., 2000; Halfon et al., 1996,). Desorption of endosulfan was higher at both acidic and alkaline pH ranges compared to neutral pH (Kumar & Philip, 2006). The authors found that in clay soil, adsorption decreased drastically when the pH was reduced. In soil column experiments, deltamethrin was essentially immobile in three different soils. Kaufman et al. (1981) suggested that for nonacidic soils, the pH may be a primary factor affecting mobility of deltamethrin. In bioassays of treated soils against *C. formosanus*, there was an interaction between effects of soil pH and clay content on effectiveness of chlorfenapyr and fipronil (Wiltz, 2010).

3.4 Organic carbon

Partitioning of insecticides between soil organic matter and soil solution affects bioavailability (Felsot & Lew, 1989). Like clay, organic matter decreases adsorption of fipronil (Bobé et al., 1997). Mulrooney and Gerard (2007) applied fipronil to 3 soils and found that *R. flavipes* mortality decreased with increasing organic carbon. Although no soil effects were found when soils were treated at label rates, pyrethroids applied at low rates were less available in soils with high OC (Henderson et al., 1998). Soil OC has been shown to affect adsorption of several non-acidic pesticides, but have little or no effect on weakly acidic chemicals (Barriuso & Calvet, 1992; Boivin et al., 2005; Worrall et al., 2001).

4. Application technique

In addition to properties of the soil and chemicals, variations in application technique can influence availability, persistence, and impenetrability of toxins. Such variables include gaps in soil treatment, thickness of treated layer, and watering method. Within certain ranges of application rates, availability increases with rate; however, the opposite is true at other rates.

Initial concentration of termiticides in soil varies from several hundred to over one thousand micrograms per gram. Kard and McDaniel (1993) reported initial concentrations of 858± 990mg/g after application to a Mississippi soil, and Davis and Kamble (1992) reported initial concentrations of chlorpyrifos as high as 1500mg/g in a Nebraska loamy sand soil. Application rate affects both initial availability and degradation rate. Saran and Kamble (2008) reported an inverse relationship between the initial concentrations of bifenthrin, fipronil, and imidacloprid and their LT50 and LT90 values against *R. flavipes*. Greater bioavailability at higher concentrations may explain similar trends reported by Smith and Rust (1992), Forschler and Townsend (1996), and Ramakrishnan et al. (2000). At low rates, fipronil has low soil affinity, but adsorption increases with concentration (Bobé et al., 1997). Kamble and Saran (2005) found that at termiticide application rates of 0.06–0.125%, there is a reversal in the fipronil adsorption process, whereby there is a decrease in adsorption coefficient with an increase in concentration, resulting in an increase in bioavailability. Chlorpyrifos exhibited a lower degradation rate when applied at ≈1,000 µg/g soil than when applied at typical agricultural levels of 0.3–32 µg/g (Racke et al., 1994). When fipronil was applied at the labeled rate for locust control (8g AI per ha), 75% degraded within 3 d (Bobé et al., 1998). However, when applied at termiticidal rates (60-125 µg AI per g), fipronil did not show much degradation, and no metabolites were detected in residue analysis after 180 d (Saran and Kamble 2008). Gahlhoff and Koehler (2001) found that concentration and treatment thickness significantly affected both mortality and penetration by *R. flavipes* into imidacloprid-treated soil, with mortality remaining low after 7 d exposure to low concentrations, as well as affecting bioavailability, high termiticide concentrations may indirectly affect degradation by negatively impacting bacterial and fungal populations, resulting in prolonged inhibition of soil dehydrogenase and esterase activities (Felsot & Dzantor, 1995). Racke et al. (1996) examined hydrolysis of chlorpyrifos in 37 soils at agricultural application rates (10mg/g) and observed that in some alkaline soils hydrolysis constituted the major degradation pathway. However, they also noted that in several soils, with pH values in the range of 7.1 to 8.5, the hydrolytic reaction was inhibited at higher concentrations (1000mg/g).

Termites can circumvent soil treatments by using untreated gaps, building materials, or debris as bridges between the surrounding soil and structure (Forschler, 1994; Smith & Zungoli, 1995; Su & Scheffrahn, 1998). Subterranean termite foragers are able to detect and avoid repellant termiticides so areas treated with pyrethroids are rarely contacted. The subterranean termites' ability to detect chemical barriers allows termite foragers to follow the edge of the pyrethroid treated area until they find a gap in the treatment (Forschler, 1994; Rust & Smith, 1993; Su & Scheffrahn, 1990b; Su et al., 1982). Thus, gaps in pyrethroid applications may actually funnel foragers toward the structures they are intended to protect (Forschler, 1994; Kuriachan & Gold, 1998). The inevitability of gaps in soil termiticide barriers is a major limitation to the efficacy of repellant liquid termiticides (Forschler, 1994; Kuriachan & Gold, 1998). Gaps may exist in a soil termiticide treatment for a number of reasons. Pre-construction treatments often contain gaps due to imperfect initial application or physical disturbance of the soil after application (Koehler et al., 2000; Su & Scheffrahn, 1990a, 1998). When an existing structure becomes infested and requires a remedial termiticide application, it is difficult to create a continuous horizontal barrier of liquid termiticide beneath the structure (Su & Scheffrahn, 1990a, 1998; Koehler et al., 2000). Finally, all termiticides degrade over time. An ageing soil treatment, applied below the foundation

before a structure was built, is inaccessible after construction and cannot be reapplied (Su & Scheffrahn, 1990a; Su, 1997; Koehler et al., 2000).

The total volume of pesticide suspension applied to soil affects penetration depth and concentration in the soil. In tests using imidacloprid and fipronil in five different soils, when equal amounts of pesticide were diluted in different volumes of water, the higher volume treatments penetrated further into the soil, but the more concentrated treatments deposited more pesticide in the top 1cm of soil (Peterson 2010). It is likely that the thicker barrier of lower active ingredient concentration would provide better protection, at least in the short term because it might be better able to withstand disturbances to the top 1 cm of soil. Additionally, termites are less able to tunnel through thicker barriers of lower active ingredient concentration than through thinner barriers of higher concentration (Smith et al. 2008). However, pesticide treatments with low initial concentrations degrade faster than those with higher initial concentrations (Bobé et al., 1998; Felsot & Dzantor, 1995; Saran & Kamble, 2008). In addition to total volume of liquid applied, initial thickness of the treated zone depends on soil and termiticide properties. Smith and Rust (1992) found that termiticidal amounts of chlordane and cypermethrin moved to soil depths of at least 7 cm, while chlorpyrifos moved to a depth of at least 30 cm.

5. Environmental factors

Both biotic and abiotic pathways have been found to be important for insecticide degradation and transformation in soils (Racke et al., 1996).

5.1 Moisture

Water can compete with pesticides for sorption sites on soil particles. Dry soils become more sorptive for both polar and non-polar chemicals (Chen et al., 2000). However, chemicals with low polarity are released when soil becomes wet (Harper et al., 1976). Repeated cycles of wetting and drying affect pesticide availability and degradation, but depend on properties of the chemical, soil, number of wetting and drying cycles, time since pesticide application, and time since wetting (Garcia-Valcarcel & Tadeo, 1999; Xia & Brandenburg 2000; Ying & Kookana, 2006; Peterson, 2007).

5.2 Temperature

Soil temperature affects termiticide bioavailability through its influence on solubility and adsorption. In addition to its effect on the physical and chemical properties of the pesticide, extreme temperatures affect the rate of microbial degradation, as described in the following section. Several studies have demonstrated that temperature affects adsorption of pesticides to soil, but that the nature of this effect varies among pesticides. Although most of the work on pesticide availability and degradation has been conducted in the temperate climates of North America and Europe, soil temperature is likely to play an important role in termiticide degradation in tropical regions. Khan et al. (1996) found that lindane adsorption to silty loam and silty clay loam soils increased with temperature. Likewise, Valverde-Garcia et al. (1988) found that higher temperatures increased the adsorption of the fungicide thiram and the organophosphate insecticide dimethoate to organic soils. Temperature may interact with pH, particularly in saturated soils. In aqueous solutions, fenamiphos, fipronil, and trifluralin degradation increased with temperature and pH (Ramesh & Balasubramanian, 1999). Other studies have demonstrated a reduction in pesticide

adsorption at higher temperatures. Dios-Cancela et al. (1990) found that sorption of the herbicide cyanazine to peats decreased with increasing temperature.

5.3 Micromial degradation

Microbial degradation occurs when fungi, bacteria, and other soil microorganisms use pesticides as food or consume pesticides along with other substances. Activity of microbes is affected by soil organic matter and texture and is usually highest in warm, moist, well-aerated soils with a neutral pH. Because microbial degradation is mediated by enzymes, temperature is important in determining the rate degradation: the rate of most reactions catalyzed by enzymes tends to double for each 10˚C increase in temperature between 10˚ and 45˚C and is greatly reduced above and below these temperatures.

Naturally-occurring pesticide-degrading microorganisms may be relatively rare in pristine environments and non-exposed agricultural soils (Bartha, 1990). Some of the pesticide-degrading microbes that have been identified include *Arthrobacter, Brevibacterium, Clavibacter, Corynebacterium, Micromonospora, Mycobacterium, Nocardia, Nocardioides, Rhodococcus* and *Streptomyces* genera (De Schrijver and De Mot, 1999).

A review of earlier work on organophosphate and carbamate insecticide degradation was prepared by Laveglia and Dahm (1977). Although organophosphates are no longer used in many parts of the world, there have been several recent studies on their degradation by microbes. Li et al. (2007) reported the isolation of a bacterium, *Sphingomonas* sp., that degrades chlorpyrifos, parathion, parathion-methyl, fenitrothion and profenofos. However, several other studies have found little microbial degradation of chlorpyrifos. Goda et al. (2010) showed that the intact cells of *Pseudomonas putida* IS168 were able to degrade fenitrothion, diazinon and profenofos when present as sole carbon sources, but failed to grow on chlorpyrifos. Trichloropyridinol (TCP), one of the main chlorpyrifos metabolites, has antimicrobial properties (Cáceres et al., 2007; Feng et al., 1997; Racke et al., 1990), possibly accounting for the scarcity of chlorpyrifos-degrading microorganisms. Degradation of pyrethroids in soil has also been extensively studied (Gan et al., 2005; Jorhan & Kaufman, 1986; Kaufman et al., 1981; Lee et al., 2004; Lord et al., 1982). Most of these studies show that microorganisms play an important role in the degradation of pyrethroid compounds in soils and sediments.

6. Termite pressure and susceptibility

In studies evaluating termite tunneling through chlordane, chlorpyrifos, or permethrin treated soil, large groups of termites were able to tunnel farther than small groups (Beal & Smith, 1971; Jones, 1990). At low population density, different colonies of *C. formosanus* either totally avoided permethrin-treated soil or tunneled slightly (Jones, 1989, 1990). Jones (1990) found that while large groups of termites tunneled more than small groups in soils treated with chlordane, chlorpyrifos, or permethrin, group size had different effects on mortality in different soil treatments. Several experiments have demonstrated correlations between termite survival rates and population density (Lenz et al., 1984; Lenz, 2009; Santos et al., 2004). At population densities below 0.1 g termites / ml, Lenz et al. (1984) found that, in the absence of termiticide treatment, survival of *Coptotermes lacteus* (Froggatt) and *Nasutitermes exitiosus* (Hill) increased with population density.

Susceptibility differences occur among termite species and colonies. Most soil termiticide evaluations have included only one target species. However, in studies comparing

responses of two or more species, there are frequently differences in susceptibility. *C. formosanus* penetrated soil treated with aldrin, chlordane, dieldrin, or heptachlor, while *R. virginicus* and *R. flavipes* failed to penetrate lower rates of the same chemicals and were killed more quickly than *C. formosanus* (Beal & Smith 1971). In a laboratory assay, chlorpyrifos, permethrin, cypermethrin, bifenthrin, isofenphos, lambda-cyhalothirn, and fenitrothion all provided equal barrier protection against *R. flavipes* (Su et al. 1993). However, in the same assay, *C. formosanus* generally tunneled deeper into sand treated with organophosphates than with pyrethroids. Penetration of sand treated with thiamethoxam or fipronil was similar for *C. formosanus* and *R. flavipes*, but thiamethoxam was more toxic to *C. formosanus* than to *R. flavipes* (Remmen & Su 2005). Osbrink and Lax (2002) evaluated seven insecticides against termites from colonies that had been previously been identified as either insecticide susceptible or tolerant, finding differences in substrate penetration and mortality among colonies and insecticides.

Termite traits other than population size or susceptibility to toxicants can increase the likelihood that soil treatments will fail to protect a structure. Aerial infestations account for a large percentage of structural infestations by *C. formosanus* (Su & Scheffrahn, 1990), making soil treatments ineffective. Additionally, *C. formosanus* colonies may seal off or avoid treated areas (Su et al. 1982) when repellent toxicants are used, but use gaps in the soil barrier to access the structure (Forschler, 1994).

7. Conclusion

Several long-term studies of termiticide persistence have been conducted. In USDA Forest Service trials, which have been conducted for the past 40 years, tests consist of treated soil plots covered by concrete slabs. Treatments are considered failures when termites penetrate >50% of field replicates to reach a wood block placed in a pipe running through the slab (Kard, 2003; Wagner, 2003). In these tests, longevity differed with geographic location and termiticide class (Mulrooney et al., 2007). Such studies have the advantage of being performed under natural soil weathering conditions for an extended period of time and provide a standard method of comparing termiticides. However, products are evaluated on a limited number of soils and it is impossible to tell if a lack of penetration into plots should be attributed to effectiveness of the termiticide or to the absence of termite pressure. To overcome this problem, other studies have included laboratory bioassays coupled with field termiticide persistence studies (Gold et al., 1996; Grace, 1991; Su et al., 1993). Unfortunately, most of these studies have evaluated relatively few soil types. Because performance is so dependent on a combination of temiticide and soil properties and weathering, more research is needed to evaluate new and existing products under a larger range of conditions. Soil termiticides have been extensively evaluated for toxicity, bioavailability, and degradation. However, reasons for termiticide failure are complex and often local in nature, indicating the need for more research and localized treatment recommendations regarding choice of toxicant, application technique, and treatment frequency.

8. References

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9

Alternatives to Chemical Control of Insect Pests

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1. Introduction

In 2011, practitioners and advocates of Integrated Pest Management (IPM) find themselves addressing agricultural, societal, and political pressures worldwide resulting from human population growth. This growth brings simultaneous burdens of sustaining a steady food supply; these include preventing losses from pests, dealing with increased human global travel, which in turn intensifies opportunities for the establishment of non-endemic pests into new ecosystems, and addressing global climate change that potentially will shift pest distributions into new areas. Concurrently, societal concerns about pesticide presence in our food and environment have resulted in political and economic pressures to reduce chemical pesticide use, or at a minimum, emphasize the development and use of products that are less toxic and more environmentally safe. These concerns drive the discovery and development of alternatives to chemical control of plant pathogens, weeds, and insect pests.

The term Integrated Pest Management has, more often than not, been identified with entomologists. Stern et al. (1959) first used the term "integrated control" to describe the potential for integration of chemical and biological control tactics. Yet from a historical view, the concept of integrating chemical control with other tactics was proposed much earlier (Hoskins et al., 1939). Furthermore, integrating multiple non-chemical tactics to control a pest has been a cornerstone of the discipline of plant pathology throughout much of its early history (Jacobsen, 1997). In fact, because plant pathologists did not have an array of corrective pesticides available to them, the development and integration of control methods that emphasized non-pesticide controls (e.g., genetic host resistance, crop rotations, tillage, and plant sanitation) for plant diseases was a necessity, not simply an option for plant disease management. In contrast, entomologists and weed scientists were more insulated from that necessity due to the availability of relatively inexpensive pesticides to correct a problem.

Several events stimulated the necessity for developing IPM programs in entomology, including those that emphasized development of non-chemical methods of insect control (e.g., cultural, biological, and physical control described herein). The chlorinated hydrocarbon, DDT, had been used for control of various insects since the 1950's. Soon after its use began, some pests began to develop resistance to DDT, including house flies, mosquitos, bed bugs, and body lice (Metcalf, 1989). The publication of Rachel Carson's book, "Silent Spring", in 1962 also generated public concern. Carson highlighted the negative

impacts that widespread use of insecticides could have on the environment and ultimately, human health. What followed was a passionate global reaction that generated intense economic and political pressure to regulate pesticide use and monitor their relative impacts on biological systems. In the United States, the Environmental Protection Agency was created and charged with regulating the registration of all pesticides through the Federal Insecticide, Fungicide and Rodenticide Act (as amended in 1972). Concerns over pesticide use also stimulated the political thrust necessary for support of IPM programs. In the United States and worldwide, IPM flourished in the following three decades and was adopted as policy by various governments (Kogan, 1998).

Today, IPM has attained many successes but fallen short on some issues. Due to the awareness and biological understanding of how insecticide resistance develops, and because insecticides are so expensive to develop, in 1984 the manufacturers of insecticides created the Insecticide Resistance Action Committee (IRAC) to encourage the responsible use of their products in a manner that minimizes the risk of insecticide in target pest populations (IRAC, 2010). New calls have been made for changing the direction of IPM in response to waning political support for funding IPM programs. Frisbie & Smith (1989) coined the term "biologically intensive" IPM, which involves reliance on ecological methods of control based on knowledge of a pest's biology. Benbrook et al. (1996) promoted the idea of moving IPM along a continuum from simple to complex, or 'biointensive". The National Research Council officially introduced the term, "Ecologically Based Pest Management", calling for a new paradigm for IPM in the 21st Century (National Research Council, 1996); eight years earlier, however, Horn (1988) outlined how principles of insect ecology could be incorporated into insect pest management strategies. More recently, Koul & Cuperus (2007) published "Ecologically Based Integrated Pest Management", essentially capturing the breadth and depth of the evolution that IPM has undergone over the past 60 years. While the scope of the "New Solutions" aspect of the NRC's charge has been challenged (Kogan, 1998; Royer et al., 1999), the term "ecologically based" has become infused into the IPM lexicon.

2. Cultural control methods to reduce insecticide applications

Cultural controls are management tools and activities that make the crop habitat less favorable for pests to survive and cause damage (Horne & Page, 2008). Cultural management practices may make the crop or habitat inhospitable to pests directly, for example, by planting cultivars resistant to pest feeding or rotating crops to deny overwintering pests their preferred food source. Cultural management practices can also make the habitat less hospitable to pests in an indirect manner by encouraging natural enemies (predators and parasitoids) to enhance biological control (see Section 3).

Cultural control is a key pest management tool available to growers because the crop variety, habitat, and selected inputs set the stage for future pest fitness and abundance. Thus, implementing preventive cultural control tactics that slow pest population growth can delay or negate the need for insecticide applications and significant plant damage. In this section we outline the major types of cultural control tactics available to growers and other pest management personnel. Our objective is to demonstrate the breadth of tactics that are used, although we do not have the space to consider them in depth. We draw examples from a diversity of well-studied plant systems from field crops to ornamental landscapes to provide examples of how they affect plant-herbivore-natural enemy interactions to reduce pest abundance and damage.

2.1 Cultural control via plant resistance

Plant resistance to herbivores is a cultural control strategy having the most direct influence on herbivore behavior, fitness, and damage. Plant resistance is achieved through three general mechanisms: antibiosis, antixenosis, and tolerance. Antibiosis is the adverse effect of plant physical or chemical traits on arthropod biology (Painter, 1951). This may include reduced size, survival, fecundity, or longevity and increased development time or mortality. Antixenosis is the effect of plant traits on herbivore behavior that reduces herbivore interactions with the plant (Painter, 1951). These effects can include reduced feeding, preference, residence time, or oviposition on plants having particular traits such as trichomes or defensive compounds. Tolerance is a plant trait that reduces the impact of herbivory on plant growth, allowing tolerant plants to sustain herbivore damage but maintain yields similar to undamaged plants (Painter, 1951).

Physical plant traits such as leaf pubescence, trichomes, and epicuticular wax, and chemical traits such as alkaloids and terpenoids have antibiotic and antixenotic effects on herbivores (Kennedy & Barbour, 1992; Painter, 1951). In the well-studied tomato production system, effects of leaf trichomes as a plant resistance trait are well documented (Kennedy, 2003; Simmons & Gurr, 2005). Trichomes and associated chemicals confer resistance to some tomato varieties against mites, aphids, whiteflies, beetles, and caterpillars (Gentile & Stoner, 1968; Heinz & Zalom, 1995; Kennedy, 2003; Kennedy & Sorenson, 1985; Simmons & Gurr, 2005). Trichomes are stiff hairs that sometimes contain chemical glands. Glandular trichomes have chemical exudates that confer resistance through antibiosis and kill or reduce longevity of pests feeding on them and entrap pests that forage on the leaves (Simmons & Gurr, 2005). Trichomes also have antixenotic effects on herbivore pests. Increasing trichome density can reduce oviposition by many species of beetles, caterpillars, true bugs, and mites. Of particular relevance is the effect of trichome density on whitefly and mites pests (Simmons & Gurr, 2005). The antibiotic and antixenotic effects of leaf pubescence on whitefly behavior and fitness have been studied in depth in a number of systems such as tomato, tobacco, cucurbits, and ornamental plants (Hoddle et al., 1998; Inbar & Gerling, 2008).

The soybean aphid offers a current example of how identifying pest resistance in crop plants can benefit IPM. Soybean aphid arrived in the U.S. from Asia in 2000 (Ragsdale et al., 2011). Since that time plant resistance conferred through antibiosis and antixenosis mechanisms has played an important role in mediating the economic impact of this pest on soybean yield (Ragsdale et al., 2011). Aphid fitness is negatively affected in resistant lines because it takes twice as long for aphids to probe into the phloem and initiate feeding (Diaz-Montano et al., 2007). Further, feeding bouts are reduced by more than 90% from less than 7 minutes per bout in resistant lines compared to greater than 60 minutes in susceptible lines (Diaz-Montano et al., 2007). Likewise, production of nymphs was reduced by 50-90% in resistant versus susceptible varieties, confirming antibiosis in resistant lines (Diaz-Montano et al., 2006; Hill et al., 2004). Antixenosis was also demonstrated in resistant varieties wherein adult aphids preferred to colonize susceptible over some resistant lines (Diaz-Montano et al., 2006; Hill et al., 2004).

In contrast to conventional breeding programs, plants can now be genetically modified to include lethal traits from other organisms, such as the bacterium, *Bacillus thuringiensis* (Bt). Bt genes are now used in many crop species to confer antibiosis in otherwise susceptible crops. Although we do not focus on this mode of plant resistance here, transgenic traits have had a tremendous effect on modern crop production and yield. However, like any

management tactic, Bt crops do not function in a vacuum and effects on natural enemies and other non-targets, secondary pest outbreaks, and evolution of pest resistance have been intensely studied (Gould, 1998; O'Callaghan et al., 2005; Shelton et al., 2002).

2.1.1 Interaction of plant resistance traits and biological control

Effects of plant resistance and biological control can be contradictory, complementary, or synergistic (Cai et al., 2009; Farid et al., 1998; Johnson & Gould, 1992). Plant resistance can work in conjunction with natural enemies to maintain low pest abundance and damage. In general, natural enemies have slower population growth rates than pests. Thus, by reducing pest population growth rates, plant resistance may help natural enemies better regulate pest populations. For example, research in wheat systems has shown that aphid-resistant wheat varieties do not have negative effects on parasitoid life history parameters such as size and development time (Farid et al., 1998). Parasitism rates may be equal or greater on resistant varieties, which when combined with reduced aphid population growth due to host plant resistance, can improve pest management dramatically (Cai et al., 2009).

Just as trichome exudates reduce herbivore survival they can also have negative effects on natural enemies. Survival and development of natural enemies may be reduced by poisoning or entrapping them, and natural enemy foraging efficiency, predation, or parasitism rate may be inhibited (Kaufman & Kennedy, 1989a, b; Obrycki & Tauber, 1984; Simmons & Gurr, 2005). For example, increasing trichome density and related changes in chemical composition of tomato leaves reduced the walking speed, parasitism rate, and survival of the egg parasitoid, *Trichogramma pretiosum* (Kashyap et al., 1991a, b). Tiny whitefly parasitoids in the genera, *Eretmocerus* and *Encarsia*, are highly affected by plant pubescence and trichome density (Hoddle et al., 1998; van Lenteren et al., 1995). Biological control may be disrupted because these parasitoids avoid highly pubescent plants. Once on the plants, pubescence reduces walking speed, foraging efficiency, oviposition, and parasitism rate (De Barro et al., 2000; Headrick et al., 1996; Hoddle et al., 1998; Inbar & Gerling, 2008).

Trichomes and other plant resistance traits also affect predator behavior and efficacy. Predatory mites used in biological control of spider mite, *Tetranychus urticae*, are readily trapped by trichomes and forage less efficiently due to reduced mobility (Nihoul, 1993a; van Haren et al., 1987). The consequence of mortality and reduced foraging efficiency is reduced biological control on cultivars with high trichome density, although the effect is also dependent on environmental factors such as temperature (Nihoul 1993a, b). Likewise, foraging efficiency of the spotted lady beetle, *Coleomegilla maculata*, and the bigeyed bug, *Geocoris punctipes*, was reduced by high trichome density, resulting in less predation of *Heliothis zea* eggs (Barbour et al., 1993, 1997). Increasing pubescence on poinsettia leaves by just 15% reduced oviposition and whitefly predation by *Delphastus catalinae* and other predators (Heinz & Parrella, 1994).

2.1.2 Herbivore resistance to plant resistance traits

Herbivores are in a constant evolutionary arms race with plants to overcome resistance traits (Ehrlich & Raven, 1964). It is not surprising then that pests have developed physiological resistance to genetically modified and conventional plant resistance traits (Gould, 1998). For example, certain soybean aphid biotypes are resistant to Rag1 or Rag2 genes that confer resistance to soybean plants (Hill et al., 2009, 2010). Evidence from

theoretical and empirical research suggests that multiple resistance traits or genes and a combination of different modes of action such as antibiosis and antixenosis should confer more stable resistance to crops. In addition, mixing resistant and susceptible varieties in the same field can reduce evolution of resistance by insect pest populations (Gould, 1986, 1998).

2.2 Cultural control via fertility management

Plant fertility and water stress play a major role in plant susceptibility to herbivore feeding, tolerance to herbivore damage, and herbivore population growth. Nitrogen can be a limiting nutrient for herbivorous insects due to the nitrogen-poor quality of their host plants (Mattson, 1980). Therefore, increasing nitrogen concentration within plants by applying fertilizer has a tendency to increase plant quality for herbivores (Mattson, 1980). Increasing foliar nitrogen can reduce pest development time and increase survival and fecundity, leading to more rapid population growth (Mattson, 1980). Research in potato crops has found that increasing nitrogen fertilization increases leaf consumption, reduces development time, and increases abundance of Colorado potato beetles (Boiteau et al., 2008). Likewise, in greenhouse ornamental production, increasing fertilization increases the fecundity, body size, and development rate of citrus mealybug (Hogendorp et al., 2006), and through similar mechanisms increases population growth rates of whiteflies, thrips, aphids, and spider mites (Bentz et al., 1995; Chau et al., 2005; Chau & Heinz, 2006; Chow et al., 2009).

In ornamental landscapes, fertilizer is often applied to improve the growth of trees and other plants and increase their resistance to abiotic and biotic stress, including herbivore feeding. However, nitrogen fertilization of trees has been shown to reduce plant resistance to many arthropod pests (Herms, 2002; Kytö et al., 1996). This reduced resistance occurs through a combination of fertilizer effects on plant nutrition for herbivores and defense against herbivores (Herms & Mattson, 1992). Herms & Mattson (1992) hypothesized that as nitrogen fertilization stimulates rapid plant growth, carbon available for production of defensive compounds is limited. Thus, over-fertilization of trees, shrubs, and other plants provides a dual benefit to many herbivores via increased nitrogen availability and decreased defensive compounds (Raupp et al., 2010).

2.3 Cultural control via pesticide selection and management

Pesticide applications are often an essential aspect of plant culture. Managing the type and frequency of applications is a cultural control tactic with well-documented implications. Insecticides can disrupt natural enemy communities and biological control via several mechanisms. Direct toxicity of pyrethroids and organophosphates to natural enemies has been documented frequently (Desneux et al., 2004b; see Galvan et al., 2005). Direct toxicity of insecticides to natural enemies results in smaller natural enemy populations on crop and landscape plants (Frank & Sadof, *in press*; Raupp et al., 2001). Insecticides also cause sublethal effects in parasitoids and predators. For example, the pyrethroid, lambda-cyhalothrin, disrupts the host location and oviposition behavior of *Aphidius ervi*, resulting in lower parasitism rates of aphids (Desneux et al., 2004a).

Non-target impacts on natural enemy communities are not limited to contact insecticides. Systemic neonicotinoids such as imidacloprid and thiamethoxam have lethal and sublethal effects on natural enemy development, fitness, and efficacy (Cloyd & Bethke, 2009; Desneux et al., 2007). These compounds can reduce survival of developing parasitoids and intoxicate

predators such as lady beetles and lacewing larvae exposed to the chemicals topically or by feeding on exposed prey (Moser & Obrycki, 2009; Papachristos & Milonas, 2008; Smith & Krischik, 1999; Szczepaniec et al., 2011). Parasitoids are also affected negatively via feeding on plant nectar or hosts exposed to the chemicals (Krischik et al., 2007; Rebek & Sadof, 2003). The consequence of disrupting natural enemy populations can be outbreaks of primary or secondary pests due to the loss of underlying biological control services (Raupp et al., 2010). Considerable work has documented this effect in field crops, orchards, vineyards, and landscape ornamentals (Penman & Chapman, 1988; Raupp et al., 2010). The effect is particularly prevalent among spider mites and scale insects that are not killed as easily as their natural enemies by insecticide applications. Pyrethroids and other broad-spectrum insecticides have direct and indirect effects on spider mites that can promote mite outbreaks. First, pyrethroids promote spider mite dispersal from treated to untreated areas of reduced competition (Iftner & Hall, 1983; Penman & Chapman, 1983). Spider mites have many predators including lady beetles, predatory bugs, lacewing larvae, and predatory mites. Pyrethroids can promote outbreaks of spider mites indirectly by killing the natural enemies that otherwise help suppress spider mite populations (Penman & Chapman, 1988).

Predatory mites in the family Phytoseiidae feed on spider mite eggs, juveniles, and adults and are effective at reducing spider mite abundance and damage on plants (McMurtry & Croft, 1997). In addition, phytoseiid mites often respond with a numerical increase to burgeoning spider mite populations via aggregation and increased reproduction. However, the abundance and efficacy of phytoseiid mites depends in large part on plant culture practices and plant characteristics. Phytoseiid mites are extremely susceptible to insecticides such as pyrethroids, organophosphates, and carbamates (Hardman et al., 1988). In many cases, phytoseiids have been found to be more vulnerable to these insecticides than spider mites (e.g., Sanford, 1967; Wong and Chapman, 1979). Therefore, by killing a disproportionate number of predatory mites compared to target pests, broad-spectrum insecticides frequently lead to spider mite outbreaks (Hardman et al., 1988). Similar dynamics have been demonstrated for scale insects, which are generally not killed by cover sprays of contact insecticides due to their protective cover. Moreover, by drastically reducing natural enemy abundance and efficacy, these insecticide applications create enemy-free space for scales, which can result in outbreak populations (McClure, 1977; Raupp et al., 2001).

Insecticide applications can directly benefit pest reproduction and survival through a process known as hormoligosis. Increased spider mite fecundity has been demonstrated after exposure to sublethal doses of pyrethroids (Iftner & Hall, 1984; Jones & Parrella, 1984). However, this is most evident in spider mites that frequently outbreak after applications of the neonicotinoid, imidacloprid (Gupta & Krischik, 2007; Raupp et al., 2004; Sclar et al., 1998; Szczepaniec et al., 2011). Outbreaks are triggered in part by negative effects on predators, but also by greater fecundity of spider mites that feed on imidacloprid-treated foliage (Szczepaniec et al., 2011). Although not commonly observed, this phenomenon points out another reason for proper insecticide management as a cultural control strategy.

2.4 Cultural control via crop rotation and planting practices

Exploiting the biological limitations of the pest to minimize insecticide applications is the essence of cultural control tactics such as crop rotation. This strategy has been used successfully to control corn rootworm for over 100 years (Forbes, 1883). Crop rotation has been highly effective as a tool to reduce Western corn rootworm, *Diabrotica virgifera virgifera,*

and Northern corn rootworm *Diabrotica barberi*, damage in corn (Levine & Oloumi-Sadeghi, 1991; Spencer et al., 2009). Corn rootworm eggs overwinter in corn fields and larvae are present to feed on corn roots the following year (Spencer et al., 2009). Therefore, rotating to a different crop such as soybeans denies food to hatching rootworm larvae (Spencer et al., 2009). Likewise, corn planted after soybeans or other crops has less rootworm damage because the field is free of overwintering eggs and larvae. However, Western and Northern corn rootworm populations eventually developed resistance to this strategy (Gray et al., 2009; Levine et al., 2002; Spencer & Levine, 2008). Northern corn rootworms circumvent crop rotation by prolonging egg diapause for two winters instead of one (Chiang, 1965; Levine et al., 1992). Therefore, larvae hatch when fields are replanted in corn two years after the eggs were laid. Western corn rootworm has become resistant to crop rotation by a behavioral rather than physiological mechanism. Western corn rootworm adults move from corn fields to soybean and other crop fields, feeding on soybean leaves and ovipositing in soybean fields (Levine et al., 2002). Selection pressure imposed by rotation of two primary crops, corn and soybeans, strongly rewarded female beetles that strayed from corn for oviposition.

Other planting practices such as delayed planting dates can also benefit pest control. Hessian fly is an introduced pest of winter wheat that has been in the U.S. since the 1700's. Prior to the development of resistant wheat varieties, growers exploited the fly's life cycle to reduce damage to winter wheat crops. Hessian fly adults become active in the fall when they oviposit in wheat and other grasses. By planting after a "fly free date" when fly activity subsides, winter wheat is protected from oviposition from the fall hessian fly generation (Buntin et al., 1991). This is a perfect example of how simple changes in plant culture can reduce the need for insecticide applications, increase yield, and provide economic benefit to growers (Buntin et al., 1992).

3. Biological control of insect pests

Many definitions of biological control have been published in the literature since the term was first used by H.S. Smith more than 90 years ago (Caltagirone & Huffaker, 1980; Cook, 1987; Coppel & Mertins, 1977; DeBach & Rosen, 1991; Garcia et al., 1988; see Huffaker & Messenger, 1976; Perkins & Garcia, 1999; Rabb, 1972; Smith, 1919). In its strictest sense, biological control is the use of beneficial organisms to reduce the relative abundance of, and damage caused by, noxious ones. This definition attributes economic rather than biological characters to organisms that fall into two categories, beneficial and noxious, based on their positive or negative impact on human-valued resources. It is also important to distinguish *biological* from *natural* control, which does not require human intervention, and from similar methods of pest control that do not involve whole (living) organisms (Huffaker et al., 1984). In fact, biological control involves interspecific, population-level processes by way of predation, parasitism, competition, or a combination of these mechanisms (van Driesche & Bellows, 1996). In practice, the effectiveness and appropriateness of biological control methods rely on real-time evolutionary forces that shape the beneficial organism's genotype, phenotype, and performance. This is not the case for similar, biologically based methods such as the application of insecticides formulated with pathogens, antagonists, or their byproducts. Furthermore, in its strictest definition, biological control does not include the deployment of pest-tolerant organisms, regardless of the source or origin of the resistance-conferring characters (e.g., Bt crops) (see Perkins & Garcia, 1999).

The history and origins of biological control have been extensively covered in previous volumes (Caltagirone & Doutt, 1989; DeBach & Rosen, 1991; van Driesche & Bellows, 1996) and is not the subject of this review. However, it is significant to note that early theory and application of biological control principles pre-date the modern insecticide era (Smith, 1919). Therefore, it is modern insecticides that became an alternative to biological control and not the other way around. In this context, biological control should not be viewed as a novel tactic but as the foundation of a successful pest management strategy involving, at minimum, the conservation of ecosystem resources to facilitate the process of pest-natural enemy colonization, host/prey finding, and ultimately, damage reduction. Although what constitutes biological control (or not) continues to be a subject of discussion and will likely evolve with new technologies, the recognition of three principal biological control methods remains unchanged. These three approaches are importation (a.k.a., classical biological control), augmentation, and conservation biological control (Smith, 1919).

3.1 Importation biological control

Importation biological control is the oldest of the three approaches (hence its alternative name, 'classical'). The first successful case of importation biological control occurred over a century ago in the control of cottony cushion scale in California citrus following importation of the vedalia beetle (Horn, 1988). The classical approach involves re-establishing the interspecific interactions (and their impact on population regulation) between pests and their natural enemies (i.e., predators, parasitoids, or insect-killing pathogens) as they occur in the pest's endemic range (Howarth, 1983). The need to re-establish these interactions arises because pests are commonly introduced into areas outside their native range where they lack natural enemies, or those that are present do not significantly impact the pest's abundance and local distribution. Since its inception, importation biological control has been used with varying degrees of success against noxious pests like cassava mealybug in Africa, Rhodesgrass mealybug in Texas, walnut aphid in California, and southern green stink bug in Australia, New Zealand, and Hawaii (Hokkanen, 1997).

The technical expertise, time commitment, and considerable expense necessary to carry out importation biological control require the involvement of specially trained university and government scientists. Importation is highly regulated in many countries, largely due to growing concern over the introduction of exotic, invasive species into new environments. In the U.S., the Animal and Plant Health Inspection Service (APHIS) oversees and coordinates importation biological control programs. The agency's charge is to preserve the safety and effectiveness of biological control primarily through post-release monitoring of biological control agents (USDA APHIS, 2011). Although there are a few documented cases of introduced biological control agents causing economic or ecological harm, societal perceptions that importation biological control is too risky are often influenced by subjectivity and misinformation (Delfosse, 2005). To minimize risk, researchers must provide evidence that introduced natural enemies are unlikely to harm crops, humans, and ecosystems. This requires substantial analysis of host feeding preference and other biological traits of prospective biological control agents (see Briese, 2005).

3.2 Augmentation biological control

The aim of augmentation biological control is to improve the numerical ratio between pest and natural enemy to increase pest mortality. It involves the release of natural enemies,

typically mass reared in an insectary, either to inoculate or inundate the target area of impact (Obrycki et al., 1997; Parrella et al., 1992; Ridgway, 1998). Inoculative releases involve relatively low numbers of natural enemies, typically when pest populations are low or at the beginning of a growth cycle or season. Inundation involves relatively high numbers of natural enemies released repeatedly throughout the growth cycle or season. Thus, inundative release of natural enemies is similar to insecticide use in that releases are made when pests achieve high enough density to cause economic harm to the crop. In both types of release, the objective is to inflict high mortality by synchronizing the life cycles of the pest and natural enemy. Hence, an effective monitoring program of pest populations is essential to the success of augmentation biological control.

Augmentation biological control has been used successfully against key pests of field and greenhouse crops. A well-known example of augmentation biological control is the use of the parasitoid, *Encarsia formosa*, for control of greenhouse whitefly (Hoddle et al., 1998). Indeed, augmentation plays an important role in greenhouse production, especially in Europe, and many natural enemies are commercially available for control of perennial greenhouse pests such as spider mites, aphids, scales, and whiteflies (Grant, 1997; Pottorff & Panter, 2009). The success of augmentative releases in greenhouses, and elsewhere, depends on the compatibility of cultural practices such as insecticide use with natural enemies (see Section 2.3). Greenhouses are often ideal sites for augmentation biological control because of the relative stability of the enclosed environment. In contrast, a critical review of augmentation biological control in field crops revealed that augmentation was typically less effective and more expensive than conventional control with pesticides (Collier & van Steenwyk, 2004). The authors found that the low success rate of augmentation biological control in field crops is influenced by ecological limitations such as unfavorable environmental conditions, natural enemy dispersal, and refuge for herbivores from released natural enemies.

3.3 Conservation biological control

Conservation biological control involves any practice that increases colonization, establishment, reproduction, and survival of native or previously established natural enemies (Landis et al., 2000). Conservation biological control can be achieved in two ways: modifying pesticide use and manipulating the growing environment in favor of natural enemies. Conservation practices have proven effective in a wide variety of growing situations ranging from small garden plots to large fields, agricultural to urban environments, and commercial to private settings (Frank & Shrewsbury, 2004; Landis et al., 2000; Rebek et al., 2005, 2006; Sadof et al., 2004; Tooker & Hanks, 2000).

3.3.1 Conserving natural enemies via modified pesticide use

Modifications to pesticide regimens include reducing or eliminating insecticide use, using pest-specific insecticides when needed, making applications when beneficial arthropods are not active, and making treatment decisions based on monitoring and the presence of vulnerable life stages. While total independence from chemical control is not feasible for most situations, reductions in insecticide use are possible through IPM programs based on rigorous pest monitoring, established treatment thresholds, and/or insect population models (see Horn, 1988; Pimental, 1997). Thus, insecticides are used only when needed to prevent crop damage that results in economic loss. When insecticide use is warranted,

adverse effects on natural enemies can be minimized by using selective, pest-specific products that are only effective against the target pest and its close relatives. Selective chemistries include microbial insecticides, insect growth regulators, botanicals, and novel insecticides with specific modes of action against target insects. Alternatively, insecticide applications can be timed so they not coincide with natural enemy activity; dormant or inactive predators and parasitoids are not exposed to broad-spectrum insecticides applied when they are dormant or inactive (van Driesche & Bellows, 1996). This strategy requires a thorough understanding of the crop, agroecosystem, and the biology and life cycle of important natural enemies in the system.

3.3.2 Conserving natural enemies via habitat manipulation

Natural enemies are attracted to habitats rich in food, shelter, and nesting sites (Landis et al., 2000; Rabb et al., 1976). Many perennial plants can provide these resources when incorporated into the system. Ellis et al. (2005) and Rebek et al. (2005) independently observed significantly enhanced parasitism of two key ornamental pests, bagworm and euonymus scale, in experimental plots containing nectar and pollen sources (i.e., resource plants). Resource plants also served as refuge for vertebrate predators of bagworms as evidenced by increased predation rates (Ellis et al., 2005). Resource plants can harbor alternative prey/host species, which sustain adult and immature natural enemies when primary prey/hosts are scarce. For example, many studies have focused on the influence of banker plants, which contain alternative prey species, on natural enemy effectiveness (see Frank, 2010).

Resource plants provide more than food to enhance natural enemy abundance in impoverished landscapes. Suitable changes in microclimate are afforded by many plants, tempering environmental extremes by providing improved conditions for natural enemy survival (Rabb et al., 1976). Candidate plants include small trees, shrubs, bushy perennials, and tall ornamental grasses with dense canopies or complex architecture. Similarly, organic mulches and ground cover plants can support large numbers of ground-dwelling predators like spiders and ground beetles (Bell et al., 2002; Mathews et al., 2004; Rieux et al., 1999; Snodgrass & Stadelbacher, 1989), which may enhance biological control of key pests (Brust, 1994). Finally, resource plants can enhance reproduction of natural enemies and provide refuge from their own enemies (Landis et al., 2000; Rabb et al., 1976).

The effectiveness of habitat manipulation to improve biological control requires careful planning and selection of plant attributes that are appropriate for the natural enemy complex present in the system (Landis et al., 2000). For example, flower morphology can significantly impact nectar accessibility by foraging parasitoids (Patt et al., 1997; Wäckers, 2004). Also important is coincidence of floral bloom with natural enemy activity. Selected resource plants should overlap in blooming periods to ensure a continuous supply of nectar and pollen to natural enemies (Bowie et al., 1995; Rebek et al., 2005). Other considerations that exceed the scope of this chapter include the influence of landscape-level attributes on biological control at different spatial scales (Kruess & Tscharntke, 1994; Marino & Landis, 1996; Roland & Taylor, 1997).

3.4 Factors affecting success of biological control

While there have been some tremendous successes, the worldwide rate of effective biological control is estimated to be between 16-25% (Hall et al., 1980; Horn, 1988; van

Lenteren, 1980). In practice, the successful application of biological control usually requires a combination of at least two of the three approaches, importation, augmentation, and conservation of natural enemies (DeBach & Rosen, 1991; van Driesche & Bellows, 1996). What drives the success or failure of biological control programs in plant crops has been the subject of many analyses, either using historical records or theoretical approaches (Andow et al., 1997; Murdoch et al., 1985; Murdoch & Briggs, 1996; van Lenteren, 1980). In general terms, biological control programs are more likely to succeed under certain production systems and environmental conditions (Clausen, 1978; van Driesche & Heinz, 2004). Biological control has been more successful in crops that: 1) are perennial versus annual; 2) grow in areas with few pests versus many pests; 3) the harvested portion is not damaged by the target pest; 4) the target pest is not a disease vector; and 5) the aesthetic damage is acceptable (e.g., some food and fiber crops versus ornamentals).

Failures in biological control programs, especially those recorded in the literature, also involve cases where the biology and ecology of the natural enemy or the pests are not well understood or altogether unknown. Historically, failures in importation biological control have occurred after errors in identification of a pest or natural enemy at the level of species, biotype, or even local strain; a mismatch in micro-environmental requirements for natural enemy growth and development; incorrectly timing natural enemy release when the production system is not conducive to establishment; or when socioeconomic or regulatory barrier have prevented adoption or implementation (Clausen, 1978; Greathead, 1976; Hall & Ehler, 1979; Knutson, 1981). Similarly, failures in augmentation and conservation biological control, although not commonly recorded in the literature, may be due to a lack of understanding of the basic biology and ecology of the species involved, the basic requirements of the production system, and any socioeconomic barriers including real or perceived costs and benefits (Murdoch et al., 1985; Perkins & Garcia, 1999; Collier & van Steenwyk, 2004). The success of biological control programs involves integrated efforts at many levels ranging from biology to economics, from research to implementation and experience, and from the farm to the community and region.

4. Physical control strategies to reduce pest incidence

Plant health can benefit greatly from preventing or limiting injury from arthropod pests from the start. Indeed, the cornerstone of an effective IPM program is prevention, which can be achieved, in part, through physical control. Physical control strategies include methods for excluding pests or limiting their access to crops, disrupting pest behavior, or causing direct mortality (Vincent et al., 2009). Physical control methods can be categorized as active and passive (Vincent et al., 2009). Active methods involve the removal of individual pests by hand, pruning out infested plant tissues, and rogueing out heavily infested plants. Passive methods usually include the use of a device or tool for excluding or removing pests from a crop. Typically, these devices serve as barriers between plants and insect pests, thus protecting plants from injury and damage. Other passive tools include repellents and traps. While traps are often used for monitoring pest abundance and distribution, many are designed as "attract and kill" technologies, which attract insect pests through color, light, shape, texture, and scent, or a combination of these factors.

The greatest disadvantage to physical control is that these methods can be laborious and time consuming, especially for crops grown in large areas. Also, a moderate degree of specialization or training is often required due to the highly technical nature of some

physical control methods. Physical control methods may also be difficult or practically impossible in some crops like large trees grown in extensive monocultures (e.g., timber production). For many crops, however, physical control of certain pests can be incorporated into established routines for managing crops. Despite the drawbacks and considering the costs, regulations, and limitations of insecticide use, physical control methods are likely candidates for inclusion in many pest management programs, especially for high-value crops (see Vincent et al. 2003). Here, we discuss briefly some examples of physical control classified by their primary function: exclusion, behavior modification, and destruction of pests.

4.1 Physical control via exclusion

Pest exclusion is a key factor in preventing pests from accessing crops, thereby reducing the economic impact of insects. Both passive and active exclusion methods have been implemented in various agricultural systems including fields, greenhouses, and postharvest facilities. Physical control via exclusion devices is perhaps most important in protected environments such as greenhouses and grain bins, where optimal temperatures and humidity, a readily available food source, and a general lack of natural enemies contribute to the proliferation of pest populations. Screens are common passive exclusion devices used in greenhouse production. Screens can prevent pest migration into greenhouses through vents and other openings, especially when insect populations build up in weeds and crops in the surrounding environment (Gill et al., 2006; Pottorff & Panter, 2009). However, screen mesh size is an important concern as fine materials with small openings inhibit entry of tiny arthropods such as thrips and mites but also restrict air flow for cooling (Pottorff & Panter, 2009). Other active methods of physical control are necessary components of greenhouse IPM. Specifically, crops should be inspected for pests prior to moving new plant materials into production areas; discovered pests are removed by hand, pruned out, or discarded and destroyed with heavily infested plants.

In the field, floating row covers can exclude important vegetable pests such as cabbage maggot, flea beetles, and cabbageworm (Rekika et al., 2008; Theriault et al., 2009). Adhesives and burlap have been used to trap caterpillar pests such as gypsy moth and cankerworms as they migrate vertically along tree trunks (Potter, 1986). Other barriers include fences, ditches, moats, or trenches. For example, V-shaped trenches have been used around potato fields to prevent movement of Colorado potato beetle into the crop from adjacent, overwintering habitat (Boiteau & Vernon, 2001; Misener et al., 1993; see Vincent et al., 2003). Efficacy of this technique relies on trench design and knowledge of the pest, specifically, the population size and the ratio of crawling to flying individuals (Weber et al., 1994; Vincent et al., 2003).

4.2 Physical control via behavior modification

IPM programs often consist of physical control methods that alter the behavior of insect pests. Behaviors such as reproduction, aggregation, oviposition, feeding, alarm, and defense can be modified in two ways: "push-pull" strategies and mating disruption (Cook et al., 2007; Zalom, 1997). The former are designed to repel (push) or attract (pull) insect pests away from a crop by exploiting their reproductive, feeding, or aggregation behavior. Although many repellents and attractants are chemically based, here we treat their use in IPM as a form of non-chemical (non-insecticidal) control.

Pheromones, or chemical lures, are used in IPM programs to monitor pest populations and modify their behavior. Specifically, pheromone traps are used to detect pest activity in a

crop and estimate their relative abundance in order to properly time an insecticide application or natural enemy release. Pheromones and other olfactory stimuli are receiving increased attention as repellents and attractants in push-pull strategies for modifying pest behavior (see Cook et al., 2007). Repellents include synthetic chemicals (e.g., DEET), non-host volatiles that mask host plant odors (e.g., essential oils), anti-aggregation and alarm pheromones, anti-feedants (e.g., neem oil), and oviposition deterrents (e.g., oviposition-deterring pheromones) (Cook et al., 2007). Herbivore-induced plant volatiles are host plant semiochemicals that induce plant defense from herbivores and attract natural enemies (James, 2003). Non-chemical repellents include reflective mulches, which have been shown to reduce damage and population density of tarnished plant bug in strawberry fields (Rhainds et al., 2001). Attractants include sex and aggregation pheromones, host plant volatiles, and feeding stimulants (e.g., baits), and oviposition stimulants (Cook et al., 2007). Other attractants are based on visual cues. For example, apple maggots are effectively controlled in apple orchards with 8-cm, red, spherical traps covered in adhesive. The attractiveness of these traps is enhanced by adding butyl hexanoate and ammonium acetate, synthetic olfactory stimulants (Prokopy et al., 1994).

Another common tactic is to use sex pheromones for mating disruption. Many insect pests rely on a species-specific, sex pheromone produced by females for mate location and recognition. Mating disruption is achieved by flooding the crop environment with the chemical signal, thus confusing males and reducing mate-finding success. This approach has been used with varying degrees of success for management of orchard and vineyard pests including codling moth, oriental fruit moth, grape berry moth, and peachtree borer (see Zalom, 1997).

4.3 Physical control via pest destruction

Insects can be killed directly through mechanical, thermal, or other means. Vincent et al. (2009) list several strategies that inflict mortality on pests including freezing, heating, flaming, crushing, and irradiating. One of the most common mechanical methods requires no specialized equipment – many gardeners derive great satisfaction from hand picking pests from a plant and crushing them. Hand removal can be used effectively for a myriad of relatively sessile landscape pests including bagworms, tent caterpillars, and sawfly larvae. Galls, egg masses, and web-making insects can also be pruned out of infested landscape plants (Potter, 1986). However, this tactic may be impractical for large trees or shrubs and dense populations of the pest. Other mechanical control options require specialized machinery. Pneumatic control involves removing pests from crops by use of a vacuum or blower and subsequently destroying them. Field crop pests such as Colorado potato beetle and lygus bug have been controlled in this manner, although care must be taken to avoid negatively impacting natural enemies (Vincent et al., 2003, 2009). Another example of mechanized destruction is the entoleter, an impact machine that is used in mills to remove and kill all life stages of insect pests (Vincent et al., 2003).

Modifying the microclimate can be effective in killing many insect pests, which cannot survive outside of optimal temperature and humidity ranges. Heat has been shown to be a very effective control method for bed bugs, which are difficult to control and are becoming more prevalent in domestic dwellings worldwide (Pereira et al., 2009). A wide variety of stored product pests can be controlled by pumping hot or cold air into the food storage facility, or modifying the storage environment with elevated temperatures and carbon

dioxide (Vincent et al., 2003, 2009). Hot-water immersion, flaming, steaming, and solar heating are other thermal control options (Vincent et al., 2003).
Electromagnetic energy has been studied for its effectiveness at killing insects (Vincent et al., 2009). Ionizing radiation has been used in quarantine facilities to treat fruit and other commodities suspected of carrying serious agricultural pests (Vincent et al., 2003). Targets of other electromagnetic methods, especially microwave treatments, include stored product pests. However, electromagnetic treatments may be limited by government regulations, cost, and the need for specialized equipment and training (Vincent et al., 2009).

5. Conclusions

Crop culture sets the stage for interactions between plants, pests, and natural enemies, and has a strong influence on the outcome of these interactions. In many cases, implementing effective cultural controls can be the most economical pest management tactic available to growers because labor and expense are incurred regardless of whether an effective cultural tactic is used. Understanding and implementing cultural practices can reduce other production expenses such as insecticides and fertilizer. Cultural control can be compatible with biological control if the myriad interactions among plants, pests, and natural enemies are well defined. Improving the predictability of biological control will rely on elevating the discipline to its proper place in applied evolutionary ecology and further refinement of the art and practice of biological control (van Lenteren, 1980; Heinz et al., 1993; Heinz, 2005). Fortunately, the organic and sustainable agriculture movements that are gaining both societal and political momentum seem to embrace the art and science of biological pest control (Edwards, 1990; Raynolds, 2000). While various physical control techniques have been used successfully in production systems, this strategy is limited by the significant labor, time, cost, and specialization required for successful control (Vincent et al., 2009). Further refinements and developments in physical control technologies hold promise for enhanced efficacy, compatibility with cultural and biological control, and profits.
As we move into the future of pest management, new challenges await. Crops are now genetically modified to produce their own "insecticides" for protection. Newly registered insecticides tend to be more target specific and often, more expensive. Older chemistries are being removed both voluntarily and involuntarily from the market. There is increasing demand for organically grown food, or food perceived as "safe" for consumption. Yet we must still feed a growing human population. More than ever, IPM researchers need to develop programs that use effective alternatives to insecticides whenever possible. We also must intensify efforts to truly integrate insecticides selectively into our IPM programs, so that they are not the predominant tool in our IPM toolbox. As such, we need to further develop principles and methods of cultural, biological, and physical control as relevant pest management tools for sustainable agricultural production.

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Part 2

Further Applications

10

Proteomic Profiling of *Escherichia coli* in Response to Carbamate Pesticide - Methomyl

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1. Introduction

Since decades, there has been mounting concern regarding the adverse health effects of environmental contaminants in general and carbamate in particular. Methomyl is a carbamate and widely used throughout the world since it is effective as "contact insecticide" as well as "systemic insecticide" for fruits and vegetables and is well known established cholinesterase inhibitor [1]. Methomyl has been classified as a pesticide of category-I toxicity [2]. Methomyl is a metabolite of thiodicarb and acetimidate is suspected oncogen, which is a metabolite in animal tissues [3]. It has been classified by the WHO, EPA (Environmental Protection Agency, USA), and EC (European Commission) as a very toxic and hazardous pesticide [4]. Methomyl is highly soluble in water and can therefore, easily cause ground water contamination in agricultural areas [5]. Bonatti *et al.*, [6] have shown genotoxic effects of methomyl in *in vitro* studies. Methomyl is potent genotoxic and is capable of inducing structural and numerical chromosomal aberration in mammalian cells [7].

Prokaryotic cells respond to environmental or chemical stress by inducing specific sites of proteins characteristic to each stress [8]. Studies on stress response and survival strategies of enteric bacteria have evolved a range of complex mechanisms, which use different regulatory structures and genetic components for their survival and virulence [9]. The stress protein induced in response to four different pesticides viz. cypermethrin, zeta-cypermethrin, carbofuran and bifenthrin were analyzed by protein profiling of *Escherichia coli* by Asghar *et al.*, [10]. Mechanisms of cellular adaptation and compensation against different kinds of toxic metals have been proposed. However, the molecular mechanisms and underlying responses of cells against various pesticides are not yet completely understood [11].

Proteomics is a technique used to investigate whole proteins expressed by an organism, tissue or a cell at a specific time point under defined environmental conditions. Nowadays, proteomics has been used for many research purposes e.g. disease diagnosis, drug target and biomarkers of pollutants [12,13] . Proteomics, transcriptomics and metabolomics are powerful tools for acquiring information on gene/protein function and regulatory networks[28]. Using proteomics, one can determine protein expression profiles related to research for both microbial isolates and communities. Proteomics provides a global view of the protein complement of biological systems and, in combination with other omics technologies, has an important role in helping uncover the mechanisms of these cellular processes and thereby advance the development of environmental biotechnologies[29].

The polyacrylamide gel electrophoresis has been used extensively for the separation of proteins in yeast, bacteria and higher organisms with the successful separation of whole cell extracts or specific proteins under selected conditions. This is an excellent method to attempt a global depiction of the cells protein profile. Thus, this technique is being extensively used to determine the *in vivo* amount of protein, its rate of synthesis, and rate its rate of degradation [13]. SDS-PAGE is an important molecular technique used for the identification of whole cell proteins and it has the advantage of being fairly simple and rapid to perform [14]. Therefore, the present investigation was undertaken to study the proteomic profiling of *Escherichia coli* on dose and durational exposure to methomyl by gel electrophoresis.

2. Materials and methods

2.1 Preparation of stock solution of methomyl

The sample of methomyl (Lannate ®) used in the experiment was supplied by E.I. Dupont India Pvt. Ltd., Haryana obtained. The stock solution of 1 M of methomyl was prepared and further diluted to give different required molar concentrations.

2.2 Maintenance and propagation of culture

The organism *Escherichia coli* was procured from NCL, Pune and the bacteria was maintained at 4°C on nutrient agar formulated by Lapage and Shelton [15] and sub cultured very fortnight.

2.3 Medium used for the study

Synthetic sewage medium (S-medium) formulated by Babich and Stotzky [16] was used as the medium for toxicity testing.

2.4 Preparation of inoculum for free cells

Pre-inoculum was prepared by inoculating a loopful of bacteria from the overnight incubated nutrient agar slant cultures on a 100 ml sterilized synthetic sewage medium and incubated for 18-24 hours at 37°C under static conditions depending on the exponential phases of bacteria under test.

2.5 Experimental procedures

Free cells: Five ml of the pre-inoculum was inoculated to 250 ml Erlenmeyer's flask containing 100 ml of sterilized S-medium amended with different molar concentrations of heavy metals. The flasks were incubated at 37°C for 96 hours under shaking conditions at 120 rpm on a rotary shaker (REMI – CIS-24). At regular intervals sample was taken out from each flask aseptically for analysis.

2.6 Isolation of protein

The bacterial cell pellet was dissolved in 100µl of lysis buffer and incubated at 37^{o}c for 15 min. the tubes were centrifuged and the supernatant was used as protein sample. PAGE according to Laemmli [17] analyzed these protein samples.

3. Results and discussion

The present investigation was attempted to elucidate the protein profiling in *Escherichia coli cells* that were exposed to different concentrations of methomyl ranging from 10^{-7} M to 10^{-3}

M of methomyl for a period of 96 hrs and at regular intervals of 24 hrs, the proteins induced were analyzed. The protein expression was observed at 29, 45, 48, 55, 63, 92 and 114 kDa at 24 hrs **(Fig. 1)**. On exposure to methomyl for 48 hrs the bands were observed at 29, 45, 48, 55, 63, 92 and 114 kDa **(Fig. 2)**. The methomyl treated for 72 hrs showed expression at 29, 39, 45, 66 and 92 kDa **(Fig. 3)** and for 96 hrs the expressions was observed at 29, 35, 39, 45, 55, 63, and 92 kDa **(Fig. 4)** respectively. The expression of proteins were more conspicuous in our result which was obligatory, since the free *Escherichia coli* cells possess antioxidant enzymes, which are induced in response to the stress and are directly exposed to methomyl [18].

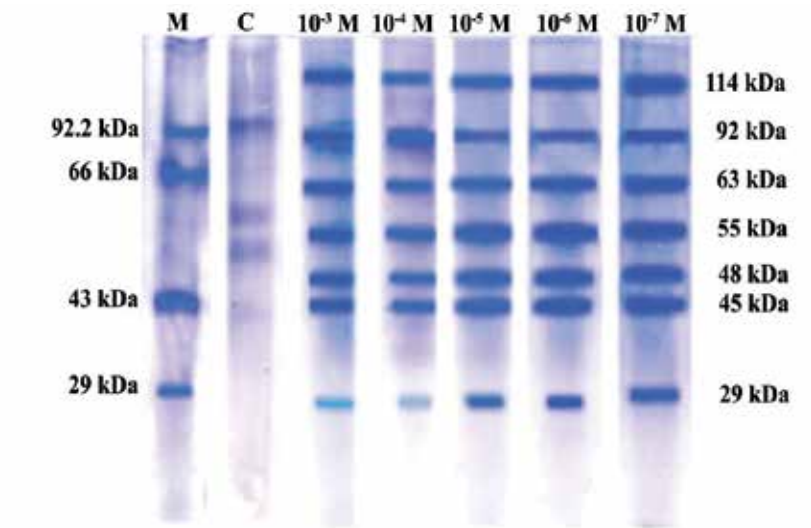

Fig. 1. Protein profile of *Esherichia coli* induced by methomyl for 24 hours.

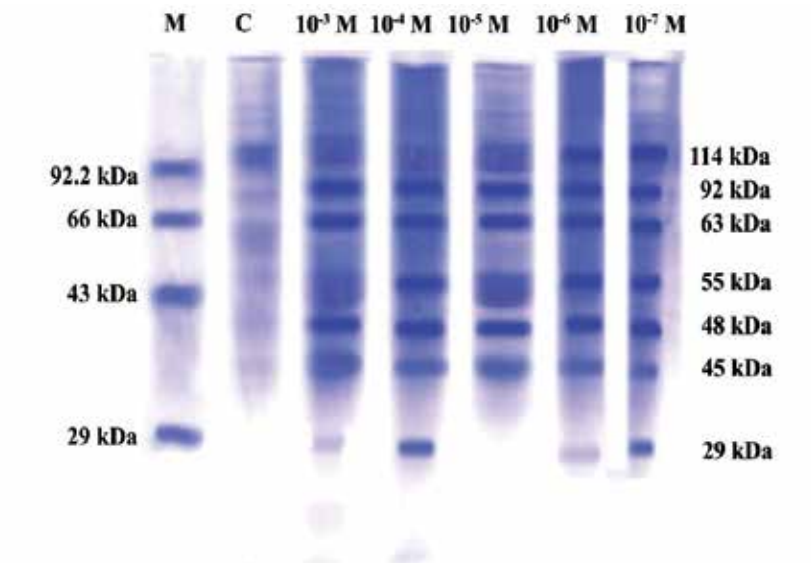

Fig. 2. Protein profile of *Esherichia coli* induced by methomyl for 48 hours.

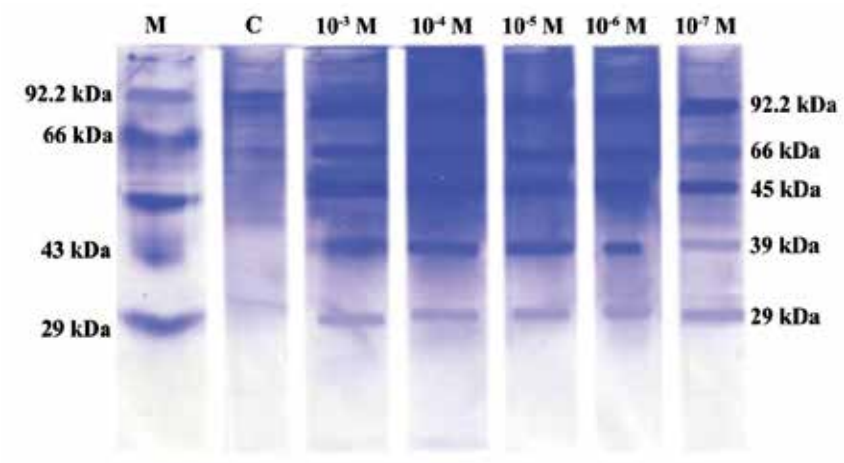

Fig. 3. Protein profile of *Esherichia coli* induced by methomyl for 72 hours.

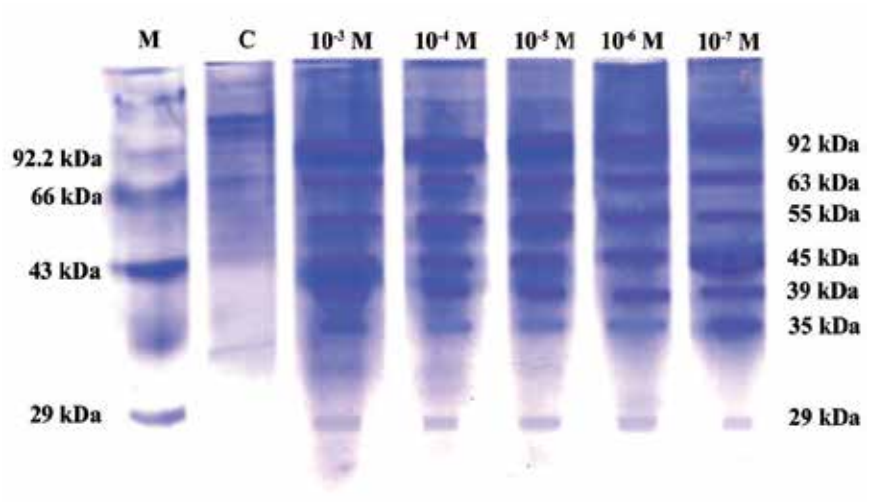

Fig. 4. Protein profile of *Esherichia coli* induced by methomyl for 96 hours.

The protein profiles were compared with the dose and duration of exposure of methomyl in *Escherichia coli* and the results revealed that the intensity of the proteins expressed increased with an increase in the dose and duration of exposure of methomyl when compared with those of the corresponding parameters of the control, indicating that the pesticide methomyl induces stress. Our results agreed with the observations made by Asghar *et al.,* [10] who analyzed the stress proteins of *Escherichia coli* induced in response to the pesticides cypermethrin, zeta-cypermethrin, carbofuran and bifenthrin.

The over expressions of some of the proteins observed in the present study at 29 and 45 kDa at all the dose and duration of exposure could be due to the fact that prokaryotic cells respond to environmental or chemical stress by inducing specific sets of proteins characteristic to each stress. It has been reported that the proteins in each set of their coding genes constitute a stimulon, such as heat shock, SOS response and oxidation stress. In some

other cases, proteins, which are associated with one stimulon, can be induced during other stresses, such as various heat shock proteins in *Escherichia coli*. These proteins are also synthesized when the cells are exposed to different physical and chemical stress. In some stimulons, exposure to non-lethal levels of a stress agent can confer protection against subsequent exposure to lethal levels of the same stress agent [19]. Similarly, in the present study, the proteins expressed at 29 and 45 kDa could be unique or could be observed in the protein profiling of other micro-organisms exposed to various physical or chemical stress.

It has been suggested that the analysis of many proteins produced during the transition into stationery phase and under stress conditions demonstrated that a number of novel proteins were induced in common to each stress and could be the reason for cross protection in bacterial cells. It is necessary to investigate the synthesis of these proteins during different stress conditions [20]. Similarly it has been mentioned that when organisms or cells are exposed to low levels of certain harmful physical and chemical agents, the organisms acquire an induced tolerance against the adverse effects [7]. Hence, in the present study the high molecular weight proteins of 114 kDa at 24 and 48 hrs respectively observed in all the doses of exposure in comparison to their corresponding controls may be ascertained to the protein selective proteolytic degradation that appears to be rather significant in homeostasis maintaining and metabolism regulation in the cell [21]. It has been reported that along with short-lived regulatory proteins, the polypeptide chains with disrupted or changed structures are selectively hydrolyzed. Such defects might arise from inaccuracy during protein biosynthesis, chemical or physical damage [22] and moreover, the extracts of *Escherichia coli* have been shown to degrade rapidly the damaged enzyme, but not the native protein, and several preliminary reports have appeared concerning the *Escherichia coli* protease that may be responsible for selective degradation of the modified proteins [23].

Although it has been reported that the starvation for individual nutrients and other stress induce a unique and individual profile of protein expression, some proteins are common to different starvation and stress factors in *Escherichia coli*. However, the proteins of one stimulon do not respond coordinately to all the starvation and stress treatments and relatively few of the starvation- inducible proteins have been found to overlap with those induced by stress. This suggests that despite the regulation of a few specific proteins being interconnected, there are major difference in the regulatory pathways controlling the expression of starvation and different stress proteins [24]. Studies in the micro-organisms have provided evidence for increased longevity, cell division rate and survival when exposed to stress [25]. Similarly in the present study, the types of stress patterns observed with the dose and duration of exposure of methomyl were identical which agreed with the earlier reports [10] that the stress proteins produced in response to two different classes of pesticides showed that the same stress patterns were obtained for different substituent chemical groups within the same class and two different classes, indicating that the gene or set of genes responsible for stress expressions were the same irrespective of the class or nature of substituent's on the pesticide.

Further, an increase in the intensity in protein expression observed in the present study may be due to the fact that the major protein modification is observed due to stress, loss of catalytic activity, amino acid modification, carbonyl group formation, increase in acidity, decrease in thermal stability, change in viscosity, fluorescence, fragmentation, formation of protein protein crosslink's, s-s bridges and increased susceptibility to proteolysis [3]. It has been revealed that the secretion of extra cellular proteins, including toxins and cellular

effectors, is one of the key contributing factors in a bacterium's ability to thrive in diverse environments [26]. Hence, the present study indicates that the protein expressions are dose and duration dependent. It has been suggested that there are many protein synthesized in common with many stress in *Escherichia coli* and some of these proteins may play a major role in the stability of the cells under different stresses. The fact that specific patterns of proteins are expressed for a particular stress has led to the use of stress proteins to monitor environmental samples for the presence of particular pollutants [27]. It has been suggested that the analysis of such stress proteins will aid in the development of more sensitive techniques for the pollutant analysis. The unique proteins could be purified and raised to enable quick detection, which could be used as biomarkers of xenobiotics in the environment [11].

4. Conclusions

The present study indicated the molecular weights of the various stress proteins induced in response to the dose and durational exposure of methomyl. Further, it indicates that the stress protein analysis is a promising alternative and more sensitive method for measuring toxic effects on the organisms at sub lethal levels. The study suggests that the proteomic profiling is a sensitive tool for environmental stress diagnosis, and that the stress proteins could be used as biomarkers for environmental pollution identification. The specific patterns of the proteins that are expressed in response to the stress induced by methomyl could be used to monitor the environmental samples for the presence of such pollutants. Although the application of gene and protein expression analysis to ecotoxicology is still at an early stage, this holistic approach seems to have several potentials in different fields of ecological risk assessment. It can be concluded that such extensive work on proteomics can be performed in understanding the proteomic/genomic response and tolerance of the microorganisms to the extreme environment.

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11

Ameliorative Effect of Vitamin E on Sensorimotor and Cognitive Changes Induced by Chronic Chlorpyrifos Exposure in Wistar Rats

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1. Introduction

The use of pesticides is inevitable in contemporary world because of their role in the improvement of food production through increase in crop yields and quality, reduction of farm labour requirements hence lowering cost of production, and improving public health through control of vector and vector-borne diseases (Weiss et al., 2004). Despite all these benefits, pesticides constitute menace to the health of man, animals and even the environment. This is because they are poorly selective and are toxic to non-target species, including humans. The segments of the population that are at the greatest risk of exposure are those that are occupationally exposed, such as agricultural workers. Despite the strict measures put in place concerning its commercialization and use, pesticides sales has increased in recent years (Carlock et al., 1999). The World Health Organization (WHO) estimated that about 3 million cases of acute intoxication and 220,000 deaths are attributable to pesticides each year with majority of these cases occurring in less developed countries (He, 2000; Clegg & van Gemert, 1999), particularly in Africa, Asia, Central America, and South America (Pancetti et al., 2007). Although many pesticides cause neurotoxicity, insecticides are the most acutely neurotoxic to humans and other non-target species compared to other pesticides (Costa et al., 2008). Association between acute exposure to pesticides and neurotoxicity is well known (Lotti, 2000) but the potential effects of chronic low-level exposure are less well established (Alavanja et al., 2004; Ambali et al., 2010a; Ambali & Aliyu, 2012).

Organophosphate (OP) compounds are one of the most widely used constituting about 50% global insecticide use (Casida & Quistad, 2004). Studies in humans showed neurological, cognitive and psychomotor impairments following cumulative exposure to OPs and organochlorines in people from agricultural communities, without history of acute poisoning (Kamel & Hoppin 2004; Kamel et al., 2007). Neurobehavioural changes following low-dose OP exposure have been reported in sheep farmers (Stephens et al., 1995),

greenhouse workers (Bazylewicz-Walczak et al. 1999), tree-fruit workers (Fiedler et al., 1997), and farm workers (Kamel et al., 2003). These studies have found deficits in measures of sustained attention, information processing, motor speed and coordination. The principal mode of insecticidal action of OPs relates to phosphorylation and subsequent inactivation of the esteratic sites of the acetylcholinesterase (AChE) enzyme. The classical role of AChE is to hydrolyze the neurotransmitter acetylcholine (ACh), effectively clearing it from the neuronal synapse and terminating impulse conduction (Farag et al., 2010). Inactivation of AChE results in the accumulation of ACh in the neuronal synapses in the central and peripheral nervous system, thereby overstimulating the nicotinic, muscarinic and central cholinergic receptors with consequent neurotoxicity. Thus, the acute neurotoxic effect of OP results in muscarinic, nicotinic and central cholinergic symptoms (Abou-Donia, 1992). However, toxicity has been reported at doses below the threshold required for inhibition of AChE (Pope, 1999; Slotkin, 2004, 2005) prompting search for other mechanisms. The induction of oxidative stress as one of the other molecular mechanisms involved in OP-induced neurotoxicity has received tremendous attention in recent years (Gultekin et al., 2007; Prendergast et al., 2007; El-Hossary et al., 2009; Ambali et al., 2010a, Ambali & Ayo, 2011a, 2011b; Ambali & Aliyu, 2012). Indeed, the enhanced production of reactive oxygen species (ROS) by pesticides has been used to explain the multiple types of responses associated with its toxic exposure (Bagchi et al., 1995; Verma et al., 2007).

Chlorpyrifos (*O*,*O*-diethyl-*O*-[3,5,6-trichloro-2-pyridyl] phosphorothioate) is a chlorinated OP insecticide that exhibit a broad spectrum of activity against arthropod pests of plants, animals, and humans, and has wide applications in both agricultural and commercial pest control (Rack, 1993). It is one of the most widely used insecticides and is applied about 20 million times per year in US to houses and lawns (Kingston et al., 1999) with 82% of adults having detectable levels of the 3,5,6-trichloro-2-pyridinol, the metabolite of CPF in their urine (Hill et al., 1995). However, the United States Environmental Protection Agency in 2000 placed ban on some its residential uses in 2000 because of the danger posed to children's health. However, CPF is still widely used as its residues have been detected in citrus fruits in some parts of the world (Iwasaki et al., 2007). Studies have shown that CPF induces neurobehavioural alterations following acute (Caňadas et al., 2005; Ambali et al., 2010a, Ambali & Aliyu, 2012) and repeated low-dose (Stamper et al., 1988; Sanchez-Santed et al., 2004; Ambali & Ayo, 2011a, 2011b) exposure. Similarly, CPF is a developmental neurotoxicant (Qiao et al., 2003; Dietrich et al., 2005; Colborn, 2006; Slotkin et al., 2006;) impairing children mental and behavioral health (Lizardi et al., 2008). Although, CPF like the other OP compounds phosphorylates and subsequently inactivate AChE, neurobehavioural and cognitive deficits have however been observed following repeated low-dose CPF exposure that cannot be attributed to the usual AChE inhibition and muscarinic receptor binding (Pope et al., 1992; Chakraborti et al., 1993; Saulsbury et al., 2009). Earlier studies have shown the involvement of oxidative stress in the neurotoxicity induced by CPF exposure (Gultekin et al., 2007; Ambali et al., 2010a; Ambali & Aliyu, 2012; Ambali and Ayo, 2011a, 2011b).

Oxidative stress, defined as a disruption of the prooxidant-antioxidant balance in favor of the former causes damage to the body tissue (Sies, 1991). Oxidative stress results from an increase in ROS, impairment of antioxidant defense system or insufficient capacity to repair oxidative damage (Halliwell, 1994; Aly et al., 2010). Damage induced by ROS which alters cellular macromolecules such as membrane lipids, DNA, and proteins results in impaired cell functions through changes in intracellular calcium or pH, and consequently leads to cell

death (Kehrer, 1993; Sally et al., 2003). The body is however endowed with cellular defence systems to combat the menace posed by the oxidants to the body. These defensive systems are accomplished by the activities of both the enzymatic and non-enzymatic antioxidants which mitigate the toxic effect of oxidants. However, under increased ROS production, the antioxidant cellular defensive systems are overwhelmed, resulting in oxidative stress. Under this type of condition, exogenous supplementation of antioxidants becomes imperative to minimise tissue damage.

Vitamin E is nature's major lipid soluble chain breaking antioxidant that protects biological membranes and lipoproteins from oxidative stress (Osfor et al., 2010). The main biological function of vitamin E is its direct influence on cellular responses to oxidative stress through modulation of signal transduction pathway (Hsu & Guo, 2002). Vitamin E primarily scavenges peroxyl radicals and is a major inhibitor of the free radical chain reaction of lipid peroxidation (Maxwell, 1995; Halliwell & Gutteridge, 1999). We have earlier demonstrated the mitigating effect of vitamin E on short-term neurobehavioural changes induced by acute CPF exposure (Ambali & Aliyu, 2012). The present study was therefore aimed at evaluating the ameliorative effect of vitamin E on sensorimotor and cognitive changes induced by chronic CPF exposure in Wistar rats.

2. Materials and methods

2.1 Experimental animals and housing

Twenty 10 week old male Wistar rats (104±4.2) used for this study were obtained from the Laboratory Animal House of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, Nigeria. The animals were housed in plastic cages and allowed to acclimatize for at least two weeks in the laboratory prior to the commencement of the experiment. They were fed on standard rat pellets and water was provided *ad libitum*.

2.2 Chemicals

Commercial grade CPF (20% EC, Termicot®, Sabero Organics, Gujarat limited, India), was prepared by reconstituting in soya oil (Grand Cereals and Oil Mills Ltd., Jos, Nigeria) to make 10% stock solution. Vitamin E (100 mg/capsule; Pharco Pharmaceuticals, Egypt) was reconstituted in soya oil (100% v/v) prior to daily use.

2.3 Animal treatment schedule

The rats were weighed and then assigned at random into 4 groups of 5 rats in each group. Group I (S/oil) served as the control and was given only soya oil (2mL/kg b.w.) while group II (VE) was dosed with vitamin E [75 mg/kg b.w. (Ambali et al., 2010b)]. Group III (CPF) was administered with CPF only [10.6 mg/kg b.w. ~1/8th LD_{50} of 85 mg/kg b.w., as determined by Ambali (2009)]. Group IV (VE+CPF) was pretreated with vitamin E (75 mg/kg b.w.), and then dosed with CPF (10.6 mg/kg b.w.), 30 min later. The regimens were administered once daily by oral gavage for a period of 17 weeks. During this period, the animals were monitored for clinical signs and death. Furthermore, at various intervals during the study period, the animals were evaluated for neurobehavioural parameters measuring motor coordination, neuromuscular coordination, and motor strength, efficiency of locomotion, learning and memory using the appropriate neurobehavioural devices. In order to avoid bias, the neurobehavioural parameters were evaluated by two trained observers blinded to the treatment schedules. At the end of the dosing period,

each of the animals was sacrificed by jugular venesection and the brain dissected, removed and evaluated for the levels of oxidative stress parameters and AChE inhibition. The experiment was conducted with the permission of the Animals Research Ethics Committee of the Ahmadu Bello University, Zaria, Nigeria and in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.4 Evaluation of the effect of treatments on motor coordination

The assessment of motor coordination was performed using the beam walk performance task as described in an earlier study (Ambali et al., 2010a) on day 0, weeks 8 and 16. Briefly, each of the rats was allowed to walk across a wooden black beam of 106-cm length, beginning at 17.2 cm width and ending at 1.0-cm width. Periodic widths were marked on the side of the apparatus. On each side of the narrowing beam, there was a 1.8-cm step-down to a 3.0-cm area where subjects may step if necessary. As the subject walked across from the 17.2 cm to the 1.0 cm width, the width at which they stepped down was recorded by one rater on each side, and this was repeated twice during each trial session.

2.5 Evaluation of the effect of treatments on motor strength

The forepaw grip time was used to evaluate the motor strength of the rats, as described by Abou-Donia et al. (2001). This was conducted by having each of the rats hung down from a 5 mm diameter wooden dowel gripped with both forepaws. The time spent by each rat before releasing their grips was recorded in seconds. This parameter was evaluated on day 0, weeks 8 and 16.

2.6 Effect of treatments on neuromuscular coordination

The effect of treatments on neuromuscular coordination was assessed using the performance on incline plane as was described earlier (Ambali et al., 2010a). Briefly, each rat was placed on an apparatus made with an angled rough wooden plank with thick foam pad at its bottom end. The plank was first raised to an inclination of 35°, and thereafter gradually increased stepwise by 5° until the subject could no longer stay and be situated horizontally on the plank for 3s, without sliding down. Angles were measured and marked on the apparatus beforehand, and were obtained by propping the plank on a vertical bar with several notches. The test was performed with the head of the rat first facing left and then right hand side of the experimenter. The highest angle at which each rat stayed and stood horizontally, and facing each direction was recorded. Two trials were performed at 2 min apart for each animal. This procedure was carried out on each animal from all the groups on day 0, weeks 8 and 16 of the study.

2.7 Evaluation of the effect of treatments on efficiency of locomotion

The ladder walk was used to assess the efficiency of locomotion as described by Ambali and Aliyu (2012). Briefly, each rat was encouraged to walk across a black wooden ladder (106 cm x17 cm) with 0.8-cm diameter rungs, and 2.5-cm spaces between them. The number of times the rat missed a rung was counted by one rater on each side. The performance on ladder walk was evaluated on Day 0, weeks 3, 7 and 11. Two trials were performed for each testing session.

2.8 Assessment of the effect of treatments on learning

The effect of treatments on learning task in rats was assessed 48h to the final termination of the study in week 17 using the step-down inhibitory avoidance learning task as described by Zhu et al. (2001). The apparatus used was an acrylic chamber 40 x 25 x 25 cm consisting of a floor made of parallel 2-mm-caliber stainless steel bars spaced 1 cm apart. An electric shock was delivered through the floor bars. A 2.5-cm-high, 8 x 25 cm wooden platform was placed on the left extreme of the chamber. Each rat was gently placed on the platform. Upon stepping down, the rat immediately received a single 1.5 amp foot shock through the floor bars. If the animal did not return to the platform, the foot shock was repeated every 5s. A rat was considered to have learned the avoidance task if it remained on the platform for more than 2 min. The number of foot shocks was recorded as an index of learning acquisition.

2.9 Assessment of the effect of treatments on short-term memory

Short-term memory was assessed in individual rat from each group using the step-down avoidance inhibitory task as described by Zhu et al. (2001) 24h after the assessment of learning. The apparatus used was the same used earlier for the assessment of learning. In this test, each rat was again placed gently on the platform and the time an animal remained on the platform was recorded as an index of memory retention. Staying on the platform for 2 min was counted as maximum memory retention (ceiling response).

2.10 Brain tissue preparation

The whole brain tissue was carefully dissected and a known weight of the brain sample from each animal was homogenized in a known volume of ice cold phosphate buffer to obtain a 10% homogenate. This was then centrifuged at 3000 × g for 10 min to obtain the supernatant. The supernatant was then used to assess the levels of protein, malonaldehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and AChE in the brain sample.

2.11 Effect of treatments on brain lipoperoxidation

The level of thiobarbituric acid reactive substance, malonaldehyde (MDA) as an index of lipid peroxidation was evaluated on the brain sample using the method of Draper & Hadley (1990) as modified (Freitas et al., 2005). The principle of the method was based on spectrophotometric measurement of the colour developed during reaction of thiobarbituric acid (TBA) with malonadehyde (MDA). The MDA concentration in each sample was calculated by the absorbance coefficient of MDA-TBA complex 1.56 x 10^5/cm/M and expressed as nmol/mg of tissue protein. The concentration of protein in the brain homogenates was evaluated using the Lowry method (Lowry et al., 1951).

2.12 Evaluation of the effect of treatments on brain superoxide dismutase activity

Superoxide dismutase activity was evaluated using NWLSS™ superoxide dismutase activity assay kit (Northwest Life Science Specialities, Vancouver, WA 98662) as stated by the manufacturer and was expressed as mMol/mg tissue protein.

2.13 Evaluation of the effect of treatments on brain catalse activity

Catalase activity was evaluated using NWLSS™ catalase activity assay kit (Northwest Life Science Specialities, LLC, Vancouver, WA 98662) as stated by the manufacturer and was expressed as mMol/mg tissue protein.

2.14 Evaluation of the effect of treatments on brain acetylcholinesterase activity

Acetylcholinesterase activity was evaluated using the method of Ellman et al. (1961) with acetylthiocholine iodide as a substrate. Briefly, the whole brain of each animal was homogenized in a cold (0–4 °C) 20 m*M* phosphate buffer saline (PBS) incubated with 0.01M 5,5-dithio-bis(2-nitrobenzoic acid) in 0.1 *M* PBS, pH 7.0. Incubations were allowed to proceed at room temperature for 10 min. Then, acetylthiocholine iodide (0.075 *M* in 0.1 M PBS, pH 8.0) was added to each tube, and absorbance at 412 nm was measured continuously for 30 min using a UV spectrophotometer (T80$^+$ UV/VIS spectrometer®, PG Instruments Ltd, Liicestershire, LE 175BE, United Kingdom). AChE activity was expressed as IU/g tissue.

2.15 Statistical analysis

Data were expressed as mean ± standard error of mean. Data obtained from the sensorimotor assessment were analyzed using repeated one-way analysis of variance followed by Tukey's posthoc test. The cognitive and biochemical parameters were analyzed using one-way analysis of variance followed by Tukey's posthoc test. Values of $P < 0.05$ were considered significant.

3. Results

3.1 Effect of treatments on clinical signs

There was no clinical manifestation recorded in the S/oil, VE and VE+CPF groups, while lacrimation, congested ocular mucous membranes and intermittent tremors were observed in the CPF group.

3.2 Effect of treatments on beam walk performance

There was no significant change ($P>0.05$) in the dynamics of beam walk performance in the S/oil group throughout the period of the study. There was a progressive decrease in the width at which VE group slipped off the beam (increase in beam walk length) throughout the study period. Although no significant change ($P>0.05$) was recorded in week 8 compared to day 0 or week 16, a significant decrease ($P<0.05$) in the width at which the VE group slipped off the beam in week 16 compared to that of day 0. There was a significant increase ($P<0.01$) in the width of slip off the beam (decrease in beam walk length) in the CPF group at weeks 8 and 16 when compared to that of day 0, and between week 16 and that recorded in week 8. There was no significant change ($P>0.05$) in the width at which VE+CPF group slipped off the beam at week 8 when compared to that recorded on day 0 or week 16 but a significant increase ($P<0.01$) was recorded at week 16 compared to that of day 0.

There was no significant change ($P>0.05$) in the width at which animals in all the groups slipped off the beam at day 0. At week 8, there was a significant increase ($P<0.01$) in the width at which the CPF group slipped off the beam compared to that of S/oil, VE or VE+CPF group. Similarly, there was a significant increase ($P>0.05$) in the width of slip in the VE+CPF group compare to that of VE group but no significant change ($P>0.05$) in the S/oil group compared to that of VE or VE+CPF group. At week 16, there was a significant increase ($P<0.01$) in the width of slip off the beam in the CPF group compared to the other groups but no significant change ($P>0.05$) in the S/oil group when compared to that of VE or VE+CPF group, and between VE group and that recorded in theVE+CPF group (Fig. 1).

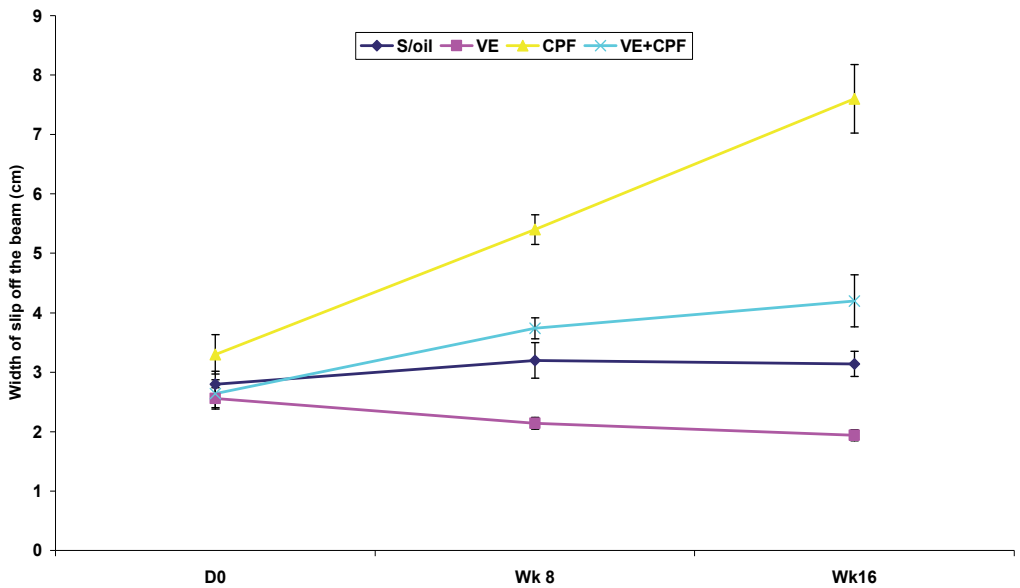

Fig. 1. Effect of chronic administration of soya oil, vitamin E and/or chlorpyrifos on the dynamic of beam walk performance in Wistar rats.

3.3 Effect of treatments on grip time

There was no significant change ($P>0.05$) in the grip time in the S/oil and VE groups throughout the study period. There was a significant increase ($P<0.01$) in the grip time of CPF and VE+CPF groups at day 0 compared to that of week 8 or 16, but not between week 8 and that of week 16. At day 0, there was no significant change ($P>0.05$) in the grip time of rats in between the groups. At week 8, there was a significant decrease ($P<0.01$) in the grip time of CPF group compared to that in the S/oil and VE groups, but not that of VE+CPF group. There was a significant decrease ($P<0.05$) in the grip time in the VE+CPF group compared to that in S/oil or VE group. There was no significant change ($P>0.05$) in the grip time in the VE group compared to that in S/oil group. At week 16, there was a significant decrease ($P<0.01$) in the grip time in the CPF group compared to that in S/oil or VE group but no significant change ($P<0.05$) compared to that in VE+CPF group. There was no significant change ($P>0.05$) in the grip time in the VE+CPF group compared to that in S/oil or VE group. Similarly, there was no significant change ($P>0.05$) in the grip time of S/oil group compared to that in VE group (Fig. 2).

3.4 Effect of treatments on incline plane performance

There was no significant change ($P>0.05$) in the angle at which the S/oil and VE groups slipped off the incline plane throughout the study period. There was a significant decrease ($P<0.05$) in the angle at which the CPF group slipped off the incline plane at weeks 8 and 16, respectively, compared to that of day 0 but no significant change ($P>0.05$) at week 8 relative to that recorded in week 16. There was a significant decrease ($P<0.01$) in the angle at which VE+CPF group slipped off the incline plane at week 16 compared to that of day 0 but no significant change ($P>0.05$) at week 8 relative to that recorded in day 0 or week 16.

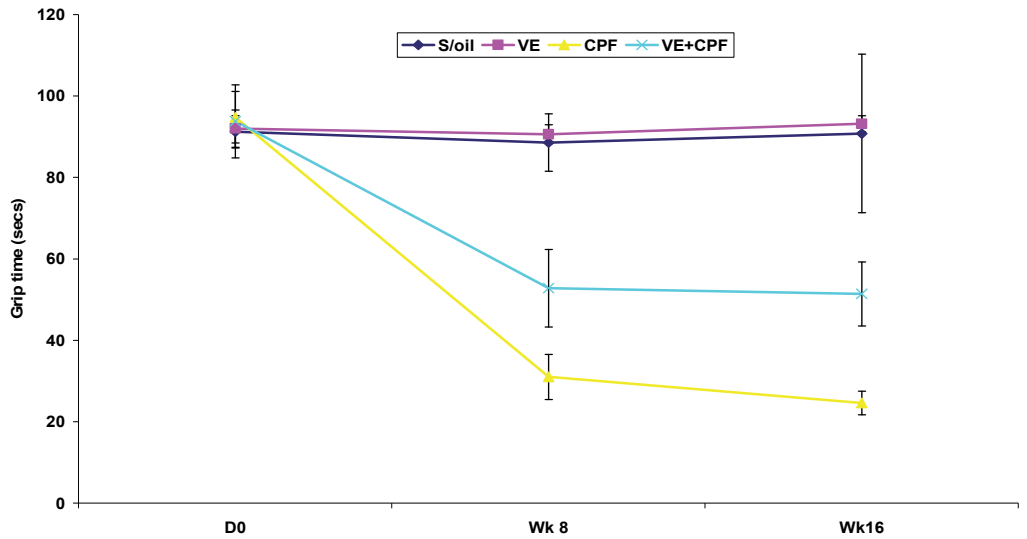

Fig. 2. Effect of chronic administration of soya oil, vitamin E and/or chlorpyrifos on the dynamic of grip time in Wistar rats.

At day 0, there was no significant change (P>0.05) in the angle of slip off the incline plane in between the groups. At week 8, there was a significant decrease in the angle of slip off the incline plane in the CPF group relative to that recorded in S/oil (P<0.05), VE (P<0.01) or VE+CPF group. No significant change (P>0.05) in the angle of slip in the VE+CPF group relative to that in S/oil or VE group, and between VE group and that of S/oil group. At week 16, there was a significant decrease in the angle of slip off the incline plane in the CPF group relative to that in S/oil (P<0.05) or VE (P<0.01) group. Although not significant, there was a 6.3% increase in the angle of slip off the incline plane in the VE+CPF group relative to that in CPF group. There was no significant change (P>0.05) in the angle of slip off the plane in the S/oil group compared to that in VE or VE+CPF group (Fig. 3).

3.5 Effect of treatments on ladderwalk performance

There was no significant change (P>0.05) in the dynamics of the number of missed rungs in the S/oil, VE and VE+CPF groups throughout the study period. There was a significant decrease (P<0.01) in the number of missed rungs in the CPF group at day 0 compared to that in week 8 or 16 but no significant change at week 8 compared to that of week 16.

There was no significant change (P>0.05) in the number of missed rungs in between the groups at day 0. At week 8, there was a significant decrease (P<0.01) in the number of missed rungs in the CPF group compared to that in S/oil or VE group. Although not significant (P>0.05), the mean number of missed rungs in the VE+CPF group was 26% higher relative to that recorded in the CPF group. There was a significant decrease (P<0.01) in the number of missed rungs in the VE+CPF group compared to that in S/oil or VE group. There was no significant change (P>0.05) in the number of missed rungs in the VE group compared to that in S/oil group. At week 16, there was a significant decrease (P<0.01) in the number of missed rungs in the CPF group compared to the VE group but no significant change (P>0.05) when compared to that recorded in S/oil or VE+CPF group. There was no significant change (P>0.05) in the VE+CPF group compared to that in S/oil or VE group.

Similarly, there was no significant change (P>0.05) in the number of missed rungs in the VE group compared to that in the S/oil group (Fig. 4).

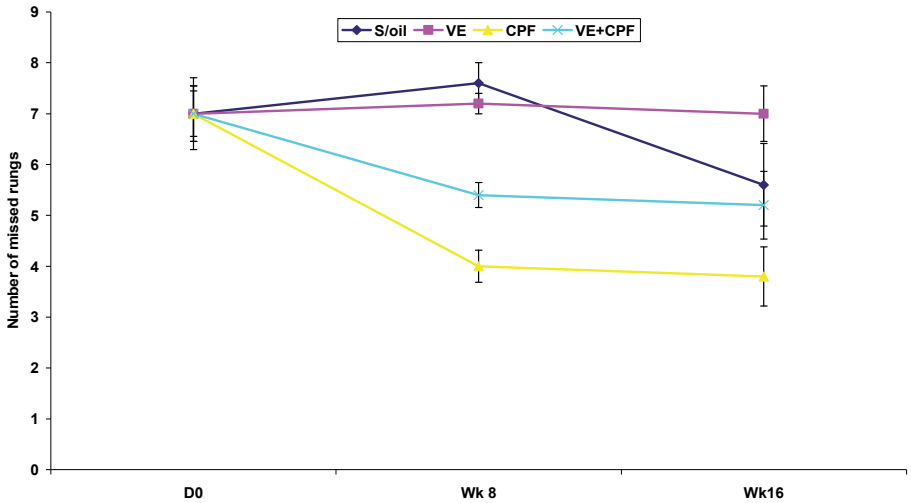

Fig. 3. Effect of chronic administration of soya oil, vitamin E and/or chlorpyrifos on the dynamics of locomotion efficiency in Wistar rats.

3.6 Effect of treatments on learning acquisition

There was a significant increase (P<0.01) in the number of footshocks applied to the CPF group relative to that recorded in the S/oil, VE or VE+CPF group. There was no significant change (P>0.05) in the number of footshocks in the VE+CPF group relative to that in S/oil or VE group (Fig. 5).

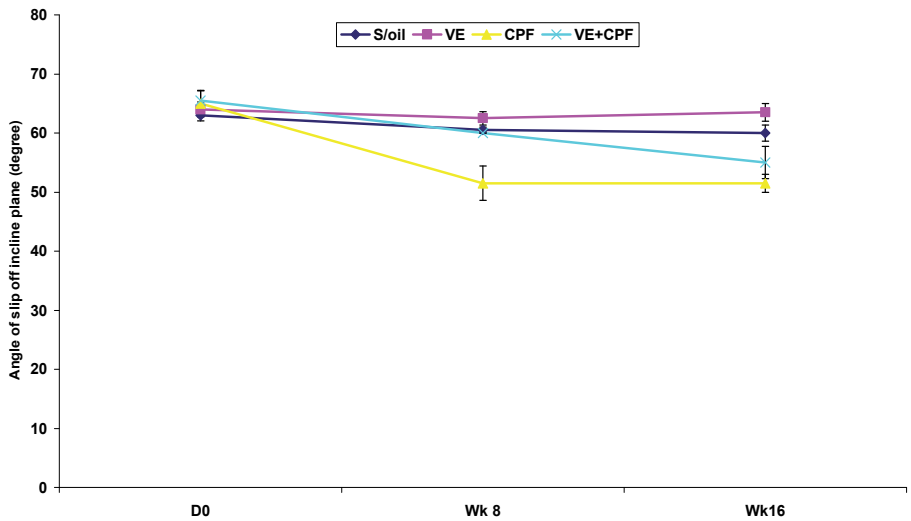

Fig. 4. Effect of chronic administration of soya oil, vitamin E and/or chlorpyrifos on the dynamics of incline plane performance in Wistar rats.

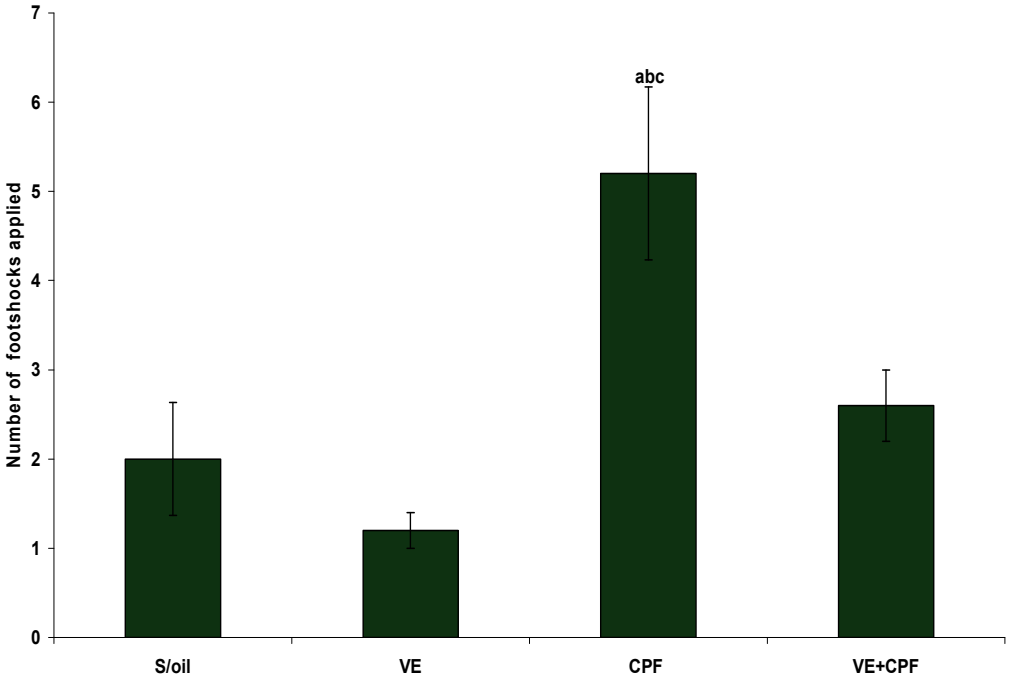

Fig. 5. Effect of chronic administration of soya oil, vitamin E and/or chlorpyrifos on the learning task in Wistar rats. [abc]P<0.01versus S/oil, VE and VE+CPF groups, respectively.

3.7 Effect of treatments on short-term memory

A significant decrease (P<0.01) in the duration of stay on platform (latency on platform) was recorded in the CPF group compared to that in the S/oil, VE or VE+CPF group. There was no significant change (P>0.05) in the duration of stay on the platform in the VE+CPF group compared to that in the S/oil or VE group (Fig. 6).

3.8 Effect of treatments on brain malonaldehyde concentration

A significant increase (P<0.01) in MDA concentration was recorded in the CPF group relative to that in the S/oil, VE or VE+CPF group. There was no significant change (P>0.05) in the brain MDA concentration in the VE+CPF group compared to that in S/oil or VE group, nor between VE and S/oil groups (Fig. 7).

3.9 Effect of treatments on brain superoxide dismutase activity

There was a significant decrease (P<0.01) in SOD activity in the CPF group relative to the S/oil, VE or VE+CPF group. No significant change (P>0.05) was recorded in SOD activity in the VE+CPF group relative to that in S/oil or VE group, nor between VE and that recorded in the S/oil group (Fig. 8).

3.10 Effect of treatments on brain catalase activity

A significant decrease (P<0.01) in brain CAT activity was recorded in the CPF group relative that in the S/oil, VE or VE+CPF group. The CAT activity in the VE+CPF group did not

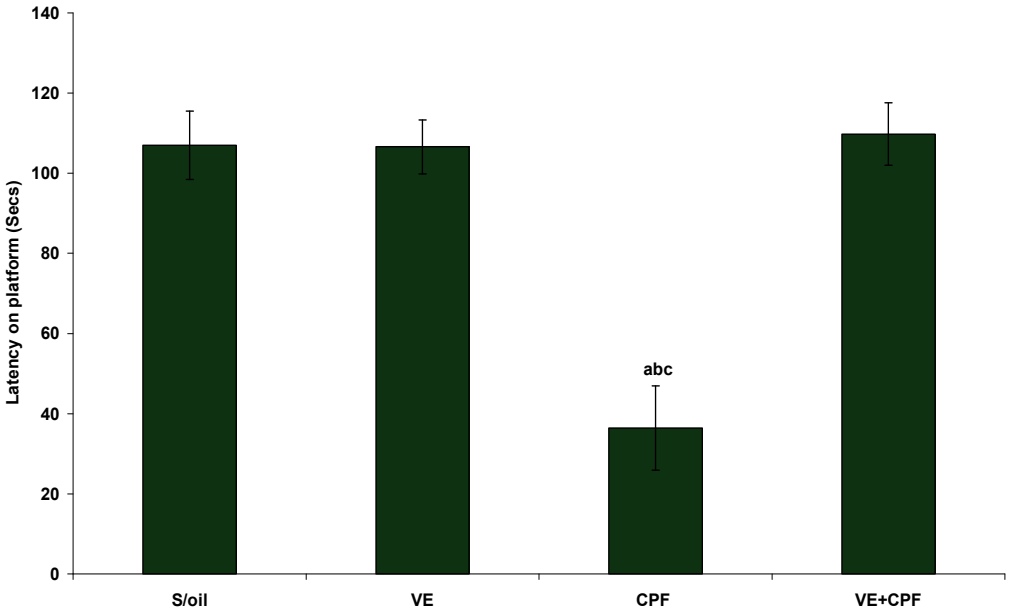

Fig. 6. Effect of chronic administration of soya oil, vitamin E and/or chlorpyrifos on short-term memory in Wistar rats. [abc]P<0.01versus S/oil, VE and VE+CPF groups, respectively.

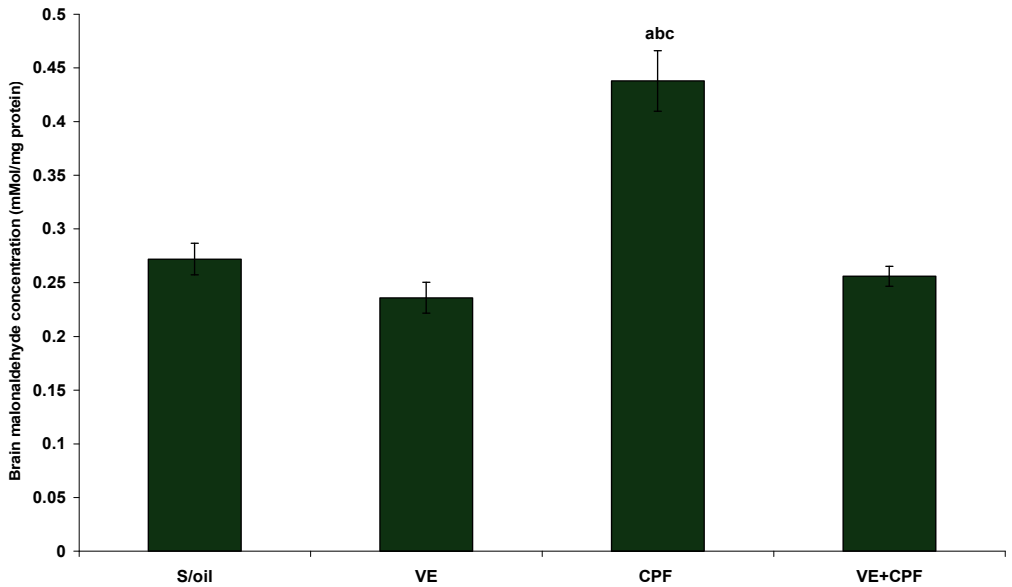

Fig. 7. Effect of chronic administration of soya oil, vitamin E and/or chlorpyrifos on the brain malonaldehyde concentration in Wistar rats. [abc]P<0.01versus S/oil, VE and VE+CPF groups, respectively.

differ significantly (P>0.05) when compared to that in the S/oil or VE group, and between VE and that recorded in the S/oil group (Fig. 9).

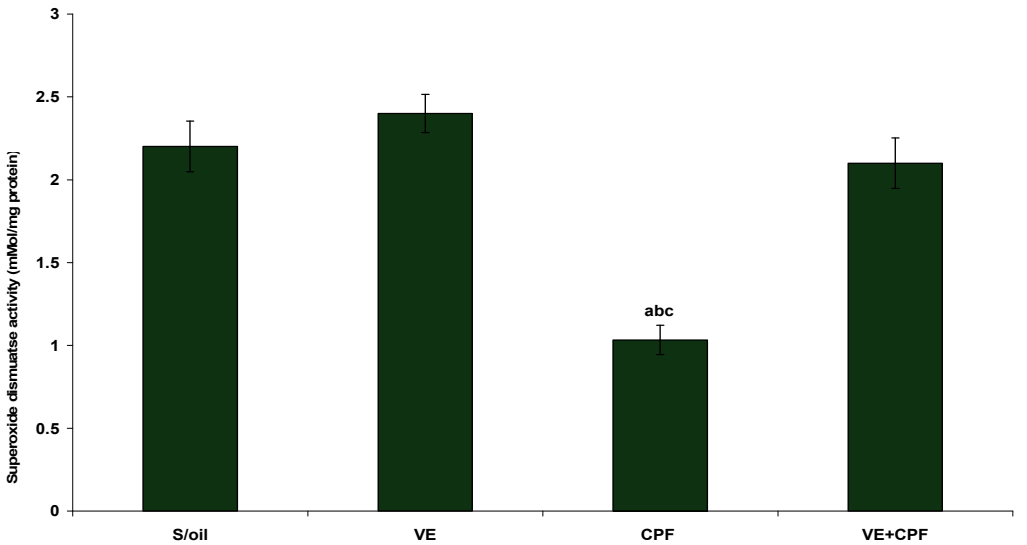

Fig. 8. Effect of chronic administration of soya oil, vitamin E and/or chlorpyrifos on the superoxide dismutase activity in Wistar rats. [abc]P<0.01versus S/oil, VE and VE+CPF groups, respectively.

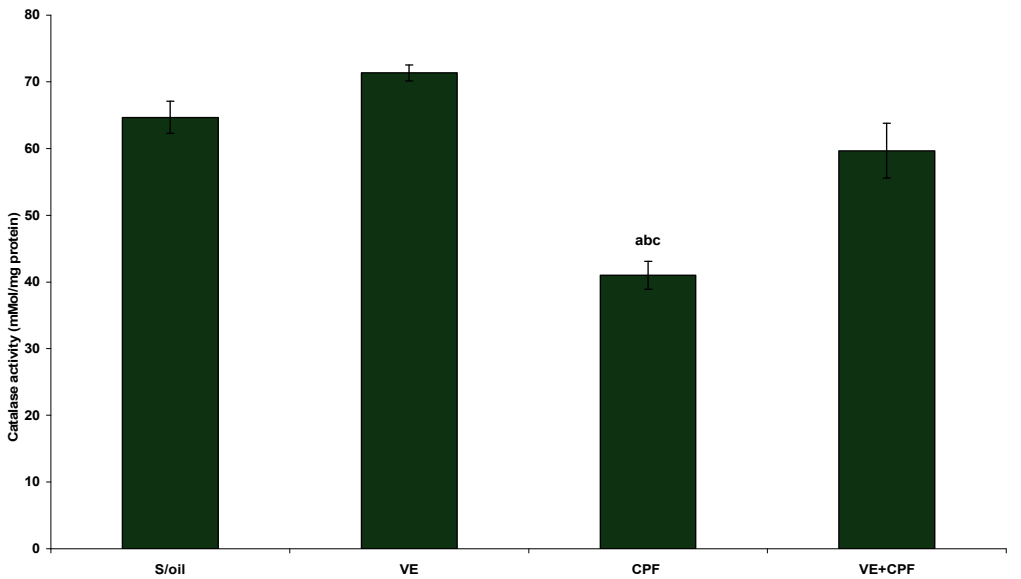

Fig. 9. Effect of chronic administration of soya oil, vitamin E and/or chlorpyrifos on the catalase activity in Wistar rats. [abc]P<0.01versus S/oil, VE and VE+CPF groups, respectively

3.11 Effect of treatments on brain acetylcholinesterase activity

There was a significant decrease in brain AChE activity in the CPF group compared to that in the S/oil ($P<0.01$), VE ($P<0.01$) or VE+CPF ($P<0.05$) group. There was no significant change ($P>0.05$) recorded in CAT activity in the VE+CPF relative to that in the S/oil and VE groups, respectively, or between VE and S/oil groups (Fig. 10).

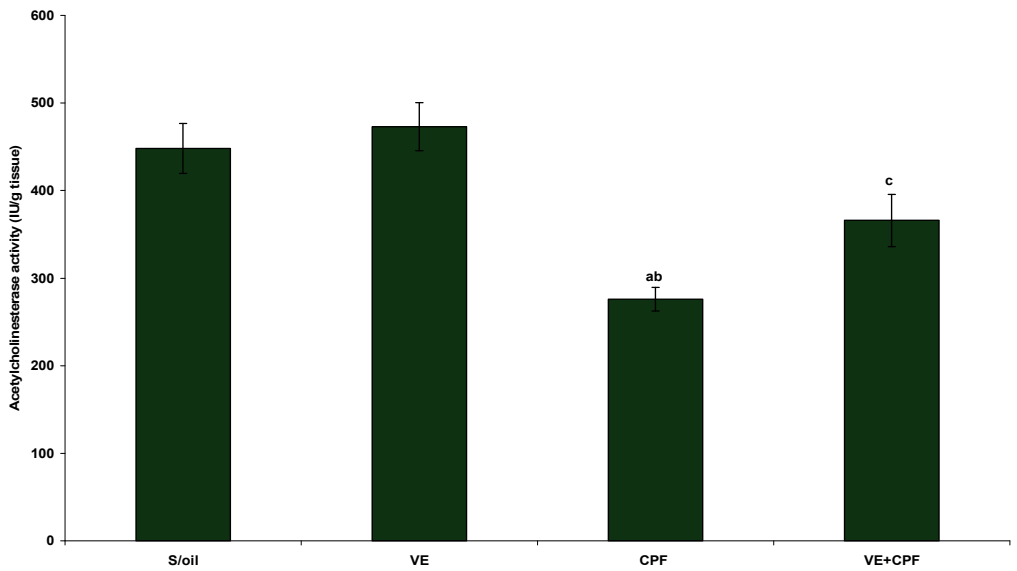

Fig. 10. Effect of chronic administration of soya oil, vitamin E and/or chlorpyrifos on the acetylcholinesterase activity in Wistar rats. $^{abc}P<0.01$versus S/oil and VE groups, respectively; $^{c}P<0.05$ versus VE group.

4. Discussion

The increase in brain MDA concentration and low SOD and CAT activities in the CPF group is an indication of the ability of this pesticide to elevate lipoperoxidative changes and thereby induce oxidative stress. This was in agreement with the findings from our previous studies (Ambali et al., 2010a; Ambali & Ayo, 2011a, 2011b; Ambali & Aliyu, 2012). The brain due to its biochemical and physiological properties is especially sensitive to free radicals, which destroy its functions and structure (Drewa et al., 1998). The brain is highly vulnerable to oxidative stress because in addition to harboring large amount of oxygen in a relatively small mass, it contains a significant quantity of metals (Fe), and has fewer antioxidant molecules than other organs (Halliwell and Gutteridge, 1999; Naffa-Mazzacoratt et al., 2001). For instance, the CNS is relatively poorly endowed with SOD, CAT, and glutathione peroxidase, and is also relatively lacking in vitamin E (Halliwell & Gutteridge, 1985). CPF is lipophilic and may enhance lipid peroxidation by directly interacting with cellular plasma membrane (Hazarika et al., 2003). The increased MDA concentration which is due to induction of free radical has been shown to alter the composition of membrane lipids, proteins, carbohydrates and DNA. Membrane lipids are vital for the maintenance of cellular integrity and survival (Jain, 1989). Peroxidation of membrane lipids results in the

inactivation of enzymes and cross-linking of membrane lipids and proteins and in cell death (Pfafferott et al., 1982; Jain et al., 1983; Jain, 1984). Furthermore, by-products of lipid peroxidation have been shown to cause profound alterations in the structural organization and functions of the cell membrane including decreased membrane fluidity, increased membrane permeability, inactivation of membrane-bound enzymes and loss of essential fatty acids (Van Ginkel & Sevanian, 1994). This lipoperoxidative changes may cause alterations in the structural and functional components of the brain neuronal cells.

The decrease in the SOD and CAT activities in the CPF group has been reported in previous studies (Tuzmen et al., 2007, 2008; Aly et al., 2010; Ambali & Ayo, 2011a) and may reflect the level of oxidative damage caused by the pesticide. SOD is involved in dismutation of the $O_2\bullet-$ to H_2O_2 and oxygen. The significant reduction recorded in the CPF group may be due to either reduction in its synthesis or elevated degradation or inactivation of the enzyme. CAT, on the other hand is known to neutralize H_2O_2 and covert it to H_2O and O_2. The significant decline in the CAT activity observed in group exposed to CPF only may be due to the reduced conversion of $O_2^{\bullet-}$ to H_2O_2 by SOD thereby resulting in the accumulation of $O_2\bullet-$. This accumulated $O_2^{\bullet-}$ inhibits the activity of CAT (Kono & Fridovich, 1982). The decline in the activity of the antioxidant enzymes following chronic CPF exposure in the present study may be due to downregulation in the synthesis of antioxidant enzymes due to persistent toxicant insult (Irshad & Chaudhuri, 2002). Furthermore, $O_2^{\bullet-}$ converts ferroxy state of CAT to ferryl state, which is an inactive form of the enzyme (Freeman & Crapo, 1982), thereby exacerbating the free radical-induced damage to the body tissue.

Pretreatment with vitamin E was shown by the present study to reduce the brain MDA concentration and increase the activities of the antioxidant enzymes, SOD and CAT reflecting its antioxidant properties. α-tocopherol prevents the peroxidation of membrane phospholipids and prevent cell membrane damage through its antioxidant action. The lipophilic character of tocopherol makes it easier to locate the interior of the cell membrane bilayer to exert its antioxidant action. Tocopherol-OH transfers a hydrogen atom with a single electron to a free radical, thus removing the radical before it can interact with the cell membrane (Krishnamoorty et al., 2007). The decreased lipoperoxidation of the membrane due to free radical scavenging effect of vitamin E may have been responsible for the restoration of SOD and CAT activities, since the vitamin may have prevent their full participation in free radical neutralization, hence preserving their activities.

The result also revealed that chronic CPF exposure caused reduction in the brain AChE activity similar to what has been reported in previous studies (Ambali et al., 2010a; Ambali & Ayo, 2011a, 2011b; Ambali & Aliyu, 2012). The ability of CPF to phosphorylate AChE results in impairment of its activity, hence the cholinergic crisis. Apart from this, the induction of lipoperoxidation may have partly contributed to the impaired AChE activity recorded in the CPF group. Oxidative stress affects the activities of various membrane-bound enzymes, including AChE (Mehta et al., 2005) via their direct attack by free radicals or peroxidation of the membrane lipids in which they are embedded (Souza et al., 2010). Besides, OH° has been shown to cause significant reduction in AChE activity in the rat brain (Tsakiris et al., 2000). Vitamin E was shown in the present study to restore the activity of AChE probably due to its antioxidant activity. Vitamin E has been shown in previous studies to restore AChE activity impaired by CPF (Yavuz et al., 2004; Ambali & Aliyu, 2012).

The lacrimation and intermittent tremors observed in the CPF group is part of the cholinergic syndrome typical of OP insecticides (Eaton et al., 2008). These cholinergic signs were due to inhibition of AChE by CPF, resulting in accumulation of ACh in the muscarinic

and nicotinic cholinergic receptors. The ability of vitamin E to remedy the CPF-induced cholinergic signs may be attributed its AChE restoration activity. Furthermore, vitamin E has been shown to increase the activity of paraoxonase 1 (Jarvik et al., 2002), an enzyme that increases the detoxification of OP compounds (Shih et al., 1998).

Beam walking across bridges of different cross-sections provides a well-established method of monitoring motor coordination and balance in rodents. The progressive increase in the width at which rats in the CPF group slipped off the beam which indicates impairment of motor coordination has been reported in previous studies (Ambali et al., 2010a; Ambali & Aliyu, 2012). Abou-Donia et al. (2002) observed similar results following repeated exposure of rats to sarin. Beam-walking performance is an integrated form of behavior requiring pertinent level of consciousness, memory, sensorimotor and cortical functions mediated by the cortical area (Abou-Donia et al., 2001). Cortical injury may therefore have been responsible for the deficit in beam-walk performance in the CPF group (Abou-Donia et al., 2001) partly due to oxidative damage. Indeed, CPF and CPF-oxon have been shown to induce apoptosis in rat cortical neuron independent of AChE inhibition (Caughlan et al., 2004). Pretreatment with vitamin E mitigated but did not completely abolish the motor coordination deficits induced by chronic CPF exposure. This is because there was a significant increase in the width at which the VE+CPF group slipped off the beam at week 16 compared to day 0. This shows that oxidative stress may not be the only mechanism involved in motor coordination deficits induced by chronic CPF exposure.

The present study has also shown a significant reduction in forepaw grip time, reflecting deficit in forepaw motor strength following chronic CPF exposure in rats. The result agreed with the finding obtained in an earlier study which showed reduction in hind limb grip strength following repeated CPF administration in rats (Terry et al., 2003). The impairment of motor strength by CPF may have also been due to the decrease in anterograde axonal transport (Terry et al., 2007) or reduced neuronal viability associated with impaired microtubule synthesis and/or function (Prendergast et al., 2007). It has also been postulated that disruption of kinesin-dependent intracellular transport may account for some of the long-term effects of OPs on the peripheral and central nervous system (Gearharta et al., 2007). Reduced hand strength (Miranda et al., 2004) and loss of muscle strength (Steenland et al., 2000) have been observed in humans following prolonged exposure to OPs. Relationship has also been established between higher OP exposure and the development of chronic fatigue syndrome (Tahmaz et al., 2003). Furthermore, the role of muscle (Ambali and Ayo, 2011b) and brain oxidative damage induced by CPF which causes impairment of neuronal viability (Ambali & Ayo, 2011a) hence reduction of motor strength cannot be over emphasized. Although there was a significant deficit in motor strength in the VE+CPF group at weeks 16 and 8 when respectively compared to day 0, the fact that there was no significant change especially at week 16 compared to S/oil and VE groups reflect improvement in motor strength in this group. This may be partly due to reduced brain and perhaps muscle oxidative damage complemented by improvement in AChE activity which improves neuronal transmission.

Chronic CPF exposure has been shown in the present study to interfere with neuromuscular coordination as shown by the decline in the incline plane performance at weeks 8 and 16. The inclined plane test has been used to evaluate integrated muscle function and strength in rodents by evaluating their ability to maintain body position on a board as its angle of inclination is increased. We have earlier demonstrated the ability of acute CPF exposure to impair short-term neuromuscular coordination (Ambali et al., 2010a; Ambali & Aliyu, 2012).

Abou-Donia et al. (2002) similarly showed the ability of the OP warfare agent, sarin to impair incline plane performance in rats. The impairment of neuromuscular coordination may be due to increase in brain oxidative changes induced by CPF, which alters the morphological and functional capacity of the brain region involved in neuromuscular coordination. Oxidative damage to the brain following CPF exposure has been reported in previous studies (Verma, 2001; Ambali et al., 2010a; Ambali & Ayo, 2011a, 2011b; Ambali & Aliyu, 2012). Furthermore, the reduction of AChE activity may have been partly involved in the impaired neuromuscular coordination recorded in the CPF group, since alterations in ACh metabolism may alter neuronal activity.

Although the incline plane performance in the group pretreated with vitamin E at week 16 was significantly lower than that obtained at day 0, the study generally showed that performance in weeks 16 and 8 in the VE+CPF group was not significantly different from that of S/oil or VE group. This shows that the vitamin mitigated the CPF-evoked deficit in neuromuscular coordination. The fact that vitamin E did not completely abolish the CPF-induced impaired incline plane performance shows that oxidative stress and restoration of AChE activity may not be the only factor responsible for the sensorimotor deficit.

The lower ladder score characterized by lower number of missed rungs observed in rats chronically exposed to CPF indicates that the legs of the rats were frequently being held stationary above the rungs for a relatively longer period. This observation demonstrated difficulty in the ability of CPF group to move fast through the obstacles, and hence a deficit in locomotor activity. The deficit in locomotor efficiency observed in the CPF group was dependent on the duration of exposure, with much more impairment recorded at week 16 compared to week 8. The results agreed with the previous findings that slowness of movement is one of the extrapyramidal symptoms (Parkinsonism) observed in humans exposed to non-specific agricultural pesticides, which increased with the duration of exposure (Ritz & Yu, 2000; Alavanja et al., 2004). Thus, the locomotion deficit in the CPF group observed in the present study is part of the sensorimotor deficits occurring in animals chronically exposed to CPF. This impaired mobility may be due to oxidative stress as oxidative damage to the muscle induced by CPF (Ambali & Ayo, 2011b) may have probably caused necrosis thereby impairing locomotion efficiency. Carr et al. (2001) attributed reduced mobility observed in OP poisoning partly to damage in the peripheral musculature, probably due to necrosis of skeletal muscle fibre. Muscle necrosis has been observed following exposure to the OP insecticide, isofenphos and the insecticide metabolite, paraoxon (Dettbarn, 1984; Calore et al., 1999). Similarly, the impaired mobility may be due to inhibition of AChE activity and the subsequent cholinergic paralysis induced by CPF. The severity of the muscle necrosis may be dependent on the level and duration of AChE inhibition (Carr et al., 2001). The amelioration of the locomotor deficits manifested in the improvement of ladder walk and characterized by increase in the number of missed rungs in rats pretreated with vitamin E demonstrated the important role played by oxidative stress and AChE inhibition in the locomotor deficit induced by CPF.

The significant increase in the number of footshocks received by the CPF group relative to the other groups indicates learning impairment. Similarly, the significant reduction in the duration the animal in the CPF group stayed on the platform indicates deficit in memory. This shows that CPF exposure even at low dose is capable of cognitive impairment. CPF-induced cognitive impairment have been reported in several studies in rats (Bushnell et al. 1991; 1994; Prendergast et al., 1997, 1998, 2007; Stone et al., 2000, Moser et al., 2005; Ambali

et al., 2010a; Ambali & Aliyu, 2012). In addition, studies in humans have shown persistent cognitive deficits in farmers and pesticide applicators repeatedly exposed to OPs but are symptom-free (Steenland et al., 2000; Dick et al., 2001). The impairment of cognition observed in the CPF group may be due to alteration in ACh metabolism due to reduction of AChE activity. Since ACh has been demonstrated to be involved in cognition, agents such as OPs which alter ACh metabolism may interfere with this role. Many studies have linked central cholinergic system to synaptic plasticity, learning and memory processes (Baskerville et al., 1997; Sachdev et al., 1998). It is believed that OP compounds play a role in memory loss by producing cholinergic dysfunction at the level of the synapse (Carr & Chambers, 1991).

Furthermore, CPF has been shown to induce cytotoxicity directly on the hippocampal cells via the induction of apoptosis, irrespective of its effect on AChE (Terry et al., 2003). Induction of apoptosis has been described as the toxic end-point of CPF neurotoxicity in the brain as it induces structural changes in the brain that may cause functional deficits, including those involved in memory and learning (Caughlan et al., 2004). Apoptosis probably resulting from oxidative damage to cellular macromolecules may have been responsible for the massive degenerative changes in the brain neurons and glial cells of rats chronically exposed to CPF that we reported in an earlier study (Ambali & Ayo, 2011a). CPF-induced oxidative stress may be central to apoptosis, since free radicals have been implicated in apoptotic death of cells (Corcoran et al., 1994; McConkey et al., 1994). Degenerative changes in the neurons leads to functional deficits as it relates to neurotransmission and other brain activities.

Vitamin E has been shown in the present study to improve learning and short-term memory impaired by chronic CPF exposure. We have earlier demonstrated the ability of either vitamin C or E to mitigate short-term cognitive changes induced by acute CPF exposure in rats (Ambali et al., 2010a; Ambali and Aliyu, 2012). The improved learning and short-term memory recorded following pretreatment with vitamin E may be due to its antioxidant and AChE restoration properties. Apart from its antioxidant function, vitamin E influences the cellular response to oxidative stress through modulation of signal-transduction pathways (Azzi et al., 1992), which may have further enhanced the neuronal function. Similarly, neuroprotective effect of vitamin E has been established in several studies (Frantseva et al., 2000a, 2000b; Pace et al., 2003; El-Hossary et al., 2009) and may have contributed in mitigating the behavioural changes induced by CPF in the present study.

5. Conclusion

The present study has shown that the impaired sensorimotor and cognitive changes induced by chronic CPF exposure mitigated by pretreatment with vitamin E are partly due to its antioxidant, neuroprotective and AChE restoration properties.

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12

Non-Chemical Disinfestation of Food and Agricultural Commodities with Radiofrequency Power

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1. Introduction

The presence of microbial and insect/mite pests in foods and agricultural commodities, particularly in fresh produce, dried foods, nuts, grains, seeds, nursery plants, ornamental flowers and in wood products (i.e. pallets), continues to be a major factor affecting their condition for safe distribution and use in local, regional and international markets. As a mean to reduce the potential of propagating non-indigenous pests, postharvest (mandatory) treatment modalities and quarantine barriers have been imposed to regulate transportation and distribution of many of these products worldwide. These regulations define strategies for the detection, control, or eradication techniques for controlling quarantine insect and mite pests.

Today, more than 6,500 nonnative species are already established in the United Sates and approximately 15% of these species are either economically or environmentally harmful (Pimentel, Lach, Zuñiga et al., 1999). Control or eradication practices for arthropod pests are mostly based on chemical pesticides, although host removal, adequate agricultural production practices, biological control agents, and sterile insect release are often techniques applied in place off or in conjunction with pesticides.

Among the most important quarantine plant pests, various exotic fruit flies have been identified in the USA as threats to more than 250 crops. On the other hand, the presence of moths in stored products represents important and unacceptable risks to many growing and expanding agricultural regions worldwide. If detected, affected commodities must be processed with effective control or eradication techniques. If unattended, losses in product's quality represent unacceptable economic losses.

Chemical pesticides, waxes, coatings, thermal treatments (heated air; hot water immersion), modified atmospheres, cold storage (refrigeration), and irradiation are some of the processes that have helped industry meet current challenges and demands. Lately, however, new consumer preferences, trends and regulatory interventions have increased the needs for minimally processed foods with low or no residual chemicals. This new trend requires that less invasive or chemical-free alternatives become available to replace or minimize the use of pesticides. Furthermore, recent concerns associated with potential terrorist threats using microbial contaminants or other pests, have increased the need to develop alternatives to

assure the safety of the food supply while minimizing economical risks associated with production and export agriculture. These combined challenges are now familiar to affected governments as well as to industry and regulators worldwide.

Historically, and with a few exceptions, pesticides have provided an ample spectrum of effective techniques to control pests and there is a continual industry trend to maintain and improve their use. However, this practice and its effects and limitations have partially fueled the emergence of organic agriculture. This in turn has prompted conventional agriculture to review its practices, its traditional processes, and to investigate new types of pesticides as well as to develop new disinfestation techniques. The incorporation of fluorine in agrochemicals to enhance stability and bioavailability is the latest attempt to increase their effectiveness while reducing their secondary impact (Jeschke, 2004). Nevertheless, their invasiveness and persistence in all environs surrounding agricultural practices continues to be resisted by consumers and by increased limiting regulations.

Past and even present industry reliance on methyl bromide fumigation for quarantine pest controls is the best and most recent example of the changing attitude that exists today with respect to invasive chemical processes. The existing ban and the new restrictions on production levels have forced agriculture to look for new and better alternatives. Fumigation, vacuum techniques and controlled atmospheres (CA) for insect (quarantine) control are marginally successful and restricted to long-storage commodities (i.e. grains, nut products, raisins) (Bond, 2007; Calderon, 1990). For perishable fresh commodities, these techniques have failed to provide the required and timely disinfestation level. Nevertheless, while somewhat successful, the needed long processing times (days or weeks) increases cost and is inadequate to fit with the logistics of marketing fresh agricultural products.

The use of low-level doses of ionizing radiation (i.e. food irradiation) is another effective and approved technique providing an alternative to disinfestation and disinfection of many commodities (Urbain, 1986). However, while technically useful and approved for certain applications, this approach prompts many public concerns and is usually and effectively resisted. Furthermore, because irradiation facilities require a high capital investment to install and operate in order to remain economically viable, it also forces the irradiation industry to operate as major centralized facilities located near high productivity agricultural areas. The seasonal nature of agriculture, however, forces the irradiation industry to meet the peak demands with excess processing capacity and to broaden off-season applications (i.e. disinfection of medical supplies) to remain viable. Consequently, the handling and distribution of to-be-treated food and agricultural commodities imposes new and severe logistical and cost adjustments to the user community. As a result, few if any agricultural export areas rely on irradiation facilities and those operating represent a small and stagnant resource for insect control.

Despite the above limitations, ionizing radiation also provide means to sterilize insects that once released in specific areas can reduce the impact of local/regional infestations.

As of today, with the exception of food irradiation, few attempts to fulfill the need for new alternatives to pesticides have been investigated using single or combined physical processes. If effective, these processes are inherently safer, eliminating the risks associated with the presence of pesticides in products and ultimately easing the current concerns with disposal issues, worker safety, and environmental impacts. Non-chemical or residue-free alternatives also provide opportunities to yield products with attributes closer to their natural sensory and nutritional properties. Furthermore, because physical processes are

solely based on the use of energy, they are naturally free of residues and therefore can serve the needs of both conventional and organic agriculture.

Since 2002, research at the University of California, Davis established the use of RF power for disinfestation as well as for many novel sanitation and preservation purposes for a variety of food, non-food and agricultural commodities. Since then, RF processing has been established as a novel methodology able to provide new alternatives for chemical-free disinfestation, disinfection and enzyme deactivation effects on various commodities (Lagunas-Solar, 2003; Lagunas-Solar, Zeng & Essert, 2003; Lagunas-Solar, Zeng, Essert et al. 2005a; Lagunas-Solar, Cullor, Zeng, et al. 2005b; Lagunas-Solar, Zeng, Essert et al. 2006a). RF disinfestation, in particular, was proven as an effective, rapid, and a reliable chemical-free alternative to pesticides and capable of large-scale processing.

Radiofrequency waves using designated, single frequencies are approved for industrial, scientific and medical uses by national (US Federal Communication Commission, FCC) and international organizations. Currently, limited but increasing commercial use in all these areas to heat-treat and dry a variety of commodities is underway. Radiofrequency power provides well-controlled, volumetric (internal) and rapid heating of a diverse variety of food and non-food commodities. Appropriate food and non-food products to be processed and heated with RF power are generally known as dielectrics (poor electric conductors) and include pests, microbes, foods and non-food agricultural commodities such as soil, packaging and wood (pallets) products.

Dielectric properties are directly related to the material's chemical (molecular) composition and due to the presence and relative abundance of dipoles like water and/or induced dipoles like proteins, lipids, and carbohydrates. Therefore, the material's ability to absorb RF power and convert it to thermal power resides at the molecular level. Because molecules are well distributed and organized within and on the surface of dielectric materials, the effect of absorbing RF power occurs throughout its volume and to a lesser extent on its surface (lower concentrations) where temperatures are slightly lower than its internal volume (< 1^{o}C). For this reason, RF processing is said to be a volumetric process, comparable to microwave heating, but in contrast with any other conventional surface thermal process known today. By comparison, the volumetric nature of RF processing provides with unique opportunities to reduce the needed thermal load (i.e. temperature over time) required for an intended effect as heat losses by radiation are larger at the surface. This volumetric property applies equally to arthropod and microbial pests as well as to the host commodity and its package.

The RF disinfestation process is rapid (seconds to minutes) and proven effective when reaches lethal thermal levels (50-60^{o}C). These levels are sufficient to provide thermal loads able to irreversibly disrupt essential and common metabolic pathways and to affect all biological stages of arthropod (and other) pests. Furthermore, as the interaction of RF photons with molecules is frequency dependent, at specific frequencies insect pests exhibit a higher heating rate than the host commodity allowing a somewhat selective heating process to be realized. This selective process minimizes processing time and lowers the overall thermal load applied to the commodity thus decreasing the potential for any adverse effects on its quality attributes.

The fundamental physical concepts and the rationale behind the RF disinfestation process, including the interactive energy-transfer and conversion mechanisms (RF to thermal power) with arthropod pests are explained below.

2. Physics of RF power

2.1 RF photons and the electromagnetic spectrum

Radiofrequency photons belong to the electromagnetic spectrum of radiant energy. The electromagnetic spectrum covers a very large range of wave photons with frequencies ranging from 10^6 to 10^{20} Hz (1 Hz = 1 cycle/sec) and wavelengths from 10^3 to 10^{-12} m. As shown below in Figure 1, this range covers radiowaves (~10^6 to 10^{10} Hz), microwaves (~10^{10} to 10^{12} Hz), infrared, visible and ultraviolet radiation (~10^{12} to 10^{16} Hz) and soft, hard X rays and gamma rays (10^{16} to 10^{20} Hz).

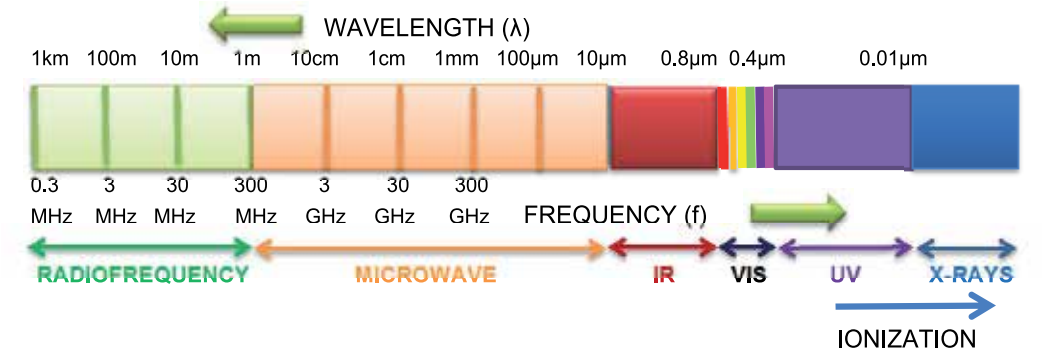

Fig. 1. Electromagnetic spectrum (simplified).

Radiofrequency power is, however, a small segment of the radiowaves region with an arbitrarily defined range of frequencies between ~ 1 MHz (300 m wavelengths) to 300 MHz (1 m wavelengths). In the defined frequency range, the RF photon energy is in the 6.6 x 10^{-28} to 6.6 x 10^{-26} J/photon (or 4.1 x 10^{-9} to 4.1 x 10^{-7} eV/photon). Therefore, RF processing involves photons of very low energy and long wavelength and therefore absorbing dipole or induced dipole molecules can only experience excitation effects (i.e. vibrational and rotational) but will not lose electrons to cause ionization or the formation of free radicals.[1]

Radiofrequency waves are produced by rapid electrical oscillations and generally are able to penetrate deep into various materials, but are reflected by electric conductors and by the ionized layers in the upper atmosphere. Like all other photons in the electromagnetic spectrum, RF photons consists of electric and magnetic waves oscillating at right angles to the direction of propagation (i.e. transverse waves) and moving through space at the speed of light (c = 2.998 x 10^8 m/sec). The combination of electric and magnetic fields originates an electromagnetic field.

The relationship between the RF photon energy and its frequency is given by Einstein's classical expression as:

$$E = hf \tag{1}$$

where: E is the photon energy (Joules);

[1] Chemical bond energies are in the range of 1 to 10 eV per bond. Therefore, RF photons (1 to 100MHz) carry one billionths to 100 millionths less energy than is required to break a single bond. Free radicals are extremely reactive (short lived) chemical species capable of inducing chemical reactions. Their formation is associated exclusively with sources of ionizing radiation (> 1 eV/photon).

h is the Planck's constant (6.626 x 10^{-34} Joules sec or 4.136 x 10^{-15} eV sec); and
f is the photon frequency (Hz or cycles/sec).

This expression indicates that all photons in the electromagnetic spectrum come as discrete quantities named "quanta" and moving at the speed of light. It also indicates that photon energy is always a multiple of Planck's constant times its frequency (cycles/sec).

Because frequency (f in Hz) and wavelength (λ in m) of an electromagnetic wave are related to the speed of light as

$$c = f\lambda \tag{2}$$

formula 1 can also be expressed as

$$E = hc / \lambda \tag{3}$$

indicating that photon energy E is inversely proportional to its wavelength λ.

2.2 Interactions of RF photons with matter

Biological materials - including foods, microbes, arthropods and many agricultural products, are non-magnetic in nature, therefore, only the electric field component of an electromagnetic wave is able to interact and strongly affect the polar and induced polar molecules in the product.

In the presence of an oscillating electric field (changing polarity at a set frequency), the interactive mechanisms of the electric field with RF active molecules (i.e. dielectrics or poor electric conductors) include: (1) reorientation of permanent dipoles (i.e. water); (2) inducing dipoles by polarization of bound charges (proteins, carbohydrates, lipids); and (3) forcing the drift (displacement) of electronic and ionic conduction charges (mineral nutrients) (Klauenberg & Miklavcic, 2000).

The above interactive mechanisms only act at the molecular level and thus the effects of RF processing is based solely on the material's chemical composition in which permanent dipoles (i.e. water) play a major role while other lower concentration non-polar or weakly polar molecules are activated in proportion to the magnitude of the electric field. Initially, and without an electric field, polar and non-polar molecules in any material are randomly oriented due to thermal excitation, which forces their multi-directional movement and spatial distribution.

When an electric field is applied, dipole (polar) molecules tend to re-orient and become aligned according to the direction of the electric field in a phenomenon known as "orientation polarization". Still, orientation is opposed by thermal excitation and therefore, the net orientation effect is proportional to the intensity of the electric field once it overcomes the random distribution of the active molecules in the RF field.

In non-polar molecules, the electric forces separate positive and negative charges a small distance thus inducing temporal dipoles. This type of induced dipole exists only when the electric field is present and occurs via electronic (displacement of electrons) or atomic (displacement of charged atoms) mechanisms known collectively as "distortion polarization".

In both cases with orientation or distortion polarization, the charges in dipoles or in induced dipoles do not cancel and, therefore, new internal electric fields are formed. Distortion polarization is temperature dependent while orientation polarization is inversely

proportional to temperature as RF active molecules must overcome the randomness from thermal excitation.
Furthermore, all polarization effects can only operate up to a limiting frequency after which if frequency increases, orientation polarization effects tend to disappear as the inertial effect of permanent polar molecules prevent reversal of their direction of motion and thus their inertial movement (i.e. momentum) cannot be overcome. The RF process is thus frequency dependent and can be optimized at certain selective frequencies matching the dielectric properties of a material (Lagunas-Solar, Zeng & Essert, 2003).
In arthropod pests, as in all biological systems, water (free and bound) and to a lesser extend proteins, lipids, carbohydrates are the major chemical constituents while mineral nutrients are at trace levels. Water is a natural permanent dipole but its degree of freedom depends on its chemical environ with free (unbound) water being the most active dipole to interact with oscillating electric fields. Bound water, on the other hand, because of its binding (coordination) with other molecules, may still be active but is somewhat restricted to respond to electric field oscillations. Proteins, including enzymes, lipids and carbohydrates are polarizable under a voltage difference and therefore become temporal induced dipoles able to experience electric field interactions and be actively involved in generating thermal energy within the material. Inorganic ions (i.e. mineral nutrients) are always charged and can be displaced by the electric fields and generate small electric currents which converts to heat through resistance (Ohm's law). Overall, although at different levels, all constituents may be actively re-oriented or displaced generating thermal energy by combination of the above different interactive mechanisms.
Although most permanent and induced dipoles are not free to drift, displacements of conduction charges or free ions under the influence of an electric field is a classical phenomenon known as ionic conductivity. Conduction effects (J_c in Amperes/m^2) are related directly to both conductivity (σ in Siemens/m^2) and the net electric field E (Amperes/Siemens) (Lea & Burke, 1998).

2.3 Mechanisms of RF heating

The ability to induce polarization effects in a material by an applied electric field and the creation of new, transient electric fields and currents within the material is characterized by a quantity noted as ε and called "dielectric constant" or "permittivity" (Klauenberg & Miklavcic, 2000). Therefore, the dielectric constant measures how easily a material is polarized to store electric energy.
However, dielectric constants are measured in relation to vacuum or air (ε_o = 1.00000 and 1.00054, respectively) as they represent the ability of a material to store electric energy (i.e. capacitance) at a given voltage as compared to vacuum or air. Therefore, relative dielectric constants for a material are given by

$$\varepsilon' = |\hat{E}_a| \,/\, |\hat{E}| \tag{4}$$

where ε' is the relative dielectric constant and $\hat{E}_a$ and $\hat{E}$ are the applied and the net electric field strengths (vectors), respectively.
In real practice, the ratio by which each mechanism intervenes in storing electric energy is accompanied by effective dissipation losses due to thermal excitation, inertial motions and due to the different binding forces in lattices or accompanying the RF active chemicals. These losses force molecules to lag behind the frequency of the oscillating electric field or

restrict drifting and thus resist movements of electric currents. These types of losses are represented by a relative complex dielectric constant (Ɛ*) which is given by the expression (Metaxas & Meredith, 1983):

$$Ɛ^* = Ɛ' - j\,Ɛ'' \quad (5)$$

In this expression, Ɛ″ is a measure of the dissipation losses per cycle and is known as the "dielectric loss factor" and j is the imaginary unit. The dielectric loss factor measures the ability of a material to convert electric energy to thermal energy purely based on polarization effects (i.e. no resistance heating) and is always positive and much smaller than the relative complex dielectric constant (Ɛ*) (Mudgett, 1986; Nyfors & Vainikainen, 1989).
Both relative complex dielectric constant and dielectric loss factors are related to the absolute dielectric constant in vacuum ($Ɛ_o$ = 8.85 x 10^{-12} F/m). For clarity, the use of the word "relative" is omitted from this point and therefore Ɛ*, Ɛ′ and Ɛ″ will be known simply as complex dielectric constant, dielectric constant and dielectric loss factor, respectively.
While most products have small dielectric loss factors, it increases rapidly with temperature but only slightly with pressure. However, these factors can vary drastically with operating frequencies but are independent of the applied electric-field magnitude.
Finally, dielectric constants are the factor by which a dielectric material increases the capacitance of a parallel-plate RF system (i.e. RF cavity, see section 3.1 below) in relation to its capacitance in vacuum or air under the same electric field conditions. Examples of Ɛ* values for selected materials are given in Table 1, below (Clarke, 2006). Worth noting is that Ɛ* values for codling moth (71.5; 84.5) and Mexican fruit fly (90; 141) are exceptionally high and similar to water and much larger than values for some host materials (i.e. nuts). Thus, RF disinfestation applications with nuts or similar products, selective (higher) heating of insects - as compared with heating of the host commodity, can be realized and is advantageous for effective insect control while lowering overall thermal loads applied to the host commodity. This phenomenon is further explained in section 3.5.2, below.

Material (Moisture %)	Temperature (°C)	Frequency	Ɛ*
Water	100	> 1MHz	80
Ethanol	20	10 MHz	24
Sand (dry)	20	1	2.5
Walnut (0%) Walnut (17%)	20 20 (60)	10 MHz 10 (27) MHz	2.0 5.0 (4.9)
Almonds (5%)	20 (60)	27 MHz	5.9 (6.0)
Codling moth	20 (60)	27 MHz	71.5 (84.5)
Mexican fruit fly	20 (60)	27 MHz	90 (141)
Douglass fir (11%) Compressed	15 15	1 MHz/10 MHz	3.2 4.3
Paper Fiber	20	1 MHz	4.5
Polyethylene (non polar)	20	50 Hz/1 GHz	2.3
Polycarbonate (polar)	20	1 MHz	3.0

(*) From: National Physical Laboratory (www.kayelaby.npl.co.uk/general_physics/2_6/2_6_5.html) and Wang et al., (2003)

Table 1. Values of complex dielectric constants for selected materials (*).

2.4 RF power dissipation as thermal power

The ability of molecules within a material to store electric energy from an operating RF system is the first step towards an effective heating process able to induce a desirable biological effect (i.e. disinfestation). As indicated above, this is expressed by the complex dielectric constant which combines dielectric properties defined by molecular composition. Therefore, the conversion of RF power into thermal power is directly related to the polarization and ionic conduction mechanisms described above. However, the fractional contribution of each interactive mechanism is determined by the frequency (Hz) of the oscillating electric field.

At low frequencies, all dipole molecules (permanent and induced) have sufficient time to follow and adjust to the reversal cycles of the oscillating electric fields. In this case, no or negligible energy dissipation losses occur due to orientation polarization effects. Under this condition, the dielectric constant is at its maximum value and the dielectric material is capable of storing a maximum energy from the applied electric field. The RF heating is then mostly due to combined polarization and ionic conduction effects.

As frequency increases, dipoles gradually lose their ability to fully adjust to the oscillations in polarity of the electric field and polarization effects lag behind and contribute less to the total polarization. To minimize this lagging, the electric field transfers its energy to the dipoles forcing them to respond faster. However, this electric-field forced adjustment reaches a limit at which no further corrections occur. At this point, lags in dipolar polarization become larger forcing the dielectric constant to fall in value while dielectric loss factors increases. Under this scenario, RF heating depends less on polarization effects but more on ionic conduction effects (displacements or drifting of charged molecules and ions) leading to resistance heating. This variation in mechanism is therefore highly influenced by commodity temperature.

The total RF power dissipation into a sample is derived from the fundamental laws of electromagnetism. For a steady-state sinusoidal electric field, the time-average of RF thermal power dissipation per unit volume of the sample is given by:

$$P_v = 2\pi\varepsilon_o f\varepsilon''_{eff}E^2_{rms} \quad (6)$$

where P_v is in watt per cubic meter (W/m^3; or $[Joules/sec]/m^3$); f is the frequency of the oscillating electric field (Hz); ε'' is the dielectric loss factor and E_{rms} is the root-mean-square of the applied electric field in Volts per meter (V/m) (Metaxas & Meredith, 1983).

The total amount of heat Q (Joules) needed for a mass m (kg) of a dielectric material to increase its temperature from an initial value (T_i) to a final temperature (T_f) (i.e. $\Delta T = T_f - T_i$) is given by the classical expression

$$Q = mC_p(T_f - T_i) \quad (7)$$

where C_p is the specific heat of the material ($Joules/kg^oC$).

Power per unit volume in formula 6 can be combined with the energy required as given in formula 7 to provide a combined formula (formula 8) leading to RF throughput as determined with RF processing parameters:

$$mC_p(T_f - T_i)/Vt = 2\pi\varepsilon_o f\varepsilon''_{eff}E^2_{rms} \quad (8)$$

which can be expressed as

$$\Delta T/t = (2\pi \varepsilon_o f \varepsilon''_{eff} E^2_{rms})/d \quad (9)$$

where the ratio $\Delta T/t$ is the rate of heating expressed as a function of processing parameters (f, E) and the other factors are associated with the product properties (Ɛ″ and d), where d (kg/m^3) is its density.

2.5 Temperature distribution and depth of penetration in RF processing

Reaching all pests in a volume of material to be disinfested is an important feature of RF disinfestation as the process must be effective over large volumes of material to assure reliable control with adequate throughputs. Temperature distribution and depth of penetration are thus important aspects that need to be considered for RF disinfestation of large volumes of commodities.

In standard volumes of boxed or palletized materials processed with a parallel-plate capacitor (see section 3, below), the intensity of the electric field is largely unaffected by the load and it contributes to similar energy absorption throughout the material. In addition, depth of penetration is an important added factor.

An electromagnetic wave incident on the surface of a dielectric material can either be reflected (i.e. reflected wave) or be transmitted into the material (i.e. transmitted wave).

In good dielectrics (including its package), a great fraction of the wave energy is transmitted but is gradually attenuated as it is converted to thermal energy. The extent (length) of the wave transmitted into the material is known as "penetration depth" (D_p) and is arbitrarily defined as the distance from the surface to the point (plane) at which its energy is reduced to e^{-1} (1/2.71 or 37%).

Because the effective loss tangent ($\tan \delta_{eff} = \varepsilon''_{eff}/ \varepsilon'$); the penetration depth can be approximated by

$$D_p = \frac{c}{2\pi f(\varepsilon')^{1/2} \tan \delta_{eff}} = \frac{c(\varepsilon')^{1/2}}{2\pi f \varepsilon''_{eff}} \quad (10)$$

Penetration depth (D_p in meters) is therefore proportional to the dielectric constant (Ɛ′) and inversely proportional to the dielectric loss factor (ε''_{eff}) and to the frequency of oscillation of the electric field. In general, at frequencies below 100 MHz, penetration depth is of the order of meters unless the dielectric loss factors are exceedingly high (Metaxas & Meredith, 1983). Despite its penetration, however, the energy distribution and thus the thermal profiles of the RF heated material must be taken into account when the process's efficacy requires a pre-defined or a narrow temperature range.

For disinfestation applications, however, the threshold temperature to assure lethal effects in all insects and mites at any biological stage is rather small (50-60^{o}C) and requires a short time (< 1 min). This allows the use of RF disinfestation in large-volume containers (i.e. pallets at 2 x 2 x 2.2 m high) as material handling techniques can also be applied to improve temperature homogeneity to narrower ranges (but assuring reaching a threshold thermal load) as some limitations are expected by penetration depth factors.

However, as explained above, changes in the dielectric behavior of the load due to increased temperature (i.e. increased dielectric loss factors) induce rapid and significant changes in the fraction of the electric energy being absorbed and converted to thermal power. Unattended, these factors could lead to severe localized, uneven heating of the packaged commodity with potential loss of quality. Therefore, process controls need to be focused into

maintaining adequate RF power densities to be applied and by controlling product temperatures during the process. Besides, product geometry, package material and its geometry, and air gaps within the material clearly contribute further to different power densities being generated in their volumes and thus temperature variations are to be expected. As lethal thermal loads in insects and mites are low (50-60^{o}C; ~ 1 min), process effectiveness is assured by reaching the relatively low lethal thermal loads needed. This occurs at levels below than those affecting quality in the host commodity and is due to the higher metabolic complexity of arthropod pests as compared with the much simpler metabolism of the host food (i.e. insects in grains) or agricultural commodity (i.e. insects in pallets).

Heat transfer and temperature distribution across a material in RF disinfestation is critical to assure effectiveness and both phenomena have been studied intensively (Giles, Moore & Bounds, 1970). In the absence of any significant mass transfer (e.g. evaporation), the temperature distribution in the medium obeys the heat diffusion law and is given by:

$$\frac{\partial T}{\partial t} = \alpha_T \nabla^2 T + \frac{\delta P}{d c_p} \tag{11}$$

where α_T is the thermal diffusivity of the medium (m^2/s), T is temperature, δP is the localized power density (W/m^3), ∇ is the Laplacian operator, d is density and c_p the specific heat (Metaxas & Meredith, 1983). The thermal diffusivity measures the ability of a material to conduct thermal energy relative to its ability to store thermal energy. Materials of large a_T will respond quickly to changes in their thermal environment, while materials of small a_T will respond more sluggishly and take more time to reach a new temperature equilibrium condition.

In RF processing, materials are usually heterogeneous, and therefore a_T plays an important role because different parts absorb RF power at different rates. For homogenous materials, a_T is less important in temperature distribution, and δP can be approximated as P_v (formula 6) for the temperature analysis. However, thermal diffusivity (or thermal mass effect) of insects and mites is not known despite the many reported studies on thermoregulation of common habitats with its surroundings. However, the rapid heating of insects with RF power (Nelson & Charity, 1972) suggests an appreciable value of thermal diffusivity for insects.

Finally, due to its direct heating effects at molecular levels, RF heating is independent of temperature differences and heat transfer coefficients, although both these factors will influence the subsequent dynamic distribution of thermal energy within the volume of the material (Hill et al., 1969).

3. Principles of RF processing

3.1 The RF cavity – A parallel-plate capacitor

In order to best realize and apply the above mechanisms in a controlled and safe RF disinfestation process, a parallel-plate capacitor is used with materials to be treated placed in between and named the "load". The process can be performed either statically (batch mode) or continuously (conveyorized mode). This type of capacitor is known as a "RF cavity" and is shown schematically in Figure 2, below.

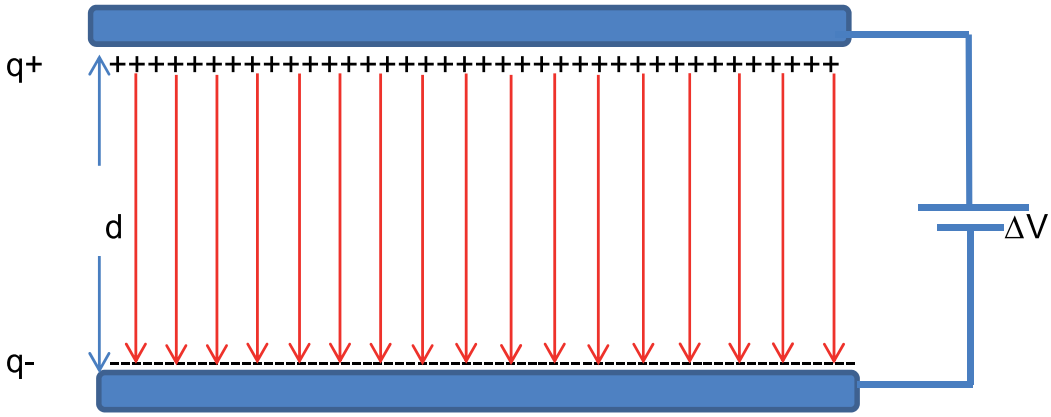

Fig. 2. A static parallel-plate capacitor (RF cavity).

The RF cavity operates with equally charged plates (top positive and bottom negative) formed when a voltage difference is applied. Electric field lines (red) are directed towards the negative (ground) plate and are equally spaced and parallel to each other. Transverse waves (not shown) are perpendicular to the electric field. When activated, however, by placing a material (load) in between, the electric field geometry is changed as field lines are distorted (i.e. fringe effects especially at low frequencies) due to the load and its package, and its intensity is decreased because of new charges created in the load. The presence of air gaps in between and on top of the packaged dielectric load also contributes to field distortion and localization effects. Therefore, an active RF cavity needs to be properly designed and managed in order to minimize the above effects and maintain field homogeneity and thus treat with adequate uniformity.
A schematic of the major features of a RF power system is shown in Figure 3, below, while a version of an operating commercial-scale prototype is shown in Figure 4.

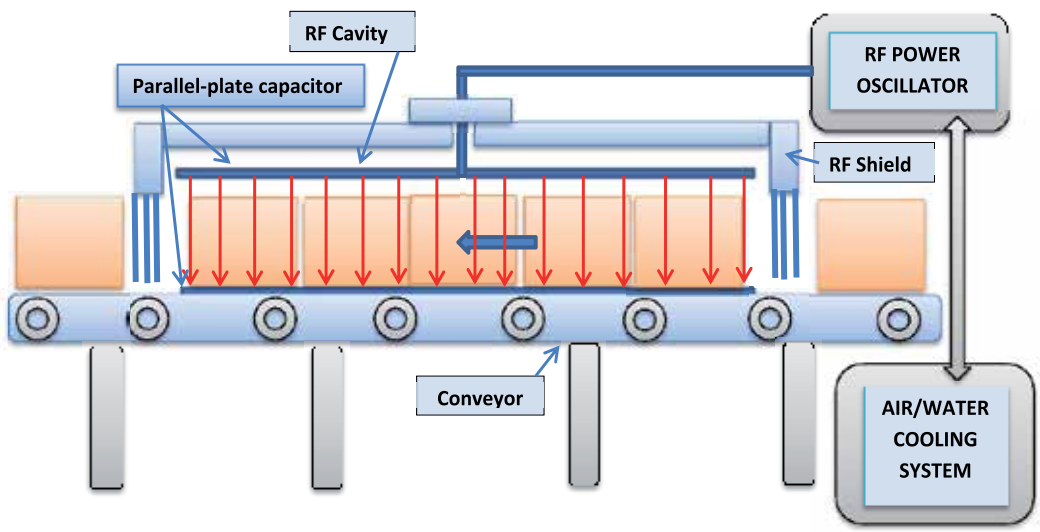

Fig. 3. Schematics of a conveyorized RF processing system.

Fig. 4. RF system for batch processing (13.15 MHz, 10 kW). Designed by UC Davis & RF Biocidics Inc.

In this latter system, the RF cavity is shielded in all directions with a metallic enclosure (shown in light blue) so as to prevent propagation or reflections of RF waves outside its boundaries and thus eliminate the potential to expose workers, the surrounding environs or interfere with other radiowaves. This basic configuration, singly or in modules, is able to operate and meet the conditions to generate and delivery RF energy safely and efficiently to food and agricultural commodities at commercial-scale levels.

The parallel-plate configuration shown in Figure 2 (above) is said to be in a static condition in which no material (other than air or vacuum) is placed in between and therefore the electric field lines are equally spaced and parallel to each other while the overall electric field is uniform except at the edges. However, when a product (load) (i.e. a dielectric) is introduced and the electric field is rapidly oscillated (changing electric polarities at every cycle) with a certain frequency, the dielectric product (load) is now capable of absorbing RF energy by a combination of the above mentioned molecular mechanisms and convert it to thermal power.

The main characteristic of RF processing (RF heating) is therefore, based on the high frequency alternating oscillating electric fields interacting with the dielectric medium (dipoles and induced dipoles) in between the plates and generating thermal energy (heat). RF heating is therefore, also known as "high frequency capacitive heating" (Piyasena et al., 2003), although as the medium in between the plates is also a dielectric material, the process is often referred as "high frequency dielectric heating" (Zhao et al., 2000).

The generation of thermal energy is due to the ability of the applied oscillating electric field to polarize and re-orient internal electric fields of charges formed in the load (material). The rotating electric field exerts torques on permanent and induced dipoles to force them into flip-flop motions. During the rapid cycling, friction and heat is generated between polarized molecules (permanent or induced dipoles) and their neighbors including lattice losses as they move. The higher the frequency of oscillations the greater is the energy available or

created to be converted to heat. However, due to lattice limitations, when the frequency is at the maximum equilibrium between rotation and inertial restrictions, it is said to be at a "Debye resonance" at which there is maximum conversion to heat. If operating frequency is beyond the ability of the molecules to react due to inertial motion, the process loose overall energy-conversion efficiency. This suggests that specific materials, due to their own unique chemical composition will present an optimal frequency at which to operate with maximum energy-use efficiency. In materials with complex or different composition (i.e. pest and host) is therefore possible to establish selective RF heating effects and establish a process with minimal energy input to the lesser dielectric component (Lagunas-Solar et al. 2006).

In addition to polarization mechanisms, a dielectric material can also be heated by the resistance to direct ionic conduction or drift mechanism as given by Ohm's law and that states that the current (I in Amperes) through a conductor between two points is directly proportional to the voltage difference (V in volts) across the two points and inversely proportional to the resistance (R in Ohms). The heating level through these mechanisms depends on the electric conductivity of the material which is generally low as dielectric (i.e. poor conductor) properties prevail.

Finally, because these mechanisms occur with equal intensity between the RF cavities (i.e. same electric field intensity) and are only dependent on the material's chemical composition, RF heating is in principle homogeneous and a volumetric (internal) method in contrast with all other surface heating methods known today. However, at a microscopic scale within biological materials, some differences do occur due to variations in chemical composition and moisture levels. These differences allow for the enhancement of the rate of heating with distinct materials and are the basis for selective RF heating effects (Zimmerman, Pilwat & Riemann, 1974).

3.2 Advantages of RF disinfestation

For disinfestation purposes, RF power provides a unique mean to heat an arthropod pest (small mass or volume) inside a host commodity (large mass or volume) volumetrically (internally) and with penetrating RF waves. This behavior is opposite to the use of conventional surface-heat methods such as infrared, dry and wet steam, or hot water where the host's surface becomes a physical barrier to the applied thermal energy. In all latter cases, the distribution of the applied heat to reach the entire volume depends on heat-transport mechanisms and time. In addition, heat is only applied at its surface. Furthermore, under these conditions, many commodities experience undesirable changes that lower product value. In contrast, because of its penetration, RF waves are effective in reaching deeply internalized pests such as eggs and larva deposited in internal cavities by borer insects, a situation in which the effectiveness of fumigants is restricted by the presence of air-locks impeding penetration of fumigants.

Radiofrequency processing is volumetric heating and its energy transfers directly to the product without the need of intermediate transfer mechanism such as conduction, radiation, or convection. This allows RF energy to be transfer to the load much faster and more effectively. The amount of input energy can be controlled by reducing the input power or switching the system on and off in order to achieve precisely the final temperature. These characteristics allow the RF process to be operated within low and high thermal boundaries, called "thermal windows". Thermal windows for RF disinfestation as compared with other biological effects (i.e. pasteurization and enzyme deactivation) are given in Figure 5, below.

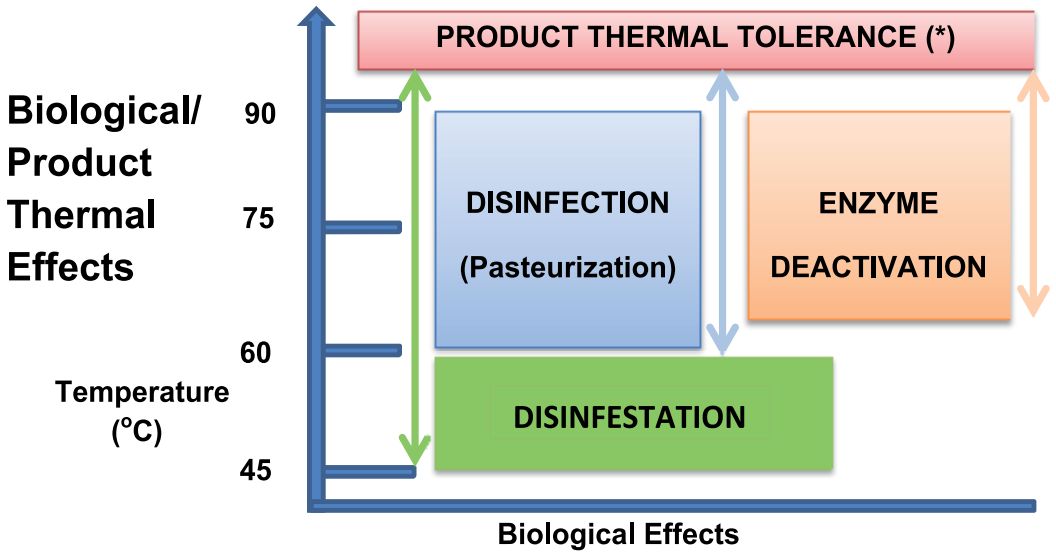

Fig. 5. Thermal Windows (colored arrows) for RF processing effects.

A thermal window represents the differential thermal sensitivity between living organisms (highly-heat sensitive) and the more heat-tolerant properties of agricultural products. Therefore, operating within a product's thermal window minimizes the impact on the host commodity. This is a critical advantage of RF processing over any conventional (surface) heating method as disinfestation effects can be well controlled because of the high sensitivity of arthropod pests to thermal energy and the higher heat tolerance of most affected foods and agricultural commodities.

By comparison with conventional heating processes, overheating the surface is very common because energy is first applied to the surface and then is conducted to its interior. Because energy loss from the surface (by radiation and/or convection) is unavoidable, significant and fast, these processes often require additional heat input on the surface in order to produce internal temperatures high enough to achieve a uniform biological effect. The host, however, received higher and usually damaging thermal loads on its surface.

As a result, the upper boundary of the thermal window (especially for the surface) is frequently exceeded causing unacceptable changes in the physiological, sensory, and quality of foods. In RF disinfestation, the surface temperature is usually lower than the internal temperature due to the heat loss from surface radiation and due to evaporation. This can be effectively prevented during RF processing by reducing evaporation (e.g. high humidity inside the chamber), by adding moisture before processing and by providing good radiation reflectors in the RF cavity design.

3.3 RF and microwave processing

Frequently, microwave heating is confused with RF heating. While fundamentally similar, microwave heating (also an energy source in the electromagnetic spectrum) is operated at 915 MHz (λ = 0.3 m) and 2,450 MHz (λ = 0.1 m), that is with higher frequency and shorter waves than RF. For most commercial scales (i.e. large amounts) of foods and agricultural products, microwave heating is not adequate also has many disadvantages in aspects of heating homogeneity, energy penetration, and energy-use efficiency. First, it does not

produce homogeneous heating because of the limited penetration of the shorter wavelength and the complex non-uniform standing wave patterns. The penetration depth of microwave is in the order of 5 cm to 10 cm for bodies with high water content, and may be higher (in several tens of centimeters) for other drier materials (Orfeuil, 1987). In addition, the electric field inside the microwave oven is not uniform due to the nature of standing waves. In fact, the enclosed electric field and power density vary with the location and the sample's shape and size. Non-uniform electric field patterns and variable power densities often lead to local (or uneven) heating in the material. Besides, the power density in microwave heating are much higher than in RF heating (due to much higher operational frequencies) and is associated with non-uniform electric fields. Therefore microwave heating normally causes local hot spots to the commodity.

In contrast, the RF process is operated at frequencies much lower than conventional microwaves hence the penetration of RF energy is greater, usually higher than 1 m and even several tens of meters at low frequencies (Orfeuil, 1987). Furthermore, the electric fields generated between two parallel plates are very uniform; therefore, RF transversal waves interact and heat the material more homogeneously (Wig *et al.*, 1999; Mitcham *et al.*, 2004).

3.4 Comparison with conventional disinfestation technologies

Today, conventional or emerging alternatives face several restrictions or their use is associated with many safety concerns many of which prompted the development of RF disinfestation as well. The contributing factors from the industry perspective are summarized below.

3.4.1 Chemical pesticides issues and concerns

Methyl bromide fumigation was for many decades the preferred treatment applied to many stored food commodities. It was used worldwide to meet quarantine and phytosanitary restrictions and quality requirements as mandated by global agriculture markets. Current alternative methods used to control insects in grains include the use of insecticides (e.g. Malathion), fumigants (e.g. phosphine, carbon dioxide) and temperature treatment (Bond, 2007).

Malathion (American Cyanamid Co., USA) is one of the safest organophosphate insecticides. Nevertheless, existing regulations demands that the treated grains should not be sold for at least 7 days nor should be eaten within 60 days after treatment to avoid potential toxic effects from residues left.

Phosphine gas is very toxic to human therefore its application requires strict controls, even though there is no residue left to the treated grains.

Other pesticides in use include Chloropicrin, 1,3-dichloropropene, Telone/Vapam, sulfaryl fluoride and hydrogen cyanide.

However, all pesticides available and those mentioned in particular are of global concern due to the potential for causing detrimental effects on animals, air, water and soil as well as potentially impacting public health and workers safety.

Conventional carbon dioxide fumigation of grains usually referred as modified atmospheres requires a lengthy treatment (i.e. days to weeks) therefore its cost is high as well as its impact on the logistics of product distribution to markets.

3.4.2 Conventional heat processing

Conventional high-temperature treatments of grains, such as hot air or hot water immersion and dry or wet steam are usually less effective to internally hidden eggs or pupae inside grain kernels. As adequate lethal temperature for insect pests need to be applied throughout the volume of the commodity, surface overheating and diminishing quality attributes usually occurs due to slow dynamics of heat transport from the outside to the core of grain kernels. Overheating also leads to the deterioration of grain quality and viability.

Because of the above, there is a clear need to develop and establish better, less or non-invasive alternatives to disinfect grains and other commodities to overcome safety concerns associated to invasive methods (leaving residues). Highly desirable is the long-stated need to reduce risks to consumers, workers and the environment as indicated by international organizations (UNEP 1998; WMO 2003).

3.5 Mechanisms of RF Disinfestation

As RF disinfestation is to initiate energy-transfer mechanisms at the molecular level, there are two possible mechanisms for the inactivation/control of insect and mites using RF power: thermal and non-thermal effects. The thermal effect of RF power is essential to the destruction of microorganisms and many studies have proven its validity (Goldblith & Wang, 1967; Fujikawa et al., 1992; Kozempel, Annous, Cook et al., 1998). The energy absorption from RF power can raise the temperature of contaminant organisms high and fast enough to induce irreversible (i.e. non repairable) biochemical damage to cells such as the denaturation of enzymes, proteins, DNA, RNA, or of other vital cellular components, as well as disruption of cell membranes (Heddleson & Doores, 1994). Reports of potential non-thermal effects (effects unrelated to heat stress) with higher-frequency dielectric heating (basically at microwave frequencies) are still controversial. While some researchers have announced these effects (Burton, 1949; Olsen, 1965; Fung & Cunningham, 1980; Cross & Fung, 1982), other researches have concluded there is little or no non-thermal effect on cells (Goldblith & Wang, 1967; Carroll & Lopez, 1969; Rosenberg & Bögl, 1987; Knutson *et al.*, 1987). However, using high-peak power RF technologies capable of delivering ultra-short pulses with very high instant power (>MW/pulse) remain as a potentially successful approach for disinfestation and in particular for fresh produce and other high-thermally sensitive commodities (Lagunas-Solar, Zeng & Essert, 2003).

3.5.1 RF disinfestation thermal effects

The cell is the fundamental unit of all living matter. Living cells (prokaryotes and eukaryotes) are basically composed of high-molecular-weight polymeric compounds (macromolecules) such as proteins, DNA, RNA, polysaccharides, lipids, and storage materials such as fats, glycogen, polyhydroxybutyrate, etc. (Madigan, Martinko & Parker, 2000). These macromolecules are only functional in the proper three-dimensional structures. The structural property is affected by thermal energy and is especially important for enzymes as they are very effective biological catalysts and involved in most of cellular reactions (Shuler and Kargi, 1992).

Because RF power generates heat at the molecular level, RF energy can effectively increase the kinetic energy of molecules and make these molecules vibrate more rapidly and violently. These molecular vibrations, up to a point, are strong enough to disrupt weak intermolecular forces, such as hydrogen bonds, salt bridges, disulfide bonds, and non-polar hydrophobic interactions in secondary, tertiary and quaternary structures of

macromolecules and denature their normal biological order and function. The most essential thermal damage that leads to cell death is the denaturation of enzymes, especially some critical enzymes responsible for DNA and RNA replications in the cell (Roti Roti, 1982). Thermal energy or heat can cause non-repairable denaturation of DNA, RNA, and sometimes create structural DNA lesions (sections of DNA contain elementary damage sites) that cause the loss of cellular genetic information (Ward, 1985).

Heat also transfers its energy to make molecules more energetic which leads to weaker hydrogen bonds and hydrophobic interactions sustaining the cell membrane, and eventually causes its disruption or collapse. The disruption of cell membrane leads to uncontrollable material exchange between the cell and its environment, which causes the cell to lose its optimum microenvironment required for its metabolisms and the cell dies eventually (Bowler & Fuller, 1987). Heat can also destroy storage materials in cells such as lipids, fats, and carbohydrates by oxidation.

Thermal energy from RF power can increase insect body temperature high enough to be lethal and destroy them (disinfestation) by causing cellular damages (i.e. cell death or dysfunctional) or body dehydration. The thermal death due to cellular damages of this multi-cellular organism is not usually the consequence of massive cell death per unit time, but it may due to the loss or disruption of cells in a certain critical tissues (Denlinger & Yocum, 1998).

Differences in species and developmental stages are also likely to influence the site of lethal thermal wounding. The more complex the biological system, the more susceptible it is to high thermal stress. Therefore, it is expected that macromolecules (e.g. proteins, DNA, RNA, lipid, fat, etc.) are more resistant to thermal stress than cellular organelles (e.g. mitochondria, nucleus, Golgi complex, etc.), cellular organelles are more resistant than cells, cells are more resistant than tissues, and tissues are more resistant than the whole organism (Ushakov, 1964; Prosser, 1986). Hence for a multi-cellular organism, lethal wounding may be inflicted from cellular damages of an organization with a high level of complexity.

The above concepts explains the prevalence of the concept "living dead" in the insect control, which means organisms are still alive but will not survive and reproduce due to cellular thermal injuries (Bowler, 1963; Chen, Lee & Denlinger, 1990). Therefore, as insect's biology is more complex than unicellular organisms (e.g. bacteria, fungi), they are expectedly more susceptible to thermal stress.

High temperature can also be lethal to insects by causing dehydration and promoting desiccation. Above a certain temperature, the critical transition temperature, the rates that insects lose water from their bodies increase dramatically (Yoder & Denlinger, 1991). Critical transition temperature values commonly range from 30 to 60°C for different species and developmental stages (Hadley, 1994). Most insects contain about 60 to 70% water in their body weights, and many can tolerate a loss of 20 to 30% of water for brief periods. The loss of water will increase the osmotic stress and concurrently increase the solute concentration within the body, presumably leading to irreversible cell damages. This also increases RF-induced ionic conduction effects in insects thus enhancing thermal energy production and thermal stress favoring lethality.

3.5.2 RF selective heating of insects

The main mechanism of disinfestation in RF selective heating is also thermal stress (i.e. heat). In the selective RF heating, a proper operating frequency is selected so that the effective dielectric loss factor (ε'') of the target material is close to its maximum value and

the load (material) can be heated fast. Because different materials have different dielectric properties (i.e. dielectric constant [Ɛ′] and effective dielectric loss factor [Ɛ″]) - both of which depend on the composition and frequency, they interact and convert RF energy into heat at different rates at the same frequency.
This leads to the potential that different materials in the same load can have different heating rates, depending on the values of their effective loss factor (Ɛ″) at that frequency. If an appropriate frequency is chosen so that contaminant organisms (e.g. arthropods, arachnids) can absorb RF energy faster than host material, those organisms can be heated much faster than other components in the same load (Lagunas-Solar *et al*., 2006; Lagunas-Solar et al., 2008). As a result, insects/mites are destroyed by heat while the host commodity is unaffected. This treatment is proposed for somewhat thermally resistance fresh products (i.e. tomatoes, avocados, apples, grapes, and broccoli) which can tolerate some low thermal loads but sufficiently high to be effective for disinfestation applications using a controlled- thermal RF treatment.
While theoretically applicable to selective RF heating of microorganisms, their small size prevents adequate absorption of the penetrating RF energy waves and thus there is no evidence today for the availability of this selective mechanism for microorganisms.
Finally, the above and other technological and consumer factors prompted the investigation on the use of RF power for disinfestation of various commodities by several authors (Ikediala et al., 2000; Wang et al., 2003; Mitcham et al., 2004; Wang et al., 2007a; Wang et al., 2007b). Results and conclusions of all these studies corroborated the advantages of RF disinfestation over available techniques and also helped identify remaining challenges (Prakash & Rao, 2002).

4. Case study: RF disinfestation of rough (paddy) rice

During long-term storage, insects can cause considerable damage to grains (and to other products, i.e. nuts), with weight and nutritional losses reducing yields and quality which reduces market values. Furthermore, deterioration of grains intended for seedling purposes may cause further losses in quality and viability (germination) thus affecting future yields in crop production.
Under current storage (bulk) conditions over long periods of time, the presence of even a few viable colonies of insect pests may result in the emergence of much larger populations as the storage conditions are favorable to insect reproduction and propagation due to the abundant presence of nutrients and lack of antagonistic organisms. In rough (paddy) rice, two major insects Angoumois grain moths (*Sitotroga cerealella* [Oliver]) and lesser grain borers (*Rhyzopertha dominica* [F.]) represent major threats as primary grain insects whose larvae feed entirely inside the kernel of the grain and eat from inside becoming more tolerant to fumigation as diffusion of gas into kernels is severely restricted or blocked by the presence of air locks (pockets). Therefore, infestation with primary insects are critically more damaging to stored grains than secondary insects that eat grains from outside and are more easily controlled with conventional fumigation or heat treatments.
As explained above (see section 3.5.2), selective heating of arthropod pests is feasible via a differential heating mechanism based upon the higher ionic conductivity in pests (see Table 1). Therefore, all biological stages of arthropod pests do heat faster than the host commodity leading to their effective biological inactivation (Wang et al., 2003). As shown in table 1 (above), insects such as codling moths and Mexican fruit flies have large dielectric constants (Ɛ* 71.5-84.5 and 90 to 141; respectively at 27 MHz). Therefore, when treated with RF power

these pests can absorb a larger proportion of the available RF energy delivered. By comparison, the host commodity is expected to have complex dielectric constants in the range of 3 to 6 for low-moisture foods (nuts, seeds, grains) or up to 60 to 70 for high-moisture foods (i.e. fruits) although considerable higher dielectric loss factors (>200) for insects have been reported under the same processing conditions (frequency) (Ikediala et al., 2000). The difference in dielectric properties between insects and host generates lower thermal effects on the commodity (Kunze, 1979).
As arthropods (arachnids as well) have similar chemical composition, selective heating effects have been demonstrated with ants, aphids, beetles, borers, bugs, fruit flies, moths, thrips, mites and arachnids confirming the validity of the selective heating process in different food hosts as well as soil and wood products (unpublished results).
Therefore, disinfestation appears as an effective RF application that can heat arthropod pests rapidly (45 to 65°C; 3-4 min) inducing lethal conditions that are well tolerated by a large variety of foods. As proven in various laboratory-scale experimentations, this approach is being developed for commercial-scale applications with RF systems designed and engineered for full optimization.

4.1 Experimental results of RF disinfestation of rough (paddy) rice

A full control of all life cycles of Angoumois grain moths (*Sitotroga cerealella* [Oliver]) and lesser grain borers *Rhyzopertha dominica* [F.]), in laboratory-scale experimentation with rough (paddy) rice as host was reported (Lagunas-Solar et al., 2008).
Samples of rough rice (13.5% and 11.0% moisture) were obtained from Pacific International Rice Mills Inc., (Woodland, CA) and were infested in separate batches (~ 10 kg each) with adult grain moth *Sitotroga cerealella* (13.5% batch) and with both *Sitotroga cerealella* and adult lesser-grain borer *Rhyzopertha dominica* (11.0% batch).
After approximately one month at 28-30°C (35-40% relative humidity) both colonies were well established showing abundant populations of all biological stages. RF disinfestation was conducted at the University of California, Davis using several processing conditions with 500 W of RF power at 20.3 MHz (Lagunas-Solar et al., 2008). Samples were treated at the same temperature (60°C) but with different times (5 and 30 min; 1 and 2 h) so as to vary thermal loads (temperature x time) delivered. Effectiveness of the RF disinfestation process was determined by assaying the emergence of adult insects found over ~40 days of periodic observations. However, as no adult insects survived any of the initial treatments, adult emergence was assumed to be due to the presence of surviving eggs, larva and/or pupas. Results from replicates in triplicate (control and treated) are shown in Figures 6 and 7, below.
The response of grain moth *Sitotroga cerealella* and lesser-grain borer *Rhyzopertha dominica* to the same RF processing conditions were different indicating that other parameters need to be considered in establishing an optimized process.
As expected, Sitotroga cerealella was found to be more sensitive to the RF disinfestation process as these insects are normally on the outside surface of the grain. While disinfestation effects were observed at all conditions (Figure 6), some adult emergence (~ 16%) was observed in the 60°C/5 min samples after ~40-day incubation and observation period. This was attributed to the partial survival of eggs at different eclosion stages prompting a delayed emergence of adult insects. In all other treatments (60°C/30min; 60°C/1h; 60°C/2h) the thermal loads were sufficiently high to cause a full control of all stages of *Sitotroga cerealella*, as no adult emerged in the treated samples.

Rhyzopertha dominica showed higher tolerance under the same processing conditions.

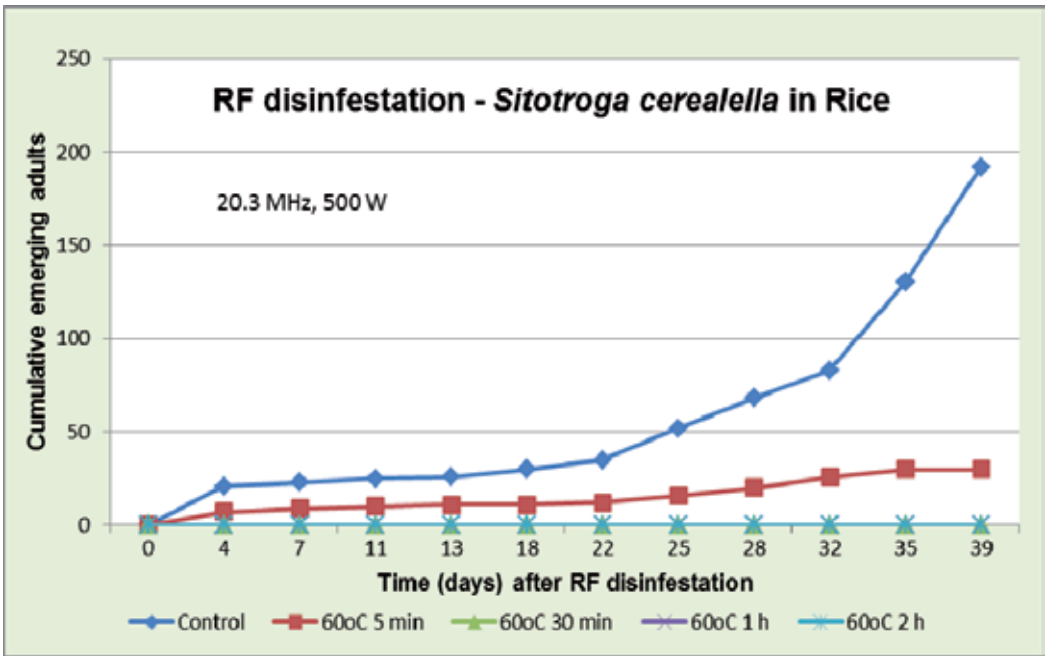

Fig. 6. RF disinfestation of *Sitotroga cereallela* grain moths in rough (paddy) rice.

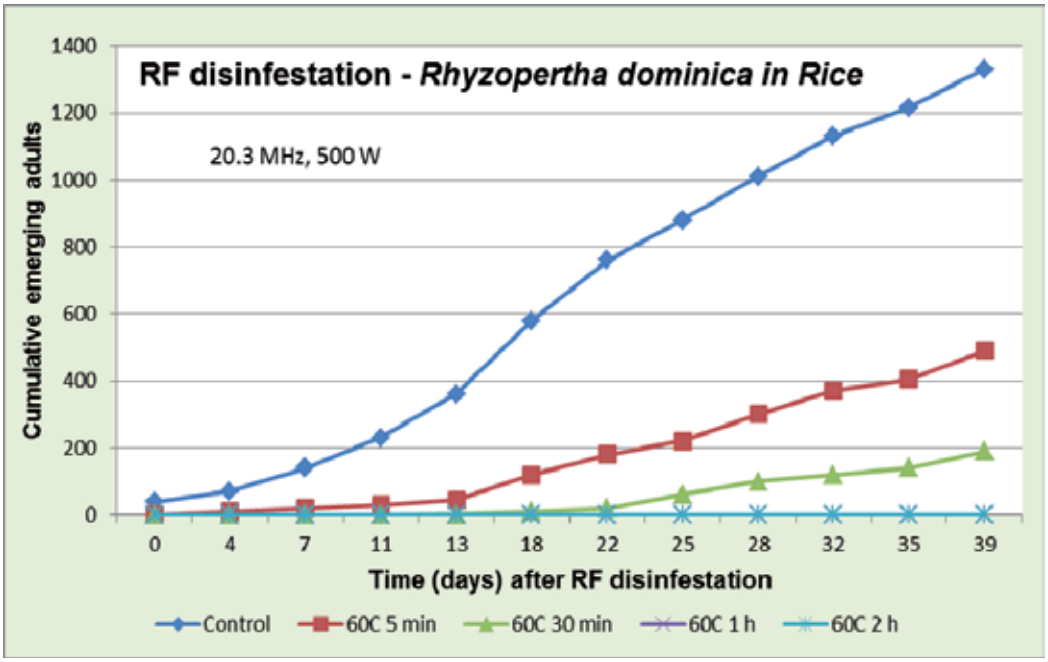

Fig. 7. RF disinfestation of *Rhyzopertha dominica* (lesser grain borers) in rough (paddy) rice.

As compared with controls (1330 adults/40 days); in the 60°C/5min batch 490 adults/40 days were observed for ~37% emergence (~63% control). As thermal load was increased, the 60°C/30 min batch showed only 190 adults/40 days (~14% emergence; 86% control). With either 60°C/1h and 60°C/2h processes, a 100% control were observed as no adults emerged during the 40-day observation period. It was concluded that the ability of *Rhyzopertha dominica* to bore into grains and deposit eggs from which larva emerged, provided additional barriers and protection due to the internalized condition of the pest.
As stated earlier, RF disinfestation is applicable under similar conditions to all arthropod pests as the interactive mechanisms utilized operate with similar molecules present in all arthropods and thus is independent from the biological speciation, developmental stages, or behavioral patterns. Optimization of the RF disinfestation process is also straightforward as thermal loads required for full control (i.e. 60°C/1h for both insects) can be achieved rapidly by increasing RF power. As only 500 W were used in previous experimentation, an operation at 10 kW would only require a 5-min processing time. Other commercial-scale conditions with increased RF power (i.e. 25-50 kW) are also possible and available (www.rfbiocidics.com) for processing larger throughputs (> 2-4 tons/h) while taking full advantage of this emerging chemical-free alternative.

4.2 Quality attributes of host commodity

The application of RF power in disinfestation applications should also consider the potential effects of the applied thermal load to the host commodity. Therefore, the potential for changes of quality attributes in RF treated rough (paddy) rice was also studied and the results are summarized in Table 2, below. These measurements were conducted using standard commercial laboratory tests and indicated no adverse effects.

Rough (paddy) rice samples				
Quality attributes (%)	**Controls**	**Batch 1 (50°C)**	**Batch 2 (60°C)**	**Batch 3 (70°C)**
Moisture	13.5 ± 0.1	13.5 ± 0.1	13.5 ± 0.1	13.5 ± 0.1
Whole kernel	79.3 ± 1.1	81.1 ± 7.9	78.3 ± 0.1	77.9 ± 0.8
Total rice	68.1 ± 0.3	68.3 ± 0.1	68.2 ± 0.1	68.0 ± 0.1
Dockage	16.9 ± 4.8	11.7 ± 1.0	12.4 ± 1.6	13.2 ± 1.7
Brown rice	81.1 ± 0.4	81.1 ± 0.4	81.3 ± 0.2	81.3 ± 0.1
Whiteness	44.2 ± 0.2	44.1 ± 0.2	44.2 ± 0.2	44.3 ± 0.3
* Mean values and standard deviations for triplicate measurements with 1-kg samples. Data courtesy of California Rice Association.				

Table 2. Quality attributes of RF disinfested rough (paddy) rice*

5. RF process economics

Commercial application of RF disinfestation is already taking place on various commodities and is combined with simultaneous disinfection (pasteurization) and enzyme deactivation effects.[2] This combination of desirable and simultaneous sanitation effects is unique to RF processing and is only dependent on the applied thermal loads (see Figure 5, above). In

[2] RF Biocidics Inc., Vacaville, CA 95688 USA (www.rfbiocidics.com)

addition, and because of its penetration, RF power is capable of processing commodities in its package (boxes, bags) thus avoiding recontamination and facilitating logistics of operation. Therefore, due to its chemical-free nature and to the combined effects, RF processing offers many advantages over single-effect technologies including those based upon applications of conventional surface-heat sources (i.e. dry or wet heat, vapor, steam, etc.). Despite its unique advantages and multiple controlling effects, the current commercial application of RF disinfestation is priced competitively in comparison with the cost of using chemical pesticides or any other physical process.

As a new and emerging option for sanitation of foods and agricultural commodities, RF operating facilities are being established to operate at or near high agriculture production areas or near key distribution centers and facilities in which RF processing can be part of the overall chain of production and distribution for local, regional and overseas markets.

6. Conclusions

The application of RF power to disinfestation provides a rapid and effective chemical-free alternative capable of replacing the use of chemical and biological pesticides and as alternative to other conventional heating processes during post-harvest management of various foods and agricultural commodities. As a physical, electricity-based process, its operation is based on well-known, designed and engineered systems capable of safe and large-scale applications. Disinfestation efficacy requires reaching a relatively low thermal-load level as RF is a volumetric heating process with interactions and heating effects starting at the molecular level and somewhat selectively. It can be readily applied to arthropods and arachnids with equal effectiveness using thermal loads well below the threshold for impacting host's quality.

The RF process - with similar and even higher thermal loads, has been demonstrated at a commercial scale for various different commodities including nut products (no effects on free fatty acids, peroxide values), other grains (Quinoa, edible seeds (Chia, pumpkins, sunflower), spices (paprika, cumin, cardamom, nutmeg, coriander, etc.) and flours (brown rice, oat, wheat, flaxseed). Therefore, RF disinfestation is an emerging process with broad applications to many potentially infested commodities and can even be extended to disinfest some heat-tolerant fruits and vegetables as the required thermal load is low and the RF disinfestation process is rapid.

Furthermore, additional energy-use savings can be realized as less RF energy would be needed to control insects (a very small load) as compared with the larger mass (load) represented by the commodity. It is postulated that this approach would result in significant operational cost reductions for RF-based disinfestation applications of a variety of foods such as grains, nut products, flours, beans, spices, and agricultural commodities such as wood products (pallets), soil and soil amendments, and tobacco.

As the needs for non-chemical (residue-free), non-thermal technologies for disinfestation (and disinfection as well) continues to be a goal in production agriculture, a new non-thermal, residue-free process named metabolic stress has recently emerged and is soon to initiate commercialization (Lagunas-Solar, Essert, Piña et al., 2006b; Lagunas-Solar & Essert, 2011). Metabolic stress, singly or in combination with RF processing, is expected to overcome some of the limitations of RF disinfestation and be able to treat commercial levels of thermally-sensitive commodities in particular fresh fruits and vegetables.[3]

[3] RF Biocidics Inc., (www.rfbiocidics.com)

Finally, because of its nature, RF disinfestation can be applied to conventionally- and organically-produced food commodities.

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13

Zero-Inflated Regression Methods for Insecticides

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Turkey

1. Introduction

The numerical abundance of many species sharing the same ecosystem very different levels of the organism and are in constant change, depending on many factors. Due to the heterogeneous strucspeciese of the life cycles of organisms and abiotic resources in the environment based on census population densities derived from overdispersion (variance is higher than means in Poisson distribution) (Cox, 1983; Cameron and Trivedi, 1998) and a large number of zero values (zero-inflated data) is observed (Yeşilova et al, 2011). In such a case, zero-inflated Poisson (ZIP) regression model is a appropriate approach for analyzing a dependent variable having excess zero observations (Lambert, 1992; Böhning, 1998; Böhning et al, 1999; Yau and Lee, 2001; Lee et al, 2001; Khoshgoftaar et al, 2005; Yeşilova et al, 2010).
Zero-inflation is also likely in data sets, excess zero observations. In such cases, a zero-inflated negative binominal (ZINB) regression model is an alternative method (Ridout et al, 2001; Yau, 2001; Cheung, 2002; Jansakul, 2005; Long and Frese, 2006; Hilbe, 2007; Yeşilova et al, 2009; Yeşilova et al, 2010). Morever, The Poisson hurdle model and negative binomial hurdle model (Rose and Martin, 2006; Long and Frese, 2006; Hilbe, 2007; Yeşilova et al, 2009; Yeşilova et al, 2010), and zero-inflated generalized Poisson (ZIGP) model (Consul, 1989, Consul and Famoye, 1992; Czado et al., 2007) are widely used in the analysis of zero-inflated data.
In this part, the analysis of data with many zeros for *Notonecta viridis* Delcourt (Heteroptera: Notonectidae) and Chironomidae species (Diptera) were carried out by means of using the models of Poisson Regression (PR), negative binomial (NB) regression, zero-inflated Poisson (ZIP) regression, zero-inflated negative binomial (ZINB) regression and negative binomial hurdle (NBH) model.

Samplings

The study was based on periodical samplings of the coastal band of Van Lake, conducted between July-September 2005 and May-September 2006. Samples were taken at totally twenty sampling points as streams entrance (6 points), settlement coastlines (7 points) and naspeciesal coastlines (7 points). Samples were taken according to Hansen et al. (2000). The

invertebrates were collected with a standard sweep net (30 cm width, 1 mm mesh) (Southwood, 1978; Rosenberg, 1997; Hansen et. al, 2000; Yeşilova et al., 2011).
Notonectid identification was made by Dmitry A. Gapon (Zoological Institute RAS, Universitetskaya nab., 1, St. Petersburg, Russia).

2. Methods

2.1 Poisson regression

The logarithm of mean of Poisson distribution (μ) is supposed to be a linear function of independent variables (x_i) is,

$$\log(\mu_i) = \left(x_i^{'}\beta\right)$$

Poisson Regression Model can be written as

$$\Pr(y_i / \mu_i, x_i) = \exp(-\mu_i)\mu_i^{y_i} / y_i!, \text{yi=0,1,...} \quad (1)$$

In equation 1, y_i denotes dependent variable having Poisson distribution. Likelihood function for PR model is, (Böhning, 1998)

$$LL(\beta / y_i, x_i) = \sum_{i=1}^{n}\left[y_i x_i^{'}\beta - \exp\left(x_i^{'}\beta\right) - \ln y_i!\right] \quad (2)$$

In equation 2, β are unknown parameters. β can be estimated by maximizing log likelihood function according to ML (Khoshgoftaar et al, 2005;Yau, 2006).

2.2 Negative binomial regression

NB regression model is,

$$\Pr(Y = y_i / x_i) = \frac{\Gamma\left(y_i + \frac{1}{\alpha}\right)}{y_i!\Gamma\left(\frac{1}{\alpha}\right)} \frac{(\alpha\mu_i)^{y_i}}{(1+\alpha\mu_i)^{y_i+\frac{1}{\alpha}}} \quad \alpha > 0 \quad (3)$$

In equation 3, α is a arbitrary parameter and indicates overdispersion level. Log likelihood function for NB regression model is (Hilbe, 2007; Yau, 2006),

$$LL(\beta,\alpha,y) = \sum_{i=1}^{n}\left[\begin{array}{l}\frac{1}{\alpha}\log(1+\alpha\mu_i) - y_i\log\left(1+\frac{1}{\alpha\mu_i}\right) \\ +\log\Gamma\left(y_i+\frac{1}{\alpha}\right) - \log\Gamma\left(\frac{1}{\alpha}\right) - \log y_i!\end{array}\right]$$

2.3 Zero inflated poisson regression

ZIP regression is [13],

$$\Pr(y_i/x_i)=\begin{cases}\pi_i+(1-\pi_i)\exp(-\mu_i), & y_i=0\\ (1-\pi_i)\exp(-\mu_i)\mu_i^{y_i}/y_i!, & y_i>0\end{cases} \tag{4}$$

In equation (4), π_i represents the possibility of extra zeros' existence. Log likelihood function for ZIP model is (Yau, 2006),

$$LL=\sum_{i=1}^{n}\begin{pmatrix} I_{y_i=0}\log\left(\pi i+(1-\pi i)e^{-\mu_i}\right)\\ +I_{y_i>0}\log\left((1-\pi_i)\dfrac{\mu_i^{y_i}e^{-\mu_i}}{y_i!}\right)\end{pmatrix}$$

$$LL=\sum_{\dot{I}=1}^{n}\begin{pmatrix} I_{y_i=0}\log\left(\pi i+(1-\pi i)e^{-\mu_i}\right)\\ +I_{y_i>0}\begin{pmatrix}\log(1-\pi i)\\ +y_i\log\mu_i-\mu_i-\log y_i!\end{pmatrix}\end{pmatrix} \tag{5}$$

$I(.)$, given in equation (5) is the indicator function for the specified event. Then μ_i and π_i parameters can be obtained following link functions,

$$\log(\mu)=B\beta \tag{6}$$

and

$$\log\left(\frac{\pi}{1-\pi}\right)=G\gamma \tag{7}$$

In equations 6 and 7, B(nxp) and G(nxq) are covariate matrixes. β and γ are respectively unknown parameter vectors with px1 and qx1 dimension (Yau, 2006).

2.4 Zero inflated negative binomial regression

ZINB regression model is [18],

$$\Pr(y_i/x_i)=\begin{cases}\pi_i+\left(1-\pi_i\right)\left(1+\alpha\mu_i\right)^{-\alpha^{-1}}, & y_i=0\\ (1-\pi_i)\dfrac{\Gamma\left(y_i+\frac{1}{\alpha}\right)}{y_i!\Gamma\left(\frac{1}{\alpha}\right)}\dfrac{\left(\alpha\mu_i\right)^{y_i}}{\left(1+\alpha\mu_i\right)^{y_i+\frac{1}{\alpha}}}, & y_i>0\end{cases} \tag{8}$$

In equation (8), $(\alpha \geq 0)$ indicates an overdispersion parameter. Log likelihood function for ZINB model is (Yau, 2006),

$$LL(\mu,\alpha,\pi;y) = \sum_i \left(\begin{array}{l} I_{y_i=0} \log(\pi_i \\ +(1-\pi_i)\left(1+\alpha\mu_i\right)^{-\alpha^{-1}} \end{array} \right.$$

$$\left. +I_{y_i>0} \log\left((1-\pi_i) \frac{\Gamma\left(y_i+\frac{1}{\alpha}\right)}{y_i!\Gamma\left(\frac{1}{\alpha}\right)} \frac{\left(\alpha\mu_i\right)^{y_i}}{\left(1+\alpha\mu_i\right)^{y_i+\frac{1}{\alpha}}} \right) \right)$$

$$= \sum_i \left(I_{y_i=0} \log(\pi_i + (1-\pi_i)\left(1+\alpha\mu_i\right)^{-\alpha^{-1}} \right.$$

$$+I_{y_i>0} \left(\log(1-\pi_i) - \frac{1}{\alpha}\log\left(1+\alpha\mu_i\right) \right.$$

$$\left. \left. \begin{array}{l} -y_i \log\left(1+\frac{1}{\alpha\mu_i}\right) + \log\Gamma\left(y_i+\frac{1}{k}\right) \\ -\log\Gamma\left(\frac{1}{\alpha}\right) - \log y_i! \end{array} \right) \right) \tag{9}$$

$I(.)$, given in equation 9 is the indicator function for the specified event. The model descripted by Lambert (1992) can be given as,

$$\log(\mu) = X\beta \quad \text{and} \quad \log\left(\frac{\pi}{1-\pi}\right) = G\gamma$$

Here, X(nxp) and G(nxq) covariate matrixes, β and γ are respectively unknown parameter vectors with px1 and qx1 dimension. Maximum likelihood estimations for β, α and γ can be obtained by using EM algorithm.

2.5 Negative binomial hurdle model

Log-likelihood for negative binomial hurdle model (Hilbe, 2007),

$$L = \ln\left(f(0)\right) + \left\{\ln\left[1-f(0)\right] + \ln P(j)\right\} \tag{10}$$

In equation (10), $f(0)$ indicates the probability of the binary part and $p(j)$ indicates the probability of positive count. The probability of zero for logit model is,

$$f(0) = P(y=0;x) = 1/\left(1+\exp(xb1)\right)$$

and
1- $f(0)$ is,

$$\exp(xb1)/(1+\exp(xb1))$$

The log likelihood function for both parts of negative binomial Hurdle Model is,

$$\text{L=cond}\{y==0,\ln(1/1-\exp(xb1)),$$

$$\ln(\exp(xb1)/(1+\exp(xb1)))$$

$$+y*\ln(\exp(xb)/(1+\exp(xb)))$$

$$-\ln(1+\exp(xb))/\alpha+\ln\Gamma(y+1/\alpha)$$

$$-\ln\Gamma(y+1)-\ln\Gamma(1/\alpha)$$

$$-\ln(1-(1+\exp(xb))(-1/\alpha))\}$$

2.6 Model selection

Akaiki Information Criteria (AIC) is goodness of criteria used for model selection. AIC,

$$AIC=-2LL+2r \tag{11}$$

In equations, LL indicates log likelihood, r indicates parameter number and n indicates sample size.

3. Results

In this study, R statistical software program was used. Insect densities were included to the model as dependent variable. Besides years, months, species and station are included as independent variables to the model. The 66 (20.63%) of the 320 dependent variable were zero valued. The distribution of the insect densities was skewed to right because of excess zeros.

Model	AIC
PR	57846.00
ZIP	47791.71
NB	3176.40
ZINB	2819.800
PH	47791.71
NBH	**2803.206**

Table 1. Model selection criteria for PR, NB, ZIP, ZINB, PH and NBH.

In PR analyses, deviance and Pearson Chi-square goodness of statistics higher than one (831.417 and 650.213, respectively). Thus, goodness of statistics represents that there is an overdispersion in insect densities. AIC model selection criteria for the models of PR, NB, ZIP, ZINB, PH, and NBH were given in Table 1. The model with the smallest AIC was NBH regression.
Maximum likelihood (ML) parameter estimations and standard errors for PR were given in Table 2.

	Estimate	Std. Error	z value	Pr(>|z|)	e^{β}
(Intercept)	6.179499	0.054470	113.449	<2e-16 ***	482.992
year	0.118847	0.013069	9.094	<2e-16 ***	1.125244
month	0.175298	0.005066	34.604	<2e-16 ***	1.191246
Station	-0.081353	0.001124	-72.357	<2e-16 ***	0.921917
species	-1.943212	0.018356 -	105.863	<2e-16 ***	0.1432735

*p<0.05, **p<0.01, ***p<0.001

Table 2. Parameter estimations and standard errors for Poisson regression.

ML parameter estimations and standard errors for negative binomial regression were given in Table 2.

	Estimate	Std. Error	z value	Pr(>|z|)	e^{β}
(Intercept)	8.52318	0.99249	8.588	4.16e-16 ***	5029.119
year	-0.15794	0.24824	-0.636	0.525	0.853901
month	-0.08205	0.09168	-0.895	0.372	0.9212259
Station	-0.08031	0.01949	-4.121	4.82e-05 ***	0.9228302
species	-1.92518	0.22452	-8.575	4.56e-16 ***	0.1458495

*p<0.05, **p<0.01, ***p<0.001

Table 3. Parameter estimations and standard errors for negative binomial regression.

ML parameter estimations and standard errors for zero-inflated Poisson regression both count model and logit model were given in Table 4 and Table 5, respectively.

	Estimate	Std. Error	z value	Pr(>|z|)	e^{β}
(Intercept)	6.017745	0.056073	107.32	<2e-16 ***	410.6515
year	0.271101	0.013047	20.78	<2e-16 ***	1.311408
month	0.162333	0.005271	30.80	<2e-16 ***	1.176252
station	-0.046859	0.001122	-41.76	<2e-16 ***	0.954222
species	-2.002676	0.018382	-108.94	<2e-16 ***	0.1349736

*p<0.05, **p<0.01, ***p<0.001

Table 4. Parameter estimations and standard errors for ZIP count model.

	Estimate	Std. Error	z value	Pr(>|z|)	e^{β}
(Intercept)	-3.92991	1.33906	-2.935	0.00334 **	0.01964544
year	0.50266	0.33705	1.491	0.13587	1.653113
month	0.04405	0.11930	0.369	0.71197	1.045035
station	0.17250	0.02994	5.761	8.36e-09 ***	1.188272
species	-0.44380	0.30013	-1.479	0.13923	0.6415937

*p<0.05, **p<0.01, ***p<0.001

Table 5. Parameter estimations and standard errors for ZIP logit model.

ML parameter estimations and standard errors for zero-inflated negative binomial regression both count model and logit model were given in Table 6 and Table 7, respectively.

	Estimate	Std. Error	z value	Pr(>|z|)	e^{β}
(Intercept)	9.47226	1.04897	9.030	< 2e-16 ***	12994.22
year	-0.13254	0.20609	-0.643	0.520132	0.8758679
month	-0.16895	0.09356	-1.806	0.070957	0.8445511
station	-0.06233	0.01806	-3.452	0.000557 ***	0.9395728
species	-2.21006	0.20855	-10.597	< 2e-16 ***	0.1096941

*p<0.05, **p<0.01, ***p<0.001

Table 6. Parameter estimations and standard errors for ZINB count model.

	Estimate	Std. Error	z value	Pr(>|z|)	e^{β}
(Intercept)	-1.21687	4.61145	-0.264	0.791872	0.2961557
year	1.64137	0.88380	1.857	0.063288	5.162237
month	-0.23791	0.22636	-1.051	0.293246	0.7882736
station	0.18398	0.05485	3.354	0.000795 ***	1.201992
species	-3.69139	3.88424	-0.950	0.341934	0.02493732

*p<0.05, **p<0.01, ***p<0.001

Table 7. Parameter estimations and standard errors for ZINB logit model.

ML parameter estimations and standard errors for Poisson hurdle both count model and logit model were given in Table 8 and Table 9, respectively.

	Estimate	Std. Error	z value	Pr(>|z|)	e^{β}
(Intercept)	6.017745	0.056073	107.32	<2e-16 ***	410.6515
year	0.271101	0.013047	20.78	<2e-16 ***	1.311408
month	0.162333	0.005271	30.80	<2e-16 ***	1.176252
station	-0.046859	0.001122	-41.76	<2e-16 ***	0.954222
species	-2.002676	0.018382	-108.94	<2e-16 ***	0.1349736

*p<0.05, **p<0.01, ***p<0.001

Table 8. Parameter estimations and standard errors for PH count model.

ML parameter estimations and standard errors obtained for the NBH count model was given in Table 8. While stations and species were significant on the insect densities, the effect of years and the effect of months were not significant on the insect densities.
ML parameter estimations and standard errors obtained for the NBH logit model was given in Table 9. The effects months, years and species were not significant on the insect densities. However, the effect of station was significant on the insect densities.

	Estimate	Std. Error	z value	Pr(>|z|)	e^{β}
(Intercept)	3.92991	1.33906	2.935	0.00334 **	50.9024
year	-0.50266	0.33705	-1.491	0.13587	0.6049194
month	-0.04405	0.11930	-0.369	0.71197	0.9569061
station	0.17250	0.02994	-5.761	8.36e-09 ***	1.188272
species	0.44380	0.30013	1.479	0.13923	1.558619

*p<0.05, **p<0.01, ***p<0.001

Table 9. Parameter estimations and standard errors for PH logit model.

ML parameter estimations and standard errors obtained for negative binomial hurdle both count model and logit model were given in Table 10 and Table 11, respectively.
ML parameter estimations and standard errors obtained for the NBH count model was given in Table 10. While stations and species were significant on the insect densities, the effect of years and the effect of months were not significant on the insect densities.

	Estimate	Std. Error	z value	Pr(>|z|)	e^{β}
(Intercept)	9.43372	1.26292	7.470	8.03e-14 ***	12502.95
year	-0.19128	0.24381	-0.785	0.4327	0.8259013
month	-0.17020	0.11124	-1.530	0.1260	0.8434961
station	-0.04587	0.02096	-2.188	0.0287 *	0.9551661
species	-2.33333	0.25071	-9.307	< 2e-16 ***	0.0969723

*p<0.05, **p<0.01, ***p<0.001

Table 10. Parameter estimations and standard errors for NBH count model.

ML parameter estimations and standard errors obtained for the NBH logit model was given in Table 11. The effects months, years and species were not significant on the insect densities. However, the effect of station was significant on the insect densities.

	Estimate	Std. Error	z value	Pr(>|z|)	e^{β}
(Intercept)	3.92991	1.33906	2.935	0.00334 **	50.9024
year	-0.50266	0.33705	-1.491	0.13587	0.6049194
month	-0.04405	0.11930	-0.369	0.71197	0.9569061
station	-0.17250	0.02994	-5.761	8.36e-09 ***	0.8415583
species	0.44380	0.30013	1.479	0.13923	1.558619

*p<0.05, **p<0.01, ***p<0.001

Table 11. Parameter estimations and standard errors for NBH logit model.

Average insect density observed in the year 2005 has shown 17% decrease in reference to the year 2006. Insect densities observed at monthly sampling ranges depending on water temperaspeciese were increased with the rise of temperaspeciese, but specifically after the month of July such intensity was decreased at the rate of 16% ($e^{-0.19128} \sim 0.8434961$) towards the month of September within the both years. It has been determined that insect intensities observed at different stations have shown differentiation at the rate of 5%. Chironomid larvae which are included in prey of notonectidae fed by different sources of food at aquatic environment have been found at rather lower density in reference to notonectid density. However, it is hard to guess that such decrement has been formed under the impact of notonectidae. Nevertheless notonectidae do not depend on a single host, their sources of food are rather wide range of variety. Small arthropods on the water surface, small crustaceans living in water, larvae of aquatic insects, snails, small fish or larvae of frog are among their preys (Bruce et al., 1990).

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It is our hope that this book will be of interest and use not only to scientists, but also to the food-producing industry, governments, politicians and consumers as well. If we are able to stimulate this interest, albeit in a small way, we have achieved our goal.

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