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Pesticides in the Modern World

Risks and Benefits

Edited by Margarita Stoytcheva

PESTICIDES IN THE MODERN WORLD - RISKS AND BENEFITS

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http://dx.doi.org/10.5772/949
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First published in Croatia, 2011 by INTECH d.o.o.
eBook (PDF) Published by IN TECH d.o.o.
Place and year of publication of eBook (PDF): Rijeka, 2019. IntechOpen is the global imprint of IN TECH d.o.o.
Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

Additional hard and PDF copies can be obtained from orders@intechopen.com

Pesticides in the Modern World - Risks and Benefits
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p. cm.

ISBN 978-953-307-458-0

eBook (PDF) ISBN 978-953-51-4425-0

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Meet the editor

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

Contents

Preface

Volume 3 of the book series "Pesticides in the Modern World" is a compilation of 29 chapters focused on: pesticides and food production, environmental effects of pesticides, and pesticides mobility, transport and fate.

The first book section (Chapters 1-6) addresses the benefits of the pest control for crop protection and food supply increasing, but also the associated risks of food contamination. The advantages and the disadvantages of pesticides using in modern agriculture, and the effectiveness of their alternatives are comprehensively reviewed in Chapter 1. The objective of Chapter 2 is to assess the impacts of agrochemicals application on plants' pests and of some essential production factors on the quality of the vegetables produced in African urban settings. Chapter 3 reports research results on the efficacy of the insecticides imidacloprid, thiamethoxam, clothianidin, methidathion and fenobucarb and of their mixtures against the citrus psyllid on citrus plants in Vietnam. Chapter 4 examines the use of pesticides in cocoa production in Ghana, demonstrates that the current unsustainable agricultural practices create environmental and economic risks, and identifies improvement options. Chapter 5 provides data on industrial chemicals and organochlorine pesticides contamination of food. Chapter 6 documents the contamination of bee products with pesticides and presents information on the sources of contamination.

The second book section (Chapters 7-20) is dedicated to the environmental pesticides impacts. Chapter 7 comments on a number of pesticides ecological effects such as: effects involving pollinators, effects on nutrient cycling in ecosystems, effects on soil erosion, structure and fertility, and effects on water quality. The impacts of the pesticides in agricultural ecosystems, in terms of pesticides resistance development are discussed in Chapter 8. The occurrence of the inorganic pesticide lead arsenate in the environment, the pathways of its uptake, the methods for lead and arsenic toxicity assessment, and the contaminated soil remediation constitute the subject of Chapter 9. The effects of arsenic exposure are commented in details in Chapter 10.

Chapters 11-13 covers the genotoxic and immunotoxic effects of pesticides on the aquatic fauna: decapods, bivalves, and teleost fish. Using zooplancton to evaluate the ecotoxicity of the main pesticide applied in paddy field is discussed in Chapter 14. Investigations on the capacity of some herbal extracts and calcium to counteract the

pesticidal effects on Indian Major Carp are reported in Chapter 15. Comments concerning the assessment of the pesticides residues in otters and ospreys, considered as good sentinels and indicator species of contamination, bioaccumulation and biomagnification of toxic contaminants in rivers, estuaries, reservoirs and lakes are provided in Chapter 16.

Chapter 17 presents cases of insecticides miss-use in rice production in West Africa and the related effects on the non-target organisms and the environment. The possible impacts of Bt maize on the development and behaviour of stem borers and their natural enemies are analysed in Chapter 18. Experimental data on the termiticidal activity of bistrifluron are reported in Chapter 19. Camouflaging of seeds treated with pesticides to mitigate the mortality of birds in grain crops is discussed in Chapter 20.

The third book section (Chapters 21-29) furnishes numerous data contributing to the better understanding of the pesticides mobility, transport and fate. In Chapter 21 are presented investigations on the mode of deposition and transformation of the organochlorine pesticides into the sediment of Lake Liangzi in Central China. Chapters 22-24 address the complex phenomenon of environmental *pollutants transport* in surface and ground waters. Studies on the presence of pesticides in treated urban wastewaters are reported in Chapter 25. The fate of the pesticides in soils, namely the interaction of ionic pesticides with model systems of soil fractions, the imidacloprid sorption and degradation processes, and the release kinetics of organochlorine pesticides in the rhizosphere are discussed in Chapters 26-28. The factors involved in the retention and degradation of pesticides in soils are analysed in Chapter 29, applying an integrated approach.

The addressed in this book issues associated with the benefits and risks of pesticides should attract the public concern to support rational decisions to pesticides use. The efforts of all the contributing authors to provide recent information are greatly appreciated.

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Part 1

Pesticides and Food

1

Role of Pesticides in Human Life in the Modern Age: A Review

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1. Introduction

Food production capacity is faced with an ever-growing number of challenges, including a world population expected to grow to nearly 10 billion by 2050 and a falling ratio of arable land to population. Based on evidences, in 1900 there were 1.6 billion people on the planet; in 1992 this had risen to 5.25 billion and by the year 2050 it will reach 10 billion. World population is increasing by 97 million per year. This explosive increase in world population is mostly in developing countries and this is where the need for food is greatest and starvation threatens human life; as, FAO[1] estimates that 500 million are already undernourished (Anon, 1990a).

Civilization has been combating weeds, insects, diseases and other pests throughout history and there are many examples of how these pests have had a major impact on humans. One of the worst examples is the Black Plaque of Europe in the fourteenth century when millions died from a bacterial disease spread by fleas from rats (Hock et al., 1991). Another example is the infamous Irish potato famine of the nineteenth century in which millions died and many more were forced to emigrate. A fungus also destroyed the entire German potato crop in the early twentieth century resulting in 700,000 deaths from starvation (Anon, 1992b).

Thus, food plays a vital and strategic role in growing global population. But, food production is encounter to different limits. For example, there is a limit to new areas to cultivate; therefore we must increase agricultural production from the areas available. However, the specialization of production units has led to the image that agriculture is a modern miracle of food production (Stoytcheva, 2011).

In our global society there is a place for people to grow and consume organic food, but if all our farmers decided against using farm chemicals, we would soon find ourselves in a grave situation. Without the use of farm chemicals, the production and quality of food would be severely jeopardized with estimates that food supplies would immediately fall to 30 to 40% due to the ravages of pests (Anon, 1990b; Anon, 1992a). While there are mountains of food in Europe and the US, this represents only 45 days food supply for the world. Only part of the problem is distribution and the ability to pay for purchases.

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[1] FAO

While the first recorded use of chemicals to control pests back to 2500 BC, it is really only in the last 50 years that chemical control has been widely used (Hock et al., 1991). Many of the earliest pesticides were either inorganic products or derived from plants (i.e. burning sulphur to control insects and mites). Other early insecticides included hellebore to control body lice, nicotine to control aphids, and pyrethrin to control a wide variety of insects. Some heavy metals like lead arsenate was first used in 1892 as an orchard spray while about the same time it was accidentally discovered that a mixture of lime and copper sulphate (Bordeaux mixture) controlled downy mildew, a serious fungal disease of grapes. It is still one of the most widely used fungicides (Hock et al., 1991).

Pesticides are an undeniable part of modern life, used to protect everything from flower gardens to agricultural crops from specific pests. Pesticides have contributed significantly to improving quality of life and safeguarding the environment. Although often taken for granted, without these important products, food production would decline, many fruits and vegetables would be in short supply and prices would rise. Some 20 to 40 percent of the world's potential crop production is already lost annually because of the effects of weeds, pests and diseases (according to the FAO reports) (WWW.CropLife America.mht). These crop losses would be doubled if existing pesticide uses were abandoned, significantly raising food prices. Even after harvest, crops are subject to attack by pests or diseases. Bugs, rodents or moulds can harm grains. In addition to increasing crop yields, crop protection products used in stored products can also prolong the viable life of products, prevent huge post-harvest losses from pests and diseases, and protect food safety for eating.

On the other hand, although pesticides are now commonplace, concerns still exist about their safety and proper use. Pesticides can be used safely and effectively. But if proper care is not taken, pesticides can harm the environment by contaminating soil, surface and ground water, and ultimately kill wildlife. Also, the modern human is constantly exposed to a variety of toxic chemicals primarily due to changes in life style. The food we eat, the water we drink, the air we breathe, and the environment we live in are contaminated with toxic xenobiotics. Humans are exposed to such chemicals while still in the womb of the mother (Lederman, 1996; Rathinam et al., 2004). Therefore, human life would be threatened not only directly by pesticides in environment, but indirectly by contaminated food chain.

However, the chapter tries to discuss about necessity of pesticides use in modern agriculture for supplying human food. Actually, traditional chemical pesticides have environmental inconvenience and disadvantages for human health; thus, the problems along with the benefits of pesticides in improvement of quality of agricultural products and food production and storage are mentioned. According to world's food demands and health hazards caused by traditional pesticides, modern and new generation of pesticides and/or alternative methods to chemicals are modified to one of the most essential needs for modern agriculture in the present age. Some of the methods are titled in this chapter.

2. Traditional pesticides

2.1 What is a pesticide?

As FAO defined, pesticide is any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances

which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies. The term includes substances intended for use as a plant growth regulator, defoliant, desiccant or agent for thinning fruit or preventing the premature fall of fruit, and substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport. A pesticide may be a chemical substance, biological agent (such as a virus or bacterium), antimicrobial, disinfectant or device used against any pest. We use pesticides to cover a wide range of chemicals used to control insect pests, plant diseases, weeds, rats or other unwanted organisms. Currently, more than 800 pesticide active ingredients in a wide range of commercial products are registered for use in agriculture to meet food supply demands (Stoytcheva, 2011; Food and Agriculture Organization of the United Nations, 2002).

Pesticides can be classified by target organism, chemical structure, and physical state (Council on Scientific Affairs, American Medical Association, 1997). Pesticides can also be classed as inorganic, synthetic, or biologicals (biopesticides) which include microbial pesticides and biochemical pesticides. Plant-derived pesticides (botanicals), which have been developing quickly, include pyrethroids, rotenoids, nicotinoids, and a fourth group that includes strychnine and scilliroside (Kamrin, 1997). In addition, Pesticides can be classified based upon their biological mechanism function or application method. Basically, agricultural pesticides are divided into five categories, depending on the target pest (WWW. Humpath.com):

i. insecticides,
ii. herbicides,
iii. fungicides,
iv. rodenticides,
v. and fumigants.

All pesticides are toxic to some plant or rodent species; at higher doses, they can also be toxic to farm animals, pets, and humans. In general, prominent insecticide families include organochlorines, organophosphates, and carbamates. Acute toxicity of insecticides for mammals ranges from low to high. Herbicides used to control weeds have low acute toxicity for mammals; and fungicides are characterized as moderately toxic (Shokrzadeh & Saeedi Saravi, 2009).

2.2 Advantages of using pesticides

A plentiful supply of fresh products is vital for a healthy population. Numerous scientific studies demonstrate the health benefits of regularly eating a variety of fresh fruit and vegetables; and consumers are increasingly aware of these benefits. Agricultural productivity is a key to ensuring that this demand can be met at an affordable price; and crop protection products help increase productivity and usable crop yields.

The crop protection industry's primary aim is to enable farmers to grow an abundant supply of food in a safe manner and prevent costs from increasing. Food production processes benefit from continual advancements in agricultural technologies and practices; in fact, a population now nearly twice as large has more food available per capita than 40 years ago. Tools such as herbicides, insecticides, and fungicides reduce crop losses both before and after harvest, and increase crop yields.

The major benefits of pesticides and their role in food production are listed below (WWW.CropLife America.mht):

- Increase food quality and quantity: Crop protection technologies allow producers to increase crop yields and efficiency of food production processes. Up to 40 percent of the world's potential crop production is already lost annually because of the effects of weeds, pests and diseases. These crop losses would be doubled if existing pesticide uses were abandoned. In addition, pesticides allow consumers to consume high-quality products that are free of insect blemishes and insect contamination. Crop protection chemicals that reduce, eliminate, and insect damage allow the consumers to purchase high-quality products free of insect fragments.
- Decrease price of food: Because the use of pesticides improves crop yields, crop protection technologies also impact the cost of food. Without crop protection chemicals, food production would decline, many fruits and vegetables would be in short supply and prices would rise. Helping to keep food prices in check for the consumer is another large benefit of pesticides.
- Human health protection: Pesticides are the most effective substances to eliminate Insects that cause human diseases such as Malaria, Dengue fever, Lyme disease, and West Nile virus loom large. Also, human health is supported against insect and fungi-borne carcinogens, like aflatoxins, which is proceeding to hepatic and other cancers.
- Environmental protection: Other positive aspects of crop protection chemicals, in responsible and safe use, include household pest control, control of vegetation in industry and infrastructure, and recreation and protection of areas against environmental pests like noxious weeds, feral animals, etc, which cause land degradation.

2.3 Disadvantages of using pesticides

Food is the basic necessity of life and food contaminated with toxic pesticides is associated with severe effects on the human health. Hence it is pertinent to explore strategies that address this situation of food safety especially for the developing countries where pesticide contamination is widespread due to indiscriminate usage and a major part of population lives below poverty line.

The four main groups of pesticides such as the organochlorine, organophosphate, carbamate, and pyrethroid insecticides (Smith & Gangolli, 2002; Ahmed et al., 2000) are of particular concern because of their toxicity and persistence in the environment; however several of the banned pesticides are still used on a large scale in developing countries and continue to pose severe health and environmental problems. Pesticide use raises a number of environmental concerns, and human and animal health hazards. Over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, including non-target species, air, water and soil (Miller, 2004). Pesticides are one of the causes of water pollution, and some pesticides are persistent organic pollutants and contribute to soil contamination. As a result, we are closely exposed to pesticides in the food and water we consume and in the air we breathe. Unfortunately these chemicals are non biodegradable, persistent and get accumulated in the environment and thus into the human food chain. Despite regulatory measures, these compounds continue to be detected in measurable amounts in the ecosystem including marine life (Smith & Gangolli, 2002).

In addition, pesticide use reduces biodiversity, reduces nitrogen fixation (Rockets, 2007), contributes to pollinator decline (Hackenberg, 2007; Haefeker, 2000; Wells, 2007; Zeissloff, 2001), destroys habitat (especially for birds) (Palmer et al., 2007), and threatens endangered

species (Miller, 2004). It also happens that some of the pest adapt to the pesticide and don't die. What is called pesticide resistance, to eliminate the offspring of this pest, will be needed a new pesticide or an increase the dose of pesticide. This will cause a worsening of the ambient pollution problem.

There is a growing concern that environmental chemicals, both natural and manmade, can cause:

- Pesticide resistance in some pests;
- Water, soil and air contamination that transfers the chemical residues along a food chain;
- Reduction of biodiversity and nitrogen fixation;
- Destruction of marine and birds' life and/or genetically defects in their next generations;
- Changes in the natural biological balances, by means of reduction of beneficial and non-target organisms and insects, including predators and parasites of pests, and honeybees.

On the other hand, the human population is exposed to these chemicals primarily through the consumption of pesticide contaminated farm products, leading to long term health hazards.

Pesticides may induce oxidative stress leading to the generation of free radicals and alteration in antioxidant or oxygen free radical scavenging enzymes such as superoxide dismutase, catalase, glutothione peroxidase, glutathione reductase and glutothione transferase (Ahmed et al., 2000).

Pesticide toxicity can result from ingestion, inhalation or dermal absorption. Also, many evidences show that pesticides are persistent in fish tissues, adipose tissue and other organs including brain cells, nervous system and endocrine glands, and even breast milk, etc (Shokrzadeh et al., 2009). Thus, continued exposure to these chemicals for a long period may result in various diseases listed below:

- Neurological, psychological and behavioural dysfunctions, including Symptoms of mild cognitive dysfunction (leading to problems in identifying words, colours or numbers and inability to speak fluently), Parkinson's disease (PD) (Uversky et al., 2002; Xavier et al., 2004);
- Hormonal imbalances, leading to infertility, breast pain, menstrual disturbances, adrenal gland exhaustions and early menopause (Xavier et al., 2004);
- Immune system dysfunction, leading to immune suppression that cause potentially serious health risks in populations highly exposed to infectious and parasitic diseases, and subject to malnutrition (Xavier et al., 2004);
- Reproductive system defects, including birth defects (Petrelli & Mantovani, 2002);
- Cancers, including brain cancers (i.e. neuroblastoma), soft tissue sarcomas (i.e. Ewig's sarcoma), and colorectal and testes carcinomas (Xavier et al., 2004);
- Genotoxicity, including DNA damage in peripheral lymphocytes (Undeger & Basaran, 2002);
- Blood disorders, including leukaemia and non-Hodgkin's lymphoma (Zahm & Ward, 1998; Zahm et al., 1997);
- and abnormalities in liver and kidneys, ...

Between specific age ranges, infants and children are at great risk from the effects of pesticides. Several studies suggest that children may be particularly sensitive to the

carcinogenic effect of pesticides. There is a potential to prevent at least some childhood cancer by reducing or eliminating pesticide exposure (Zahm & Ward, 1998).

3. Modern alternatives to traditional pesticides

Until about four decades ago, crop yields in agricultural systems depended on internal resources, recycling of organic matter, built-in biological control mechanisms and rainfall patterns. Pesticides started a revolution in agriculture and quality improvement methods. The state of the art in pesticides continues to evolve and progress as time passes. But, in these years pesticides were very toxic and left residues in the environment for a long time. On the other hand, loss of yields due to pests in many crops (reaching about 20-30% in most crops), despite the substantial increase in the use of pesticides (about 500 million kg of active ingredient worldwide) is a symptom of the environmental crisis affecting agriculture.

However, farm products must obviously be free from pesticide contamination, which is possible primarily through organic farming. In addition, global social awareness of proper and minimal need based use of these chemicals, to some extent may reduce health related problems (Altieri, 1995).

Therefore, many countries established adequate regulatory safeguards over the manufacture, sale and use of the pesticides. For this reason, complex and costly studies were conducted to indicate whether the material is safe to use and effective against the intended pest. Despite the applications, some human disasters like which occurred in Bhopal and China, and long term side effects on human and animal life style and environmental contamination amplify the approach to find and produce modern pesticides with lower problems and/or to perform alternative applications to traditional pesticides.

IPM[2], the use of multiple approaches to control pests, is becoming widespread and has been used with success in countries such as Indonesia, China, Bangladesh, the U.S., Australia, and Mexico (Miller, 2004). IPM attempts to recognize the more widespread impacts of an action on an ecosystem, so that natural balances are not upset (Daly et al., 1998). New pesticides are being developed, including biological and botanical derivatives and alternatives that are thought to reduce health and environmental risks. In addition, applicators are being encouraged to consider alternative controls and adopt methods that reduce the use of chemical pesticides.

Pesticides can be created that are targeted to a specific pest's life cycle, which can be environmentally friendlier. For example, potato cyst nematodes emerge from their protective cysts in response to a chemical excreted by potatoes; they feed on the potatoes and damage the crop.[81] A similar chemical can be applied to fields early, before the potatoes are planted, causing the nematodes to emerge early and starve in the absence of potatoes (WWW. Wikipedia.com).

The major alternatives to traditional chemical pesticides are listed below:

i. Natural pesticides,
ii. Biological pest control,
iii. Plant genetic engineering,
iv. Interfering with insect breeding,
v. Application of composted yard waste,

[2] Integrated pest management

vi. Cultivation practices,
vii. Release of organisms that fight the pests,
viii. Interfering with insects' reproduction,
ix. Soil steaming, etc.

In the last 10 years, one line of research has been in the area of natural pesticides. This typically means that certain botanical plant oils have been processed, combined, or concentrated into pesticides. These plant oils have a unique action that targets a key neurotransmitter receptor called octopamine which is found in all invertebrates (i.e. insects), but not in mammals.

Alternatives to pesticides are available and include methods of cultivation, use of biological pest controls (such as pheromones and microbial pesticides), plant genetic engineering, and methods of interfering with insect breeding (Miller, 2004). Application of composted yard waste has also been used as a way of controlling pests (McSorley & Gallaher, 1996). These methods are becoming increasingly popular and often are safer than traditional chemical pesticides. In addition, EPA is registering reduced-risk conventional pesticides in increasing numbers.

Cultivation practices include polyculture (growing multiple types of plants), crop rotation, planting crops in areas where the pests that damage them do not live, timing planting according to when pests will be least problematic, and use of trap crops that attract pests away from the real crop. In the U.S., farmers have had success controlling insects by spraying with hot water at a cost that is about the same as pesticide spraying (Miller, 2004).

Release of other organisms that fight the pest is another example of an alternative to pesticide use. These organisms can include natural predators or parasites of the pests. Biological pesticides based on entomopathogenic fungi, bacteria and viruses cause disease in the pest species can also be used (Miller, 2004).

Interfering with insects' reproduction can be accomplished by sterilizing males of the target species and releasing them, so that they mate with females but do not produce offspring (Miller, 2004). This technique was first used on the screwworm fly in 1958 and has since been used with the medfly, the tsetse fly, and the gypsy moth. However, this can be a costly, time consuming approach that only works on some types of insects (Miller, 2004).

Another alternative to pesticides is the thermal treatment of soil through steam. Soil steaming kills pest and increases soil health.

3.1 Effectiveness of alternatives to traditional pesticides

Some evidence shows that alternatives to pesticides can be equally effective as the use of chemicals. The experiences resulted from some countries used alternatives emphasize that reduction of pesticide use, application of composted yard waste with high carbon to nitrogen ratio to agricultural fields, etc were highly effective at r increasing crop yield. As a result, today's pesticides and alternative methods are safer and more effective in controlling pests than ever before in our history.

3.2 Problems of modern pesticide systems

As agricultural modernization progressed, the ecology-farming linkage was often broken as ecological principles were ignored and/or overridden. In fact, several agricultural scientists have arrived at a general consensus that modern agriculture confronts an environmental crisis. A growing number of people have become concerned about the long-term

sustainability of existing food production systems. Evidence has accumulated showing that whereas the present capital- and technology-intensive farming systems have been extremely productive and competitive; they also bring a variety of economic, environmental and social problems. Evidence indicates, however, that excessive reliance on monoculture farming and agro-industrial inputs, such as capital-intensive technology, pesticides, and chemical fertilizers, has negatively impacted the environment and rural society. Most agriculturalists had assumed that the agroecosystem/natural ecosystem dichotomy need not lead to undesirable consequences, yet, unfortunately, a number of ecological diseases have been associated with the intensification of food production. They may be grouped into two categories:

i. diseases of the ecotope, which include erosion, loss of soil fertility, depletion of nutrient reserves, salinization and alkalinization, pollution of water systems, loss of fertile croplands to urban development;
ii. diseases of the biocoenosis, which include loss of crop, wild plant, and animal genetic resources, elimination of natural enemies, pest resurgence and genetic resistance to pesticides, chemical contamination, and destruction of natural control mechanisms.

4. Conclusion

Agricultural and veterinary chemicals are vital to our welfare and the protection of the health of our families and pets. Unless, and until, better, more efficient and more cost effective means of pest control are developed, farm chemicals will remain a major weapon in our constant battle against pests. Production would drop drastically, and food would be of poorer quality, more expensive and in short supply. Many pets and farm animals would suffer and die needlessly. World's economy and our standard of living would rapidly decline. In addition, to elevate the human life quality level and to protect public health against even mortal effects of chemicals, new generations of pesticides and alternatives to traditional chemical pesticides are applied to produce healthier and larger amount of various food.

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2

Quality of Vegetables and Pests Control in African Urban Cities

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1. Introduction

Urban farming or Urban gardening (Urban Agriculture) is the practice of farming in a city environment. This practice of food production takes place on rooftops, in backyards, in community gardens and in vacant public spaces in industrial countries (JOB S Ebenezer, 2010). In the industrialized world, urban farming largely disappeared in this century in spite of the recent development of the green roof movement, but in the developing world it has persisted and since the 1970's has shown signs of increase (Nelson., 1996). Today, in the developing world especially in African countries, more and more people are migrating from rural to urban settings adding to the increase in global population in urban cities. Such growing urbanization has increased the demand for quantity and quality food production and consumption in the cities. The contribution of urban agriculture to these cities has the potential to improve livelihoods and provide economic growth and stability to the population (Nugent, 1997; Garnett, 1996). Also, organic practices can be further promoted in urban agriculture by transforming nutrient rich waste from landfills into organic fertilizer and returning it to the land (Nancy Simovic, 1998).

In Côte d'Ivoire, migration from rural areas brings into the urban areas many persons with very little formal education. This may result in unemployment and under-employment of a sizable number of people. Urban agriculture may be a way to occupy the inner city youth, and new migrants.

Urban agriculture has the potential for creating micro-enterprises that can be owned and operated by the community members with little initial investment capital.

Horticulture is a vital economic sector for most African countries. Côte d'Ivoire fruits and vegetables export to EU (European Union) countries are estimated to over 360, 000 tons. In 2007, Burkina Faso exported more than 925, 000 tons of fresh green beans. In Mali tomatoes production was over 17,000 tons and okra reached 8,600 tons. Despite the economic potential, the horticultural sector including urban agriculture is confronted to pests' attacks and phytosanitary problems. It needs to comply with the pesticides regulations and the quality control (traceability) standards which are now required by most industrials and export countries. Hence, the importance of the present initiative to study the problematic of "The Quality of Vegetables and the Pests Control in African Urban Farming".

The main objective of this study is to assess the impact of pest on urban farming Lettuce, Spinach, and Turnip production, the application of agrochemicals for plant protection, and the quality of irrigation water. The specific objectives are (1) to evaluate the impact of agrochemicals application on plants' pests, (2) to determine their economic incidence, (3) to monitor irrigation water quality, and (4) to control some essential production factors which are indicators of a good standard quality production.

2. Materials and methods

The study was conducted near the "M'POUTO village "located around the lagoon Ebrié next to the district of Riviera-Golf of the city of Abidjan, the economic capital of Côte d'Ivoire.

Photo 1. M' POUTO village; district of Riviera-Golf in Abidjan City. Côte d'Ivoire

The experimental zone is in full sub-equatorial climate with surrounding vegetation mostly composed of tall grasses and scattered bushes (Photo 1).
The area is characterized by hydromorphic and sandy soils (DUCHAUFOUR Ph, 1997). The climatic conditions of the study zone is characterized by four seasonal cycles:
A big or long rainy season from May to July and a small shorter rainy season from October to November followed by a long dry season from December to April and a short dry season from August to September. The average annual rainfall is about 2500 millimeters with a relative humidity of 80 to 90 %. The maximum and minimum average air temperatures are respectively 33°C and 21°C.

2.1 Plants

Subsistence crops are defined as crops that may be rich in proteins or carbohydrates grown by a farmer principally to feed his or her family, with little or nothing left over to sell while urban farming crops are considered as crops supplying luxury items intended for privileged people (MESSIAEN C.M., 1989);. Our study concerned three urban farming vegetable crops namely: Lettuce, Spinach and Turnip.

2.1.1 The Lettuce, *Lactuca sativa L*

The Lettuce, *Lactuca sativa* **L. (Asteraceae or Compositaceae)** is the more consumed vegetable in the world. There are approximately 149 varieties worldwide (CHAUX C et al., 1994). There are two main classes of lettuce: non-head forming lettuces such as the " celtuce " or " lettuce - asparagus " and the head-forming lettuces such as the " Batavia " or " curly " cabbage lettuce (Photo 2).

Seeds germination is normal between 0°C and 25°C, and sunlight plays a major role in the growth and the development process. Lettuce has a high water demand (E.J. RYDER et al, 1976), and grows well in different types of soils presenting a steady structure with good water holding capacity. In general, lettuce is a moderately heavy consumer of nutrients. Seedlings of lettuce are planted at 2 to 4 leaf-stage in well-prepared seedbeds (trays of earth) ploughed at depth and mixed to manure. The application of fertilizer (NPKS) is often necessary and must be incorporated in the soil before planting. The growth cycle is very variable (45 to 100 days) depending on the variety. Agrochemicals applications (insecticides and fungicides) on the lettuce cultures against the pest attacks are often done in the middle and end of cultural cycle.

Photo 2. Lettuce salad: *Lactuca sativa (*Batavia)

2.1.2 The spinach, *Spinacia oleracea L*

The spinach, *Spinacia oleracea L.* **(Chénopodiaceae)**, is named " the prince of vegetables " (VERGNIAUD P. 1976). It is an annual plant generally cultivated as biennial in vegetable gardens (Photo 3). The plant develops initially, on a very short axis, a rosette constituted of fifteen (15) to twenty (20) leaves. These leaves are lengthily petiolate with full limb more or less blighted. Mineral fertilization (NPK) is often necessary according to expected yields. But the poultry's liquid manures and dejections abundantly brought are very largely sufficient to face exports of mineral elements. Watering must be sufficiently abundant to satisfy the water needs of the plant. The diseases and pest management of the plants must be carefully and frequently controlled (LAUMONNIER R., 1978). Also, weeding is very important and a thinning can be practiced in case of a very dense germination and seedlings. Spinach usually matures in 35 to 45 days. The plant may be harvested from the time there are 5-6 leaves on the plant right before the seed stalk develops (Photo 3). The phytosanitary protection of the plants intervenes in middle and end of cycle (FABIEN SEIGNOBOS et al., 2000).

Photo 3. Spinach: *Spinacia oleracea L*

2.1.3 The turnip, *Brassica rapa L. var. rapa*

The turnip, *Brassica rapa L. var. rapa*, **(Brassicaceae)** is produced in specialized market gardening. The plant is normally bi-annual (photo 4). In its vegetative stage it is constituted of a basal rosette made of about fifteen leaves with real green limb and bristling with rough hairs (photo 4).

According to the varietal type, it has a tuberous root of flattened, conical or cylindrical form and of variable color (white (photo 4), yellow, black or two-tone) (LAURENCE S et al., 2009). One notes about thirty varieties but the range of the varieties currently cultivated is rather restricted (Tokyo hybridizes F1, Chinese turnip…). Turnips are primarily cultivated in full field by direct seeding on fertile well-prepared seedbeds.

The needs in mineral elements are important and sustained fertilization (NPK) is needed before planting and during the growth cycle. The application of manure must be done before planting and preferably on the previous crop in a rotation.

The growth cycle is 40 - 70 days dependent on climatic conditions and varieties. Turnips are harvested as young roots by successive thinnings. The diseases control and protection of turnips must be regular due to frequent pest attacks.

Photo 4. Turnip: Brassica rapa L. (White – Turnip)

2.2 Experimental plots

The used method is the visual trapping by colored traps to estimate the presence, the quantity and the quality of the individual species (RIBA et al., 1989; FISCHER et al., 1987). Two types of traps were put in every plot of land:

- An air trap at the level of the foliage (see Fig 1).
- A ground trap on the surface of the ground**.**

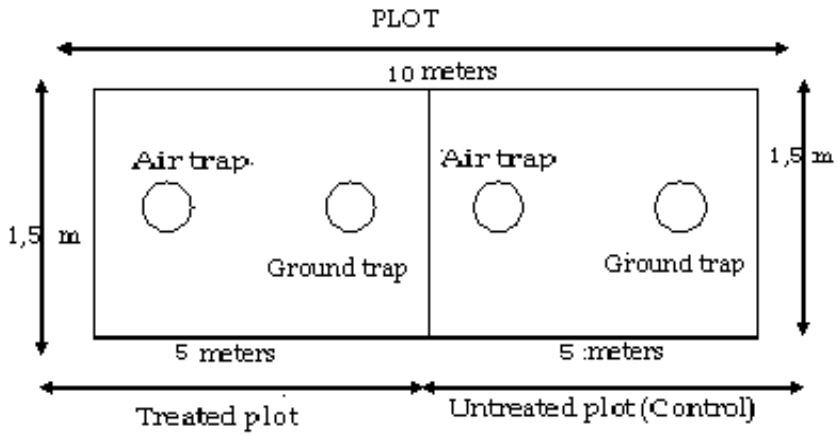


Fig. 1. Experimental Plots with air and ground traps

All these traps contain some soapy water which captures insects. The harvest of the grips is made every two days with change of the trapping liquid. Insects are kept in glass jars containing some alcohol (70 degrees) before being sent to the laboratory.

The ground is raised to form wide mounds or ground trays of 10 meters long by 1, 5 meters wide Blocks.

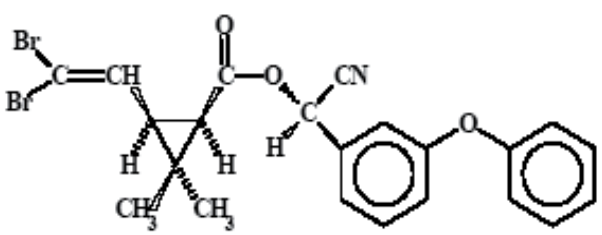
Every Block is formed by two plots of land or beds of 5 meters by 1, 5 meters each (see Fig1,). One of the plots of land is treated and the other one is untreated and constitutes the Control plot (blank). All in all, four Blocks and eight plots of land were realized: two Blocks for Lettuce, one Block for Spinach and one Block for Turnips. Every plot of land contains two traps.

2.3 Agrochemicals

2.3.1 Deltamethrin: trade name DECIS (K -OTHRINE)

Molecular formula: $C_{22}H_{19}Br_2NO_3$ (WHO., 1990a, 1990b)

Structural formula:



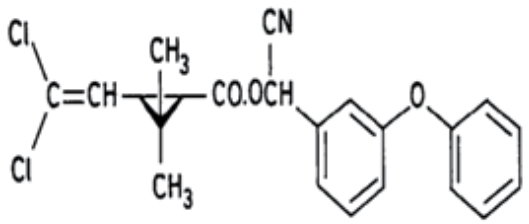
(*S*)- α-cyano-3-phenoxybenzyl (1*R*,3*R*)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylate (*IUPAC*)

Decis 25 EC is an emulsifiable concentrate of formulation (25g/l). It is approved for a wide variety of insects including acarina, thysanoptera and arthropods pests of the horticultural plants **(DEMBELE A.; 2000)**. We made a first application on the salad lettuce at the stage 27 days at a concentration of 0.042 g/l (25 L of Decis in 15 l of water) for 400 m². The stage 39 days corresponds to the second treatment by Deltamethrin with the same concentration of 0,042 g/l and by Maneb with the concentration of 5 g/l representing 93,75 grams of CALLIMAN 80 WP for the sprayer of 15 liters for 400 m². The stage 45 days: corresponds to the third treatment by Maneb with the same concentration of 5 g/l.
For turnip, at the stage 18 days we have done the first treatment by Deltamethrin with the concentration of 0,025 g/l (15 milliliters of DECIS 25 EC in 15 liters of water) for 400 m². The Stage 30 days corresponds to the second treatment by Deltamethrin with the same concentration of 0,025 g/l and by Maneb with the concentration of 5 g/l representing 93,75 grams of CALLIMAN 80 WP for the sprayer of 15 liters. The Stage 38 days of turnips received the same treatment as the stage 30 days.

2.3.2 Cypermethrin: trade name Cypercal 50 EC

Molecular formula: $C_{22}H_{19}C1_2NO_3$ (WHO., 1979)

Structural formula:



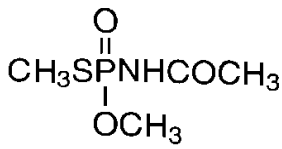
RS)**-I-cyano-3-phenoxybenzyl (1***RS*)**-*cis-trans*-3- (2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (***IUPAC*)**.**

Cypermethrin 50 EC is an emulsifiable concentrate of formulation (50 g/l). It is approved for a broad spectrum of harmful insects (Caterpillars, Thrips, Heliothis and white flies).
We carried out the first application on spinaches at the stage 18 days; the amount of application is of 0.133 g/l (40 ml of CYPERCAL 50 EC. in 15l of water) for 400 m². At the stage 30 days the spinaches received an amount of treatment of 0,133 g/l in addition to 5 g/l of maneb (93.75g of CALLIMAN 80 WP in 15 l of water) for 400 m².

2.3.3 Acephate: trade name Orthen 75 SP

Molecular formula: $C_4H_{10}NO_3PS$ (WHO., 1976)

Structural formula:

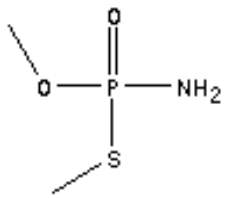


O,S-**dimethyl acetylphosphoramidothioate (IUPAC)**

Orthen 75 SP is a water-soluble powder of formulation 75% acephate. It is a systemic pesticide. Methadomiphos ($C_2H_8O_2NPS$) is a metabolite of acephate and it is also systemic **(DEMBELE A.; 2000)**.

Molecular formula: $C_2H_8O_2NPS$

Structural formula:



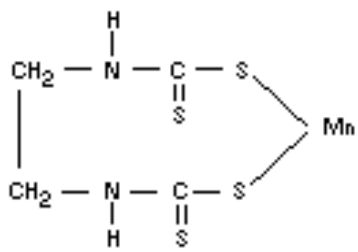
O,S,**-dimethyl phosphoramidothioate (IUPAC)**

These two organophosphorous pesticides are both effective against a broad range of insect pests (sucking, biting, and mining insects) on such vegetable and crops as cabbages, cotton, tobacco, sugar beet, head lettuce. It is used as a pre-harvest spray at 0.5-1.5 kg/ha. With this amount, protection against the insects vermin is obtained from 7 to 21 days.
The first application on lettuce is done at at stage 27 days with the amount of 2 g/l (40 g of Orthen 75 SP in 15 l of water) for 400m².
At the stage 39 days the amount of treatment of lettuce is 2 g/l in addition to 5 g/l of maneb for 400 m².

2.3.4 Maneb: trade name Calliman 80 WP

Molecular formula: $C_4H_6N_2S_4Mn$ (WHO-1993)

Structural formula:



Manganese ethylene-1,2-bisdithiocarbamate (IUPAC)

Calliman 80 WP is a wettable powder of formulation 80% of maneb (dithiocarbamate), an effective protective fungicide against the main foliar diseases (Anthracnose, Mildew, Alternaria, Rhizoctonia, cercospora, Sclerotinia and Septoria). It should be applied before and after seeding on all three vegetables at amount of 5 g/l. Moreover the salad lettuce received a treatment at the stage of 45 days.

2.4 Plants phytopathology and pests monitoring

After each treatment, every two days we proceed:

- To the description of the general characteristics of the plants, especially the leaves, and we look out for visible signs of attacks and diseased plants.

- To the counting of the insects captured in the traps for follow-up of the dynamics of the recolonization following the various treatments.

The final identification of the fungus was made after observation of the samples under a microscope (enlarged to a size 400 times) and according to known keys of identification (BOTTON.B et al, 1990; KIFFER. E et al, 1997).

2.5 Irrigated water monitoring

We sowed under the fume hood raw water of boring in the Petri glass, on culture medium sterilized. The analysis consisted of identifying thermotolerant Coliform and fecal Streptococci and counting of the colonies of red or pink coloring of 2 to 3 millimeters (mm) in diameter. The criterion of assessment is fixed to $2x10^3$.

3. Results and discussion

3.1 Pests assessment on lettuces

The trapping on the level of lettuces allowed the identification of six (6) Orders grouping together in total forty two (42) families of insects. They are: 10 families of Beetles, 9 families of Hymenopterans, 10 families of Dipterans, 7 families of Hemiptera, 3 families of Lepidoptera and 3 families of Orthoptera. Seventy nine per cent (79 %) of these families are present on the untreated plot (blank) whereas 69 % meet on the treated plot with Deltaméthrin (Tabl 1, 2, 3) (Graphic 1).

We counted 79% of pests in the untreated lettuce plots against 69% in the deltamethrin treated plots with the Hemiptera representing the most important group of devastators pests. The pests recolonization of the field plots was done 12 days after the first application of acephate against 8 days with deltamethhrin. The agrochemicals application makes it possible to reduce by 50% the losses of production of the Lettuces salad (Graphic 1).

THE COLEOPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Buprestidae	P	+	-	-	-
Carabidae	T	-	+	-	+
Chrysomelidae	P	-	+	+	+
Coccinellidae	T	+	-	+	+
Elateridae	P	+	-	-	-
Hydrophilidae	P	-	+	-	+
Scarabaeidae	P&T	+	+	+	+
Staphylinidae	T	+	-	+	-
Tenebrionidae	T	-	+	-	+
Cicindelidae	T	-	-	-	+

(-) = Absent (+) = Present P = Pest insects T = Non -Target Insects N = Neutrals

Table 1. Order and Insects families' identified on lettuces

THE HYMENOPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Pompilidae	N	-	+	-	-
Ichneumonidae	T	+	-	-	-
Halictidae	T	+	-	-	-
Vespidae	T	+	-	-	-
Formicidae	T	+	-	-	+
Cynipidae	P	+	-	-	-
Cephidae	P	-	-	+	-
Crabronidae	T	-	-	+	-
Encyrtidae	T	-	-	+	-
THE DIPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Agromyzidae	P	+	+	+	-
Drosophilidae	N	+	+	+	-
Sarcophagidae	N	+	-	-	-
Muscidae	P&N	+	-	+	-
Dolichopodidae	T	+	-	+	-
Chironomidae	N	+	-	+	-
Diopsidae	P	-	-	+	-
Stratiomyidae	N	-	-	+	-
Tephritidae	P	-	-	+	-
Phoridae	N	-	-	+	-
THE HEMIPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Cicadellidae	P	+	+	+	+
Cicadidae	P	+	-	+	-
Miridae	P	+	-	+	+
Piesmidae	P	+	-	+	-
Coreidae	P	-	-	-	+
Membracidae	P	-	-	+	-
Lygaeidae	P	-	-	+	-

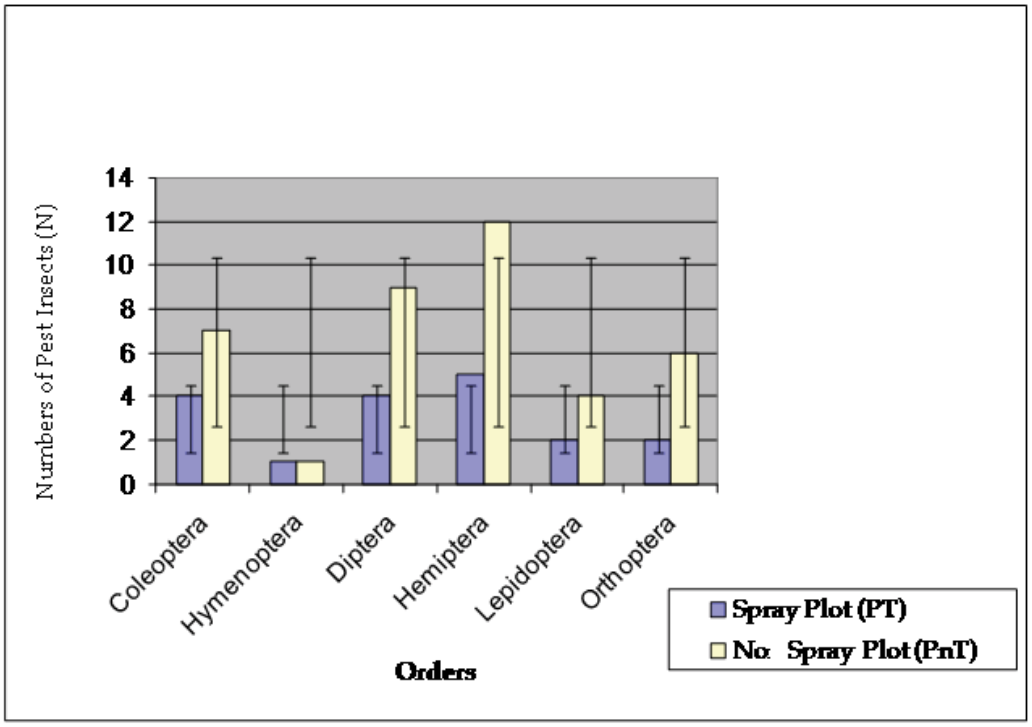
(-) = Absent (+) = Present P = Pest insects T = Non -Target Insects N = Neutrals

Table 2. Order and Insects families' identified on lettuces

THE LEPIDOPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Pieridae	P	+	-	-	-
Noctuidae	P	+	-	+	-
Yponomeutidae	P	-	-	+	-
THE ORTHOPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Gryllidae	P	-	+	-	+
Acrididae	P	+	-	+	-
Gryllotalpidae	P	-	-	-	+

(-) = Absent (+) = Present P = Pest insects T = Non -Target Insects N = Neutrals

Table 3. Order and Insects families' identified on lettuces



Graphic 1. Pests Control on Lettuces

3.2 Pests sssessment on spinaches

On the spinach Seven (7) Orders grouping together in total Thirty seven (37) families of insects were identified. They are: 9 families of Beetles, 9 families of Hymenoptera, 10 families of Diptera, 4 families of Hemiptera, 2 families of Lepidoptera, 2 families of Orthoptera, and 1 family of Isoptera.

84 % of these families were present on the Untreated against 57 % on the treated plot with Cypermethrin.

THE COLEOPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Carabidae	T	-	+	-	+
Cicindelidae	T	-	+	-	+
Scarabaeidae	T	+	+	+	+
Hydrophilidae	P	+	+	+	+
Staphylinidae	T	+	-	+	-
Coccinellidae	T	+	-	+	-
Ténébrionidae	T	-	-	+	+
Chrysomélidae	P	-	-	+	-
Elateridae	P	-	-	+	-
THE HYMENOPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Pompilidae	N	-	+	+	+
Ichneumonidae	T	-	+	-	-
Tenthrédinidae	P	+	-	-	-
Sphecidae	T	+	-	+	-
Crabronidae	T	+	-	+	-
Vespidae	T	-	-	-	+
Formicidae	T	-	-	+	+
Nyssonidae	T	-	-	+	-
Bethylidae	T	-	-	+	-

(-) = Absent (+) = Present P = Pest insects T = Non- Target Insects N = Neutrals

Table 4. Order and Insects families' identified on Spinach

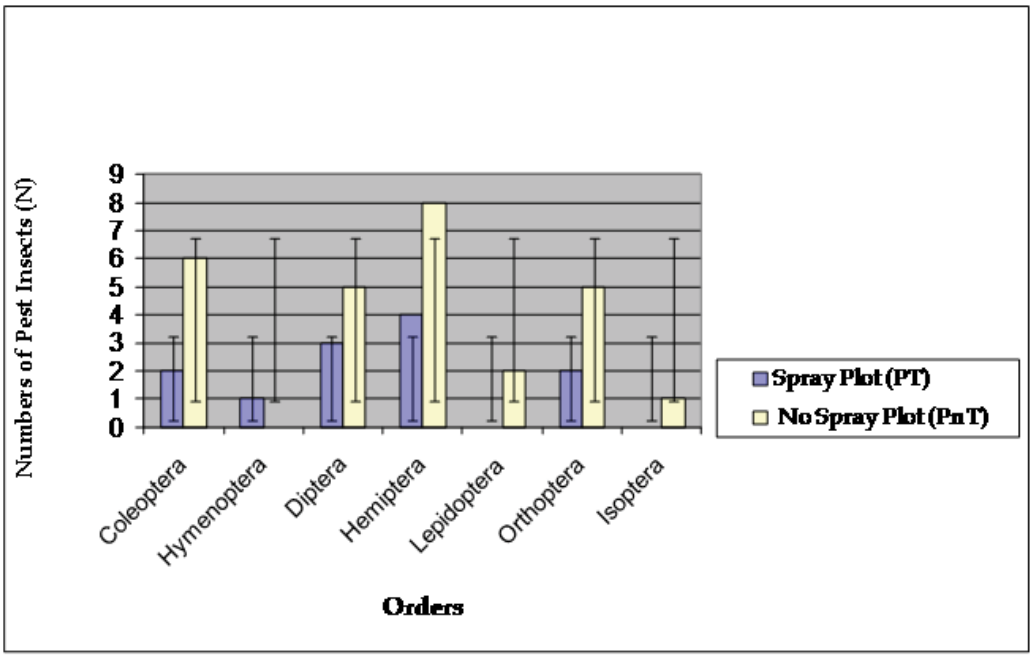
THE DIPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Tachinidae	T	-	+	-	-
Agromyzidae	P	+	+	+	-
Dolichopodidae	T	+	-	-	-
Diopsidae	P	+	-	-	-
Stratiomyidae	N	+	-	+	-
Muscidae	P&N	+	-	-	-
Sarcophagidae	N	-	-	+	-
Anthomyiidae	P	-	-	+	-
Calliphoridae	N	-	-	+	-
Phoridae	N	-	-	+	-
THE HEMIPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Cicadellidae	P	+	+	+	+
Piesmidae	P	+	-	+	+
Cicadidae	P	+	-	+	-
Tingidae	P	-	-	-	+

(-) = Absent (+) = Present P = Pest insects T = Non- Target Insects N = Neutrals

Table 5. Order and Insects families' identified on Spinach

We obseved 84% of pests in untreated plots against 57% in plots treated with the cypermethrin, with the Hemiptera representing the most important group. The recolonization by the pests was done 10 days after the first application of cypermethrin or Lambdacyhalothrin. Agrochemicals application makes it possible to reduce by 25% the losses of production of the spinaches (Graphic 2, photo 5).

Photo 5. Application of pesticides doesn't respected GAP



Graphic 2. Pests Control on Spinaches

3.3 Pests assessment on turnips

On the turnips we identified six (6) Orders making a total of 34 insect's families. They are: 7 families of Beetles, 9 families of Hymenopterans, 8 families of Dipterans, 4 families of Hemiptera, 4 families of Lepidoptera and 2 families of Orthoptera.

91 % of these families were present on the untreated plots whereas 62 % were on the treated plot (spray plot) with Deltamethrin (Tabl 6, 7, 8,).

THE COLEOPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Coccinellidae	T	+	-	+	+
Buprestidae	P	+	-	-	-
Carabidae	T	-	+	-	+
Hydrophilidae	P	-	+	-	+
Staphylinidae	T	+	-	+	-
Cicindelidae	T	-	+	-	+
Chrysomelidae	P	-	-	+	+

(-) = Absent (+) = Present P = Pest insects T = Non -Target Insects N = Neutrals

Table 6. Order and Insects families' identified on the turnips

THE HYMENOPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Pompilidae	N	-	+	+	+
Ichneumonidae	T	-	+	-	-
Sphecidae	T	+	-	+	-
Crabronidae	T	+	-	+	-
Vespidae	T	+	-	-	-
Nyssonidae	T	-	-	+	+
Bethylidae	T	-	-	+	-
Cephidae	P	-	-	+	-
Chalcididae	T	-	-	+	-
THE DIPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Sarcophagidae	N	+	-	+	+
Muscidae	P&N	+	-	+	-
Agromyzidae	P	+	+	+	+
Drosophilidae	N	+	-	+	+
Stratiomyidae	N	-	+	+	-
Lonchaeidae	P	-	-	+	-
Cecidomyiidae	P	-	-	-	+
Mycetophilidae	T	-	-	-	+
THE HEMIPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Cicadellidae	P	+	+	+	+
Cicadidae	P	+	-	+	-
Miridae	P	-	+	-	+
Aphididae	P	-	-	+	+

(-) = Absent (+) = Present P = Pest insects T = Non -Target Insects N = Neutrals

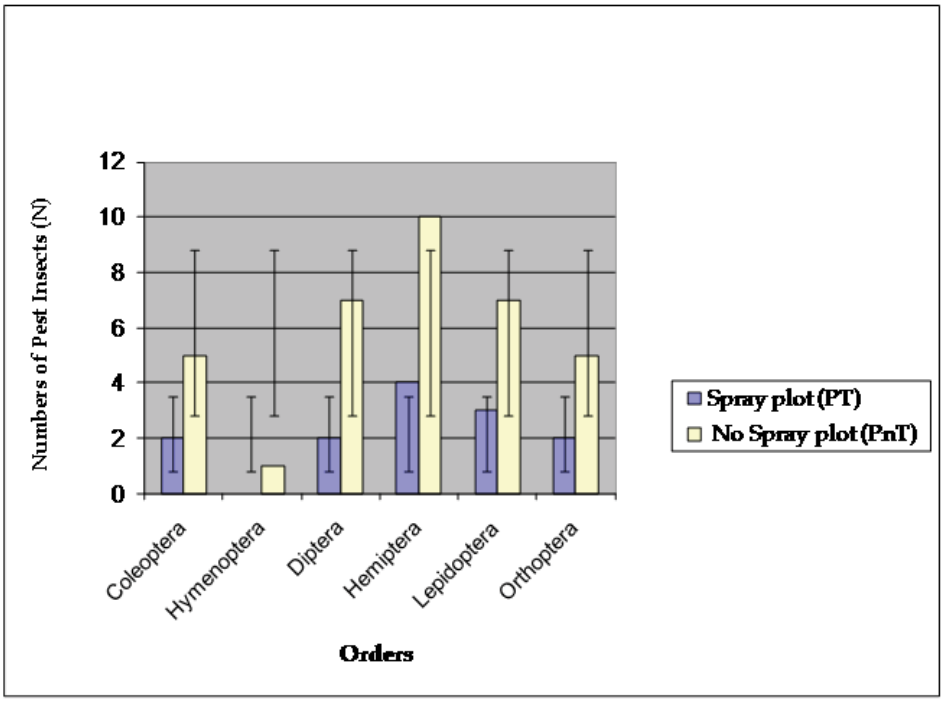
Table 7. Order and Insects families' identified on the turnips

THE LEPIDOPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Noctuidae	R	+	+	+	+
Pieridae	R	-	-	+	-
Lycaenidae	R	-	-	+	-
Yponomeutidae	R	-	-	+	-
THE ORTHOPTERA ORDER					
FAMILIES	RELATION	Treated Plots		Untreated Plots	
		Areas Traps	Grounds Traps	Areas Traps	Grounds Traps
Acrididae	R	+	+	+	+
Gryllotalpidae	R	-	-	-	+

(-) = Absent (+) = Present P = Pest insects T = Non -Target Insects N = Neutrals

Table 8. Order and Insects families' identified on the turnips

We observed 91% of pests in untreated plots against 62% on the plots treated with deltarmethrin, and the Hemiptera also set up the most important group of pests. The pests recolonisation was done 8 days after the first application of deltarmethrin (Graphic 3). The first application of agrochemicals makes it possible to reduce by 42% the losses of turnip production.



Graphic 3. Pests Control on Turnips

Overall the application of Agrochemicals significantly reduced the number and the species of pests on the treated plots. The Coleoptera and the Hymenoptera contain the main species of the predatory and natural enemies thus auxiliary (no target insect) of plants protection, having a significant impact on the dynamics of the populations of pests. The preservation of these different auxiliaries is necessary for a sustainable management of natural resources. Deltamethrin has a good level of selectivity with a superior advantage for the management of pests and the environment over the Acephate which, has a low selectivity but a wide range of effectiveness against insect pest and good residual activity. The preservation of the auxiliaries of culture in spite of the chemical treatment is essential considering the important role that they play in the maintenance of agro-ecological balances.
The Lepidoptera (larva), Orthoptera and Hemiptera represent the most important group of insect pests, which attack and cause the highest damage in vegetable gardening of lettuce, spinach and turnip. However, the considerable differences in number of captured insects and pests found between the treated and untreated field plots show that a targeted application of agrochemicals against these groups of pests is efficient.
The majority of the groups of pests which attach and cause important damage on turnip, spinach and lettuce can be controlled by the application of agrochemical products applying good agricultural practices (GAP) compatible with the protection of the environment and the preservation of non-target organisms.

Photo 6. Pesticides Plastic container on the plot (Maneb)

However, one of the biggest problems encountered by vegetable producers is their lack of sufficient knowledge about how to use safely the agrochemicals. Very large numbers of empty pesticide containers are left lying in the fields because of the lack of collection and disposal facilities and constitute acute potential hazards for the environment and the fauna due to the left-over of toxic pesticides in the containers (Photo6).
The producers are not sufficiently aware of the risks of pesticides accumulation in vegetables, and the possible health problems for consumers being exposed to these risks. They are also often confronted with the problems of accessibility to agricultural credits.

3.4 Plants phytopathology and water monitoring

We identified only one pathogenic fungus on the lettuce (9 % of production). It is Cladosporium *sp of The* Amastigomycota *Divion*; *Group of Deuteromycete;* Hyphomycetes' Class and *Gender of Cladosporium.* This fungus is the agent responsible of Cladosporium gray mold, but the preventive spraying of Maneb (photo 6) gives efficient protection on the lettuce.

The irrigation water is characterized by the presence of micro-organisms such as Thermotolerant Coliform and the fecal Streptococci. Their numbers areas respectively one hundred fifty (150) times and one thousand (1000) times higher than the criteria for international standard allowed for irrigation water quality in agricultural fields (Table 9 and Photo 7).

BACTERIA	RESULTATS	CRITERIA
Thermotolerant Coliform/g	3.10^5	2.10^3
Faecal Streptococci/g	10^6	10^3

Table 9. Microbiology Monitoring of Irrigated Water

Photo 7. Irrigated water quality is doubtful

The microbiological analysis of the irrigation water highlighted an overload of thermotolerant Coliforms and fecal Streptococci. These bacteria which are not normally pathogenic are usually used to indicate the possible presence of pathogenic microfauna organisms. Thus their very high number compared to the threshold recommended shows a low water quality (Photo 7).

The strong presence of these indicator bacteria suggests a probable presence in the irrigation water of very dangerous pathogenic parasites that could develop and cause very important damages to the plants, farmers and the consumers.

The contaminated vegetables can cause a certain number of diseases. Particularly, the contaminated salads are sources of bacterial diseases such as the typhoid and paratyphoid

fevers (Salmonella typhi/paratyphi) whose origin comes from the excrements of the patients or healthy carriers (MESSIAEN C.M, 1989). Other bacteria of the Salmonella species can also cause collective intoxications. The periodically endemic Cholera in the tropical countries, maybe transmitted by soiled salads. Also the bacterial dysenteria (Shigella dysenteriae) can be transmitted by soiled vegetables believed contaminated by the excrements. The preventive protection against these diseases is often done by vaccination. But the use of hygienic measures like disinfections with chloramphenicol, bleach into the water or the potassium permanganate ($KMnO_4$) is of primary importance.

4. Conclusion

The insecticides of biological origin represent an asset but their major disadvantage in addition to their high costs, is their instability with storage. They quickly lose their effectiveness and consequently any competitiveness. But the need for both safe and natural food products while respecting nature and maintaining a healthy environment is a very important concept to be considered in Integrated Pest Management (IPM). IPM can be defined as a combination and the reasoned use of all the methods which makes it possible to control or to maintain the populations of pests to a threshold economically bearable. And if the consumers estimate that the products are of the first rate quality, they will not hesitate to pay for the full price. Finally, one can reach a great effectiveness in the improvement of plants protection by associating the conservation of auxiliary insects with the application of agrochemicals and biotechnology. Our developing countries will be able certainly to benefit from this progress.

5. Acknowledgments

I wish to thank the M'Pouto village of Riviera-Golf in Abidjan City urban farming producers for their collaborative contribution in this work.

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3

Differential Efficacy of Insecticides According to Crop Growth: The Citrus Psyllid on Citrus Plants

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1. Introduction

Citrus greening is a destructive disease of citrus trees in subtropical and tropical regions. The pathogen of this disease in Asian countries, *Candidatus* Liberibacter asiaticus, is transmitted by the citrus psyllid, *Diaphorina citri* Kuwayama (Huang et all, 2004). Since no direct control of the pathogen has been established yet, the current management of this disease relies on the control of the vector, especially by insecticides (Halbert & Manjunath, 2004; Yang et al., 2006). Many studies have been performed to examine the efficacy of various insecticides on the control of psyllids, revealing that neonicotinoids such as imidacloprid or thiamethoxam are very effective on the psyllid (Hayashikawa et al., 2006; Yasuda et al., 2006; Srinivasan et al., 2008; Gatineau et al., 2010; Ichinose et al., 2010a). These insecticides are characterised by their long-continued residue effect on the pest, up to several months (Yasuda et al, 2007), and their reducible effects on the psyllid population can continue even after the concentration of the insecticides is decreased below the lethal level (Boina et al., 2009).

Important issues in the use of insecticides are 1) how frequently an insecticide should be applied in year, 2) how much the insecticide should be used in each application, and 3) when the insecticide application should be performed in the year. For the third case, insecticides will be unnecessary when target pests are few due to their seasonal activities or other factors that affect the pest population. For example, the application of insecticides in winter in Florida allows the omission of insecticide uses in subsequent several months when psyllids are likely increased (Quresh & Stansly, 2010). On the other hand, more frequent insecticide application may be required for the control of the psyllid in the tropics where the insect can maintain its population at higher densities throughout the year. Gatineau et al. (2010) reported that sufficient reduction of psyllids was attained either by monthly application of imidacloprid or fortnightly application of fenobucarb in citrus orchards of southern Vietnam. The weather in this region is characterised by the tropical climate with

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two distinct seasons in the viewpoint of precipitation, dry season with low precipitation from November to April and wet season with frequent heavy rains from May to October. Serious problem in the management of citrus greening by insecticides in southern Vietnam is raised in the cultivation of king mandarin, which is the most predominant cultivars in southern Vietnam. When trees grew in the stage to yield fruits, usually one and half to two years after planting, the efficacy of neonicotinoids is significantly reduced or even lost despite either the dose or the mode of application (Ichinose et al., 2010b). The control efficiency on the psyllid on grown trees was not implemented by either the increase of insecticide dose up to 100 times or the change of the application mode, not only soil drench but also trunk injection, trunk painting, or leaf spray. Thus, studies were urgently needed to establish control methods for the psyllid on grown trees.

The following five experiments were thus performed for the control of the psyllid on the grown king mandarin tree: 1) the residue effect of five insecticides, imidacloprid, thiamethoxam, clothianidin, methidathion and fenobucarb, were tested by the spray on the whole body of citrus trees cultured in a net house; 2) two application modes, soil drench and spray, of imidacloprid and methidathion were compared in a net house; 3) imidacloprid, methidathion or the mixture of these two insecticides were sprayed on three year old citrus trees, and their efficacy on the psyllid and aphids were counted for four weeks after the application. In the course of these experiments, the selling of methidathion was banned in Vietnam. Hence, we replaced this insecticide for dimethoate, which were tested in the following experiments: 4) imidacloprid, dimethoate, the mixture of these two insecticides, pymetrozine, or dinotefuran was applied on three years old trees and the mortality of psyllids were compared between these treatments; 5) imidacloprid, dimethoate or the mixture of these two insecticides were applied on three years old citrus trees and psyllids and other citrus pests were counted for four weeks after the application. More efficient uses of insecticides were attempted to establish not only for the control of the psyllid but also other citrus pests.

2. Experiments on the control of psyllids by insecticides

All experiments in this report were carried out in the province of Tien Giang, southern Vietnam, about 70 km south-west to Ho Chi Minh. The altitude is generally less than 10 m. This region lies in the tropical zone with two seasons: wet season with relatively higher temperatures from May to October and dry season with relatively lower temperatures from November of the year to April of the succeeding year. Means of annual, minimum and maximum temperatures are 28.0ºC, 23.7ºC, and 32.3ºC, respectively. Annual precipitation is about 2500 mm. Total area for fruit crops in the Mekong Delta Region of Vietnam, more southern areas than Ho Chi Minh, is 282,200 ha, and 77,300 ha (27.4%) area is used for citrus. The most common variety of citrus crops cultivated in this region is king mandarin, which is sensitive to citrus greening (Koizumi et al, 1997). Mean area of farm per family is about 0.5 ha. Various managements for citrus greening are performed in this region, and the most efficient management seems to be the combination of the use of disease-free seedlings and bimonthly application of neonicotinoids (Ichinose & Kano, 2006). Only king mandarin, *Citrus nobilis* Loureiro, was used in our studies, which were carried out under the research project for CG between Japan International Research Centre for Agricultural Sciences (JIRCAS) and Southern Horticultural Research Institute of Vietnam (SOFRI) owing to the predominance of king mandarin in this region and high economic return compared to the investment for the cultivation. All trees were originated from seedlings produced at SOFRI under the condition of no invasion of CG. These seedlings are referred to as disease-free seedlings hereafter.

2.1 Quickness in and longevity of insecticide efficacy

This experiment was attempted to evaluate the efficiency of one organophosphate, methidathion, and one carbamate, fenobucarb. These two insecticides were compared with three neonicotinoids, imidacloprid, thiamethoxam and clothianidin, since the efficacy of the latter insecticides had been examined and their efficacy was already known (Ichinose et al., 2010a). Hence, using seedlings of king mandarin, residue effect of five insecticides was tested: imidacloprid (0.20 g/tree), thiamethoxam (0.17 g), clothianidin (0.19 g), methidathion (0.40 g) and fenobucarb (0.50 g). In the end of February 2009, 30 disease-free seedlings of king mandarin were purchased at the division of seedling production of SOFRI, and 18 seedlings were randomly chosen among them. These selected seedlings were randomly separated into six groups with the same number, three, and each group was treated on 5 March 2009 by no insecticide or any of the above insecticides. In the application, three solutions were prepared for each neonicotinoid treatment: neonicotinoid was dissolved in water to obtain a solution of 20 ml in the total volume so that the dose in the solution would be as in the above quantity. Each 20 ml solution was applied to each seedling on the soil surface on the pot. Methidathion and fenobucarb were diluted by water 1000 times in volume, thus 400 ml and 500 ml respectively, and each solution was sprayed on the whole body of seedlings in each treatment. The above treatments provided a completely randomized design with three replicates in each treatment. These seedlings were maintained in a net house at SOFRI.

One leaf was collected from each tree on the day of the application before spraying, one, two, five, 10, 15, 30, 45, 60 and 90 days after the application. Each leaf collected on each day was placed in a transparent plastic cup of 500 ml just after the collection. Psyllids were collected one day before each leaf-collection day and left in a laboratory for one day. These psyllids transferred in a plastic box with plaster of paris of 1 cm thickness in the bottom for humidity and kept there for about one day for the starvation condition. Just after the preparation of the leaf, two psyllids were randomly selected from the box and released in each cup. The survival of each psyllid in each cup was recorded at every hour until six hours after the release, at 8, 12, 24, 36, 48 and 72 hr. The residue effect of the insecticides was determined by the mortality of psyllids in each cup. The mean of the mortality of the psyllid in the control cup at each observation hour on each observation day was calculated and used to correct the psyllid mortality in cups with insecticide-treated leaves at the same observation time on the same day by using the Abbott's correction formula. The detail of the mortality calculation is described in Ichinose et al. (2010a).

2.1.1 Efficacy of methidathion and fenobucarb

It is difficult to determine the effective mortality attained by insecticide for the management of CG. At this moment, a mortality of 80% was taken as an efficient one in this study, and the effective period was conveniently defined as the days during which mean mortalities were over this level.

Methidathion showed quick, high lethal effect from one to 10 day within 12 hr after the application (Fig. 1A), but such high mortalities were not attained in 15 d after the application (Fig. 1B). High mortalities were not seen in fenobucarb treatment throughout this experiment. Although mortalities were increased in 12 h after the release on 1 to 10 d after the application, the mean mortality was lower than 80 % (Fig. 1C). Although the mortality at 72 hr after the release on the 15th d was a little higher than 80 %, no high mortalities were seen at any time on any day after this day (Fig. 1D). Both methidathion and fenobucarb completely lost its efficacy on the 45th d.

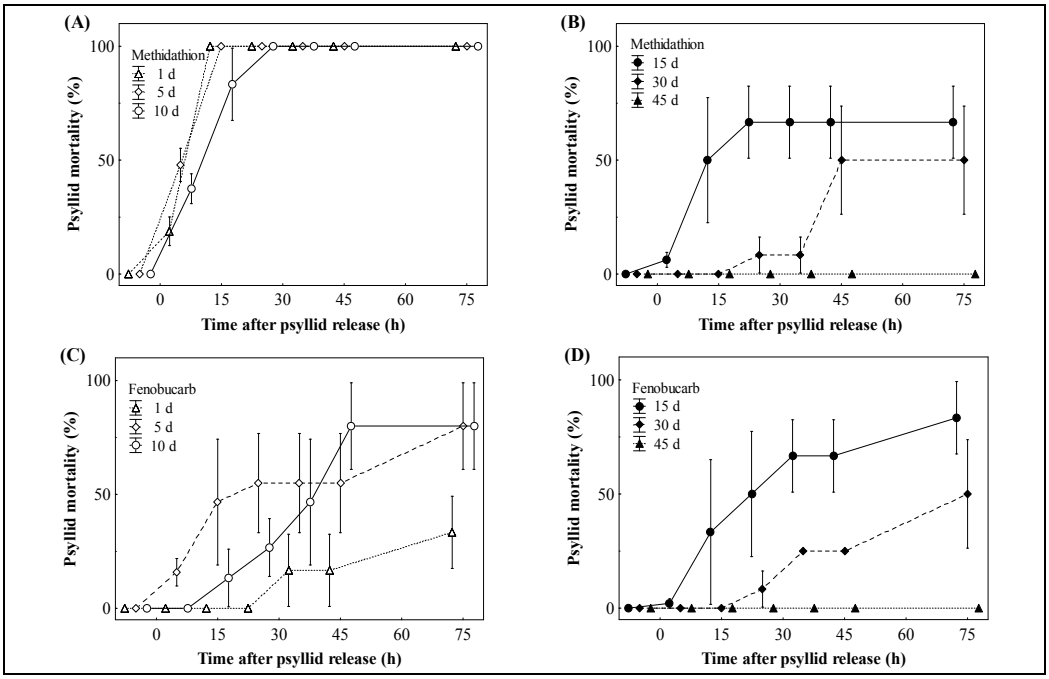

Fig. 1. Mortality of psyllids released on leaves treated with thiamethoxam one to 10 d (A) and 30 to 90 d (B) after the application. Similarly the mortality of psyllids on leaves treated with fenobucarb is shown in C and D, respectively. All mortalities were corrected by the Abbott's formula with using the data of the control treatment. Bars indicate the standard errors of the means.

2.1.2 Efficacy of neonicotinoids

The mortality of psyllids released on leaves treated with imidacloprid was not high until 24 h after the release of psyllids on the leaf until the 10th day after the application (Fig. 2A). Although the mortality reached 100 % after 72 hr even on the 1st to 5th day, it took also 72 hr to reach this mortality on the 60 d and no such high mortalities were attained thereafter (Fig. 2B). Both thiamethoxam and clothianidin needed 10 days after the application to reach high mortalities,> 80 %, at 72 h after the release (Fig. 2C, E) and no high mortalities were attained before this time throughout the experiment (Fig. 2D, F). Mortalities on the 90th day were lower in both thiamethoxam and clothianidin. Hence, three neonicotinoids showed similar quickness of both the lethal effect and the residue effect, although imidacloprid seemed to affect quickly psyllids in the early periods after the application.

These results indicate that the application of methidathion with a frequency of every 10 days would control psyllids best among the insecticides examined. If any of three neonicotinoids are used, similar results in the control of psyllids could be expected among them. Application time of every two month can be recommended. Although the probability of the transmission of the CG pathogen by the psyllid is not known well, methidathion would reduce the infection more efficiently than the neonicotinoids owing to its quickness to reach the 100% lethal effect. If adults eclosed from nymphs grown on infected trees would

transmit the pathogen more efficiently than those infested by the pathogen after eclosing (Inoue et al. 2009), the efficacy of the CG reduction would be similar between methidathion and imidacloprid. Since three neonicotinoids showed similar residue effect but imidacloprid seemed to be better in the quickness, imidacloprid was examined in the succeeding experiments.

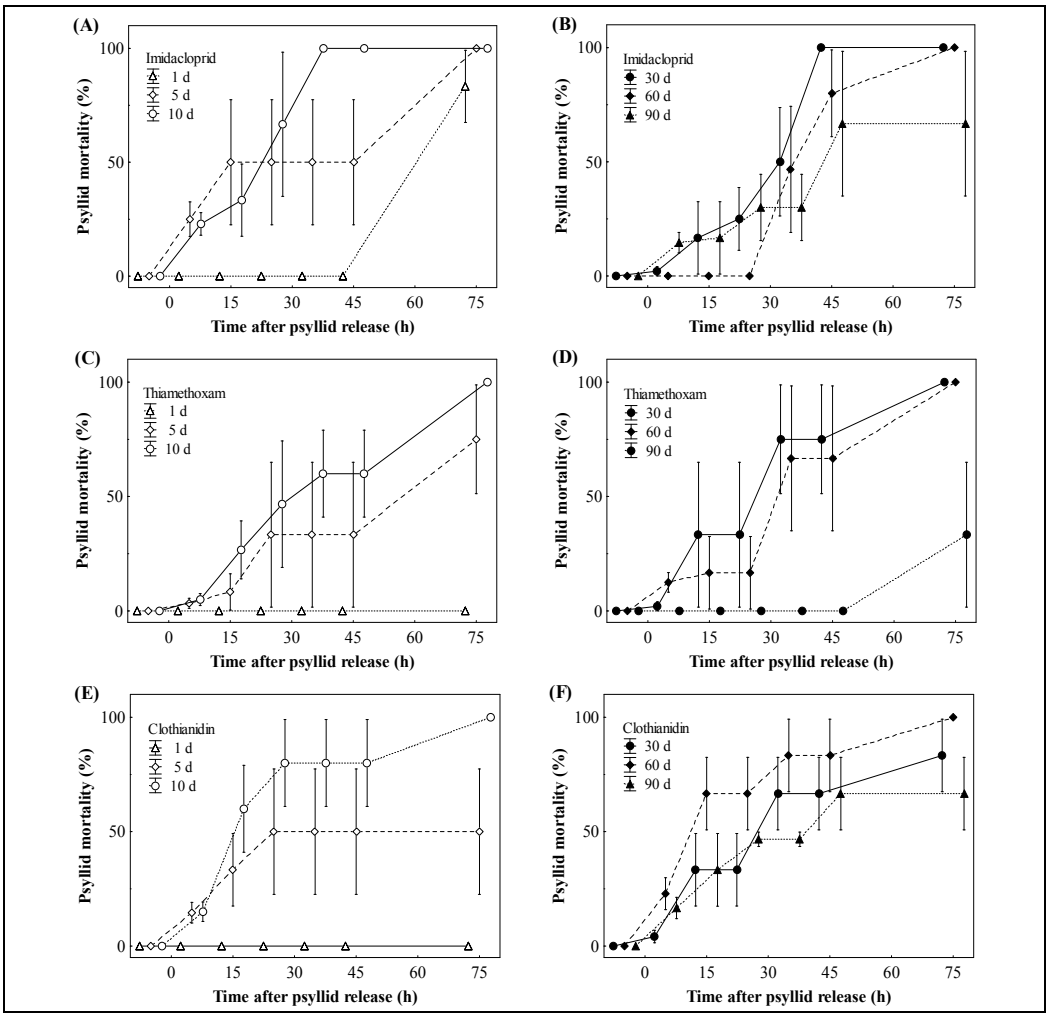

Fig. 2. Mortality of psyllids released on leaves treated with imidacloprid one 10 d (A) and 30 to 90 d (B) after the application. Similarly mortalities of psyllids on leaves treated with thiamethoxam and clothianidin are shown in C-D and E-F, respectively. All mortalities were corrected by the Abbott's formula with using the data of the control treatment. Bars indicate the standard errors of the means.

2.2 Effect of the application mode of insecticide: spray and soil drench

In this experiment, it was attempted to test the application mode of imidacloprid and methidathion. In particular, focused issue was the extension of the residue effect of

methidathion by soil drench. Insecticides sprayed on leaves would be flown away off by raining, while those applied on soil surface by drenching would be less affected by rain. Thus, the efficacy of these insecticides applied by leaf spray or soil drench was compared. On 18 March 2008, 25 seedlings of king mandarin were purchased at SOFRI, and were separated into five groups in the same number. Any of the following treatment was randomly distributed to the groups: no insecticide, spray of 0.2 g imidacloprid in 300 ml water, soil drench of 0.2 g imidacloprid diluted to 20 ml by water, spray of 0.40 g methidathion in 400 ml water, and soil drench of 0.40 g methidathion diluted to 20 ml by water. On the day of the seedling purchase, seedlings were treated as in the above experiment, and the evaluation of the efficacy of each insecticide by each application mode was evaluated similarly.

2.2.1 Comparison of application mode

The psyllid mortality at 12 h after the release was generally low, except for imidacloprid by spray or drench on the 10th day after the application and methidathion by spray from first to fifth day (Table 1). If mortality over 80% was taken as "effective" residue effect, the best efficacy at 12 h was attained by methidathion by spray (Table 1). The mortality at 24 h was still under the effective level in the treatment of imidacloprid by spray or drench on any day except for the 10th day, although the days over this level in the methidathion treatment by spray was extended until 10th day. Methidathion by soil drench was still under the level on any day. The psyllid mortality at 72 h after the release was over the effective level on fifth to 30th days in the treatment of imidacloprid by spray or drench. Methidathion by spray was effective at this time on the first to 15th days after the application, but this insecticide by soil drench was effective only on 15th day.

Insecticide applied	Application mode	Time (h)	Max mortality (%)	Start (d)	End (d)
Imidacloprid	Spray	12	100.0	10	10
Imidacloprid	Drench	12	100.0	10	10
Methidathion	Spray	12	100.0	1	5
Methidathion	Drench	12	50.0	n/a	n/a
Imidacloprid	Spray	24	100.0	10	30
Imidacloprid	Drench	24	100.0	10	30
Methidathion	Spray	24	100.0	1	10
Methidathion	Drench	24	66.7	n/a	n/a
Imidacloprid	Spray	72	100.0	5	30
Imidacloprid	Drench	72	100.0	5	30
Methidathion	Spray	72	100.0	1	15
Methidathion	Drench	72	83.3	15	15

Table 1. The maximum mean of the psyllid mortality observed at 12 or 72 hours after the release on leaves treated with imidacloprid or methidathion applied by leaf spray or soil drench. The first day when the mean mortality was over 80 % and the last day when the mean was reduced below the level again are shown as start and end, respectively. The treatment in which a mortality over 80% was not attained is shown by "n/a".

These results indicate that imidacloprid could effectively control psyllids in 72 h from fifth day on after the application, but the effective level would be only on 10th day unless it would take 24 hours to transmit pathogen efficiently. Imidacloprid by spray did not show quick lethal effect even at 72 h after the release of psyllid until fifth day after the application. It provided similar results with this insecticide by soil drench in both quickness and residue effect. This suggests that either permeability or systemics of imidacloprid would be delayed even on leaves. Methidathion by soil drench should be avoided, since this application would not bring good controls in either quickness of efficacy or longevity. In this point, methidathion by spray could be expected for better management than imidacloprid owing to its quickness in the termination of psyllids after infestation on plants. However, the relatively shorter residue effect would need more frequent application of the insecticide which would not be preferred by farmers. Although either imidacloprid or methidathion was not effective if they were applied on grown trees in field (Ichinose et al., 2010b), it has been reported that imidacloprid effectively control psyllid populations in field (cf. Srninivasan et al., 2008; Boina et al., 2009; Gatineau et al., 2010). Thus, the application of imidacloprid and methidathion in field was examined also in this study.

2.3 Application of methidathion and imidacloprid in field

The results of the above two experiments indicate that methidathion can quickly eliminate psyllids probably within 24 hr after application but the efficiency could continue shorter than a half month. On the other hand, neonicotinoids, especially imidacloprid, could maintain high efficacy for longer than one month, although it would take five to 10 days to reach such high efficacy and three days would be needed to attain effective control level. These results also provide an expectation that the mixture of these two insecticides would have quick and long-lasting efficacy on the psyllid. Thus, the efficacy of individual insecticides and the mixture of them were examined in field. Two orchards, located in Cai Be, about 100 km south-west of Ho Chi Minh, were selected for this study. The area size of each orchard was extended about 0.35 ha, in which three year old king mandarin were planted in 2.5 m distance both between trees and lines. The total numbers of king mandarin trees in these two orchards were 386 and 378. The experiment in this section was carried out from July 2009 to June 2010. Before the study, both orchards were divided into four parts, in each of which three lines lied and 16 to 18 trees were planted on each line. Hence, these divided parts had 54 to 56 trees. Any of the following four treatments was randomly distributed to a part of each orchard: no insecticide application as control; spray of 0.40 g methidathion dissolved in 400 ml water per tree; spray of 0.20 g imidacloprid dissolved in 400 ml water per tree; spray of the mixture of 0.40 g methidathion and 0.20 g imidacloprid in 400 ml water per tree. Based on the results in the second experiment, high residue effect in the plots with imidacloprid was expected to be maintained for one month, although residue effect in the plots with only methidathion would be kept high less than two weeks. Thus, the insecticide spray was carried out once in the early of every month during the study period. In this experiment, 15 trees were selected systematically: every seven trees from the first tree were examined. For the evaluation of insecticide efficacy, psyllid adults were counted individually, and nymphs were counted in a unit of colony. Colony was defined as a group of nymphs on one new

shoot. Besides the count of the psyllid, aphids and scale insects were also counted in a colony unit as in the psyllid nymph. The efficacy of each insecticide treatment was evaluated in the numbers of these pests, compared with the control treatment. These counts were performed within three days after the insecticide application and once every week thereafter until the end of the month. The mean numbers of psyllid adults and nymph colonies, aphid and scale colonies were calculated for individual selected trees over all samplings. These numbers were used for statistical analyses to evaluate the effects of treatments on these insects.

2.3.1 Insecticide efficacy on the psyllid

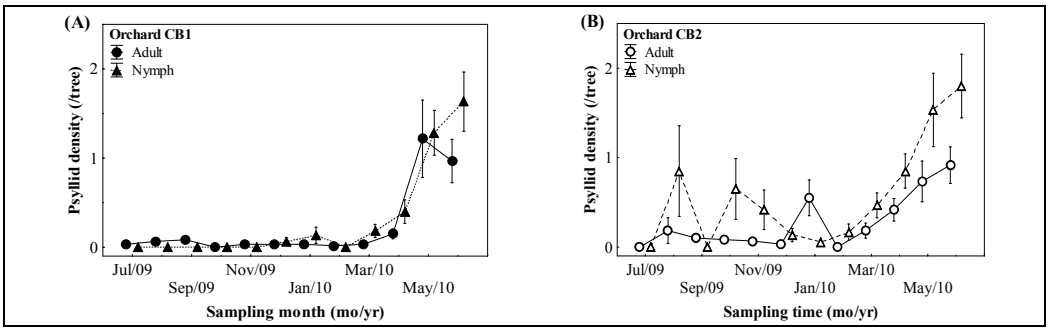

Fig. 3. Densities of psyllid adults and nymph colonies in the plot without any insecticide for the control of psyllids in two old orchards, CB1 (A) or CB2 (B). Bars indicate the standard errors of the means.

The means of adult and nymph colony counts on each tree in every month were calculated. Psyllids in the control plot without insecticide in two orchards were expected to be free from the influence of insecitide. Then, the densities of both adults and nymph colonies in these plots were compared between sampling times. Both of adults and nymphs increased in April to June 2010, but were generally much fewer in the other months (Fig. 3 A, B).

The population of psyllid adults and nymphs were compared between treatments with using the data obtained after April 2010. In these analyses, the means of these counts were calculated for every sampling time to trace the time-dependent change of the residue effect of insecticides. The effects of insecticide treatment on the psyllid was evaluated by MANOVA, in which the means of the psyllid counts were compared among orchards and treatments. The numbers were significantly different between orchards ($F_{2, 111}$ = 4.229, P = 0.017) but not between treatments ($F_{6, 222}$ = 1.680, P = 0.170). The interaction of orchard and treatment was not significant ($F_{6, 222}$ = 1.349, P = 0.237). Although there were significant differences in the numbers of psyllids between treatments, apparent differences in the numbers were hardly found in the change of the population density of the psyllid between the treatments through the above sampling periods (Fig. 4A - F). Furthermore, no distinct decreases in the psyllid numbers were seen at the first sampling time just after each insecticide spray. These results means that these insecticides by spray did not effectively reduce psyllids in the field, although the application mode and dose both followed those recommended as effective in the former experiments.

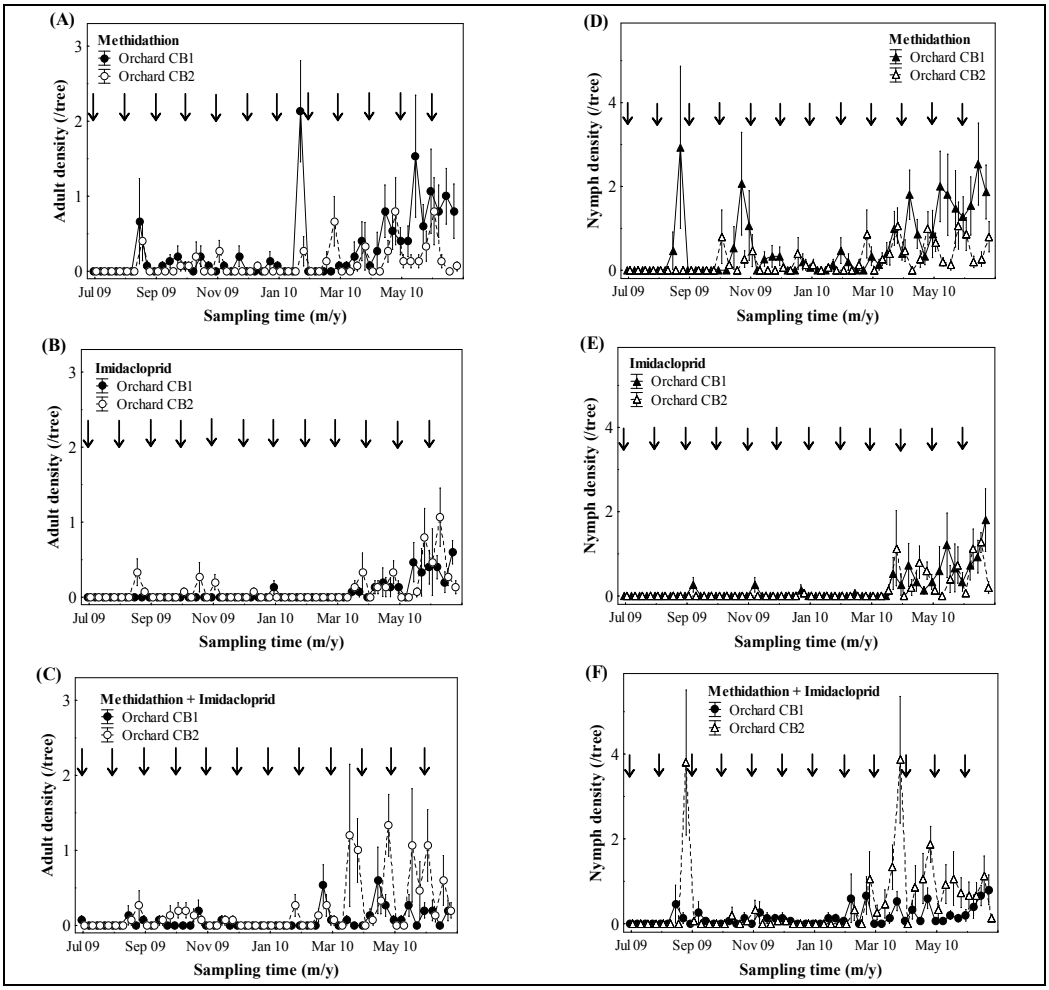

Fig. 4. Densities of adults (A-C) and nymph colonies (D-F) in two orchards in Cai Be, southern Vietnam. Each orchard was divided into four plots, and one was used as control without any insecticide and the other three plots treated by either methidathion 0.40 g/tree, imidacloprid 0.20 g/tree or the mixture of these two insecticides in two orchards. Arrows show the time when insecticides were sprayed. Bars indicate the standard errors of the means.

2.3.2 Insecticide efficacy on the aphid and scale insect

Aphids increased in November 2009 to March 2010 in both orchards, although a smaller increase was seen in July 2009 in CB2 (Fig. 5A, B). In both orchards, monthly application of insecticides reduced the aphid population, but the efficacy continued for one to two weeks only in both orchards. In particular, the aphid population seems to have been less affected by imidacloprid. On the other hand, scales insects increased until november 2009, and showed little increase thereafter (Fig. 5C, D). In CB2, scales were fewer throughout this study than in CB1. Thus, the scale population was effectively suppressed just after the application of methidathion, but the residue effect was maintained for less than two weeks. The application of imidacloprid led the scale rather to increase more than the no-insecticide treatment.

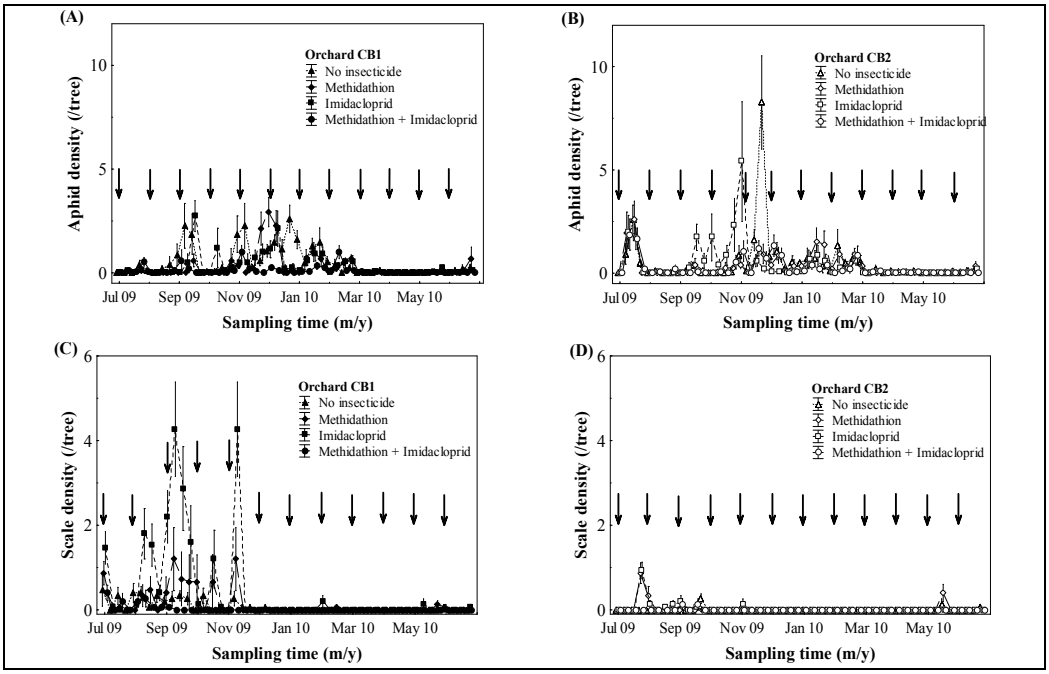

Fig. 5. Densities of aphid (A, B) and scale insect (C, D) colonies in two two-years old orchards, where seedlings had been planted either in May 2007 (CB1) or in November 2007 (CB2). Each orchard was divided into four plots, to each of which any of the four treatments were randomly distributed: no insecticide use for the control of psyllids, 0.40 g/tree methidathion by spray, 0.20 g/tree imidacloprid by spray, or 0.40 g methidathion and 0.20 g imidacloprid/tree by spray. Arrows indicate the time when insecticides were sprayed. Bars show the standard errors of the means.

Univariate ANOVA was used to evaluate the effect of treatment on the aphid and scale. The mean numbers of the aphid colony was significantly different both between orchards ($F_{1, 112}$ = 4.958, P = 0.028) and between treatments ($F_{3, 112}$ = 3.803, P = 0.012). The interaction of these two variables was significant ($F_{3, 112}$ = 2.754, P = 0.046). Similarly, the number of scales were significantly different both between orchards ($F_{1, 112}$ = 12.983, P < 0.001) and between treatments ($F_{3, 112}$ = 8.936, P < 0.001). The interaction of these two variables was significant ($F_{3, 112}$ = 3.718, P = 0.014).

These results indicate that aphids could be effectively controlled by either methidathion or imicloprid, but the efficacy would be continued for two weeks at longest. Scale insects would be controlled effectively by methidathion, but imidacloprid should be avoided for the control of the scale.

2.4 Efficacy of insecticides replaced for methidathion

Unfortunately, the sale of methidathion was discontinued in 2010 in Vietnam. According to local vendors, this was not due to any problem of methidathion per se, but farmers were likely to avoid using this insecticide due to its relatively higher prices in the market of Vietnam. Irrespective of the reason of the ban, it was urgently needed to find other insecticides that could be replaced for methidathion. Candidate insecticides should have

been those which could be easily purchased in Vietnam and would be expected to show similar efficacy as methidathion. For these conditions, dimethoate was selected, since it belongs to the same insecticide group with methidathion, "organophosphate". Two other insecticides, dinotefuran and pymetrozine, were also examined as well. For this experiment, 12 two-year old king mandarin trees were randomly selected in an orchard, located in Cai Be, about 120 km south-east to Ho Chi Minh. The mean (± SEM) of circumference of these trees just above the grafted part was 156.2 ± 22.8 mm. This experiment was consisted of four treatments, no insecticide, dimethoate, dinotefuran and pymetrozine, each of which was randomly distributed to three trees, producing three replicates for each. The doses of dimethoate, dinotefuran and pymetrozine were 0.40 g/tree, 0.20 g/tree and 0.50 g/tree, respectively. These insecticides were dissolved in water to become 400 ml in total volume, and sprayed on the whole body of the tree. The spray was executed on 11 May 2010, and there was no precipitation for longer than one month after this day. Two leaves of each tree were collected on the day just before the insecticide application, one, two, five, 10, 15, 20, 30, 45, 60, and 90 days after it. These leaves were treated as in the previous experiments for the test of insecticide efficacy. The mean of psyllid mortality on two leaves of each tree on each collection day was calculated, and the mean was used as the mortality which was achieved at the observation time on the day. The mean of the mortality on the control tree at a given time on a given day was calculated, and incorporated into the Abbott's formula to correct the mortality on insecticide-treated leaves at the same time on the same day.

2.4.1 Insecticide efficacy on the psyllid

High lethal effect of dimethoate was attained only at 72 hr after the release of psyllids only one day after spray, and decreased thereafter (Fig. 6). The psyllid mortality at 12 hr on this day was only about 60%. The results mean that dimethoate was inferior in both quickness and residue of the efficacy on the psyllid to methidathion. Hence, it could be expected that field application of dimethoate would show mortalities as high as methidathion on one day after the spray, and its effectiveness would be much lower thereafter with allowing the psyllid population to be recovered earlier. Dinotefuran showed similar effect as dimethoate until this day, but its residue effects was likely to be higher than dimethoate thereafter. The mortality of psyllids at 72 hr by dinotefuran was smaller than 80% after five days. These results in this experiment indicate that both quickness and residue effects of dinotefuran

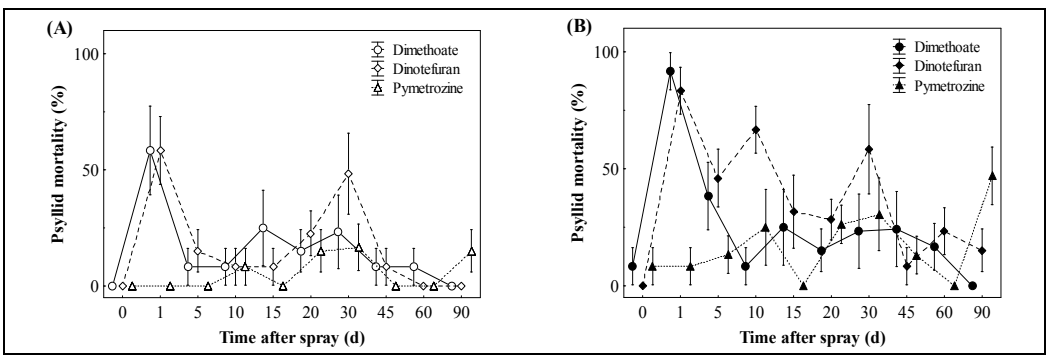

Fig. 6. Mortality of psyllids at 24 (A) and 72 (B) hours after released on leaves collected on the days after application of dimethoate, dinotefuran, or pymetrozine. The psyllid mortality was corrected by the Abbott's formula. Bars indicate the standard error of the mean.

were lower than those of imidacloprid, and thus dinotefuran should be much less effective on the control of psyllids in field than that of imidacloprid. The lethal effect of pymetrozine was always lower through this experiment, never attaining mortality over 50%. Based on the results, dimethoate and imidacloprid were examined in the following experiment.

2.5 Efficacy of dimethoate in field

The efficacy of dimethoate on the psyllid in field was examined as in the experiments of methidathion. The two orchards which had been used for the experiment of methidathion were also used for this experiment. Each of the two orchards was divided into four plots, to each of which any of the four treatments was randomly distributed: no insecticide, spray of 0.80 g dimethoate per tree, spray of 0.20 g imidacloprid, and spray of 0.80 g dimethoate and 0.20 g of imidacloprid per tree. These treatments were carried out in the early of every month from July 2010 to November 2010. The dilution and application mode of insecticides were same as in the experiment of methidathion. Psyllids, aphids, and scale insects were counted in a couple of day after the insecticide application, and once a week after then. This experiment was carried out from July 2010 to November 2010.

2.5.1 Insecticide efficacy on the psyllid

Adults and nymphs increased in July to September 2010, but few were present in the other months in both orchards (Fig. 7 A, B). Although adults were likely to be more in CB1 than in CB2 and nymphs were more in CB2 than in CB1 in the early period of this experiment, no apparent differences in these numbers were seen between the two orchards thereafter.

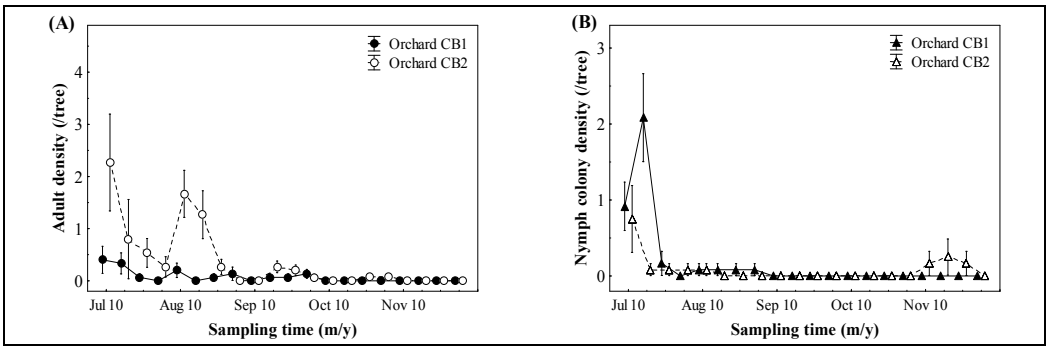

Fig. 7. Densities of adult (A) and nymph colonies (B) of the psyllid in plots without any insecticide for the control of psyllids in two old orchards, where seedlings had been planted either in May 2007 (CB1) or in November 2007 (CB2). Each orchard was divided into four plots, to each of which any of the four treatments were randomly distributed: no insecticide use for the control of psyllids, 0.40 g/tree dimethoate by spray, 0.20 g/tree imidacloprid by spray, or 0.40 g dimethoate and 0.20 g imidacloprid/tree by spray. Bars indicate the standard errors of the means.

Despite few occurrences of psyllids in the control plot after September, both adults and nymphs were counted at higher densities in plots with insecticide treatments in these months (Fig. 8). The first and second applications of dimethoate was followed by the increase of nymph colonies in both orchards, although following applications of this insecticide resulted in the decrease of both adults and nymph colonies for less than two weeks after the application (Fig. 8A, D). Adults in the plot with imidacloprid treatment

maintained low densities throughout this experiment in CB1. Distinct reduction of nymph colonies by imidacloprid was seen only in August, however, and nymph colonies rather increased in the other months (Fig. 8B, E). The application of the mixture of these two insecticides resulted in decreases of both adult and nymph psyllids throughout this experiment, except for the nymph in July in CB2.

The effects of insecticide treatment on the psyllid was evaluated by MANOVA, in which the means of the counts of adult and nymph psyllids per sampling time were culculated for individual trees and compared among orchards and treatments. The numbers were significantly different between orchards ($F_{2,\ 98}$ = 23.016, P < 0.001) but not between treatments ($F_{6,\ 196}$ = 2.058, P = 0.060).The interaction of orchard and treatment was significant ($F_{6,\ 196}$ = 3.272, P = 0.004).

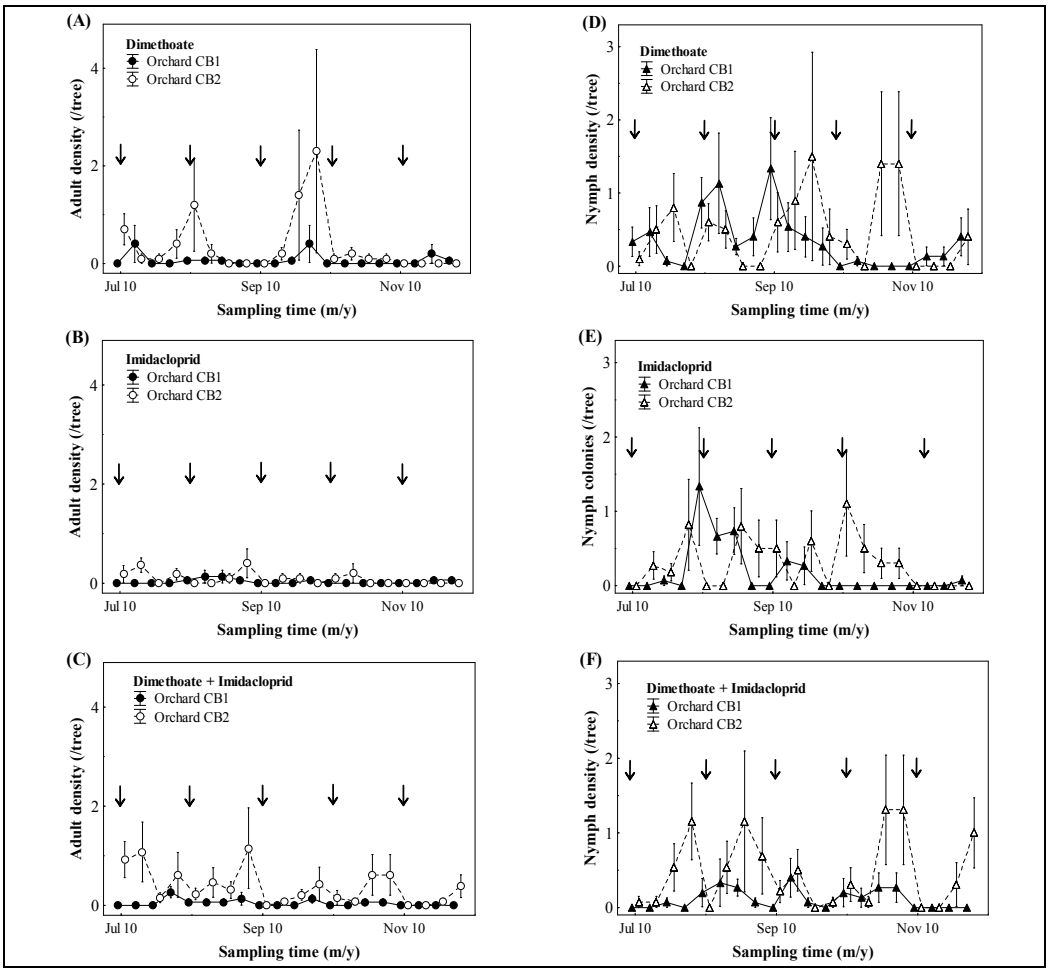

Fig. 8. Densities of adults (A-C) and nymph colonies (D-F) in plots treated by either dimethoate 0.40 g/tree, imidacloprid 0.20 g/tree or the mixture of these two insecticides in two orchards, where seedlings had been planted either in May 2007 (CB1) or in November 2007 (CB2). Arrows show the time when insecticides were sprayed. Bars indicate the standard errors of the means.

Any insecticide did not show long residue effects in this experiment. Furthermore, psyllids were increased in plots with insecticide treatments when few or no psyllids were found in the control plot. In particular, psyllids were likely to be more in insecticide-treated plots after September 2010 than in the control plot, even though psyllids were reduced just after the insecticide application. The interative factors in the increase in these plots are still unknown. One possible explanation seems to be predators of the psyllid that had been eliminated by insecticide application and their populations could not be recovered soon. Other interesting results were the difference in the the efficacy of imidacloprid on adults and on nymph colonies. Adults were well controlled by imidaclprid, but nymphs appreared to be free of the insecticide effects. However, adults in the plot with the treatment of the mixture of two insecticides were as many as those in the plot with only dimethoate. In the experiment of methidation application in field, imidaclprid did not show such a control effect on either the adult or the nymph. The few occurrences of adults in this experiment seem to have been involved in other confounding factors. Furthermore, adult psyllids were likely to be more in CB2 than in CB1, although no apparent differences in the nymph numbers were seen. Thus, any insecticide effectively reduced psyllid populations just after the application, but the residue effect could not continue for longer than two weeks.

2.5.2 Insecticide efficacy on the aphid and scale insect

In the control plot, the increase of aphids was not necessarily correspondent between two orchards (Fig. 9A). In each orchard, only one increase was observed: in September in CB1 and in July in CB2. On the other hand, scale insects increased similarly in these two orchards: one increase in August and the other in September to October (Fig. 9B). These densities were compared with those in plots with insecticide treatments. Generally, aphid populations after insecticide application were reduced in both orchards, but increased in one to two weeks later (Fig. 10A, B). In October to November, their populations were recovered in the plots with dimethoate or imidacloprid treatment in two weeks after the application. Although scale insects were found more in CB2 than in CB1, poulation densities of the scale insect were likely to be higher in the plots with the treatment by only imidacloprid in both orchards (Fig. 10C, D). Application of dimethoate effectively suppressed the scale population, but imidacloprid led the scale rather to increase more than the no-insecticide treatment. The population of scale insects were particularly higher in CB2

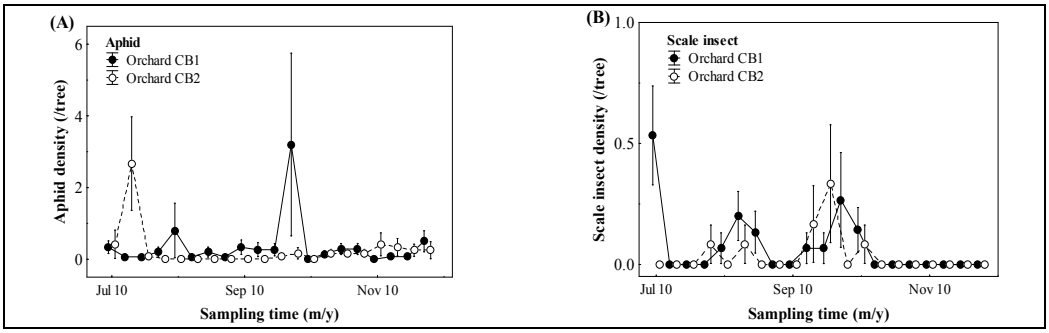

Fig. 9. Densities of aphid (A) and scale insect colonies (B) in plots without insecticide treatment for the control of psyllids in two orchards, where seedlings had been planted either in May 2007 (CB1) or in November 2007 (CB2). Bars indicate the standard errors of the means.

during the late August to early October than in the control plots. In the plots with the mixture of both insecticide, populations were maintained lower than the others.

Univariate ANOVA was used to compare the effects of insecticide treatments on the aphid or scale insect population. The aphid population density was significantly different between treatments ($F_{3, 99}$ = 4.078, P = 0.009) but not between orchards ($F_{1, 99}$ = 0.253, P > 0.05). The interaction of these two variables was not significant ($F_{3, 99}$ = 0.319, P > 0.05). The population density of scale insects was significantly different both between orchards ($F_{1, 99}$ = 6.206, P = 0.014) and between treatments ($F_{3, 99}$ = 3.443, P = 0.020). The interaction of orchard and treatment was not significant ($F_{3, 99}$ = 0.506, P > 0.05). These results indicate that aphids could be effectively controlled by either dimethoate or imidacloprid but the efficacy would be continued for two weeks at longest. Imidacloprid would more effectively control aphids than dimethoate. Scale insects would be controlled effectively by dimethoate, but imidacloprid should be avoided for the control of the scale. Hence, when aphids are dominant and scales insects were few, either dimethoate or imidacloprid could be used. However, when scales were abundant, imidaclprid should be avoided even for the control of aphids, and dimethoate should be selected. If both pests are abundant, the mixture of these insecticides would be considered. It should be noted that the residue effects in any insecticide application would continue for two weeks at longest.

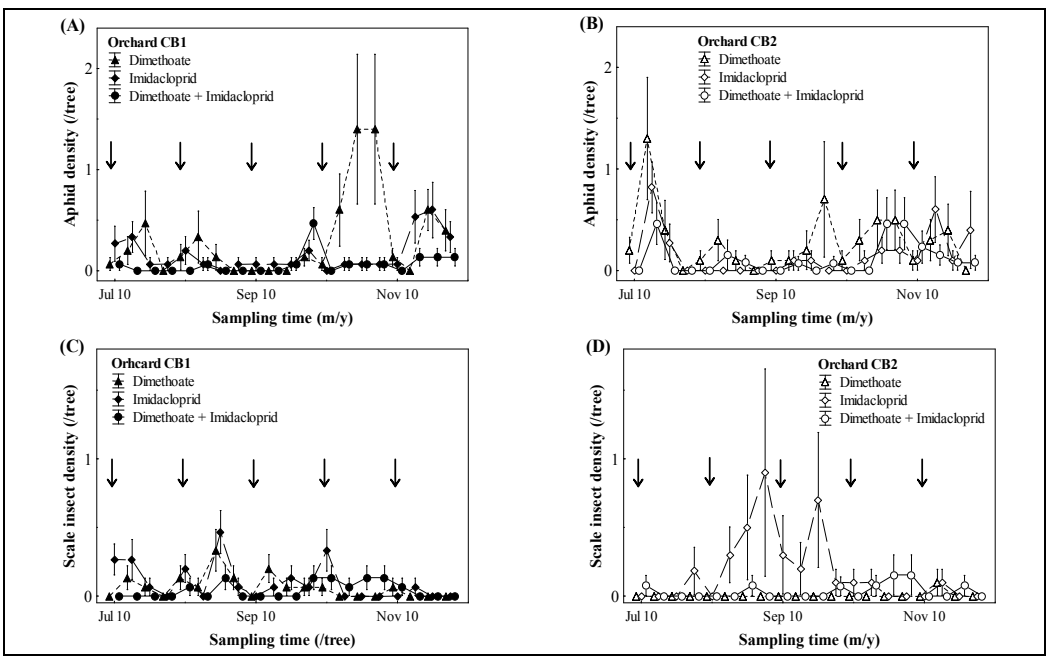

Fig. 10. Densities of aphid (A, B) and scale insect (C, D) colonies in two orchards, where seedlings had been planted either in May 2007 (CB1) or in November 2007 (CB2). Each orchard was divided into four plots, to each of which any of the four treatments were randomly distributed: no insecticide use for the control of psyllids, 0.40 g/tree dimethoate by spray, 0.20 g/tree imidacloprid by spray, or 0.40 g dimethoate and 0.20 g imidacloprid/tree by spray. Arrows indicate the time when insecticides were sprayed. Bars show the standard errors of the means.

3. Conclusion

When trees are young, usually until one and a half years after planting in southern Vietnam, neonicotinoids by soil drench are expected to control psyllids effectively for two months after the application. Methidathion by spray can keep high residue effects for a half month for the control of psyllids on seedlings. This insecticide can quickly kill psyllids in 12 hr after their infestation on plants, and neonicotinoids did not attain such quick effect on the psyllid. This is the advantage in the use of methidathion. Dimethoate showed similar lethal effects on the psyllids as methidathion, although both its quickness and residue effects were inferior to those of methidathion. Field application of imidacloprid, methidathion, dimethoate, and the mixture of imidacloprid with any of the two organophosphates showed that these insecticides showed similar effects on the control of psyllids: their residue effect was maintained for less than two weeks and no insecticides succeeded in attained high lethal effect to eradicate psyllids on trees even in a couple of days after the spray. It should be noted that dimethoate could not control aphids as much as imidacloprid or its mixture with dimethoate but imidacloprid would lead the increase of scale insects after the application. The application of any insecticide examined in this study did not lead these pests to be eradicated for even one week after the application. These results indicate that the application of insecticides cannot be expected to attain perfect protection of citrus trees from CG infection once the tree grew. Furthermore, since nymphs could increase in two weeks after the application without elimination, even secondary infection would not be avoided when citrus trees have grown to a stage of fruit yielding. The application of insecticide would only reduce more or less the probability of the second infection of trees by CG in the orchard.

4. Acknowledgment

This study was performed under the collaborative research project, no. 3241, of Japan International Research Centre for Agricultural Sciences (JIRCAS) with the Southern Horticultural Research Institute of Vietnam (SOFRI). Prof. Emeritus Su, H.-J. at the Taiwan University provided us with precious suggestions in the study. Dr. Chau, N. M. supported our studies both officially and scientifically. Dr. Koizumi, M. and Dr. Kano, T. suggested us about works on citrus both in Japan and other countries. Dr. Yonemoto, M. and Mr. Ogata, T. at JIRCAS provided information necessary for citrus cultivation. Dr. Hoa, N. V. and Mr. Dien, L. Q. gave us information of citrus in Vietnam. Miss Nga, V. T. and Miss Oanh, T.T.K. helped our works both in laboratory and field. We would like to express our sincere thanks to them.

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4

Use of Pesticides in the Cocoa Industry and Their Impact on the Environment and the Food Chain

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1. Introduction

Cocoa, *Theobroma cacao L.*, is a major cash crop cultivated in the tropical regions of West Africa, the Caribbean, South America and Asia. In West Africa, where over 70% of the world's cocoa is produced – with about 21% coming from Ghana - it is a significant component of the rural economy, as the industry is dominated by large numbers of small-holder peasant farmers who depend on the crop for their livelihood (Acquaah, 1999; Appiah, 2004). Like all living organisms, the cocoa plant can also be attacked by a wide range of pests and diseases. When this happens expected production targets are not met, and the economies of the producer nations are adversely affected. Preventive and curative measures are therefore necessary in the cocoa industry to maintain and even increase output (Akrofi and Baah, 2007).

While non-chemical means of managing pests and diseases in the industry are widely recommended for health and other reasons, the use of some amounts of chemicals in the form of fertilizers, insecticides and fungicides is unavoidable in the effective management of cocoa farms (Moy and Wessel, 2000; Opoku et al., 2007; Adjinah and Opoku, 2010). Their use is therefore expected to increase with time. Indeed in the twenty-year period from 1986-2006, the use of fertilizer world-wide increased by almost 250% (UNEP, 1991). The same trend applies to pesticides, although they are more difficult to monitor partly because of the secrecy that goes with the continued production and use of banned substances. The trends suggest quite clearly however, that much of the increase in world food production can be attributed to the response of crops to increased use of fertilizers and pesticides (UNEP, 1991). Fortunately, there has always been a clear appreciation of the potential deleterious effects of the chemicals used in the cocoa industry since the 60s, and standards have been set by FAO and WHO for acceptable levels of residues in the beans exported to other countries.

The goal of maintaining high levels of agricultural productivity and profitability while reducing pesticides use presents a significant challenge. There are repeated cases of excessive levels of pesticide residues being found in agricultural produce and the safety of these products has become an issue of concern. Recently, changes in regulations in the European Union (EU), North America and Japan have called for a reflection on crop protection practices in cocoa and other commodity crops (ICCO, 2007). The quality of cocoa

imported into the EU and elsewhere will be assessed based on traces of pesticides and other substances that have been used in the supply chain.
The cocoa bean has a high content of butter or fat which absorbs the active ingredients in insecticides. The acceptable levels of active ingredients in foods are determined by the committee on Pesticide Residue of FAO/WHO, known as the Codex Alimentarius Commission, CAC. Created in 1963 the CAC implements the Joint FAO/WHO Food Standards Programme which is aimed at protecting the health of consumers and ensuring fair trade practices in the international food trade (Moy and Wessel, 2000). The commission has set maximum levels of residue poisons in commodities going through the international market, including cocoa. If for any reason the residual levels in any commodity exceed the Codex levels, that particular commodity could be rejected by the importing country. Secondly, the accumulation of any chemicals in the cocoa fat may change the taste of the beans and eventually that of the chocolate made from them. This is known as tainting. It is therefore, the task of entomologists to ensure that recommended chemicals do not leave any residues, and that the dosage is the minimum that would give the optimum control under the agricultural conditions in the country.
In Ghana, significant gains have been made in the control of pests and diseases of the cocoa industry through the nationwide use of pesticides under government sponsorship and supervision. The growing global concerns about the effects of the increasing use of agricultural chemicals on farmers, consumers of agricultural produce and the ecology require a re-examination of the issues related to their application in the cocoa industry. This chapter examines the use and the impact of pesticides in cocoa production in Ghana - where data has been accumulated - as a representative country of the industry. The potential ecological impacts of chemicals in the cocoa industry are analyzed, using the modern tool of life-cycle assessment (Ntiamoah and Afrane, 2009). Life-cycle assessment, LCA, has gained such prominence in the environmental management discipline that the International Standards Organization has developed standards for its implementation (ISO 14040-14043, 1997-2000b). This particular analysis is based on primary farm-level data collected from a nationally representative sample of cocoa farmers, published data, results from research institutions, the Ghana Cocoa Board and other relevant sources.

2. The Ghanaian cocoa industry in brief

In Ghana, cocoa has played an important role in the economy of the country for over one century. Although the crop was believed to have been brought to the colonial Gold Coast - as Ghana was then known - from Fernando Po, an island in the Gulf of Guinea, off the coast of Gabon, in 1879 and from Sao Tome in 1886, records show that in 1891, only twelve years after it first arrived here, cocoa was being exported as a cash crop (Acquaah, 1999, Adjinah and Opoku, 2010). From the 1910/1911 season, Ghana became the leading cocoa producer in the world, a position it held until 1977, when it was overtaken by the Ivory Coast. The country went from being the number one cocoa producer to a period in the early 80s when, as a result of drought, bushfires, low producer prices, diseases and general economic malaise, Ghana fell to the twelfth position and produced less than 160,000 metric tonnes in the 1983/1984 season (Adjinah and Opoku, 2010).
Cocoa became attractive as a cash crop in Ghana because of the lower cost involved in its cultivation, compared to a popular crop like palm, as well as the favourable natural conditions that existed in the forest belts. Cocoa could be grown along with other crops and

when soil conditions deteriorated the land could be left to the cocoa trees and other tracts tilled in the shifting-cultivation systems of farming (Acquaah, 1999). Because of the prominence that the crop had began to gain in the economy, even before World War II, government was seriously alarmed when the swollen shoot disease was discovered in 1936. In the process of combating this disease, a permanent research center was established at Tafo, in the Eastern Region, and product quality inspectorate, grading of beans, extension services and proper engagement of farmers in the growth of the crop were initiated (Acquaah, 1999). Since then government has continued to offer technical assistance, financial incentives and inputs like fertilizer and pesticides to cocoa farmers.

Over the last decade, as a result of government intervention, cocoa production has picked up, reaching a peak of 740 thousand metric tonnes in the 2005/2006 season (Aryeetey *et al*, 2007). Constituting 7.3% of the Gross Domestic Product of the country, it is second only to gold, which first overtook cocoa as the highest foreign exchange earner in 1992; a trend which still continues. Agriculture contributes about 35% of Ghana's Gross Domestic Product (GDP) and 60% of total employment. The Cocoa Industry is the single largest contributor to agricultural GDP (16.5 %). It is estimated that about 65% of the country's agricultural workforce work either directly or indirectly in the cocoa industry. In Ghana cocoa is grown on small farms owned by individuals and families in the forest zones of Ashanti, Brong Ahafo, Western, Eastern and Volta regions. Thus the livelihood of about two million farmers and their dependants, mostly in the rural areas, depend directly on cocoa (Opoku *et. al,* 2006).

2.1 Cocoa processing in Ghana

Although serious attempts have been made to process them locally, the majority of cocoa beans produced in the country are still exported. Government put a policy in place to process at least 50% by the end of the last decade. The enabling conditions created in free zone enclaves, led to the attraction of private foreign processing companies and the expansion of state-owned facilities. According to data from International Cocoa Organization, ICCO, 200,000 metric tonnes of cocoa grindings were achieved in Ghana in the 2009/2010 season. Compared to the production figure given in Table 1 for the same season, this constitutes about 32% of the beans produced. This means the government's target for grindings was not achieved.

In spite of their peripheral role in the standard household menu - mainly as a dessert or snack, food products made from cocoa go through a long line of operations not normally found with other processed foods, as depicted in Figure 1 (Awua, 2002). Ripe cocoa pods are plucked from the trees and gathered together on clearings in the cocoa farms. After about ten days, all available hands, young and old, gather together to assist in the splitting of the pods and removal of the beans with their hands. (According to Owusu-Manu (1977), this could be a critical stage in the contamination process, with pesticides getting transferred from the workers to the wet beans.) The wet beans are collected together in a heap and covered with plantain leaves and plastic sheets for fermentation. After fermentation, the beans are dried in the sun on bamboo mats to a desired moisture content of around 7.5%.

After dried cocoa beans have been received at the processing plant, they are inspected and thoroughly cleaned of all extraneous matter, such as sticks, stones, metal fragments, dust, loose shells, small fragments and clumps of cocoa beans. The cleaning process consists of a series of operations involving sieves, brushes, airlifts and magnetic separators to remove the

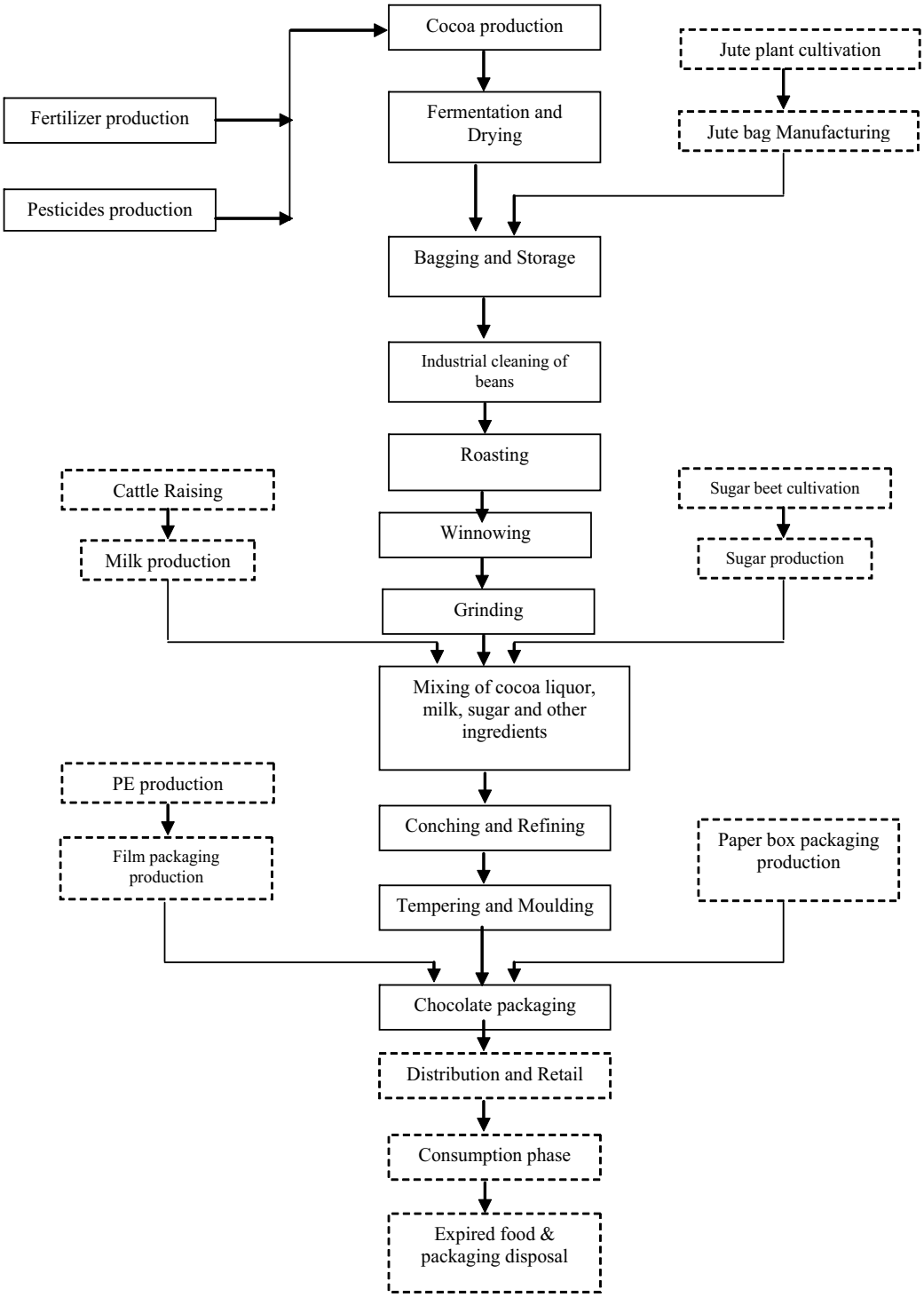

Fig. 1. Process Flowchart for Chocolate Production

unwanted materials. The cleansed cocoa beans are roasted at temperatures between 90-170^{o}C, using a petroleum-based fuel or electricity. This process is needed to develop the chocolate flavour, reduce the moisture content further, and loosen the shells for subsequent removal. The nibs (cotyledons) become friable and generally darker in colour in the process. At the next stage, the shells are separated from the nibs in a process known as winnowing. Winnowing machines use a multi-layered sieve frame with meshes of different sizes, one above the other, with the largest mesh on top. The roasted and crushed beans are ground into a paste known as cocoa liquor or cocoa masse. The grinding process is achieved in two or three stages, using a combination of mills. The cocoa liquor obtained is heat-treated in storage tanks at temperatures of between 90-100^{o}C for aging and microbial destruction.
The cocoa paste could be pressed in a hydraulic device to extract cocoa butter. The cake released after pressing is passed through kibbling machines, which break them into smaller pieces, and are packed into four-ply multi-walled paper sacks lined with polyethylene. These are ready for sale and shipment as kibbled cake. The cocoa butter, on the other hand, may be mixed with the other ingredients of chocolate, namely, butter, sugar, milk and emulsifiers. The chocolate mix is subjected to additional processes known as conching and tempering. Conching removes residual moisture, while tempering transforms the thick semi-liquid mix into a solid product through heat treatment. After this process the chocolate is poured into moulds of different shapes and then packaged for the market. Knowledge of the material and energy requirements of each of the processes as shown in figure 1 is necessary to perform the LCA analysis needed to determine the environments impacts.

2.2 The place of cocoa in the food chain

While the soporific effect of cocoa drinks is widely known, recent research activities have unearthed additional more important health benefits which have enhanced further the attractiveness of cocoa products generally. There are three types of chocolate: dark, milk and white chocolates. Most of the benefits of chocolate consumption are associated with the dark brand. In the last decade, studies have shown that chocolate consumption can play an important role in the reduction of risks or delaying the development of cardiovascular diseases, cancer and other age-related diseases. It has also been linked positively to anti-carcinogenic activity in human cells, hypertension, diabetes and sexual weakness. It's newly found reputation as an aphrodisiac, stems from the ability of its sweet and fatty nature to simulate the hypothalamus, which induces pleasure sensation and affects the level of serotonin in the brain (Afoakwa, 2008).
Cocoa products contain flavonoids and amino acids, and these have been cited as the source of its beneficial effects, while carbohydrates, theobromine and lead have been mentioned as responsible for the negative effects. The flavonoids belong to a large and complex group of compounds called polyphenols and are found in plant products, mainly fruits and vegetables. The phenols in cocoa products have been associated with antioxidant properties, reduction in migraine, protection of arteries from plaque formation and prevention of LDL formation two hours after consuming dark chocolate and perceptible lowering of blood pressure. Some studies have also linked chocolate consumption to muscle recovery and delayed brain function decline (Reuters, 2007). Protein is broken down in the body to form twenty amino acids needed by the body. Eight of these are called essential, which means they are not made by the human body itself and must be supplied from outside. Fourteen of the twenty amino acids found in the body, including the eight essential ones, have been

found in cocoa. In addition to building cells and repairing tissues, amino acids also have antioxidant properties, and they form antibodies to combat invading bacteria and viruses (Awuah, 2002).
While international standards are such that the pesticides used in the field can hardly find their way into chocolate, a number of documented negative effects have been associated with some of the natural and absorbed constituents of cocoa. Perhaps the major one is obesity. It is believed that the amounts of dark chocolate that needs to be consumed in order to experience the good benefits of the product could lead to obesity and its resultant negative effects. Although it is not supported by scientific studies, it is also believed that chocolate consumption can lead to acne (*www.chocolate.gourmetrecipe.com*). The heavy metal, lead, is known to maintain a high solubility in chocolate, and this may lead to lead poisoning (Rankin *et al*, 2005). Chocolate is also known to be toxic to some animals like horses, dogs, parrots, cats and small rodents, because they are unable to metabolize the theobromine which is found in chocolate (Drolet *et al*, 1984; Blakemore and Shearer, 1943).

3. Pests and diseases of cocoa

The increasing world population cannot be sustained without the use of pesticides in food production. Their usage therefore benefits not only farmers but also consumers. Pesticides are used to reduce food losses not only during production, but also during the post-harvest storage stage (Moy and Wessel, 2000). The general pest control strategy is for the intervention to destroy the pests feasting on the crops, but at the same time not to damage the produce so much as to render them unhealthy or unprofitable. This means looking for the thin line which separates good practices from bad. Good agricultural practice (GAP) requires good timing and proper application. The crops are sprayed on the advice of specialists at an opportune time in the reproductive cycle of the pest, when the highest numbers could be eliminated. Also in order to maintain the activities of friendly insects the area of application of the insecticides should be clearly delineated.
The cocoa tree and its pod can be attacked by different species of insects, fungal diseases and rodents (Entwistle, 1972). The major diseases affecting cocoa in Ghana are given in Table 1. The most important of these are *Phytophthora* pod rot, commonly called "black pod", and locally known as '*akate*'; and the swollen shoot virus, also known locally as '*cocoa sasabro*'. The black pod rot, a fungal disease which appears as characteristic brown necrotic lesions on the pod's surface and as rotting of the beans, does the most damage to cocoa. An estimated 30% of annual cocoa production is lost to it, especially during years of high rainfall. At 2005 cocoa bean prices this is an estimated US$1.5 billion in lost revenue (www.icco.org). Other estimates put the loss specifically at 450 thousand metric tonnes annually, while 250, 200 and 50 thousand MT are lost to witches' broom, capsids, and the swollen shoot virus (CSSV), respectively (*www.dropdata.org*). Witches' broom and frosty pod rot are predominant in Latin America, while the black pod and CSSV are common in West Africa. These diseases are counted by breeding disease-resistance species, sanitation and the use of fungicides (Bastos, 1996; Opoku *et al*, 2007).
Most insects which attack cocoa are of the bug or *miridiae* family. This is a large family of insects of which capsids, the most well-known, have achieved their notoriety from the degree of havoc they can wreck on cash crops like cocoa. They feed on plants by piercing the tissue and sucking their juices. Capsids are small, terrestrial insects, usually oval-shaped or elongate and measuring less than 12 mm. They were identified as pests at the turn of the last century and are the main insects that feed on cocoa in Africa (Mahot et al., 2005).

Disease	Type of Infection (Causal agent)	Symptoms
Black pod	Fungus (*Phytophthora spp.*)	Pod rots, go brownish-black. Beans destroyed in immature pods. Could result in die-back
Brown root rot	Fungus (*Fomes noxius*)	Leaves fall prematurely and die-back of twigs occurs. Fungus fruit bodies on root and dead trunks. Soil is affected
Cocoa necrosis	Virus (Cocoa necrosis virus)	Leaves show bands of transparent lesions often with perforated centers
Collar crack	Fungus (*Armillaria mellea*)	Longitudinal cracking of trunk from ground level to about 1.2m upwards, fills with cream-coloured mycelium
Collar rot	Fungus (*Ustulina zonata*)	Defoliation and death of plants. White fan-shaped patches of mycelium are produced underneath bark and roots
Cushion gall	Fungus (*Calonectria rigidiuscula*)	Excessive production of buds at the nodes
Vascular Streak Die-back	Fungus (*Oncobasidium theobroma*)	Leaves turn yellow and fall prematurely. Smaller branches wither starting from the tips
Horse hair blight	Fungus *(Marasmius equicrinis)*	Network of black threads which spread throughout the canopy, smothers shoots growth
Mealy pod	Fungus (*Trachysphaera fructigena*)	Pods turn brown, becomes encrusted with white to pinkish mealy growth of the fungus
Mistletoe	Flowering Plant (*Tapinanthus bangwensis*)	Parasitic flowering plant on host branches. Part of branch withers
Pod rot	Fungus (*Botryodiphlodia theobromae*)	Appears as brown necrotic areas with concentric rings of black spots. Pods are later covered with black sooty powder
Red rust	Alga (*Cephaleuros mycoidea*)	Reddish patches on leaves and twigs; leaves are shed prematurely
Swollen shoot	Virus (Cocoa swollen shoot virus)	Swelling of chupons and twigs; leaves develop yellow patterns, get crinkled and malformed
White Root	Fungus (*Fomes lignosus*)	Premature defoliation, death of twigs, pods are small
White thread Blight	Fungus (*Marasmius scandens*)	Leaves are covered and killed in a network of white mycelial threads

Source: Offei *et al*. (2005)

Table 1. Diseases of Cocoa in Ghana

3.1 National cocoa pests and diseases control programme

Throughout the 90's, the tonnage of cocoa produced annually rarely exceeded 400,000 metric tonnes. This situation was attributed to a variety of causes, although the prevalence of pests and cocoa diseases was seen as the main reason. Crop losses due to mirids alone

were estimated at between 25-35% per annum. To reverse this trend, the government of Ghana in the year 2000 introduced the national Cocoa Diseases and Pests Control Programme, CODAPEC, popularly known as "mass spraying", to combat the resurgence of mirids and black pod diseases on cocoa farms. This opportunity was also to be used to train farmers and technical personnel in the scientific methods of pests and diseases control (Adjinah and Opoku, 2010). Participants were trained in the dosage of the various pesticides, dangers of exposure to pesticides, importance of the use of protective clothing, observance of personal hygiene, environmental safety issues, first-aid, techniques of application and handling and disposal of empty containers. Lessons were given through radio programmes, town meetings and 'training-of-trainers' workshops. Table 2 gives the brands of pesticides, approved by the Cocoa Research Institute of Ghana (CRIG), which are currently in use on Ghanaian cocoa farms under the CODAPEC programme and their application frequency.

Pesticide used	Active ingredient	Method of application	Frequency
Fungicides			
Ridomil 72 plus WP	12% metalaxyl, 60% Cuprous oxide	Knapsack sprayer	3 times during each cocoa season
Nordox 75 WP	86% Cuprous oxide, 14% inert		
Funguran OH WP	Cuprous hydroxide		
Champion WP	77% cupric hydroxide		
Kocide 101 WP	Cupric hydroxide		
Fungikill WP	Cupric hydroxide + metalaxyl		
Metalm 72 Plus WP	Cuprous oxide + metalaxyl		
Insecticides			
Akatemaster	Bifenthrin	Knapsack sprayer	Twice during each cocoa season
Actara	Thiamethoxam		
Cocostar 210 EC	Bifenthrin + Pirimiphos-methyl		
Confidor 200SL	Imidacloprid		
Carbamult	Promecarb		

Table 2. Pesticides approved for used in the control of mirids and black pod disease under the CODAPEC programme

The black pod control programme covered all cocoa-growing districts in the Volta, Brong Ahafo and parts of Western, Ashanti and Eastern Regions. Spraying against mirids, on the other hand, covered the Central, Eastern and parts of Western and Ashanti Regions. Spraying gangs were established at each spraying centre. A gang of ten (for black pod control) and six (for mirids control) had a supervisor each responsible for the general execution of the programme at the unit level. One mechanic was attached to a group of 20 gangs to oversee the maintenance and repairs of the spraying machines. The farmers, who

were direct beneficiaries of the exercise, were themselves responsible for the sanitation practices, i.e. brushing, pruning, shade management and removal of diseased pods from the farms. They also provided water for spraying and were expected to monitor the activities of the sprayers on the farm. The spraying is carefully done using a portable petrol-engine-driven knapsack mist-blowers, which combines the idea of low-volume application of sprays with the principle of using fan-driven air to carry the spray up into the trees.

As a result of this initiative, between the period 2002-2004, nearly 600,000 ha involving about 360,942 farms and 330,121 individual farmers, were sprayed three times each season against the black pod diseases, while an estimated 826,141 ha involving 470,801 and 446,593 farmers were sprayed twice each season in the mirids control exercise. From the 2001/2002 season when beans output of 380,000 metric tons was recorded, production jumped to about 500,000 metric tonnes in the 2002/2003 season and almost doubled in the 2003/2004 season to an all-time high of over 736,000 metric tonnes.

Started ten years ago, the mass spraying exercise has now become a permanent fixture in all the 72 geographical districts in the cocoa-growing areas with the following breakdown: 21districts for black pod spraying only, 35 districts for mirids only, and 16 for both programmes. District Task Forces (DTF) and Local Task Forces (LTF), have been formed in each operational district and local area, respectively. The DTF manages the project at the district level and is in charge of gang recruitment, storage, distribution of inputs and logistics and general supervision. The LTF on the other hand, handles project management at the village level and is responsible for the planning and execution of the programmes at that level.

Table 3 gives the seasonal cocoa production figures along with the amounts of fertilizers and pesticides which have been used in Ghana in recent years. The table indicates clearly that cocoa production has increased significantly in the last decade, but it has been at the expense of more pesticides and fertilizers. Data obtained from COCOBOD indicate that fourteen different kinds of insecticides and fungicides have been used for spraying farms since the start of the mass spraying exercise. Even with the limited data provided, the increase in pesticide usage per unit weight of cocoa over the period is evident. The same trend applies to fertilizer usage. Serious attention must be paid to these trends beyond the normal concerns with maximum residue limits (MRLs) which international traders focus on. The impact of these prodigious amounts of chemicals used in cocoa production on the environment as a whole can be determined through life-cycle analyses (Ntiamoah and Afrane, 2009).

Crop Season	Cocoa Production (10^6 kg)	Total[a] Fertilizer Used (10^6 kg)	Pesticides Usage[a]		Fertilizer used per MT	Fungicides used per MT
			Insecticides (liters)	Fungicides (MT)		
2004/05	601.9	-	1023.6	1120.0	-	1.86
2005/06	740.4	-	745.0	759.4	-	1.03
2006/07	614.5	70.1	590.0	1120.0	0.11	1.83
2007/08	729.0†	55.8	1020.0	1290.0	0.08	1.77
2008/09	662.0†	105.0	1760.0	1800.0	0.16	2.72
2009/10	632.0†	130.0	2300.0	1997.7	0.20	3.16

†Source: ICCO Quarterly Bulletin of Cocoa Statistics, Vol. XXXVI, No. 4, Cocoa year 2009/2010. Published: 30-11-2010; all others in this column from, The State of the Ghanaian Economy, 2007. [a] Source: COCOBOD, Ghana.

Table 3. Seasonal Cocoa Production, Fertilizer and Insecticide Usage in Ghana, 2004-2010

While non-chemical means of managing cocoa pests and diseases are widely recommended, the need for agro-chemicals to manage cocoa pests and diseases is unavoidable and will continue for years to come. However, the effects of continued exposure of users of pesticides, environmental risks, issues of pest resistance and possible hazards for consumers require a re-examination of the benefits of pesticide application and the risks involved. Hence the introduction of Good Agricultural Practices (GAP) to considerably mitigate, if not eliminate, the problems associated with the excessive and unnecessary application of pesticides. High residue levels and tainting of the beans could lead to their rejection on the international market. Testing for residues is carried out following internationally agreed and validated methods (Moy and Wessel, 2000). Though some insecticide residues are sometimes found in the shells, they are hardly found in the nib which is used in chocolate manufacture.

4. Socio-economic impacts of pesticides use on the cocoa industry

In terms of output the CODAPEC programme was a tremendous success, because it was able to resuscitate cocoa production in Ghana. The country continues to benefit not only because of increased output, but also because of the high prices the crop is currently enjoying on the international market. Thus the benefits to the economy as a whole were obvious. What was not so obvious was the direct benefit to the cocoa farmers.

In order to assess the impact of the programme on these farmers, Abankwa *et. al* (2010), conducted a study in a typical cocoa-growing district, Ahafo Ano South, located at the north-western section of the Ashanti Region of Ghana. The study found that while the farmers could not take their children to better basic schools, they were able to afford school uniforms and other basic educational needs for them. They also found that farmers were able and more willing to visit hospitals instead of self-medicating or using herbal treatment. The improvements brought about by the programme seemed to benefit more farmers with higher levels of education, the study showed.

One poignant conclusion of the study was that, while the price of cocoa was reviewed upwards every year over the first five years of the programme, these increments did not translate into increased purchasing power of farmers. They were not able to afford assets like radios, televisions, mattresses and vehicles any better, five years after the programme was started. Table 4 gives the nominal and actual farmers' income over the period 2001-2005. While the nominal figures trend upwards annually as a result of the increases in cocoa price, the actual income (calculated using CPI of 1997 as base) goes down every year due to the effect of inflation.

Year	Consumer Price Index	Nominal Income	Actual Income
2001	216.4	631.5	294.6
2002	246.2	679.9	276.2
2003	311.8	753.3	241.6
2004	351.2	805.5	229.4
2005	404.3	939.3	232.3

Table 4. Variations in Farmers' Income, 2001-2005 (Source: Abankwa *et al.* 2010)

From the point of view of COCOBOD, the implementers of the programe, the mass spraying exercise has been a roaring success, because of the increased yield it has generated, the renewed enthusiasm for cocoa cultivation that it has awoken in farmers, and also because of the 60,000 direct jobs it has created for sprayers, supervisors and mechanics in the rural areas of the country (Adjinah and Opoku, 2010). According to the Seed Production Unit of COCOBOD, demand for planting materials has gone up significantly because new farms are been established and old ones rejuvenated (Adjinah and Opoku, 2010). Farmers now clearly see cocoa farming as a profitable venture, especially with the continued reduction in inflation and the general improvement in the economy.

5. Potential ecological impacts of pesticides use in cocoa production

A proper assessment of the effect of pesticides and other chemicals used during cocoa production and processing on the environment and human health, has to begin with an effective quantification of the chemicals released into the environment and their impact on various aspects of human life and the environment. For this purpose, one of the widely accepted modern methods for examining the environmental impacts associated with a service or a product is the life cycle assessment (LCA) technique.

5.1 Life cycle assessment methodology

The Society of Environmental Toxicology and Chemistry (SETAC), defines LCA as:
"an objective process to evaluate the environmental burdens associated with a product, process or activity by identifying and quantifying energy and materials used and wastes released to the environment; to assess the impact of those energy and material uses and releases to the environment; and to identify and evaluate opportunities to effect environmental improvements. The assessment includes the entire life cycle of a product, process or activity, encompassing extracting and processing raw materials; manufacturing, transportation and distribution; use, re-use, maintenance; recycling, and disposal" (Consoli *et al*, 1993).
The International Organization for Standardization (ISO) has also provided very relevant input to the definition of LCA. According to ISO 14040 (1997), LCA is
"a compilation and evaluation of the inputs, outputs and the potential environmental impacts of a product system throughout its life cycle. A product system is a collection of materially or energetically connected unit processes, which performs one or more defined functions".

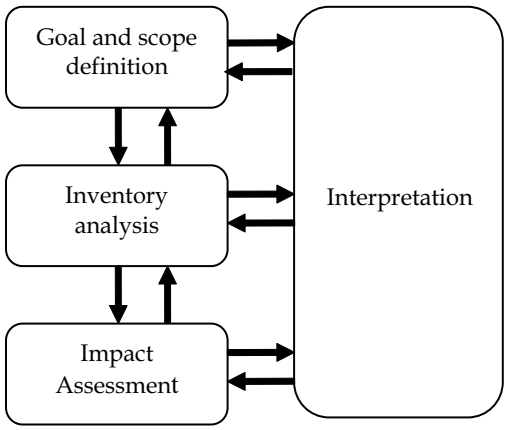

Fig. 2. Components of a Life Cycle Assessment (ISO 14040)

The standard LCA methodology consists of four stages, namely, goal and scope definition, inventory analysis, impact assessment and interpretation of results. These are represented pictorially in Figure 2. The goal and scope definition means a clear statement of the reasons for performing the study, the intended use of the results and the specification of the basic parameters of LCA study, such as the functional unit, system boundaries, allocation rules, data quality and simplifications. According to ISO 14040:1997, the functional unit is defined as 'the quantified performance of a product system for use as a reference unit in an LCA study'. For a product this usually simply involves specifying the weight, volume or number of a unit amount. Thus it has to be clearly defined and measurable. The primary purpose of the functional unit is to provide a reference to which the input and output data can be normalized in a mathematical sense.

The LCI stage involves collecting data concerning resource usage, energy and materials consumption, emissions and products resulting from each activity in the production system. As mentioned above, all these in- and out flows are calculated on the basis of the functional unit. In the third phase, the LCIA phase, the data collected is classified into specific categories and aggregated. This stage is composed of several mandatory elements and there are also optional elements for normalization, grouping or weighting of the indicator results and data quality analysis techniques. Finally, the life cycle interpretation is a procedure to identify, qualify, check and evaluate the information from the results of the LCI and/or LCIA of a product system. It is important to appreciate the reversible nature of an LCA study. It may be necessary, at some point, to go back to the previous stage to question and probe the results obtained. This is commonly done in LCA analysis, and arrows have been turned round to emphasize this point.

In this study, LCA was conducted following the guidelines stated above to determine the potential environmental impacts of producing 1 kg of chocolate in Ghana. The boundaries of the system studied have been shown in Figure 1, the process flow chart for chocolate production. (Those processes with broken boundaries were excluded from the analysis.) The inputs and outputs data collected in this work from the field and standard LCA databases are summarized in Table 5. Using the ISO series and CML 2001 database from the Centre for Environmental Science at the University of Leiden for impact assessment, the results given in Table 6 were obtained for the quantified impact scores for the selected relevant environmental impact categories (Ntiamoah and Afrane, 2009). Data storage and analysis were performed using the GaBi 4 LCA software.

The overall scores show that freshwater aquatic ecotoxicity and human toxicity are the most significant environmental impacts made by the process. In order to examine closely the contribution of the various stages of production to the overall impacts, Figure 3 was plotted. The percentage contribution of each stage to the total impact score of each category is given in this figure. The cocoa production stage was identified as the key life-cycle stage in terms of environmental impacts, as it makes the largest contribution to five out of the eight environmental impact categories considered in the study. The figure shows that it this is the most predominant contributor to eutrophication, ozone depletion, freshwater aquatic ecotoxicity, human toxicity, and terrestric eco-toxicity, with average contributions greater than 95%. Indeed the production and use of fertilizers and pesticides account for almost all the environmental burdens in the cocoa production stage. The significance of each of these environmental categories which are prominent, in cocoa production will be examined in turn.

INPUTS/OUTPUTS	Amount	Unit
Energy Inputs		
Electricity, (from national grid)	3.1716E-01	MJ
Diesel	5.3142E-02	Kg
Petrol	8.9967E-03	Kg
Materials Inputs		
Water	5.1274E+00	Kg
Fertilizer (N:P:K 0: 22:18 + 9CaO + 7S + 6MgO)	1.4590E-01	Kg
Pesticides		
-Fungicides	7.4200E-03	Kg
-Insecticides	8.0000E-04	Kg
Land use	3.9218E-05	Ha
Products/By-Products		
Chocolate	1.0000E+00	Kg
Cocoa Liquor	3.1948E-01	Kg
Cocoa Butter	2.3125E-01	Kg
Cocoa Cake	2.6875E-01	Kg
Cocoa Powder	7.5000E-02	Kg
Cocoa Shells	9.8000E-02	Kg
Air Emissions		
Dust (PM2,5 - PM10) [Particles to air]	2.5000E-03	Kg
Sulphur dioxide [Inorganic emissions to air]	8.0000E-03	Kg
Heavy metals to air	3.5745E-05	Kg
Carbon dioxide [Inorganic emissions to air]	2.3790E-01	Kg
Carbon monoxide [Inorganic emissions to air]	8.4100E-03	Kg
Pesticides to air	8.1308E-04	Kg
Water Emissions		
Biological oxygen demand (BOD)	5.0437E-12	Kg
Chemical oxygen demand (COD)	9.8212E-12	Kg
Nitrates	3.7500E-15	Kg
Oil & Grease	1.0000E-14	Kg
Phosphates	4.4204E-14	Kg
Total dissolved solids	5.1525E-12	Kg
Total suspended solids	4.1287E-12	Kg
Heavy metals to fresh water	7.4761E-04	Kg
Pesticides to fresh water]	3.6880E-03	Kg
Soil Emissions		
Pesticides to soil	9.4477E-04	Kg
Heavy metals to agricultural soil	4.1870E-05	Kg

(Source: Ntiamoah and Afrane, 2009.)

Table 5. Summary of input/output data for the production of 1 kg chocolate from Ghanaian cocoa beans, 2004/2005 season.

Environmental Impact Category	Overall Impact Score	Unit
Acidification Potential (AP)	9.7351E-03	kg SO_2
Eutrophication Potential (EP)	9.1568E-04	kg PO_4^{3-}
Freshwater Aquatic Ecotoxicity Potential (FAETP)	5.0797E+00	kg *DCB
Global Warming Potential (GWP)	3.5602E-01	kg CO_2
Human Toxicity Potential (HTP)	4.4426E+00	kg *DCB
Ozone Layer Depletion Potential (ODP)	4.9805E-09	kg *R11
Photochem. Ozone Creation Potential (POCP)	9.3002E-04	kg Ethene
Terrestrial Ecotoxicity Potential (TETP)	6.3796E-03	kg *DCB

*DCB is 1, 4 dichlorobenzene, *R11 is trichlorofluoromethane.

Table 6. Characterization results (overall impact scores) for the production of 1 kg chocolate in Ghana, obtained by using the CML 2001 method

Eutrophication

Eutrophication or nutrification is a measure of the over-fertilisation of soils and contamination of water-bodies with nutrients. In waters it causes excessive algal growth and negative modification of the aquatic ecosystems resulting in oxygen depletion and death of certain species. In soils, on the other hand, it promotes monocultures and loss of biodiversity (Heijungs *et al* (1992) and Guinee *et al* (2001)). Since nitrogen and phosphorus are the limiting nutrients for most of the aquatic systems, leaching of these nutrients into water-bodies results in eutrophication. High nitrate levels have been found in drinking water in developing countries. This has been linked to a disease known as *methaemoglobinaemia*, commonly referred to as the blue-baby syndrome, in agricultural areas (Pretty and Conway, 1988; Conway and Pretty, 1988). Although incidence of this disease in Ghana has not been reported in the literature, to the best of our knowledge, given the large amounts of fertilizer being used in cocoa production, possible contamination of water bodies need to be a matter of concern to stakeholders in the industry.

Freshwater Aquatic, Terrestial and Human Toxicity

From the results of Figure 2, not only are freshwater aquatic and human toxicity limited almost exclusively to the cocoa production stage, but they have the highest numerical values in the figure, which makes them more significant than the others. Terrestial toxicity, though not of the same magnitude as the other two, is nevertheless important. Toxicity to humans, flora and fauna is caused by a variety of substances, ranging from carcinogens to persistent toxins such as heavy metals which find their way into the food chain. The probability exists for harmful chemicals directly or indirectly poisoning some organisms and ultimately eliminating them from the ecosystem, and thereby restricting the biodiversity of the region and upsetting the food chain.

Acidification

Acidification is an indication of the gradual degradation of the soil and it is caused by acid solution formed when pollutants generated from the combustion of fuels are released into the atmosphere. In technical terms, it is caused by the build-up of protons in soils and lakes. Hauschild and Wenzel, (1998) describe it as a fall in the capacity of the soil to neutralize the acids that run through it. Higher acidity in certain types of soils can lead to the mobilisation of different fixed ions, which are then absorbed by plants to their detriment. Water which

seep through acidic soils can harm aquatic ecosystems in the different lakes and rivers and in some severe cases, acidic water has been known to leave some water-bodies lifeless (Mannion and Bowlby, 1992). Acidification can be caused directly by acids and indirectly by acidic anhydrides (sulphur dioxide and trioxide and oxides of nitrogen) and ammonia.

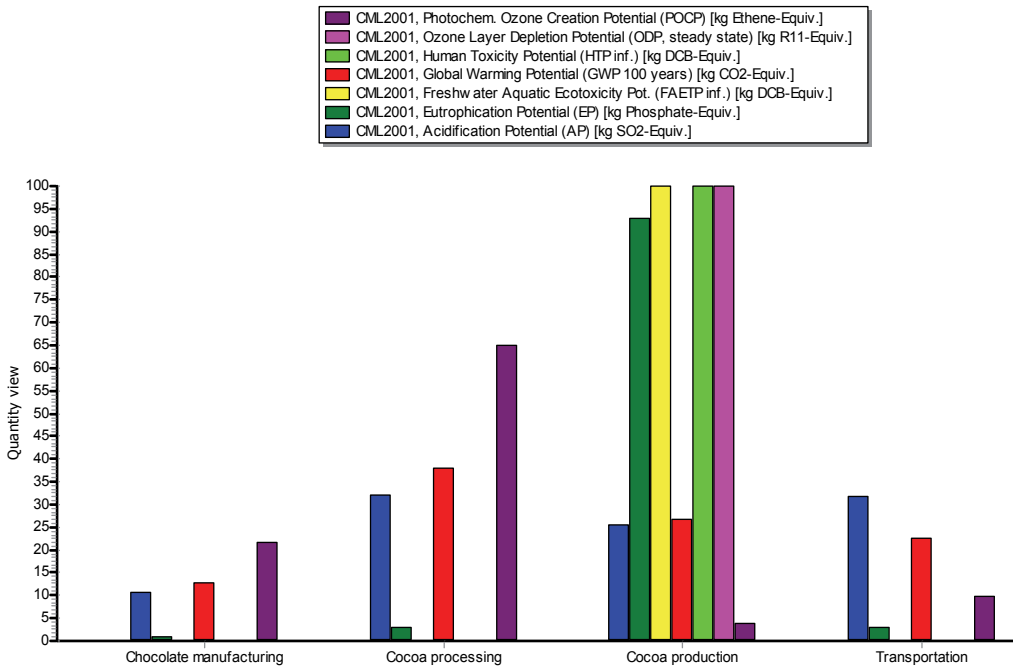

Fig. 3. Relative contribution of different stages of the life-cycle to the various environmental categories

Ozone layer Depletion Potential

The thinning of the ozone layer in the stratosphere is allowing increased levels of ultraviolet radiation to reach the earth, leading to diseases in humans (skin cancer and cataracts) and adverse effect on ecosystems. Ozone layer depletion is caused by the emission of halons and CFCs during the production of pesticides. These processes are based on complicated reaction systems, including both solid phase and gaseous phase reactions, and a limited number of substances are involved (Hauschild and Wenzel, 1998). Most notably methane, nitrous oxide, water vapour, chlorine and some bromine compounds, are responsible for the breakdown of ozone molecules. Human activities have increased the amount of substances involved in the breakdown of ozone and especially stable, long-lived chlorine and bromine containing hydrocarbons (i.e. chlorofluorocarbons or CFCs, tetrachloromethane, trichloroethane, etc.) are believed to contribute significantly. Fortunately the contribution to ozone-layer thinning, as a result of cocoa production and processing, turns out to be the least significant, according to Figure 3.

6. Conclusion

The use of pesticides is often advisable and sometimes essential when a crop is threatened. Integrated pest management is a concept which is now generally known and widely

accepted, and it is hoped that the judicious use of pesticides will be accepted as an integral part of pest management strategy. Technologies are presently available for the safe use of pesticides in cocoa and awareness of their correct and proper use needs to be stimulated (Bateman, 2008). However, introducing Good Agricultural Practices to the more than three million (often illiterate) smallholder farmers in the world cocoa economy is a major challenge. Ghana is making some strides in this area.

The clear indications are that the current agricultural practices for cocoa production are not sustainable, from both the environmental and economic perspective. Continued increase in the costs and amounts of chemicals put into the environment does not portend well for the future of this cash crop. The study has shown that current pests and diseases control practices in Ghanaian cocoa production which rely primarily on chemical methods, though well administered, results in more environmental damage. In the long term integrated pest management (IPM), which encourages natural control of pest populations, promises to reduce the use of pesticides. Some of the techniques used in this approach include enhancing natural enemies, planting pest-resistant crops, and, when absolutely necessary, efficient and judicious use of pesticides.

Pesticides continue to be attractive to most farmers and governments because they are simple to use, compared to the IPM methods, and returns on investments are not only good, but are predictable. A switch to IPM must be preceded by careful planning, and intensive education and training at the farm level, along with continuing research. In addition, promoting IPM will definitely require adjusting those subsidies and policies that encourage extensive pesticide use; otherwise farmers may not be able to resist the temptation of going back to their old ways.

7. Acknowlegements

The readiness of the staff of CODAPEC, Research and HI-TECH Divisions of COCOBOD, Accra, and the Cocoa Research Institute of Ghana at Tafo, to assist during the data collection stage was commendable and is hereby acknowledged.

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5

Industrial Contaminants and Pesticides in Food Products

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1. Introduction

In our modern world a large number of man-made chemicals are being used. As a consequence their widespread presence in the environment is becoming increasingly well documented (Vethaak et al, 2002; Peters et al., 2008). They are found in a vast range of consumer products and include plasticizers, emulsifiers, flame retardants, perfluorinated compounds, artificial musks and organotin compounds. While they have undoubtedly improved the quality of our lives, a consequence of their intensive use is a widespread presence in the environment. Human exposure to these compounds may be through contact with consumer products containing such chemicals as additives, but also through food products. Since many of these compounds have a lipophilic nature there is a potential for bio-accumulation through the food chain especially in products with a high fat content. This is reflected in the presence of persistent organic compounds such as organochlorine pesticides and polychlorinated biphenyls that can be found in food products although there use has been seized many years ago. Many of these compounds have also been found in human blood indicating that humans are exposed to these chemicals (CDC, 2001, 2003; Guenther et al., 2002). This exposure may be through different routes. One is the use of these chemicals as additives in consumer products such as carpets, curtains, toys and electronic equipment. The exposure of these chemicals in house dust indicates the potential for human exposure. Another route for human exposure is, of course, through food products. Since many of these compounds have a lipophilic nature, they can be bio-accumulated through the food chain especially in products with a high fat content. This study focused on the presence and concentrations of a number of typical man-made chemicals in food products that many of use daily. The chemicals considered in this study are: brominated flame retardants (BFR's), phthalates, artificial musks, alkylphenols (AP's), organochlorine pesticides (OCP's), polychlorobiphenyls (PCB's), organotin compounds (OT's) and perfluorinated compounds (PFC's).

2. Methods and materials

2.1 Sampling and sample pre-treatment

All samples, mostly fresh food products were purchased in regular shops in nine European countries including the Netherlands, the United Kingdom, Germany, Finland, Sweden,

Spain, Poland, Italy, Estonia and Greece. Samples were sent to the laboratory where laboratory samples were prepared and stored at -18°C until analysis. In general, solid food samples were cut into small pieces and homogenised with a blender. If not the entire sample was used or homogenised, proportional sub-sampling was applied and the collected sub-samples were homogenised. Milk was acidified with formic acid and the solid part containing the proteins and fat was separated from the liquid phase. Both parts were stored for analysis. Orange juice was centrifuged and vacuum filtrated and the solid and liquid parts were stored for analysis. A selected number of chemical parameters were determined in each sample, based on expectations and reports in the literature.

2.2 Chemical parameters

The chemical parameters determined in this study are listed in table 1, including the abbreviations that are used throughout the text and in the result tables. Note that not all parameters are determined in all samples.

2.3 Analytical procedures

For the determination of the OCP's, PCB's, BFR's, phthalates and artificial musks, a weight sub-sample of the homogenised laboratory sample was mixed with anhydrous sodium sulphate in a mortar and spiked with internal standards. The internal standards used were ^{13}C-labelled standards for PCB's and BFR's, ^{2}D-labelled standards for OCP's and phthalates, and a surrogate standard for the artificial musks. The samples were Soxhlet extracted for 16 hours using a mixture of 10% diethyl ether in hexane. For milk and orange juice a proportional amount of the liquid phase was pre-extracted with hexane and this hexane extract was used in the Soxhlet extraction of the solid part of these samples. Olive oil was directly diluted in hexane. One procedural blank, consisting of 40 g anhydrous sodium sulphate, was included in every batch of 10 samples. All extracts were concentrated to a volume of 50 ml and split into two equal parts of 25 ml. For the determination of the OCP's, PCB's and BFR's, one part of the extract was washed several times with sulphuric acid of increasing concentration to remove the major part of the lipids. The remaining extract was concentrated and purified over a glass chromatographic column packed with florisil and capped with anhydrous sodium sulphate to isolate the fraction containing the OCP's, PCB's, PBDE's and HBCD. The eluent was concentrated to a small volume and a syringe standard (1,2,3,4-tetrachloronaphthalene) was added. This final extract was analysed on an Agilent 6890 series gas chromatograph coupled to an Agilent 5973 mass spectrometer (GC/MS) and equipped with a HP-5-MS, 30 m × 0.25 mm (i.d.), film thickness 0.25 μm, fused silica capillary column. The mass spectrometer was operated in the selected ion monitoring mode and typically two or three characteristic ion masses were monitored for each analyte. The samples were analyzed for the following OCP's; α-, β- and γ-hexachlorohexane (HCH), hexachlorobenzene (HCB), α- and β-chlordane, o,p'-, p,p'-DDE, o,p'-, p,p'-DDD and o,p'-, p,p'-DDT: The following PCB congeners: 18, 28/31, 22, 41/64, 44, 49, 52, 54, 56/60, 70, 74, 87, 90/101, 99, 104, 105, 110, 114, 118, 123, 138, 141, 149, 151, 153/168, 156, 157, 158, 167, 170, 177, 180, 183, 187, 188, 189, 194, 199 and 203: The following PBDE congeners: 17, 28, 32, 35, 37, 47, 49/71, 66, 75, 77, 85, 99, 100, 119, 126, 138, 153, 154, 156, 166, 181, 183, 184, 190, 191, 196, 197, 206, 207 and 209 and HBCD.

For the determination of the phthalates and artificial musks the second part of the extract was purified using a dimethylformamide-hexane partitioning to remove lipids. In this

Organochlorine pesticides (OCPs):	**Phthalates:**
α-hexachlorohexane (α-HCH)	di-methyl phthalate (DMP)
β-hexachlorohexane (β-HCH)	di-ethyl phthalate (DEP)
γ-hexachlorohexane (γ-HCH) = lindane	di-isobutyl phthalate (DiBP)
hexachlorobenzene (HCB)	di-butyl phthalate (DBP)
α-chlordane	benzylbutyl phthalate (BBP)
β-chlordane	di-(2-ethylhexyl) phthalate (DEHP)
o,p'-DDE	di-isononyl phthalate (DiNP)
p,p'-DDE	di-isodecyl phthalate (DiDP)
o,p'-DDD	
p,p'-DDD	
o,p'-DDT	
p,p'-DDT	

Polychlorinated biphenyls (PCBs):		**Polybrominated diphenylethers (PBDEs):**	
PCB-18	PCB-118	BDE-17	BDE-138
PCB-28/31	PCB-123	BDE-28	BDE-153
PCB-22	PCB-138/158	BDE-32	BDE-154
PCB-41	PCB-141	BDE-35	BDE-156
PCB-44	PCB-149	BDE-37	BDE-166
PCB-49	PCB-151	BDE-47	BDE-181
PCB-52	PCB-153/168	BDE-49/71	BDE-183
PCB-54	PCB-156	BDE-66	BDE-184
PCB-56/60	PCB-157	BDE-75	BDE-190
PCB-64	PCB-167	BDE-77	BDE-191
PCB-70	PCB-170	BDE-85	BDE-196
PCB-74	PCB-177	BDE-99	BDE-197
PCB-87	PCB-180	BDE-100	BDE-206
PCB-90	PCB-183	BDE-119	BDE-207
PCB-99	PCB-187	BDE-126	BDE-209
PCB-101	PCB-188		
PCB-104	PCB-189	**Other brominated flame retardents:**	
PCB-105	PCB-194		
PCB-110	PCB-199	tetrabromobisphenol-A (TBBPA)	
PCB-114	PCB-203	hexabromocyclododecane (HBCD)	

Perfluorinated chemicals (PFCs):	**Artificial musks:**
perfluoro-octanoic acid (PFOA)	galaxolide (HHCB)
perfluoro-octane sulphonate (PFOS)	tonalide (AHTN)
perfluoro-octane sulfonamide (PFOSA)	musk xylene (MX)
perfluoro-nonanoic acid (PFNA)	musk ketone (MK)
perfluoro-decanoic acid (PFDA)	
perfluoro-undecanoic acid (PFUnA)	
perfluoro-dodecanoic acid (PFDoA)	**Organotin compounds:**
perfluoro-tetradecanoic acid (PFTrA)	
	mono-butyltin (MBT)
Alkylphenols (AP):	di-butyltin (DBT)
	tri-butyltin (TBT)
nonylphenol isomers (NP)	mono-octyltin (MOT)
octylphenol isomers (OP)	di-octyltin (DOT)

Table 1. Chemical parameters determined in this study including abbreviations used in the text and result tables

partitioning the hexane extract is extracted with dimethylformamide (DMF) to isolate the phthalates and artificial musks. After removal of the hexane layer, water is added to DMF fraction and the analytes are re-extracted into fresh hexane. This extract was concentrated and purified over a glass chromatographic column packed with florisil and capped with anhydrous sodium sulphate to isolate the fraction containing the phthalates and artificial musks. The eluent was concentrated to a small volume, the syringe standard was added and this final extract was analysed with the identical GC/MS system as described above. The mass spectrometer was operated in the selected ion monitoring mode and typically two or three characteristic ion masses were monitored for each analyte. The samples were analyzed for the following phthalates; di-methyl- (DMP), di-ethyl- (DEP), di-isobutyl- (DIBP), di-butyl- (DBP), benzylbutyl- (BBP), di-(2-ethylhexyl)- (DEHP), di-isononyl- (DINP) and di-isodecyl phthalate (DIDP): The following artificial musks; musk ketone (MK), musk xylene (MX), tonalide (AHTN) and galaxolide (HHCB).

AP's and the brominated flame retardant tetrabromobisphenol-A (TBBPA) were isolated using a steam distillation procedure described by Guenther et al., 2002. In the round bottom flask of the steam distillation apparatus typically 10 g of the laboratory sample was spiked with an internal standard and mixed with 250 ml Milli-Q water to which was added 1 ml of concentrated hydrochloric acid and 20 g of sodium chloride. During the overnight distillation process the organic phenols are isolated in the organic solvent, in this case hexane, in the backflow cooling system of the apparatus. The hexane extract is isolated, dried with anhydrous sodium sulphate and concentrated. Following drying the extract was reduced in volume nearly to dryness under a stream of nitrogen and re-dissolved in 1 ml of methanol. During concentration of the hexane extract care has to be taken to avoid losses of the AP's and rinsing of the glass surfaces with methanol is necessary. The methanol extract is analysed with liquid chromatography coupled with mass spectrometry (LC/MS) in the selected ion monitoring mode.

For the determination of perfluorinated compounds, specifically perfluoro-octanoate (PFOA), perfluoro-octane sulphonate (PFOS) and perfluoro-octane sulphonamide (PFOSA) sub-samples of 5 gram were collected in 50 ml poly-propylene tubes and extracted using acetonitrile. The samples were centrifuged and the clear liquid is decanted and purified over a glass chromatographic column packed with florisil, silica, LC-NH_2 and activated carbon. The residue in the poly-propylene tube is extracted two times more and each extract is decanted over the same chromatographic column, thus combining the purified extracts. 0.5 ml octanol is added as a keeper and the extracts are concentrated to a small volume.

3. Results

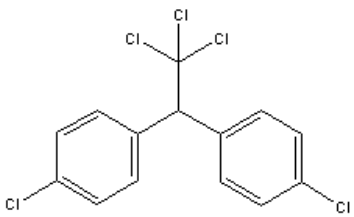
3.1 Validation of analytical methods

The analytical methods used were validated previous to the execution of this study. The parameters determined were linearity, repeatability, recovery from the rainwater matrix and method detection limits (MDL). For the determination of the organochlorine pesticides, polychlorinated biphenyls, brominated flame retardants, phthalates, artificial musks and organotin compounds internal standards were added to each sub-sample prior to analysis. For the perfluorinated compounds two relevant samples are spiked with the compounds of interest and analysed. The recovery of the internal standard and spikes were in the range of 70 to 140% with the exception of artificial musks where recoveries in the range of 56 to 87% were found, and the organotin compounds where recoveries were in the range of 67 to 99%.

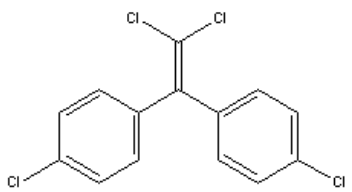
With the exception of PCBs and organotin compounds, the results are not corrected for the recovery of the internal standards or spikes since the spikes used were not compound specific and their recovery is only used to evaluate the performance of the method. With each series of ten samples a blank sample was included. For the blank analysis the complete analytical procedure was followed, including all chemicals and solvents, but no sample was added. Blank results were only found for the phthalates DEHP, DIBP and DBP, The results were corrected for these blank values and the detection limits were raised to 10 ng/g for DIBP and DBP, and to 20 ng/g for DEHP.

3.2 Organochlorine pesticides

Organochlorine pesticides (OCPs) include compounds like DDT, lindane, hexachlorobenzene and chlordane, among others. DDT is a well-known agricultural insecticide that has been used extensively on a global basis for over 40 years. Although their manufacture and application are now largely prohibited or restricted in industrialized western countries because of their toxicity and persistence, they can still be found in environmental and biological matrices due to their persistence. Pesticide exposure has been associated with arthritis, diabetes, neurobehavioral changes and DNA damage Cox et al., 2007; Lee et al. 2007; Jurewicz et al., 2008; Rusiecki et al., 2008). The structures of p,p'-DDT and it's breakdown product p,p'-DDE, are shown below.



p,p'-DDT



p,p'-DDE

As a part of its monitoring program, the Food and Drug Administration (FDA) determines the levels of pesticide residues in a wide variety of foods typically consumed by Americans. Over the past ten years, these surveys have detected DDE and other OCPs in a variety of foods including meat, fish and shell fish products, eggs, root vegetables, legumes (beans, peas, and peanuts), some fruits, and leafy greens. In 1999 DDE or DDT were detected in 22% of the 1,040 food items analysed in the FDA Total Diet Study (FDA, 1999). The results for the 2003 Total Diet Study indicate DDT, but mainly DDE in 18% of the various food items in concentrations ranging from 0.1 to 11 ng/g (FDA, 2003). Those for chlordane and lindane range from 0.1 to 3.8 and from 0.1 to 8.4 ng/g product. In general, the concentrations as well as the frequency of detection of OCPs were lower in the 2003 study. The results of the OCPs in this study are presented in table 2 at the end of this section, and graphically in figure 1. OCPs are found in 17 of the 25 samples. The predominant OCPs that are detected are p,p'-DDE and HCB both found in 15 of the 25 samples. In addition o,p'-DDE and cis-chlordane were found in one and two samples respectively. The maximum concentration found for p,p'-DDE was 5.6 ng/g in a sample of pickled herring. The median concentrations for p,p'-DDE and HCB were 0.43 and 0.14 ng/g. Compared to the FDA's Total Diet Study, p,p'-DDE and HCB are found more frequently but in lower concentrations. This compares with a recent study of Schecter et al. who reported p,p'-DDE in 23 out of 31 samples in concentrations ranging from 0.06 to 9.0 ng/g with a median value of 0.51 ng/g (Schecter et al., 2010).

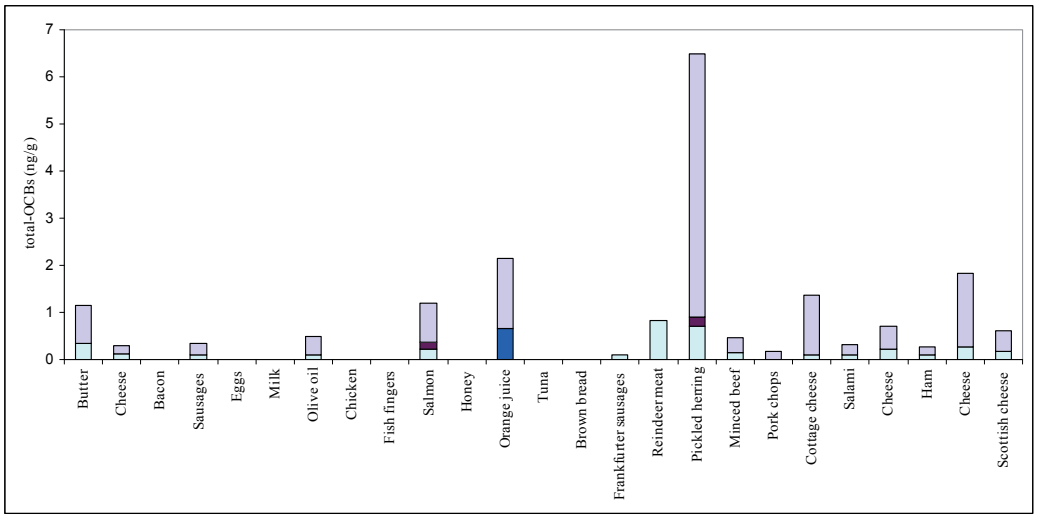
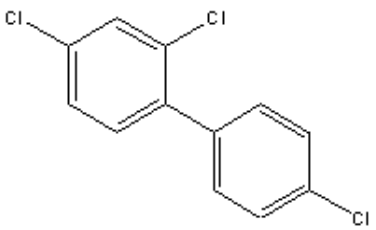


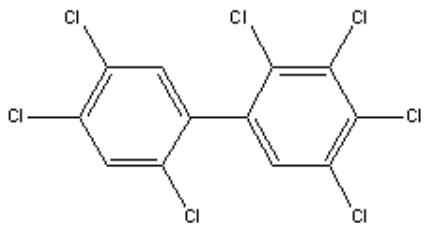
Fig. 1. Graphic presentation of the total-OCP concentration in 25 food items

3.3 Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are marketed as cooling or insulating fluids for transformers, as softeners in the varnish and adhesive industries, and as hydraulic fluids. These compounds are not combustible, are heat resistant and make good solvents. On the other hand there is a severe toxic effect of PCBs, which damage the organs responsible for metabolism and also the nervous system (Guo et al., 1995; Ribas-Fito et al., 2001). Because of their persistence, PCBs are widely spread in the environment and, due to their good lipo-solubility, are easily deposited and concentrated in human, animal and plant tissue. The structures of two of the seven indicator PCBs, PCB-28 and PCB-180 are shown below.



2,4,4'-trichlorobiphenyl (PCB 28)



2,2',3,4,4',5,5'-heptachlorobiphenyl (PCB 180)

In the Total Diet Study of 2003 the levels of the sum of i-PCBs (e.g. PCB-28, PCB-52, PCB-101, PCB-118, PCB-138, PCB-153 and PCB-180) ranged from 6 to 70 ng/g product. The Estonian Environment Research Centre determined levels of the sum of i-PCBs in Estonian butter and found a relative narrow concentration range of 5.2 to 8.8 ng/g lipid, translating to a range of 4.2 to 7.0 ng/g on a wet weight basis (EU, 2004). A study of the European Food Safety Association (EFSA) of different food types sampled across Europe between 1997 and 2003 reports median concentrations for the sum of i-PCBs ranging from 1.4 to 12 ng/g. Only fish oil showed a higher median concentration of 52 ng/g for the sum of i-PCBs (Gallani et al., 2004). The results for the PCBs in this study are presented in table 2 at the end of this

section. A graphic summary is given in figure 2. PCBs are found in every sample with PCB-18, -28 and -52, with the highest frequency. The highest PCB concentrations were found in the pickled herring and the salmon with concentrations for the sum of i-PCBs of 13 and 3.3 ng/g. The highest concentration of an individual PCB was for PCB-153, 5.5 ng/g in the salmon. The results found are comparable with those reported by EFSA for different food types sampled across Europe.

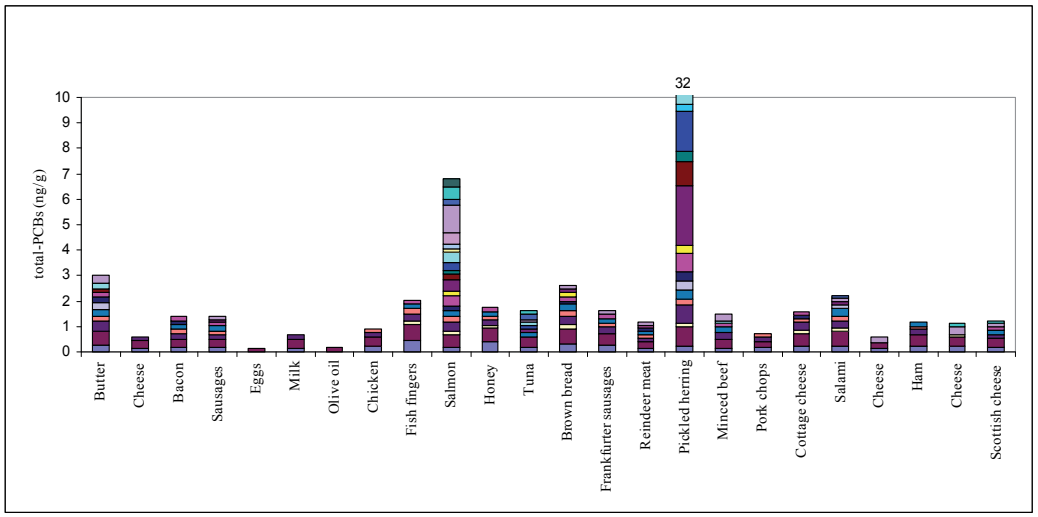
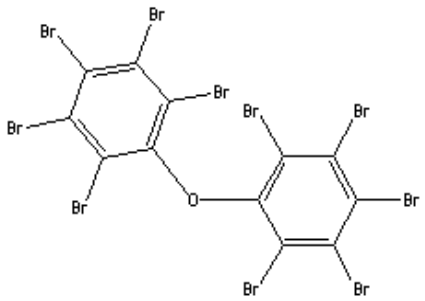


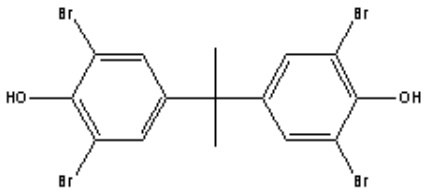
Fig. 2. Graphic presentation of the total-PCB concentration in 24 food items

3.4 Brominated flame retardants

Brominated flame retardants (BFRs) are widely used in electronic household equipment, plastics, textile and polyurethane foam in furniture and cars for safety reasons. Of the brominated products, about one-third contain tetrabromobisphenol-A (TBBPA) and derivatives, another third contains various bromines, including hexabromocyclododecane (HBCD) and the last third contains polybrominated diphenylethers (PBDEs). All three types of BFRs are determined in this study. The PBDEs are commercial mixtures with different degrees of bromination and used as additives to fireproof polymers. HBCD is a cyclo-aliphatic brominated chemical introduced as a replacement for the PBDEs and with the same applications. TBBPA is mainly used as a reactive (chemically bound) flame retardant in epoxy polymers such as printed circuit boards in electronic equipment. The structure of BDE-209 (better known as deca-BDE), HBCD and TBBPA is shown below. In a study by the Dutch National Institute for Public Health and the Environment the levels of various PBDEs, HBCD and TBBPA in 84 food products were determined (de Winter-Sorkina et al., 2003, 2006). With the exception of fish products PBDEs were absent or present in low concentrations (<0.1 ng/g) in food products. For the fish products the concentrations of the congeners BDE-28, -47, -99, -100, -153 and -154 ranged from 0.1 to 14 ng/g. BDE-209 was found in none of the 84 products while TBBPA was found in 7 products in concentrations ranging from 0.1 to 3.4 ng/g. Surprisingly, HBCD was found in 28 of the 84 samples in concentrations ranging from 0.1 to 8.9 ng/g product. TBBPA and HBCD were predominantly found in fish products, especially in eel.



decabromo diphenylether (BDE 209)



tetrabromo bisphenol-A (TBBA)

A recent report on the results of a round robin exercise for BFRs in environmental, human and food samples gives some results for herring and salmon. The highest concentrations are found for BDE-47, up to 9.3 ng/g in herring and 0.89 ng/g in salmon. Those for the BDE-28, -99, -100, -153, -154 and -183 are in the range of 0.1 to 1.3 ng/g. BDE-209 and HBCD were not detected in these samples. The results for PBDEs in typical market basket studies show some differences, while studies in Spain (Bocio et al., 2003) and Japan (Ohta et al., 2002, 2008) show a predominance of the tetra- and penta-BDE with maximum concentrations up to 0.34 ng/g, an American study reports PBDE levels up to 3.1 ng/g product with surprisingly a predominance of BDE-209 which comprised as much as 50% of the total PBDE content in some of the samples (Schecter et al., 2004). The results for the BFRs in this study are reported in table 3 at the end of this section, and graphically in figure 3. PBDEs were found in 19 of the 24 samples. BDE-209, TBBPA and HBCD were found in none of the samples. BDE-47, -32 and -99 seem to be the predominant and the highest concentration for an individual PBDE was 0.65 ng/g found for BDE-47 in the sample of Scottish Cheese. Surprisingly, and different from other studies, only a limited number of BFRs were found in the fish products, in salmon no BFRs were found at all. The total PBDE concentrations ranged from 0.15 to 1.2 ng/g with the highest concentration in the sample of minced beef. The concentrations found in this study are therefore comparable with those found in the Spanish and Japanese study and much lower than those found in the American food study.

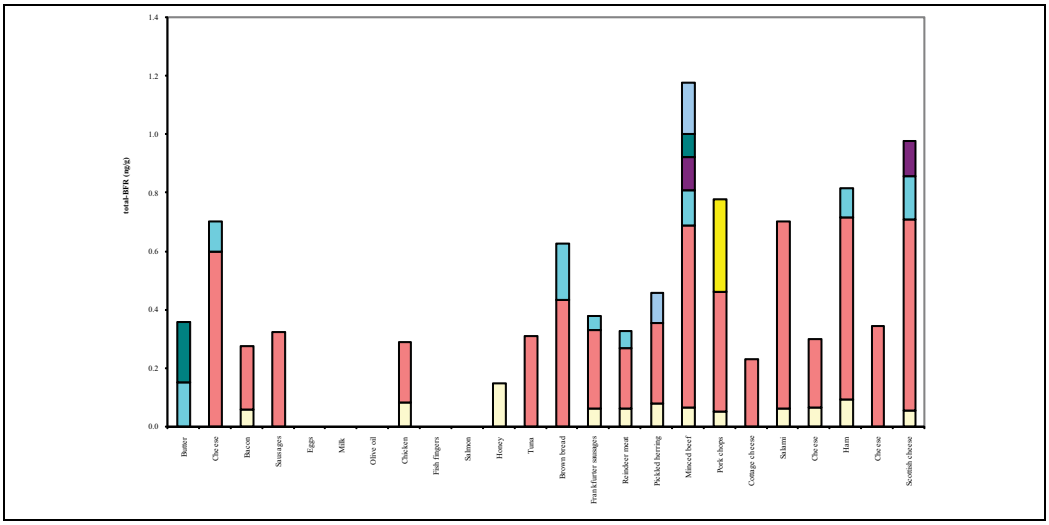
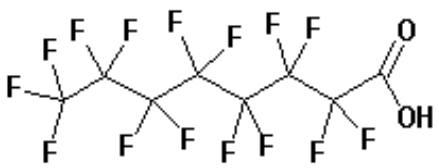


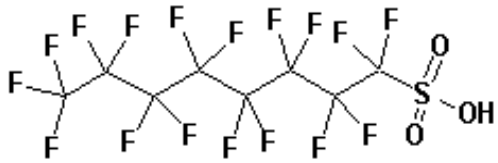
Fig. 3. Graphic presentation of the total-BFRs concentration in 24 food samples

3.5 Perfluorinated compounds

Perfluorinated compounds (PFCs) are synthetic compounds characterised by an alkyl chain in which the hydrogen atoms are completely replaced by fluorine atoms. PFCs are heat stable, very resistant to degradation and environmental breakdown and have an amphiphilic nature (they repel water as well as oil). Because of these properties PFCs are used a myriad of applications, such as non-stick pans, stain and water repelling coatings for clothing, furniture and paper with typical brand names as Teflon, Gortex, Stainmaster and Scotchguard (3M-company, 1999). PFCs accumulate in the environment and they have been detected far from manufacturing plants in birds, marine plants and mammals from the Arctic to the Pacific and Indian Oceans and in land creatures in Europe and the USA (Kannan et al., 2002; Martin et al., 2003, 2004). In addition PFCs have been found in human blood (Kannan et al., 2004; Peters, 2005). The structures of the two most common PFCs, PFOA and PFOS are given below.



perfluoro-octanoic acid (PFOA)

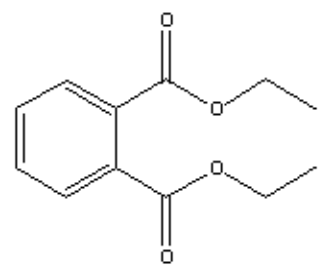


perfluoro-octane sulfonate (PFOS)

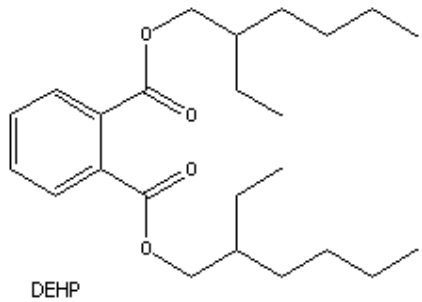
Many studies focus on biota such as fish and birds and only limited information about levels of PFCs in food seem to be available. In 2001 the Centre Analytical Laboratory performed a study for the 3M-company as part of a Multi-City Study. PFCs were found in a limited number of samples. PFOS was found in five samples, four whole milk samples and a ground beef sample in concentrations up to 0.85 ng/g. PFOA was found in seven samples, two ground beef samples, two bread samples, two apple samples and one green been sample in concentrations up to 2.35 ng/g (Centre Analytical Laboratory, 2001). The results for PFCs are given in table 3 at the end of this section. PFOS and PFOSA are found in only one of the five samples that were analysed. The concentrations found in the sample of pickled herring, are 1.3 ng/g for PFOS and 0.2 ng/g for PFOSA. PFOS is widely detected in the environment, animals and humans and therefore expected. Surprisingly, Schecter et al. did not find PFOS, but do find PFOA in 50% of the analysed samples in concentrations ranging from 0.02 to 1.8 ng/g (Schecter et al., 2010).

3.6 Phthalates

Phthalates are one of the most ubiquitous classes of chemical contaminants in our everyday environment as a consequence of their high volume uses in open applications. They are used as plasticizers to increase the flexibility of high molecular weight polymers (mainly in PVC), as heat-transfer fluids and as carriers, and can be found in ink, paint, adhesives, pesticides, vinyl flooring (Vethaak et al., 2002), but also in cosmetics and personal care products. Consequently, the potential for human exposure is very high. Di-(2-ethylhexyl) phthalate (DEHP) and di-ethyl phthalate (DEP) are two of the most common used plasticizers. DEHP is nowadays gradually replaced by iso-alkyl phthalate mixtures like di-isononyl phthalate (DINP). The chemical structure of DEP and DEHP is shown below.



DEP



DEHP

There is not much information about concentrations of phthalates in food. Most attention has been focused on phthalates in plastic wrapping materials for food products. An older study dating from 1994 deals with the determination of DEHP in milk, cream, butter and cheese (Sharman et al., 1994). DEHP was found in all these products in concentrations ranging from 330 to 980 ng/g. More recent information is available from the UK Food Standard Agency and is concerned with the presence of phthalates in infant formulae (Joint Food Safety and Standards Group, 1998). Seven phthalates including DEHP were determined in 39 samples of infant formulae. In 12 of the 39 samples none of the phthalates were found. In the remaining samples di-butyl phthalate (DBP), benzylbutyl phthalate (BBP) and DEHP were found. DEHP was the most abundant individual phthalate in concentrations ranging from 50 to 440 ng/g product. DBP was found in concentrations up to 90 ng/g and BBP up to 15 ng/g product. Concentrations of other phthalates were less than 10 ng/g. The results for phthalates in this study are presented in table 3 at the end of this section, and graphically in figure 4. 16 of the 19 samples analysed for phthalates did contain one or more of these compounds. In eggs, milk and orange juice no phthalates were detected. DIDP was the only phthalate that was not found in any of the samples. As expected DEHP is the predominant phthalate found in 16 of the 19 samples with concentrations ranging from 20 to 24,000 ng/g. It should be mentioned that the latter concentration is an exception and was found in the sample of olive oil. The neck of the olive oil bottle contains a polymer spout that may be responsible for the high DEHP concentration in the olive oil. Other phthalates that are frequently found (>50% of the samples) are DBP and BBP, be it in lower concentrations than DEHP.

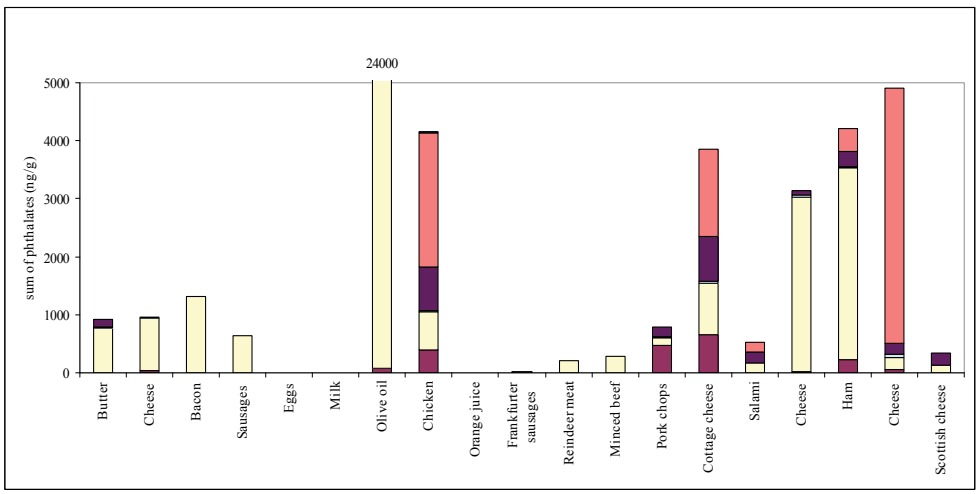
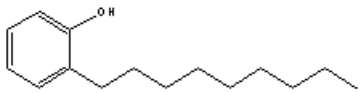


Fig. 4. Graphic presentation of the sum of the eight phthalates in 19 food items

3.7 Alkylphenols

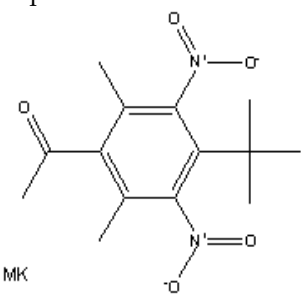
Alkylphenols, but primarily alkylphenol ethoxylates are used as additives in plastics and as surface-active ingredients in industrial detergents and emulsifiers. The ethoxylates are produced by a condensation reaction of alkylphenols with ethylene oxide. Alkylphenols commonly used are nonylphenol (NP) and to a lesser extent octylphenol (OP), in both cases pre-dominantly the para-substituted isomers (>90%). Alkylphenols are the common products of bio- or chemical degradation of the ethoxylates. The chemical structure of n-NP is shown below.



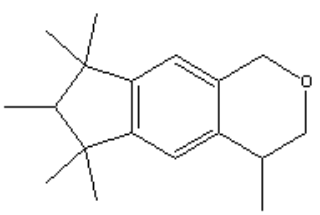
As with the phthalates only little information seems to be available about levels of alkylphenols in food. Guenther et al. determined NPs in 60 different commercially available foodstuffs and concluded that NPs are ubiquitous in food (Guenther et al., 2002). The concentrations of NPs (sum of the isomers) varied between 0.1 and 19.4 ng/g product and were found in all samples. Despite the lipophilic properties of NPs, high concentrations of NPs were not only found in fatty foods but also in non-fatty food products. In another study OP and NP were determined in composite foods (Fernandes et al., 2003). OP was found in only one sample in a concentration of 8.7 ng/g while NP was found in concentrations up to 25 ng/g. In a previous TNO study alkylphenols were determined in wrapped fresh meat and cheese products (Geenen, 2003). Since the alkylphenols were determined in slices of the product collected directly below the foil or wrapper, the results are not representative for the entire product. OP was detected in none of the samples while NPs were found in five of the eight sub-samples in concentrations ranging from 9 to 590 ng/g. For one sample the whole food item was analysed resulting in a much lower concentrations in the order of 1 ng/g for NP. The results for alkylphenols in this study are given in table 3 at the end of this section. NP was found in 2 of the 19 samples, the samples of butter and bacon in concentrations around 5 ng/g. OP was found in none of the samples. Although the concentrations are in the range of what Guenther found, the results are different because the frequency of detection in this study is far lower. Perhaps this is a result of the way sub-samples are collected since higher concentration may be found in top-layers beneath the packaging foil.

3.8 Musk compounds

In nature, musk is a compound produced by a gland in male deer which has been used in perfumes, but the increasing demand resulted in the production of artificial musk compounds. The most well-known are nitro musks like MX and MK that are nowadays replaced by polycyclic musks like AHTN and HHCB. Musks are used as additives for perfumes, in detergents and soaps, in body lotions and deodorizers. The structure of MK and HHCB is presented below.



MK

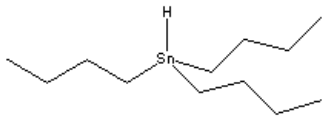


HHCB

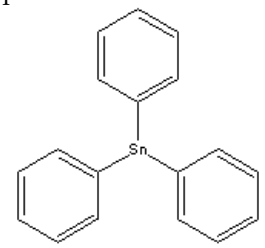
As far as we know there are no reports or studies concerning the presence of artificial musks in food or food products. However, since 1981 it is known that artificial musks can be found in fish (Yamagishi et al., 1981, 1983; Rimkus & Wolf, 1995; Fromme et al., 2001; Gatermann et al., 2002), and as a results artificial musks may be present in fish products. The results for musks in this study are given in table 3 at the end of this section. The nitro-musks MK and MX are not found in the four fish products analysed for artificial musks. The polycyclic musks AHTN and HHCB are found in two of the samples, the samples tuna and pickled herring in a maximum concentration of 0.56 ng/g for the latter sample. As in other environmental matrices and human blood the HHCB concentrations are about twice that of the AHTN concentrations. That the concentrations are lower than those reported for fish in the literature is probably because most literature studies report results for fish in waterways connected to sewer effluents and not for typical marine fish species.

3.9 Organotin compounds

The main OTCs to be found in food are likely to be tri-substituted compounds, tributyltin (TBT) and triphenyltin (TPT), which have been used extensively as biocides in wood preservatives, in antifouling paints for boats and as pesticides. Mono- and di-substituted OTC's (dibutyltin, mono-n-octyltin and di-n-octyltin) are used as stabilizers in PVC plastics, and di-alkyltins have been approved as PVC stabilizers for food contact materials. OTC's tend to accumulate in fish and other aquatic organisms and tri-alkyltins are bio-degraded to di- and mono-alkyltin compounds and therefore these may be found also in addition to the tri-substituted OTCs. The structures of TBT and TPT are presented below.



tributyltin (TBT)



triphenyltin (TPT)

Based on an EU SCOOP report the European Food Safety Authority estimated that the median concentrations of TBT, DBT and TPT in fish and fishery products are 7.0, 2.5 and 4.0 ng/g product (EC, 2003; EFSA, 2004). The EU SCOOP report contains very few data on DOT, which were always below the limit of detection. The results for organotin in this study are given in table 3 at the end of this section. Organotin were found in three of the four samples that were analysed. The highest concentration of 9.0 ng/g was found for mono-butyl tin (MBT), a degradation product of TBT in the sample of tuna. Di-butyl tin (DBT) and TBT were also found in this sample. The pickled herring and the fish fingers contained butyl-tin as well as octyl-tin compounds.

3.10 Result tables of the concentrations of determined parameters in 25 samples of food

Tables 2 and 3 contain the full results of the study. Note that not all parameters are measured in all samples. In those cases the positions in the table are left blank. If concentrations of the parameters in a sample were below the detection limit, this is indicated with a "<" sign. The limits of detection for each parameter are printed directly after the compound name of the parameter. All results are expressed in ng/g product.

compound	limit of detection	cheese 1	cheese 2	cheese 3	scottisch cheese	cottage cheese	butter	eggs	milk	bacon	sausages	chicken breast	frankfurthers	reindeer beef
	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g
Organochlorine pesticides														
α-HCH	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
β-HCH	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
γ-HCH	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
HCB	0.1	0.12	0.22	0.26	0.18	0.10	0.34	<	<	<	0.10	<	0.10	0.83
α-chlordane	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
β-chlordane	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
o,p'-DDE	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
p,p'-DDE	0.1	0.18	0.48	1.6	0.43	1.3	0.79	<	<	<	0.25	<	<	<
o,p'-DDD	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
p,p'-DDD	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
o,p'-DDT	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
p,p'-DDT	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
Polychlorinated biphenyls														
PCB 18	0.1	0.13	0.15	0.22	0.16	0.24	0.25	<	0.12	0.19	0.16	0.20	0.26	0.14
PCB 28/31	0.1	0.31	0.22	0.37	0.36	0.49	0.56	0.16	0.36	0.32	0.35	0.40	0.44	0.26
PCB 22	0.1	<	<	0.11	<	0.14	<	<	<	<	<	<	<	<
PCB 41/64	0.1	<	<	<	<	<	0.27	<	<	<	<	<	<	<
PCB 44	0.1	<	<	<	0.18	<	0.27	<	<	0.18	0.24	<	0.18	0.14
PCB 49	0.1	<	<	<	<	0.14	0.18	<	<	0.19	0.13	0.13	0.16	0.13
PCB 52	0.1	0.16	<	<	0.15	0.29	0.40	<	0.21	0.21	0.16	0.17	0.27	0.15
PCB 54	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 56/60	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 70	0.1	<	<	<	0.12	0.16	0.20	<	<	0.21	0.12	<	0.17	0.11
PCB 74	0.1	<	<	<	<	0.14	0.22	<	<	0.11	<	<	<	0.12
PCB 87	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 90/101	0.1	<	<	<	<	<	<	<	<	<	0.11	<	<	<
PCB 99	0.1	<	<	<	<	<	0.14	<	<	<	<	<	<	<
PCB 104	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 105	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 110	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 114	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 118	0.1	<	<	<	<	<	0.23	<	<	<	<	<	<	<
PCB 123	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 138	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 141	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 149	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 151	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 153/168	0.1	<	0.22	0.31	0.16	<	0.29	<	<	<	0.12	<	0.14	0.11
PCB 156	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 157	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 158	0.1	<	<	0.12	0.10	<	<	<	<	<	<	<	<	<
PCB 167	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 170	0.2	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 177	0.2	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 180	0.2	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 183	0.2	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 187	0.2	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 188	0.2	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 189	0.2	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 194	0.2	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 199	0.2	<	<	<	<	<	<	<	<	<	<	<	<	<
PCB 203	0.2	<	<	<	<	<	<	<	<	<	<	<	<	<

Table 2. Results for organochlorine pesticides and polychlorobipenyls expressed in ng/g product.

compound	limit of detection	minced beef	pork chops	salami	ham	fish fingers	salmon	tuna	pickled hering	honey	orange juice	brown bread	olive oil
	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g
Organochlorine pesticides													
α-HCH	0.1	<	<	<	<	<	<	<	<	<	<	<	<
β-HCH	0.1	<	<	<	<	<	<	<	<	<	<	<	<
γ-HCH	0.1	<	<	<	<	<	<	<	<	<	<	<	<
HCB	0.1	0.14	<	0.10	0.10	<	0.22	<	0.7	<	<	<	0.10
α-chlordane	0.1	<	<	<	<	<	0.13	<	0.2	<	<	<	<
β-chlordane	0.1	<	<	<	<	<	<	<	<	<	<	<	<
o,p'-DDE	0.1	<	<	<	<	<	<	<	<	<	0.65	<	<
p,p'-DDE	0.1	0.33	0.17	0.21	0.17	<	0.83	<	5.6	<	1.5	<	0.40
o,p'-DDD	0.1	<	<	<	<	<	<	<	<	<	<	<	<
p,p'-DDD	0.1	<	<	<	<	<	<	<	<	<	<	<	<
o,p'-DDT	0.1	<	<	<	<	<	<	<	<	<	<	<	<
p,p'-DDT	0.1	<	<	<	<	<	<	<	<	<	<	<	<
Polychlorinated biphenyls													
PCB 18	0.1	0.14	0.16	0.22	0.24	0.45	0.19	0.20	0.23	0.38		0.29	<
PCB 28/31	0.1	0.36	0.23	0.59	0.43	0.63	0.49	0.38	0.76	0.55		0.61	0.19
PCB 22	0.1	<	<	0.14	<	0.13	0.11	<	0.13	0.11		0.16	<
PCB 41/64	0.1	<	<	0.14	<	<	<	<	0.38	<		<	<
PCB 44	0.1	0.24	<	0.29	0.17	0.22	0.24	0.19	0.33	0.19		0.21	<
PCB 49	0.1	<	0.12	0.19	0.11	0.21	0.23	<	0.22	0.15		0.19	<
PCB 52	0.1	0.25	0.20	0.28	0.24	0.27	0.36	<	0.74	0.19		0.35	<
PCB 54	0.1	<	<	<	<	<	<	<	<	<		<	<
PCB 56/60	0.1	<	<	<	<	<	0.18	<	0.31	<		0.14	<
PCB 70	0.1	0.13	<	<	<	0.14	0.38	<	0.71	0.16		0.17	<
PCB 74	0.1	<	<	<	<	<	0.19	<	0.37	<		0.11	<
PCB 87	0.1	<	<	<	<	<	0.12	<	0.41	<		<	<
PCB 90/101	0.1	<	<	0.13	<	<	0.47	0.14	2.4	<		0.15	<
PCB 99	0.1	<	<	<	<	<	0.24	<	0.95	<		<	<
PCB 104	0.1	<	<	<	<	<	<	<	<	<		<	<
PCB 105	0.1	<	<	<	<	<	0.14	<	0.59	<		<	<
PCB 110	0.1	<	<	<	<	<	0.30	0.14	1.6	<		<	<
PCB 114	0.1	<	<	<	<	<	<	<	<	<		<	<
PCB 118	0.1	0.11	<	<	<	<	0.40	0.11	1.8	<		<	<
PCB 123	0.1	<	<	<	<	<	<	<	0.27	<		<	<
PCB 138	0.1	<	<	0.10	<	<	0.24	0.20	1.5	<		<	<
PCB 141	0.1	<	<	<	<	<	<	<	0.30	<		<	<
PCB 149	0.1	<	<	<	<	<	0.49	<	2.8	<		<	<
PCB 151	0.1	<	<	<	<	<	0.17	<	0.92	<		<	<
PCB 153/168	0.1	0.26	<	0.13	<	<	1.0	0.12	5.5	<		0.13	<
PCB 156	0.1	<	<	<	<	<	<	<	0.22	<		<	<
PCB 157	0.1	<	<	<	<	<	<	<	<	<		<	<
PCB 158	0.1	<	<	<	<	<	0.50	0.16	3.2	<		<	<
PCB 167	0.1	<	<	<	<	<	<	<	0.19	<		<	<
PCB 170	0.2	<	<	<	<	<	<	<	0.56	<		<	<
PCB 177	0.2	<	<	<	<	<	<	<	0.56	<		<	<
PCB 180	0.2	<	<	<	<	<	0.32	<	0.90	<		<	<
PCB 183	0.2	<	<	<	<	<	<	<	0.43	<		<	<
PCB 187	0.2	<	<	<	<	<	<	<	1.6	<		<	<
PCB 188	0.2	<	<	<	<	<	<	<	<	<		<	<
PCB 189	0.2	<	<	<	<	<	<	<	<	<		<	<
PCB 194	0.2	<	<	<	<	<	<	<	<	<		<	<
PCB 199	0.2	<	<	<	<	<	<	<	<	<		<	<
PCB 203	0.2	<	<	<	<	<	<	<	<	<		<	<

Table 2. (continued) Results for organochlorine pesticides and polychlorobipenyls expressed in ng/g product. Empty spaces, as PCBs in orange juice, indicate that the parameter was not determined in this sample.

compound	limit of detection	cheese 1	cheese 2	cheese 3	scottisch cheese	cottage cheese	butter	eggs	milk	bacon	sausages	chicken breast	frankfurthers	reindeer beef
	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g
Brominated flame retardents														
BDE-17	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-28	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-32	0.05	<	0.07	<	0.06	<	<	<	<	0.06	<	0.08	0.06	0.06
BDE-35	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-37	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-47	0.05	0.75	0.3	0.43	0.82	0.29	<	<	<	0.27	0.41	0.26	0.33	0.26
BDE-49-71	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-66	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-75	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-77	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-85	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-99	0.05	0.10	<	<	0.15	<	0.15	<	<	<	<	<	0.05	0.06
BDE-100	0.05	<	<	<	0.12	<	<	<	<	<	<	<	<	<
BDE-119	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-126	0.05	<	<	<	<	<	0.21	<	<	<	<	<	<	<
BDE-138	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-153	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-154	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-156	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-166	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-181	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-183	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-184	0.05	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-190	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-191	0.1	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-196	0.2	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-197	0.2	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-206	1	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-207	1	<	<	<	<	<	<	<	<	<	<	<	<	<
BDE-209	5	<	<	<	<	<	<	<	<	<	<	<	<	<
Perfluorinated chemicals														
PFOA	0.2													
PFNA	0.2													
PFDA	0.2													
PFUnA	0.2													
PFDoA	0.2													
PFTrA	0.2													
PFOS	0.2													
PFOSA	0.2													
Phthalates														
DMP	1	<	<	<	<	<	<	<	<	<	<	4.9	<	<
DEP	1	<	<	<	<	<	5.6	<	<	<	<	24	<	<
DIBP	10	<	<	4400	<	1500	<	<	<	<	<	2300	<	<
DBP	10	<	76	190	200	780	132	<	<	<	<	760	<	<
BBP	1	21	32	50	9.9	25	17	<	<	2	<	11	<	<
DEHP	20	910	3000	210	130	890	770	<	<	1320	640	670	20	210
DINP	20	31	26	59	<	660	<	<	<	<	<	390	<	<
DIDP	20	<	<	<	<	<	<	<	<	<	<	<	<	<
Alkylphenols														
NP	2	<	<	<	<	<	5.1	<	<	5.5	<	<	<	<
OP	2	<	<	<	<	<	<	<	<	<	<	<	<	<
Artificial musks														
AHTN	0.1													
HHCB	0.1													
MK	0.1													
MX	0.1													
Organotin compounds														
MBT	0.2													
DBT	0.2													
TBT	0.2													
MOT	0.2													
DOT	0.2													

Table 3. Results for brominated flame retardants, perfluorinated chemicals, phthalates, alkylphenols, artificial musks and organotin compounds expressed in ng/g product. Empty spaces, as for the artificial musks, indicate that the parameter was not determined in this sample.

compound	limit of detection	minced beef	pork chops	salami	ham	fish fingers	salmon	tuna	pickled herring	honey	orange juice	brown bread	olive oil
	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g
Brominated flame retardents													
BDE-17	0.1	<	<	<	<	<	<	<	<	<		<	<
BDE-28	0.1	<	<	<	<	<	<	<	<	<		<	<
BDE-32	0.05	0.07	0.05	0.06	0.09	<	<	<	0.08	0.15		<	<
BDE-35	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-37	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-47	0.05	0.78	0.52	0.81	0.78	<	<	0.39	0.35	<		0.55	<
BDE-49-71	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-66	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-75	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-77	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-85	0.05	<	0.32	<	<	<	<	<	<	<		<	<
BDE-99	0.05	0.12	<	<	0.10	<	<	<	<	<		0.19	<
BDE-100	0.05	0.11	<	<	<	<	<	<	<	<		<	<
BDE-119	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-126	0.05	0.08	<	<	<	<	<	<	<	<		<	<
BDE-138	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-153	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-154	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-156	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-166	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-181	0.05	0.18	<	<	<	<	<	<	0.10	<		<	<
BDE-183	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-184	0.05	<	<	<	<	<	<	<	<	<		<	<
BDE-190	0.1	<	<	<	<	<	<	<	<	<		<	<
BDE-191	0.1	<	<	<	<	<	<	<	<	<		<	<
BDE-196	0.2	<	<	<	<	<	<	<	<	<		<	<
BDE-197	0.2	<	<	<	<	<	<	<	<	<		<	<
BDE-206	1	<	<	<	<	<	<	<	<	<		<	<
BDE-207	1	<	<	<	<	<	<	<	<	<		<	<
BDE-209	5	<	<	<	<	<	<	<	<	<		<	<
Perfluorinated chemicals													
PFOA	0.2					<	<	<	<			<	
PFNA	0.2					<	<	<	<			<	
PFDA	0.2					<	<	<	<			<	
PFUnA	0.2					<	<	<	<			<	
PFDoA	0.2					<	<	<	<			<	
PFTrA	0.2					<	<	<	<			<	
PFOS	0.2					<	<	<	1.3			<	
PFOSA	0.2					<	<	<	0.2			<	
Phthalates													
DMP	1	<	<	<	1.1						<		<
DEP	1	<	<	<	<						<		<
DIBP	10	<	<	165	400						<		<
DBP	10	<	170	183	260						<		<
BBP	1	<	13	12	17						<		340
DEHP	20	290	140	160	3300						<		24000
DINP	20	<	470	<	230						<		72
DIDP	20	<	<	<	<						<		<
Alkylphenols													
NP	2	<	<	<	<						<		<
OP	2	<	<	<	<						<		<
Artificial musks													
AHTN	0.1					<	<	0.18	0.29				
HHCB	0.1					<	<	0.27	0.56				
MK	0.1					<	<	<	<				
MX	0.1					<	<	<	<				
Organotin compounds													
MBT	0.2					0.5	<	9	<				
DBT	0.2					<	<	1.1	0.6				
TBT	0.2					<	<	0.2	0.8				
MOT	0.2					0.3	<	<	0.8				
DOT	0.2					<	<	<	1.2				

Table 3. (continued) Results for brominated flame retardants, perfluorinated chemicals, phthalates, alkylphenols, artificial musks and organotin compounds expressed in ng/g product. Empty spaces, as for the brominated flame retardants in orange juice, indicate that the parameter was not determined in this sample.

4. Conclusions

In this study the concentrations of a number of typical man-made chemicals in food or food products were determined. The compound groups of interest were organochlorine pesticides, polychlorinated biphenyls, brominated flame retardants, phthalates, alkylphenols, artificial musks, perfluorinated compounds and organitin compounds. The results show that many of these compounds are present food in the range of 0.1 to 10 ng/g with the exception of phthalates for which the typical concentrations are two orders of magnitude higher. Organochlorine pesticides were found in the 17 of the 25 samples. The main organochlorine pesticides found in food are p,p'-DDE, a metabolite of DDT, and HCB in concentrations up to 5.6 ng/g. Polychlorinated biphenyls were found in all samples with predominance for PCB-18, -28 and -52. The sum of the indicator-PCBs ranged from 0.16 to 13 ng/g and total PCBs up to 32 ng/g. The highest concentrations were found in fish. Brominated flame retardants were found in 19 of the 24 samples with predominance for the tetra- and penta-PBDEs, especially BDE-47, -32 and -99. Total PBDE concentrations ranged from 0.15 to 1.2 ng/g with the highest concentration found in meat and not in fish as in other studies. BDE-209, HBCD and TBBPA were not found in any of the samples. The prefluorinated compounds PFOS and PFOSA were found in one of the four samples analysed, a fish sample, in concentrations of 1.3 and 0.2 ng/g. The predominant phthalates in food were DEHP, DBP and BBP. Phthalates were found in 12 of the 19 samples. DEHP concentrations ranged from 20 to 24,000 ng/g, the latter for a sample of olive oil, with a median concentration of 640 ng/g. Median concentrations for DBP and BBP were 200 and 17 ng/g. Alkylphenols were detected in 2 of the 19 samples, in both cases nonylphenol in concentrations around 5 ng/g. Of the artificial musks the polycyclic musks HHCB and AHTN were found 2 of the 4 samples in concentrations up to 0.56 ng/g for HHCB. As in other matrices the AHTN concentrations are about half those of HHCB. Organotin compounds were found in three of the five samples. Apart from TBT and its metabolites DBT and MBT, two samples also contained octyltin compounds.

5. Acknowledgement

We kindly acknowledge the support of the WWF in the execution of this study.

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6

Pesticide Residues in Bee Products

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1. Introduction

"The man has no divine right over the food. He must compete for this with weeds, diseases, insects and other organisms" (Grodner, 1996).

With the above quote, Grodner clearly reflects the situation in the area production and processing of food. More than 10.000 species of insects and mites, 1.500 species of fungi and 600 plant species have been identified as harmful to agriculture (Grodner, 1996; Pimentel et al., 2000). The production of plant and animal products requires the use of large quantities of chemicals (plant protection agents, veterinary drugs, fertilizers, etc.), which could lead to increased production and improved quality, as a consequence. The quality of the final product is usually reflected to particular visual parameters like color, size and general appearance. In the case of food, however, many questions are raising about their safety. The reason is the possible presence of chemical residues detected in the final product. Nowadays, consumer safety is a major priority for governments of developed countries and food safety is a criterion for the trading and prices on the market. Reports in media about alimentary scandals cause anxiety to consumers and turning a part of the market to organic products, which are considered free, or at least less contaminated by hazardous substances. In recent years, reports in media have grown at an alarming rate and any references to the consumer aimed at creating impressions, achieved by overemphasizing the disadvantages of chemical use and mainly problems related to environmental pollution and its impact on human health. In contrast, reports in media referring to the advantages of using chemicals are minimal to nonexistent. For example, the absence of appropriate chemical agents to combat rodents lead to the first epidemic of the bubonic plague disease and the death of 65.000.000 people. Moreover, the starvation in Ireland began due to the fungal disease *Phytophthora infestans*, which destroyed potatoes causing 1.000.000 deaths from 1845 to 1851 (Knutson et al., 1990). Besides health problems that preoccupied humans in the past, the economic impact on different groups of consumers will be significant in case of pesticides withdrawal. Specifically, the weekly expenses for food are expected to rise by 44% for consumers of low average income. The economies of countries with intensive agriculture will be stroke because of the decline in exports of grains and products like cotton. Finally, undesirable environmental effects are expected due to the increasing of cultivated land and the erosion problems that could be observed because of the limited growth of the root

system of plants (Knutson et al., 1990). In contrast to the above, there are few cases of pesticides proved dangerous to public health. A typical example is the chlorinated hydrocarbon DDT, which was previously used against mosquitoes. The active substance DDT contributed greatly to reduce the spread of diseases like malaria, but was withdrawn in 1970 as it was considered dangerous for human and environment safety.

Active substances are classified into five groups according to their toxicity. The first group (Ia) includes the extremely toxic agricultural plant protection agents, while the four other groups of substances are listed in order of decreasing toxicity (Ib, II, III). The fifth group (U) includes substances that are unlikely to become toxic to humans (International Programme on Chemical Safety, 2004). The adverse effects of these compounds may be observed in a short period (acute toxicity) or after a long time (chronic toxicity). In any case, it should be noted that, although the annual reported number of deaths by poisoning is 355.000, only a part of these poisonings, which is not specified in the World Health Report, are due to pesticides (WHO The World Health Report, 2003). Moreover, all cases of poisoning are due to accidents, like accidental ingestion or inhalation of chemicals, and not by food intake. However, the possibility of indication of various effects in consumer health through chronic toxicity cannot be ignored. Toxicity of a plant protection product depends on various factors, including chemical structure, temperature and humidity conditions, dose, duration of exposure, mode of action and the kind of exposure, like ingestion, inhalation, dermal etc. Different groups of pesticides and veterinary drugs are likely to be responsible for causing malaise, sore eyes, abnormalities to skin and respiratory system. Moreover, some pesticides and veterinary drugs are suspected of causing certain types of cancer, teratogenicity, chromosomal abnormalities and the weakening of the immune system of humans (Banerjee, 1999). The toxicity of various active substances, which can be detected in bee products, varies according to their chemical synthesis. In any case, poisoning or deaths due to the presence of toxic substances exclusively to bee products have not been reported. An exception is the death of infants, which was caused by *Clostridium botulinum* (Arnon, 1980). Even in these cases, however, the responsibility of honey has not been proven clearly, as this clostridium appears in the environment widely (Midura, 1996). In addition, 65% of infants that became ill had not eaten honey at all (Arnon et al., 1979). In any case, appropriate infant feeding prohibits the consumption of honey until the age of one year to eliminate possible poisoning from toxins of this micro-organism.

Another way of classification of active substances, relates to the subject of this investigation, and is based on bee toxicity. In general, active substances are classified as very toxic, moderately toxic and non-toxic to bees. The most significant impact on bee colonies has been observed after treatments with plant protection products during the blooming. Most deaths occurred during the stage of forager worker bee that collects nectar and pollen. Moreover, larvae and domestic bees die because of pesticide residues detected in pollen. Although in many cases the concentration of pesticide found in pollen is not lethal, it is however likely to cause paralysis of bees, irritability, killing and replacing of the queen bee and generally abnormal behavior. This behavior can also be caused by substances that do not kill bees directly (e.g. carbaryl as active ingredient of Sevin®) but are transferred into the hive by foragers and affect the entire population (Sanford, 1993). The long–term persistence of many pesticides in stored pollen has also serious impact of bees' survival. Arsenic from paris green and calcium arsenate was present in pollen stored in comb analyzed six months after application. Methomyl residues persisted in honeybee combs for eight months. Methyl parathion from Penncap M, persisted in combs samples of stored pollen for 7 to 14 months after use and carbaryl similarly persisted over winter for 7-9 months (Erickson et al., 1983)

Neonicotinoids like imidacloprid were also detected in stored pollen (Gregorc & Bozic, 2004; Chauzat et al., 2006). Also, the type of formulation and application of the pesticide in relation to toxicity caused to the bees proved particularly important. For example, the standardization of the active ingredient methyl parathion in microcapsules kills 13 times more bees than the formulation of the same substance as an emulsified solution. Furthermore, wettable powder or dust proved less dangerous than microcapsules, but also caused more deaths than aqueous or emulsified solutions (Sanford, 1993).
Under the development of framework concerning consumer safety, the European Union created a warning system called RASFF (Rapid Alert System for Food and Feed), which reports hazardous foods and feeds, identified in the markets of the Member States. Bee products like honey and royal jelly have been reported occasionally. The main reason why they have been reported is the detection of residues of antibiotics that have been used by beekeepers to fight various diseases of bees.

2. Contamination of bee products

There are two ways of contamination of bee products with various chemicals; the indirect and the direct contamination. The indirect way reflects the transporting of toxic substances by foragers bees during the collection of nectar, honeydew, water, pollen and propolis. Many studies concern the contamination of hive products by agrochemicals and heavy metals, while few concern the presence of nitrofurans, toxins and PCBs in beehive products. The direct way, which is the most important, regards the contamination of bee products by acaricides, antibiotics and volatile pesticides caused by beekeeping practices.

2.1 Indirect contamination of bee products

Many researchers supported the theory that the transferring of pesticides from fields to beehive is prevented in various ways. The bees' death at the field, the lost of orientation of the foragers, the reluctance of guard bees to permit the entrance to foragers with contaminated nectar, the retaining of contaminated food in bees' stomach, the stopping of further elaboration of contaminated nectar by hive bees and the removal of affected bees from the hive are natural provisions against general contamination of honey (Johansen & Mayer, 1990; Atkins, 1992). Contrary to the above-mentioned cases, older studies reported that worker bees may carry high concentrations of pesticides into their beehive. In some cases the concentration of pesticides in the load was 25 times greater than the lethal dose of the bee (Jaycox, 1964).

2.1.1 Pesticides

Pesticides used on various crops are classified into groups based on their chemical structure (organophosphates, pyrethroids, organochlorines, carbamates, neonicotinoids etc.), mode of action (systemic, contact), target (insecticides, acaricides, herbicides, fungicides, bactericides, nematicides) and synthesis (synthetic or natural). The residues of pesticides detected in beehive products are classified in the groups of insecticides (organochlorines, organophosphates, carbamates and neonicotinoids), acaricides, fungicides and herbicides.

2.1.1.1 Organochlorine pesticides (OCPs)

This specific group of insecticides is considered particularly hazardous because of its ability to bioaccumulate into the food chain, to remain stable for many years and to move into the

environment in every potential way (air, water, soil, biota). The case of bioaccumulation of DDT in the environment is the most characteristic, while the concentration detected in the higher levels of the food chain is 10,000,000 times greater than that detected into the water. In recent decades there had been many efforts worldwide to prevent the use of substances belonging to the group of persistent organic pollutants (POP), which includes many organochlorine compounds. The continuous transfer of semi-volatile compounds from tropical regions of the world to the colder poles is suspected for long-term effects on living beings (Carson R., 1962). Chlorinated hydrocarbons are detected in high concentrations in various products, because of their low rate of degradation. Wax is the beehive product more likely to be contaminated by organochlorine insecticides because of its strong lipophilic character. Moreover, OCPs were proved to remain stable during the conversion of old combs into new (Jimenez et al., 2005). The problem is magnified by the import of wax from continents where the use of chlorinated hydrocarbons is still permitted like Asia and Africa. The encouraging news is that the percentage of honey contaminated with chlorinated hydrocarbons dropped from 96.1% to 52.3% during the decades 1980 and 1990.

2.1.1.2 Organophosphorus pesticides (OPPs)

This specific class of pesticides is of relatively high toxicity for humans and was first studied and used as an asphyxiating gas during the Second World War. Organophosphorus compounds are not stable in the environment and are not bio-concentrated and this is probably the main reason why they were detected rarely and at lower concentrations into beehive products. Most of published information concerns the compound methyl parathion, which has been used in agricultural crops as preparation in the slow-release form of microcapsules. Residues of this chemical were detected in honey and pollen (Atkins & Kellum, 1984). The microcapsules stick on the dense coat of bees, transferred into their hive and stored along with pollen. Pollen is the main component of the diet of larvae. The presence of polluted pollen might cause poisoning and eventually death to brood of bees. In a survey conducted on pollen from France, residues of parathion and methyl parathion were found in 1.2% and 4.9% of the samples, respectively. The average concentration for parathion was 0.019 mg kg^{-1} and for parathion methyl was 0.025 mg kg^{-1} (Chauzat et al., 2006). Blasco et al. (2003) detected only heptenophos in 4% of honey samples that were analyzed, out of 23 organophosphorus pesticides that they researched. Heptenophos concentrations in this survey ranged from 0.08 mg kg^{-1} to 0.23 mg kg^{-1}. Finally, Balayiannis and Balayiannis (2008) detected the organophosphorus compounds chlorfenvinphos, chlorpyriphos and phorate in honey originated from Greece, in concentrations ranged from 0.7 μg kg^{-1} to 0.89 μg kg^{-1}.

2.1.1.3 Carbamate pesticides

Carbamate insecticides have a similar mode of action with organophosphates but their insecticidal activity is more selective and depends to a certain extent on the insect species. Some fungicides and herbicides belong to this family. These substances are highly volatile in the environment and in some cases they were detected in beehive products. Concentration of carbamate residues detected in pollen ranged from 0.126 mg kg^{-1} to 0.265 mg kg^{-1} for the active ingredient carbaryl, while the maximum concentration of carbofuran was 0.14 mg kg^{-1} (Chauzat et al., 2006). Concentration of carbaryl, carbofuran, pirimicarb and methiocarb residues, in most cases is considered low and does not exceed 0.071 mg kg^{-1}. In only one Spanish honey the concentration of carbofuran was 0.645 mg kg^{-1} (Blasco et al., 2003).

Nevertheless, the concentration of carbamate residues is low in pollen and honey, while no residues have been reported in other beehive products.

2.1.1.4 Neonicotinoid pesticides

Most studies reported on neonikotinoids insecticides, refer to the active ingredient imidacloprid. This substance was proved toxic to bees, but the concentrations of residues detected in honey were very low (0.001 mg kg^{-1} to 0.005 mg kg^{-1}) (Bonmatin et al., 2003; Maus et al., 2003; Schmuck et al., 2001). In many cases residues did not exceed the limit of quantification (<0.002 mg kg^{-1}) (Rogers & Kemp, 2003; Schöning & Schmuck, 2003; Stadler et al., 2003; Faucon et al., 2004). Detected residues of imidacloprid in pollen were 0.005 mg kg^{-1}, while detection rate was 49.4% (Bonmatin et al., 2003). The hazard quotient (application rate in grams per hectare/LD_{50}) of neonicotinoids is far below the trigger value of 50, but the most important is the chronic toxicity that they cause to bees. The long-term exposure to neonicotinoid after the behaviour of bees, reduce their reproduction capacity and lead of population decline. The low detectable concentrations in combination with the low toxicity of imidacloprid in humans are reassuring for consumer safety. On the contrary, the effects of imidacloprid residues on bees should be further explored. The high toxicity for bees makes neonicotinoid residues suspicious about the death of many forager bees collecting nectar from sunflower, corn and cotton crops. The implementation of neonicotinoid active substances in seed of plants like cotton, corn and sunflower led to a theory that this specific class of pesticides is responsible for the "colony collapse disorder" syndrome (CCD). The CCD is defined as the sudden depopulation of a beehive and the rapid collapse of the colony. The causes of this phenomenon are not clear yet. The suspicion is directed at the mite *Varroa destructor* Anderson & Trueman, while others blame the protozoan *Nosema ceranae*. Additionally, suspicion directed at poisoning of bees by neonicotinoid insecticides and at various forms of radiation (telephony, wireless networks etc.) as well. In fact, research on the toxicity of neonicotinoids to the bee, proved tolerance of the insect body at normal concentrations identified in honey, pollen and nectar (Schmuck et al., 2001; Faucon et al., 2004). More recent research is directed at the effect of imidaclorpid to the orientation of bees (Bortolotti et al.; 2003).

2.1.1.5 Fungicides

Fungicides are toxic substances that are used to kill or inhibit the growth of fungi that cause economic damage to crops and endanger the health of domestic animals or humans. Most fungicides are toxic to humans and can cause both acute and chronic problems if absorbed into food. Kubik et al. (1999; 2000) studied the possibility of contamination of beehive products with residues of chemicals used on apple and cherry trees. Vinclozolin, iprodione and thiophanate methyl residues were detected in honey and pollen collected from cherry flowers, while captan and difenuconazole were detected in beehive products collected from apple trees. Specifically, the average concentration levels of vinclozolin in honey were determined at 0.107 mg $kg^{-1.}$ A recent review of vinclozolin by the US Environmental Protection Agency has concluded that the chemical or its breakdown products are associated with the development of testicular tumors in rats. The mean concentrations of residues of other active compounds were 0.0006 mg kg^{-1}, 0.009 mg kg^{-1}, 0.023 mg kg^{-1} and 0.059 mg kg^{-1} for captan, difenuconazole, iprodione and methyl thiophanate respectively. In all cases, the concentration was lower in honey, than in stored pollen (Kubic, 2000).

2.1.2 Antibiotic residues due to agricultural use

Antibiotics can find their ways to bee products not only from beekeepers but also from the environment. Bees collect and transfer readily in their hive bactericides that are used against *Erwinia amylovora*. Out of 166 Greek citrus honeys that had been analyzed the 146 of them were found having antibiotic residues of soulphonamides and streptomycine originating from the therapeutical products that had been used in citrus plants (Karampournioti, 2004). Similarly in South Germany 40 samples out of 183 (21%) were found having residues of that source (Wallner, 1998). Moreover, Brasse (2001) identified the antibiotic streptomycin in 27 out of 128 honey analyzed samples. Bees may also transfer antibiotics through water since sulphanimide and tetracyclines are used in drinking water from poultry farms, rabbit cages and other animals. The manure of pigs and cows treated with sulphonamides or sulpha-compounds could also be the vector. Some herbicides products, like Asulan may be degradated to sulphanilamide and bees with nectar can transfer it into the hive (Bogdanov & Edder, 2004; Kaufmann & Kaenzig, 2004). Finally bees may rob honey from colonies of other apiary that had been treated by antibiotics and by this way can contaminate their product in detectable levels.

2.2 Direct contamination of bee products

Active substances used by beekeepers themselves are likely to contaminate bee products with undesirable residues. Acaricide and antibiotic preparations are used in order to control the mite *Varroa destructor*, American foulbrood, Nosemosis and other diseases. Moreover, several volatile insecticides were used in the warehouse, in order to fend lepidopteron *Galleria mellonella* Linnaeus, which is responsible for considerable damages to stored combs.

2.2.1 Acaricide residues

The use of synthesized substances for crop protection and livestock is the easiest and most effective way for beekeepers to control mites. Acaricides like amitraz, cymiazole, bromopropylate, tau-fluvalinate, flumethrin, coumaphos and malathion have been used by beekeepers all over the world. Many preparations like Apistan (a.i. tau-fluvalinate), Perizin (a.i. coumaphos), CheckMite+ (a.i.coumaphos), Bayvarol (a.i. flumethrin) and Apiguard (a.i. thymol) gain approval in most European countries. There are substances like amitraz that got approval only in certain countries and others like malathion that have not been approved at all.

2.2.1.1 Amitraz

Structure: It belongs to the group of formamidines
Action: Non-systemic insecticide and acaricide, which causes stimulation of neuronal activity killing the target.
Preparation: The main commercial formulation is the Taktik, used in livestock and particularly horses and sheep. Other preparation used: Mitak and Bye Bye.
Ways to use in beekeeping: Fumigation, Spray.
Acceptable Daily Intake (ADI): 0.003 mg kg^{-1} body weight per day or 0.18 mg per person per day (EMEA, 1999).
MRL for honey: EU established maximum residue levels (MRL) for amitraz residues in honey. The MRL of amitraz established to 0.2 mg kg^{-1} including the parent compound and its metabolites containing 2,4-dimethylaniline moiety. It should be noted that despite the establishment of the MRL, amitraz residues in honey are not acceptable in some countries

because of the lack of approval for beekeeping use. Therefore, in this case the limit corresponds to the Limit of Quantification, which is 0.01 mg kg^{-1}.
The use of active substance amitraz is widespread in several European countries and the United States. Moreover, the effectiveness of this substance against varroa is satisfactory. The residues of the active substance is not often detected because of the rapid degradation of amitraz, which takes place within three weeks in blossom honey and four weeks in honeydew honey. The difference in degradation interval was attributed to the lower pH of blossom honey, which accelerates the chemical reactions of decomposition (Corta et al., 1999). The active ingredient amitraz is usually detected in cases where the preharvest interval is very short. A study reports as final degradation product of amitraz in honey, the 2,4-dimethyl-aniline, which is classified as hazardous to public health (Taccheo et al., 1988a). Recently, several samples of pears found to contain significant concentrations of amitraz and its metabolites. This fact, as well as indications about carcinogenic effects of the substance, led to a series of inspections and repeated alerts reported on RASFF of EU (Rapid Alert System of Food And Feed). To date, no published RASFF on residues of amitraz in honey have been reported. Finally, a reference work published in USA, observed the development of resistance of varroa to amitraz (Eljen et al., 2000).

2.2.1.2 Coumaphos

Structure: It belongs to the group of organophosphorus insecticides-acaricides
Action: Substance with systemic action that causes death in insects and mites by affecting cholinergic synapses of the central nervous system.
Preparations: The active ingredient coumaphos prepared by Bayer as three different formulations; Perizin, CheckMite+ and Asuntol. The last is the only one without authorization for beekeeping use.
Ways to use in beekeeping: The active substance used as an aqueous solution or a controlled release film. Perizin is used as an aqueous solution applied as drops between the frames. Spraying or adding to food can also be used for the application of this preparation. Special mention should be made to the use of coumaphos in the form of controlled release strips (CheckMite+). Primarily, application of CheckMite+ took place in the U.S., by providing a limited number of films in beekeepers of every State (Sanford & Flottum, 1999). In Europe, CheckMite+ was granted authorization in 2006. The major advantage of this preparation is that it also controls the small hive beetle *Aethina tumida* Marey.
ADI: 0.25 mg kg^{-1} body weight per day or 15 mg per person per day (EMEA, 2001).
MRL for honey: the established MRL for coumaphos in the EU is 0.1 mg kg^{-1}.
Coumaphos does not control mites exclusively through contact, like most acaricides used in beekeeping, but it has a systemic action as well. The advantage of this way of action is the greater efficacy, and the rapid dispersion throughout the whole area of the hive. However, the disadvantage of substances with systemic action like coumaphos is the great persistence. According to a study, bees produce wax with residues of coumaphos, even six months after the application of the substance into the hive (Wilhelmina, 1992).
The persistence and dispersion of coumaphos in the hive after the application of CheckMite+ was studied by Karazafiris et al. (2008). According to that study, concentration of coumaphos residues was great in honey frames, which were in contact with strips. In some cases, residues exceeded the value of the established MRL. Moreover, it was observed that the concentration of acaricide in honey was at the level of MRL even 103 days after the removal of the strips. Therefore, the exclusion of frames that are in contact with the strips

could lead to a drastic reduction of residual coumaphos concentrations in the final product. On the contrary, it was observed that the concentration of coumaphos residues in honey chamber was significantly lower and in no case exceeded the MRL. Finally, the time between application of the preparation and the collection of honey affected the amount of residues. Gajduskova et al. (1990), studied the contamination of bee products under different methods of application, found that higher concentrations of coumaphos levels were recorded when the substance was added to the syrup rather than the usual method of dripping the chemical into the hive. Coumaphos proved very stable in honey, moved quickly to the wax because of its strong lipophilic character and remained there in significant concentrations even after melting of the wax (Krieger, 1991). Reports that mites became resistant to coumaphos have already been published (Maggi et al., 2009; Petis, 2004).

2.2.1.3 Flumethrin

Structure: It belongs to the group of pyrethroids.
Action: Non-systemic insecticide-acaricide which acts in contact through the stomach. As the majority of pyrethroid pesticides, flumethrin is characterized as a broad range pesticide presenting low toxicity to mammals. Through its action, flumethrin disrupts the functioning of the Na^+ pump and therefore the equilibrium of Na^+/K^+ across the membrane.
Preparation: Bayvarol is one of the approved preparations for use in beekeeping. Production Company is the Bayer CropScience.
Beekeeping use: Flumethrin applied in the form of controlled release strips.
ADI: 1.8 mg kg^{-1} body weight per day or 108 mg per person per day (EMEA, 1998).
MRL for honey: The very low concentration required per hive and the low water solubility, are the main reasons why no detectable residues were detected in honey after the recommended use. That is the reason why no MRL has been established for this substance (EMEA, 1998). According to recent studies, mites became resistant to pyrethroids (Milani, 1995; Thompson, 2003).

2.2.1.4 Tau fluvalinate

Structure: It belongs to the group of pyrethroids.
Action: This is a broad range non-systemic insecticide-acaricide that acts by contact through stomach. The way of action of tau fluvalinate is similar to that of the flumethrin.
Preparation: There are three preparations used by beekeepers containing tau fluvalinate: Apistan, Mavrik and Klartan. Out of the three, only Apistan has an approval for beekeeping use.
Beekeeping use: The use of the authorized preparation is in the form of controlled release strips (Apistan).
ADI: 0.5 mg kg^{-1} body weight per day or 30 mg per person per day (EMEA, 1998).
MRL for honey: EU established no MRL for tau fluvalinate residues in honey, as the concentrations of detected residues were extremely low, based on the experimental results included in the file submitted (<0.01 mg kg^{-1}) (EMEA, 1998).
The use of Apistan strips is likely to lead to accumulation of residues, if they have been left in the hive for more than 6 weeks. Balayiannis and Santas (1989) reported an increased persistence of residues in stored honey, compared with the honey in combs. This is apparently owed to the non-transfer of tau fluvalinate in wax. Tau fluvalinate is the most lipophilic of all compounds used in beekeeping. This property combined with the high stability of the substance in the wax contributes to the drastic increase in the concentration

of residues in the honeycombs (Tsigouri et al., 2004). Tau fluvalinate has been used by beekeepers for many decades. Nowadays, the beekeepers have stopped using it because the mites developed resistance towards this chemical. (Elzen et al., 2000; Milani et al., 1995; Thompson et al., 2003).

2.2.1.5 Bromopropylate

Bromopropylate is one of the oldest compounds that had been used against Varroa under the commercial product FOLBEX-VA. Its use was totally abandoned in Switzerland in 1991. An analysis made 19 years later showed that bromopropylate was still in beeswax in high concentrations and scientists believe that more than 20 years will pass before it is expected to fully disappear from beeswax.
Structure: It belongs to the group of chlorinated derivatives of benzene.
Action: bromopropylate is a broad spectrum, non-systemic insecticide-acaricide with high residual activity, which acts by contact and inhibits the synthesis of ATP.
Preparations: The name of the commercial preparation is Folbex VA, manufactured by Giba-Geigy.
Beekeeping use: The use of this preparation is in the form of fumigant.
ADI: 0.03 mg kg^{-1} body weight per day or 1.8 mg per person per day.
MRL for honey: no MRL exists for this chemical at E.U. Bromopropylate was used in crops such as pome fruits, stone fruits and plants of Solanacea family. The agricultural use has led to an establishment of MRL under the provisions of EU Regulation 396/2005. This new MRL corresponds to a concentration of 0.1 mg kg^{-1} and concerns the contamination of bee products through the use of agricultural pesticides.
Bromopropylate is not toxic to bees, while its metabolite 4,4-dibromobenzolic acid is likely to be detected. The use of bromopropylate was particularly widespread in Central Europe. The observation that bromopropylate degradation is slow and, therefore very stable in honey and wax, forced Europeans beekeepers to start using acaricides that are more environment and consumer friendly. According to a survey conducted by Taccheo et al. (1988b), concentration of bromopropylate residues was greater in honey from an uncapped comb than from a capped one. Moreover, the burning of fumigant strips in an empty floor above the hive reduced the concentration of residues in honey (Taccheo, 1988b). The use of bromopropylate has stopped in many countries and no samples with residues of bromopropylate were found in Greek honey (Karazafiris et al., 2007).

2.2.1.6 Malathion

Structure: It belongs to the group of organophosphorus compounds.
Action: malathion is broad spectrum, non-systemic insecticide-acaricide which acts through stomach. The way of action is similar to that of the organophosphate acaricide coumaphos. In addition, malathion oxidized to malaoxon, which is a substance with high toxicity to insects and mites. This chemical reaction does not occur in the body of mammals, limiting the toxicity of the acaricide for humans.
Preparations: Malathion
Beekeeping use: Malathion has never had approval for beekeeping use. Despite this, many beekeepers use it as spraying material or as powdered sugar. Due to the high bee toxicity, malathion requires special attention during application. A slightly increased dose can be fatal for the colony, especially when used as a solution.

ADI: 0.03 mg kg^{-1} body weight per day or 1.8 mg per person per day.
MRL for honey: No MRLs have been established in honey, and therefore the threshold corresponding to the LOQ is 0.01 mg kg^{-1}.
Two different studies were conducted by Thrasyvoulou et al. (1988) and Balayiannis et al. (1989) concerning the time of degradation of malathion in honey. Both two studies proved that the time of degradation of malathion is three months. In a survey conducted in 50 samples of Greek honey, 4% were found contaminated in concentrations that did not exceed 0.005 mg kg^{-1} (Thrasyvoulou et al., 1988). Futhermore, malathion was detected in 23 out of 593 honey samples analyzed in the laboratory of Apiculture-Sericulture, Aristotle University of Thessaloniki during the years 2003-2006 (Karazafiris et al., 2005). In Cuba, Pelayo et al. (1987) detected malathion in 12 out of 110 samples. The concentration of the active substance did not exceed 0.02 mg kg^{-1} in any case.

2.2.1.7 Cymiazole

Structure: It belongs to the group of iminophenyl thiazolidine.
Action: Cymiazole is another substance, like coumaphos, that used in beekeeping and has systemic action.
Preparation: The name of the commercial preparation is Apitol and is manufactured by Giba-Geigy.
Beekeeping use: Cymiazole can be applied in different ways.
ADI: 1 mg kg^{-1} body weight per day or 60 mg per person per day (EMEA, 1996).
MRL for honey: MRL that was established for cymiazole was 1 mg kg^{-1}, but latest E.U. regulations established new MRL that corresponds to LOQ (0.01 mg kg^{-1}).

2.2.2 Antibiotic residues

The term antibiotic originally refers to any agent with biological activity against living organisms; however, ‘‘antibiotic” nowadays refers to substances with antibacterial, anti-fungal, or anti-parasitical activity. There are currently about 250 different substances registered for use in medicine and veterinary medicine (Kuïmmerer & Henninger, 2003).
Antibiotics such as tetracycline, chloramphenicol, sulfathiazole, streptomycin, tylosin, erythromycin etc, are commonly used by beekeepers, in order to control European Foulbrood Disease (EFB), American Foulbrood Disease (AFB) and Nosemosis caused by *Paenibacilus larvae larvae*, *Streptococcus pluton* bacteria and fungus of the genus *Nosema*, respectively. The use of antibiotics is not allowed in beekeeping since no MRLs have been set for honey. Some countries, like Switzerland, UK and Belgium, have established action limits for antibiotics in honey, which generally lie between 0.01 to 0.05 mg kg^{-1} for each antibiotic group. An action limit is the concentration of antibiotics in honey, above which the sample is considered non-compliant. The presence of antibiotic residues in honey and other hive products is not accepted in Europe for products imported from third countries. In case a product is found contaminated with antibiotics then it should be destroyed and the producer should be penalized. In the U.S.A., Canada and Argentina, preventive treatments with antibiotics are considered a routine procedure to control AFB. As a result, various strains of *P. larvae* have developed resistance to antibiotics, such as oxytetracycline (OTC). Such strains have been isolated in Argentina (Alippi, 2007) as well as in many areas of the U.S.A. (Miyagi et al., 2000). Generally, the presence of antibiotics in the environment

especially in foods may lead to the rapid emerge of resistant bacterial strains and consequently to the demand of new substances to replace the old. Moreover, the emergence of resistant bacteria involves the use of powerful antibiotics leading to serious consequences in the normal flora of the human body.

2.2.2.1 Chloramphenicol

Chloramphenicol is a potent antibiotic that has limited uses; it has been declared carcinogenic and causing fatal aplastic anemia, which makes it an unacceptable substance for use in production of food products where any residue may be found. Several reports document human fatalities resulting from ophthalmic preparations containing chloramphenicol, with exposure dozes that could be found in residues in food (Settepani, 1984). Chloramphenicol was detected in bee products, honey and royal jelly, imported from China and India. In 2002, alerts appeared from the U.S., Canada, and Europe that honey samples from China often contained traces of the antibiotic chloramphenicol with a range of 0.3 to 34 μg kg^{-1} (LOD= μg kg^{-1}). Since China did not have stringent controls on veterinary use of various antibiotics, this drug had been used (along with streptomycin) by the Chinese beekeepers to control a bacterial epidemic that affected bee hives (Dharmananda, 2003). The EU, in an effort to protect consumers, banned the import of Chinese products of animal origin since 2004. Also in Switzerland, chloramphenicol residues detected in thirteen out of 75 (17%) of commercially obtained honey samples, ranged between 0.4 and 6.0 μg kg^{-1} (Ortelli et al., 2004).

2.2.2.2 Tetacyclines

Tetracycline is used by beekeepers in order to control AFB and EFB. Normally, it degrades in 6-10 weeks (Matsuka & Nakamura, 1990; Gilliam et al., 1979). In some cases tetracycline was detected in honey, even after three years, because of the high dose used by beekeepers (Shakaryan & Akopyan, 1973). Acidity, viscosity and organic acids of honey contribute to the stability of antibiotics (Gilliam et al., 1979). The treatment of the hive with antibiotics results in tetracycline residues in honey and wax (Gilliam et al., 1979; Corner & Gochnauer, 1971). In two studies of Shakaryan & Akopyan (1972 & 1973), 1.2% of the initial concentration of the antibiotic residues remained stable even after the heating of honey for three successive times in 90°C (30 minutes). Tetracyclines (tetracycline, oxytetracycline, chlortetracycline, doxycycline) have been found in honey in various countries. In a study conducted in Greece, tetracycline residues were found in 23% of the spring floral honey samples tested (Karazafiris et al., 2007). In another study, out of 251 greek honey samples, 29% were found contaminated with tetracycline residues ranged from 0.018 to 0.055 mg kg^{-1} (Saridaki-Papakonstadinou et al., 2006).

2.2.2.3 Sulfonamides

The sulfonamides are analogues of para-aminobenzoic acid, which include sulfapyridine, sulfadimidine, sulfadiazine, sulfamethoxazole, sulfadimethoxin, sulfamethopyridazine, sulfadoxine, sulfamethoxypyridazine, sulfadoxine and sulfamethopyrazine. They are suspected to cause aplastic anemia, like chloramphenicol. It is the most stable antibiotic in honey (Bonvehi & Pajuelo, 1983). In the past, sulfathiazole was detected regularly in honey produced in the European countries. Beekeepers used sulfa-drugs in order to control AFB and EFB and in some cases Nosemosis.

In 2002, sulfa drugs were detected in 3 out of 91 samples of honey collected from the Belgian market. Moreover, 12 out of 203 honey samples collected in 2003 were contaminated by residues of sulfonamides (Reybroeck et al., 2004).

2.2.2.4 Streptomycin

The problem with streptomycin is that it may cause ototoxicity and nephrotoxicity. It is considered more dangerous than oxytetracycline and less hazardous than sulfathiazole and chloramphenicol regarding side effects. According to the Food Standards Agency of UK, an Indian honey was found to be contaminated by streptomycin in 2003 (Mayande, 2007).

2.2.2.5 Fumagillin

This is the active ingredient of the preparation Fumidil used by beekeepers to treat nosemosis. It could cause teratogenesis and have genotoxic effects (Stanimirovic et al., 2007). Nowadays, it is not permitted to use fumagillin in Europe and no MRLs have been established, neither for honey nor for any other products of animal origin.

2.2.2.6 Monitoring of antibiotics in bee products

Many other antibiotics have been used worldwide. One of these is tylosine, which got an approval for use in the U.S.A. in the form of preparation Tylan. Moreover, beta-lactams are suggested to be the ideal antibiotic group in terms of efficiency and lack of residues to the final product.

Fifty chestnut, pine, linden and multifloral honey samples from Southern Marmara region of Turkey were analysed for erythromycin residues by Liquid Chromatography-Mass Spectrometry. Four of the honey samples were contaminated with erythromycin residues at concentrations ranging from 50 to 1776 μg kg^{-1} (Gunes et al., 2008).

A percentage of 1.7% out of 3855 honey samples of European market, which was analyzed for antibiotic residues, were non compliant with the EU standards. Antibiotics were detected in the honey samples in a range of 3–10.820 μg kg^{-1}, 5–4.592 μg kg^{-1}, 5–2.076 μg kg^{-1}, 0.1–169 μg kg^{-1}, 0.3-24.7 μg kg^{-1}, 2–18 μg kg^{-1}, 1-504 μg kg^{-1} for streptomycin, sulfonamides, tetracyclines, chloramphenicol, nitrofurans, tylosine and quinolones respectively (Diserens, 2007).

In the period 2000-2001, samples of honey of Belgian market were monitored for the presence of residues of antibiotics. Streptomycin was detected in 4 out of 248 (1.6%) samples that, tetracycline in 2 (2.8%) and sulfonamides in 3 (4.2%) out of 72 samples analyzed. No residues of β-lactams and chloramphenicol were detected. In imported honey samples, streptomycin was detected in 51 out of 108 samples (47.2%), tetracyclines in 29 out of 98 samples (29.6%), sulfonamides in 31 out of 98 samples (31.6%) and chloramphenicol in 40 out of 85 samples (47.1%). Residues of β-lactams were not detected in any sample (Reybroeck, 2003).

A total of 57 samples of royal jelly were collected from beekeepers and the Chinese market. The royal jelly was analyzed for seven fluoroquinolones used in beekeeping (ciprofloxacin, norfloxacin, ofloxacin, pefloxacin, danofloxacin, enrofloxacin, and difloxacin). Ofloxacin, ciprofloxacin and norfloxacin residues were detected in concentrations ranging from 0.012 to 0.056 mg kg^{-1}. Difloxacin was found at a concentration of 0.047 mg kg^{-1} in one sample (Zhou et al., 2009).

2.2.3 Residues of volatile insecticides in bee products

The greater wax moth *Galleria mellonella* is a serious pest of stored combs and weak colonies. Adult female wax moths enter hives and lay their eggs on wax combs or in small crevices between wooden parts of the hives not easily accessible to honey bees. After few days the larvae hatch and begin feeding on bees-wax, pollen, cast larval skins and other remains in cells. This devastating activity of wax moths leads to great financial losses every year in the field of beekeeping.

Strong colonies are the best control against the wax moth in the field. In comb storage chests, technical, physical, biological and chemical methods have been used to control the pest. The most effective method to avoid the destruction of combs from wax moth is their continuous maintenance in temperatures of the refrigerator, or their passing from the freezer for a short time. Cantwell and Smith (1970) confirmed that temperature lower than -18 °C destroys all stages of the wax moth insect (egg, immature forms and adult). Although this treatment requires expensive facilities, it is successfully applied nowadays protecting the honeycombs from the wax moth without contaminating the beehive products.

In addition, biological and environment-friendly control method were developed such us the male sterile technique with gamma-rays (Jafari et al., 2010), the trapping of moths by using pheromone (Flint & Merkle, 1983) and the use of the bacterium *Bacillus thuringiensis* that kills the wax moth larvae when it ingests the spores (Burges & Bailey, 1968; Burges 1997; Charriere & Imdorf, 2004).

Chemical methods, includes substances that are considered friendly to environment like methyl salicylate, clove oil, formic acid, sulphur, acetic acid, basil oil and other have been used (Wilson, 1965; Williams, 1980; Owayss & Abd-Elgayed, 2007). Most of these compounds are dangerous for bee brood and human health, while they require repeated application and may react and destroy the metal parts of the combs. Besides these, 1,2-dibromo-ethane (DBE), 1,4-dichloro-benzene (p-DCB), naphthalene had been used for many years in different countries even though their use causes significant contamination of bee products.

DBE is a manufactured chemical. In nature, it is produced in small amounts in the sea water, where it is formed, probably by algae and kelp. It is dissolved in water and by this way it can stay in groundwater and in soil for a long time. In air it breaks down quickly. This substance has been used as a pesticide in soil, and on citrus, vegetables, and grain crops. EPA has banned most of these uses since 1984. The same organization has also set a limit of 0.05 $\mu g.cm^{-3}$ of 1,2-dibromo-ethane in drinking water (ATSDR, 1992).

The compound p-DCB is one of the three di-chloro-benzene isomers (1,2-DCB, 1,3-DCB and 1,4-DCB), which is commonly used as a space deodorant i toilets and for moth control. It is a volatile colorless to white crystalline material with a mothball-like, penetrating odor and it is commercially, the most important isomer (ATSDR, 2006).

Naphthalene is a white solid substance that evaporates easily. Its major use is in the manufacture of polyvinyl chloride (PVC) plastics and it is also used in moth repellents and toilet deodorant blocks. That use of naphthalene accounted for 73% and 60% of commercial demand for naphthalene in Japan and the United States, respectively in 1999, (ATSDR, 2005).

No MRL's in honey for the above three compounds were defined until 2005 when the European regulation 396/2005 EC set the limit at 10 $\mu g\ kg^{-1}$ for substances for which no MRL had been established. This limit for p-DCB was also the Swiss Tolerance Limit (STL)

and was already used as action level in Greece. ADI values for DBE, p-DCB and naphthalene, range according to Table 3. Besides killing the moth those chemical are absorbed by the wax and when bees store honey into combs, they are transferred into the product. Laboratory comb-melting experiment showed that p-DCB is not removed from wax during the comb recycling (Bogdanov et al., 2004). Residues up to 0.002 mg kg^{-1} may be detected in honey due to the use of precontaminated wax. Residues of p-DCB exceeding 0.01 mg kg^{-1} indicate contamination of bee product by beekeeping practices. Countries that have reported problems with residues from the above volatile insecticides are Germany, Switzerland, Greece and Turkey (Wallner, 1992; Bogdanov et al., 2004; Tananaki et al., 2005; Beyoğlu & Omurtag 2007).

Wallner (1992), stated in his paper that the problem of p-DCB residues in Germany is serious, since 50% of the analyzed honey samples had been found contaminated from 3 to 50 µg kg^{-1}. He noticed that p-DCB is very stable in honey and it cannot evaporate from the sealed glass containers. Finally, he stated that beeswax works like a sponge as it has large capacity for fat-soluble active compounds. The more the p-DCB crystals are added to combs the higher is the substance stored in the wax. The evaporation of p-DCB from wax is impossible even after prolonged ventilation.

Compound	ADI (mg kg^{-1} bw day^{-1})	
	US EPA	Canadian health
1,2-dibromoethane	0.009	0.009
1,4-dichlorobenzene	0.03	0.11
naphthalene	0.02	0.02

Table 1. ADI of three compounds that have been used against wax moths

Bogdanov et al. (2004) analyzed Swiss commercial honey samples during five years period for p-DCB residues and they found that the contaminated samples ranged from 14% to 46% (fig. 1). The percentage of the imported samples was lower, on average 7%. Although there is no MRL for p-DCB, Switzerland has established a "Swiss tolerance value" (STV) for honey at 10 µg kg^{-1}. From the total 173 Swiss and 287 imported honey samples, 13% and 0.8% exceeded the STV respectively.

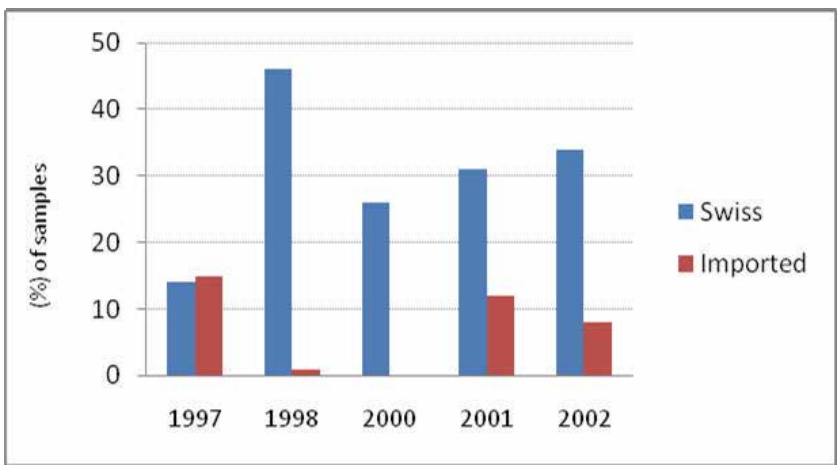


Fig. 1. Residues of p-DCB in honey samples in Switzerland (Bogdanov et al., 2004)

Contamination of bee products by chemicals that are used against wax moths was also noted in Greece by Tananaki et al. (2005). Initially a multi-method had been developed for the determination of DBE, p-DCB and naphthalene and then this method had been applied in twenty five honey samples produced in different areas of Greece. The 8% of the samples had detectable amounts of DBE, 92% had p-DCB and 88% had naphthalene residues. Concentrations of naphthalene, p-DCB and DBE that exceeded 10 μg kg^{-1} were measured in 6.7%, 32% and 8% of tested samples, respectively.

After confirming the mass contamination, beekeepers had been informed to stop the treatment with those chemicals and to destroy all the combs that had been treated before. Meanwhile a monitoring program for the residues of volatile insecticides in Greek honey was initiated by laboratory of Apiculture – Sericulture of Aristotle University. A total of 1,519 samples were analyzed during the period 2004 – 2010 (Tananaki et al., 2006). From those, 209 samples were bought from Greek supermarkets (commercial) while 1,310 were collected from beekeepers or from their associations (bulk honey). Results of this research are indicated in Fig. 2.

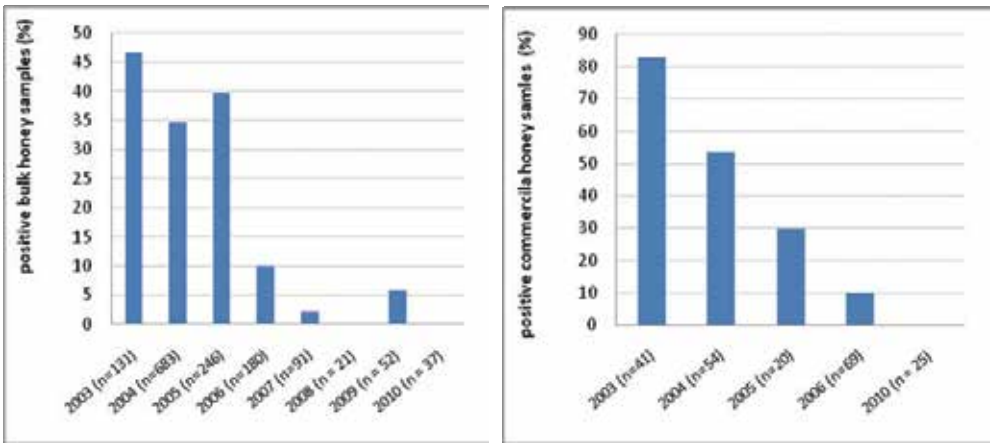
Comparing the results of eight years' monitoring of p-DCB, a considerable reduction of residues is observed both in commercial and bulk honey samples. During the first year the 82.9% of commercial samples had residues more than 10 mg kg^{-1} which is the established action limit in Greece since 2005. In the following three years this percentage decreased gradually and finally p-DCB wasn't detected at concentrations more than 10 mg kg^{-1} in 2010. Similar behavior was observed for the samples collected from beekeepers. These results demonstrate that the Greek beekeepers' efforts to restrict the problem and to find alternative solutions for the control of the wax-moth (*Galleria mellonela*) have been accomplished.

The great percentage of commercial samples in all years of study have either no detectable amounts or below 10 μg kg^{-1} DBE. Only one sample was found exceeding 40 μg kg^{-1} in year 2003. This sample had 60.5 μg kg^{-1} DBE, which is the maximum concentration found in samples bought from stores. Samples that had been collected from beekeepers had higher concentration of DBE than the commercial ones. This is because commercial samples usually are mixtures from different producers. During year 2003, a percentage of 9,9% of the samples exceeded the level of 10 μg kg^{-1} and a maximum value of 132.5 μg kg^{-1} was found in one of them.During the following two years this percentage decreases to 1.9% and 2.8% respectively, but still some beekeepers continue to use the chemical as indicated by the high concentration of 331.2 μg kg^{-1} detected in one sample in 2004.

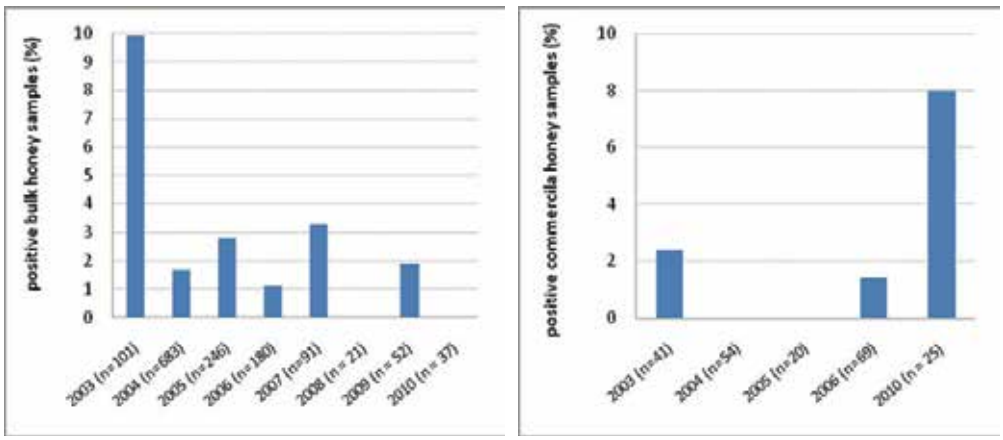
Figure 2 summarizes the results of naphthalene residues in honey from the Greek market and from beekeepers as well. Contrary to p-DCB and DBE, naphthalene was found in more commercial samples than in samples from beekeepers during the first year of monitoring program. This could be attributed to blending of Greek commercial honeys with imported honey originating from countries where naphthalene is still used to control wax-moth. During the following years the residues in commercial samples dropped below 10 $\mu g.kg^{-1}$ and very few beekeepers' samples were contaminated at higher levels. The highest concentration of naphthalene found in one sample was 523.6 $\mu g.kg^{-1}$ in 2004.

Tananaki et al. (2006) also found differences in the level and the frequency of contamination among different types of honeys. Honey produced during the spring honey flow (blossom and fir honeys) was contaminated in a higher percentage than the honey produced later in the season (thymus and pine honey). Thymus and blossom honey have higher contamination in naphthalene than other types of honey. This might happen because both thymus and blossom are the types of Greek honey that are probably mixed with imported honey. Paleologos et al., (2006), Tsimeli et al., (2008) and Harizanis et al., (2008) have also analyzed samples of Greek honey with similar results.

1,4-dichlorobenzene



1,2-dibromoethane



Naphthalene

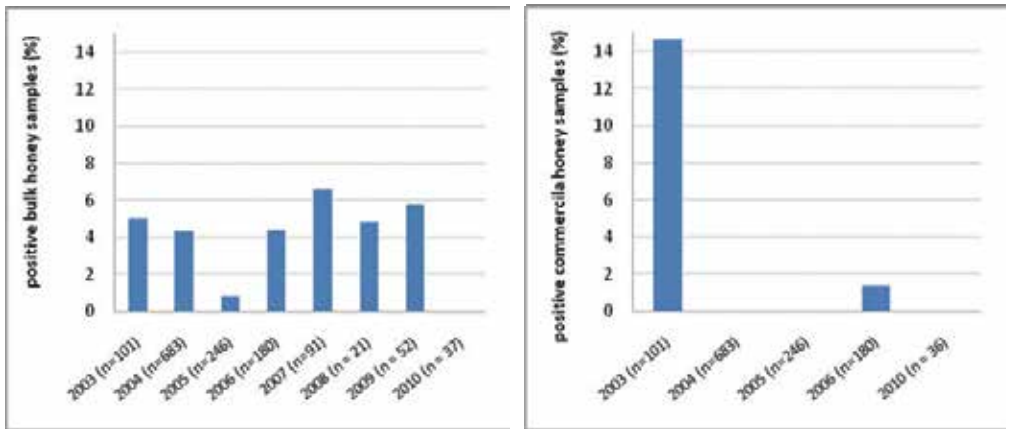


Fig. 2. Residues of volatile insecticides in Greek honeys

Residues of p-DCB were also detected in royal jelly. Tananaki et al. (2009) found that the concentrations of p-DCB in honey were significantly lower than in the royal jelly; in some cases, royal jelly had some hundred times more residues than honey from the same comb. The maximum concentration of p-DCB found in royal jelly was 1,520 μg kg^{-1}. Bogdanov et al. (2004) checked the p-DCB residues in wax. They analyzed wax samples from manufactures during the years 1994 -1998 and 2002 and they found residues in 66% of the wax sample, in concentrations from 0.7 to 74.9 mg kg^{-1}. The concentrations of p-DCB in new wax after melting of old combs were the same with those of the old combs. This indicates that p-DCB is not being removed from wax during the comb recycling process.

2.3 Methods of analysis of pesticide and acaricide residues detected in bee products

The need of monitoring residues of acaricides used by the beekeeper in conjunction with the need to monitor the contamination of bee products from other sources, such as pesticides used on crops and environmental pollutants, makes the development of appropriate methods of analysis obligatory. Furthermore, it is necessary to analyze products like honey and pollen randomly in order to find any violations of existing legislation on the part of producers or sellers. The suitability of each method lies in its ability to give a reliable result of the concentration of residues. A complete method should usually includes four sub-stages, which are described as:

- Sampling
- Sample Preservation
- Sample Preparation
- Analysis

The particular physicochemical properties of each product (moisture, fat, protein content etc.) in conjunction with specific physicochemical properties of each substance (polarity, volatility, etc.) do not permit the use of one methodology for the determination of all active substances in all products. Various techniques have been reported in order to clean –up the sample and isolate the analyte.

2.3.1 Sampling

The first step of an analysis is the sampling. Specifically, the meaning of a sample is to take a part of the product, which should be as representative as possible. The way of the sampling varies, depending on the type of sample. In homogeneous samples such as water, the sampling is simple and does not require complicated procedures. On the contrary, heterogeneous samples such as fruits, vegetables and animal products require additional measures during sampling in order to reduce the uncertainty. The contribution of sampling to the total uncertainty is so great that in some cases approaches 40%. The EU issued a special directive on the sampling of food (2002/63/EK) and requires the accurate implementation of the official control laboratories.

2.3.2 Sample preservation

The second stage of the analysis is the preservation of the sample. The common practice of laboratories is the storage of collected samples for a period ranging from some hours to years. Storage conditions must ensure the preservation of the sample during the period required for the analysis. Food should normally be preserved under freezing conditions,

until the day of analysis, in order to minimize the evaporation or chemical reactivity of these compounds.

2.3.3 Sample preparation and analysis

The next step includes the preparation and the analysis of the sample. The analysis is performed directly, i.e. without pretreatment of the sample, where a direct measurement is possible (e.g. measurement of moisture in honey). In most cases, however, a preparation of the sample should take place before the analysis. The preparation of the sample includes the removal of interferences and the isolation of compounds of interest. This step is necessary in methods of analysis for residues of pesticides and veterinary drugs. Especially in the case of residue analysis, this stage is divided into separate stages that vary in terms of the number and type. Typically, these steps are five and consist of:

- The homogenization, which may includes a stage of subsampling.
- The extraction, including the isolation of analytes in a suitable solvent.
- The removal of a significant amount of solvent in order to make the stage of purification of the sample easier and faster (optional step).
- The cleaning of the sample in order to remove interferences that prevent the proper evaluation of chromatograms.
- The final concentration of solvent, allowing qualitative identification of analytes and the minimization of the quantitative limits.

The cleaning of the sample is the most complicated stage. That is the reason why various techniques have been used. The main techniques used for the preparation and analysis of honey samples are summarized in a review of Rial-Otero et al. (2007). Techniques that have been used in order to achieve the determination of acaricide and pesticide residues in bee products are:

- Solvent Extraction (SE). This is the first technique developed in order to detect pesticide residues. In this technique, the sample is dissolved in water, or mixtures of water and alcohols. After the dilution of the sample, an extraction with suitable organic solvents takes place, in order to collect the analyte and remove a large portion of co-extractives components. Several methods of the SE used combined with acidification of the sample (Waliszewski et al., 1998; Waliszewski et al., 2003; Bernal et al., 1997) or the use of ultrasound (Jimenez et al., 2000; Rezic et al., 2005), in order to improve the efficiency. Due to the use of large quantities of organic solvents, the SE is particularly aggravating for the environment and the health of laboratory staff. Moreover, the cost is quite high due to the large quantity of supplies. Finally, many hours are required for analysis of a sample and the automation of the process is very difficult. Despite these drawbacks, the SE has been used with satisfactory results in various methods of analysis of honey (Jimenez et al., 2002; Menkissoglu-Spiroudi et al., 2000; Taccheo et al., 1988a), royal jelly (Balayannis, 2001), pollen and bees (Bernal et al., 1997) for the determination of pesticide and acaricide residues.
- Accelerated Solvent Extraction (ASE). This technique includes steps of extraction with organic solvents at predetermined conditions of pressure and temperature. In ASE, the extraction solvent is carried out in a special device and the extraction under steady environmental conditions (pressure and temperature) allows efficient and reproducible isolation and collection of analytes. The quantities of solvents used in

this technique are very small compared to the SE and the automation of the extraction procedure much easier. Disadvantage of this technique is the high cost of required equipment. However, the small amount of solvent and the possibility of automation make it possible to recover the cost within a short period of time (especially for laboratories that analyze large numbers of samples). The ASE has been used successfully in many cases of food and water analysis by EPA (Chuang et al., 2001). There is only one study on the analysis of bee products with ASE. Results indicated good efficacy in the determination of acaricides in honey by the use of High Performance Liquid Chromatography (HPLC). The recovery rates of this method ranged from 58% to 103% and limits of quantification ranged from 0.01 mg kg^{-1} to 0.2 mg kg^{-1} (Korta et al., 2002). ASE is likely to be referred in the literature with the names of PLE (Pressurized Liquid Extraction), PSE (Pressurized Solvent Extraction) and PFE (Pressurized Fluid Extraction).

- Supercritical Fluid Extraction (SFE). The SFE is a technique similar to the ASE with similar advantages and disadvantages, while the equipment used for this technique is rather expensive (Mitra, 2003). The difference between the two techniques lies in the type of solvent, which is carbon dioxide (CO_2) for the SFE. Adding a small amount (1-10%) of an organic solvent (such as methanol, ethanol, etc.) improves the efficiency of extraction of more polar compounds, which otherwise would be very small. Two types of supercritical fluid extraction techniques, called static and dynamic were developed. In the case of static SFE, the solvent enters the cell, which contains the lyophilized sample and remains an exact time at constant pressure and temperature conditions. However, in the dynamic SFE, the flow of solvent into the cell remains constant and stable for perfectly accurate time and at constant pressure and temperature conditions. The final extract is transferred to a vial containing an organic solvent. The SFE is a rapid technique that requires very small quantities of organic solvents and does not contaminate the environment significantly. Unlike ASE, there are several publications on the analysis of residues in honey using SFE. Rissatto et al. (2004) developed a method to analyze samples of honey combined SFE system and gas chromatography. The limit of quantification was 0.01 mg kg^{-1}, while recovery rates ranged from 75% to 94 %. In a second study conducted by Atienza et al. (1993) the average recovery rates ranged from 53% -94% while the RSD of the method ranged from 1.3% to 1.6%. In one case, this technique was used for the analysis of organophosphorus and carbamate insecticide residues in bees. The recovery rate exceeded 75% for all substances except omethoate (Jones & McCoy, 1997).
- Gel Permeation Chromatography (GPC). This technique allows the separation of different components based on their size (larger particles move faster). Gels of various porosity and organic solvents are used in order to achieve the separation. Usually, this technique is used to remove lipids, proteins, polymers and other macromolecules contained in the sample. Especially for the pesticide analysis, the technique is suitable for removing high boiling point compounds, which are deposited to the inlet of gas chromatography. Rossi et al., (2001) have used the GPC on the analysis of residues in bees. The recovery was satisfactory for 25 of 29 substances analyzed (percentage recovery ranged from 70.9% to 106.8%). In contrast, the recovery rate for active substances pirimicarb, ethiofencarb, methiocarb and fenoxycarb was 38.7%, 48.6%, 46.6% and 58.4% respectively.

- Stir Bar Sorptive Extraction (SBSE). This extraction technique is using an appropriate stirring bar, which adsorbs the analyte. The bar was either eluted with suitable organic solvents or placed directly to the inlet of gas chromatography systems (Baltussen et al., 1999). Particularly, the bar is made by stainless steel coated with a thin layer of glass and poly-dimethyl siloxane (PDMS), which adsorbs the analyte in the sample (Popp et al., 2001). It is very important for the efficiency of extraction to be accurate in temperature and extraction time. The greater the precision, the more improved the repeatability of the method. In the final phase, the bar is placed in a special unit, which in turn is attached to the inlet. The adsorbed substances led to the column and detector with a carrier gas flow rate increasing with temperature. There is also the option for the bar to be extracted with organic solvents (e.g. acetonitrile), which constitute the final sample for chromatographic analysis (Sanchez-Rojas et al., 2008). SBSE has been used on bee products with good results compared the SPME. Based on data given by Blasco et al. (2004), the SBSE is more efficient as a technique than SPME, while accuracy and repeatability are much better. More specifically, the limit of quantification was 0.04 mg kg^{-1} for SBSE technique, while those for SPME techinique ranged from 0.8 mg kg^{-1} to 3.0 mg kg^{-1}. Moreover, the recovery of SBSE ranged from 40% to 64%. Finally, the relative standard deviation of repeatability did not exceed 10% in both cases.
- Solid Phase Micro Extraction (SPME). The SPME is a relatively modern technique, developed by Pawlyszin et al. (1997). The principle of this technique relies on the use of a fiber, which adsorbs the analyte, which then eluted to the inlet of gas chromatography systems. The SPME technique is suitable for the determination of volatile compounds in liquid or solid samples. SPME can be used in two ways. The first method involves an extraction by sinking the fiber into the sample solution directly. This is an advantage in terms of sensitivity and the number of identified substances. The second way relates to cases of extraction in the supernatant layer of sample. The advantage of this method is the higher level of purity of the final sample (Arthur & Pawliszyn, 1990; Louch et al., 1992; Zhang & Pawliszyn, 1993; Page & Lacroix, 1993). Both SPME and SBSE are based on the logic of the adsorption of chemicals in various absorbents, which in the first case is a fiber (SPME), and in the second a bar (SBSE). Another important parameter, which can greatly improve the results of SPME, is pH (Volante et al., 2001). The adjustment of pH by using buffers could improve efficiency or reduce the time of extraction. It should be noted that there is a variety of fibers, which differ in the type and thickness of the adsorbent material. The advantages of SPME include: (i) the lack of use of organic solvents, (ii) the purest final samples, (iii) the minimization of time, (iv) the good linearity of the method, (v) the non-requirement for full adsorption of the analyte & (vi) the relatively simple automation (Pawliszyn, 1997). The major disadvantage of SPME is the low efficiency for the semi-volatile or non-volatile compounds and the inability to repeat the analysis of a sample (same bottle). SPME was used for the detection of pesticide and acaricide residues in honey. More specifically, the fiber of SPME was immersed in an aqueous solution of honey and remained there until equilibrium of the analyte between the fiber and the environment was achieved. After this, the fiber was removed and placed in the inlet of gas chromatography in order to desorb the

ingredients. The period of immersion of the fiber, as well as the temperature was strictly defined and determined by tests during the development of the method. The technique of solid phase microextraction has been applied for the determination of OCP, OPP, pyrethroid and acaricide residues in honey (Blasco et al., 2004; Yu et al., 2004; Jimenez et al., 1998). In a comparative study, two different types extraction fibers (PDMS 7 mm, PDMS 100 mm and PA 85mm) were tested. The fiber made of PDMS proved significantly superior in terms of reproducibility, sensitivity, linearity and time of extraction obtained (Jimenez et al., 1998).

- Matrix Solid Phase Dispersion (MSPD). The MSPD includes a stage of dilution of the sample in an organic solvent (e.g. methanol) and mixing a quantity of the solution with a sorbent, which is usually C_{18} or Florisil. Next phase involves addition of solvents (hexane, ethyl acetate, etc.), working as means of extraction and elution. After good homogenization in an ultrasonic bath and centrifugation, the extract is collected and analyzed in chromatography systems. The advantages of this technique include the limited use of solvents and the rapid process of the sample. Although MSPD was a promising technique, it is expected to be replaced by QuEChERS, which is a new method of analysis described in next paragraph. The MSPD is rarely used in the analysis of acaricide and pesticide residues in bee products. However, there are few studies used MSPD and gas chromatography for the detection of pesticides in honey. Limits of quantification in these studies were lower than 0.015 mg kg^{-1} for any pesticide, while the recovery ranged between 60% and 113% (Albero et al., 2001; Sanchez et al, 2002).
- QuEChERS. The name of the technique derives from the characteristics of this method, which is described as Quick, Easy, Cheap, Effective, Rugged and Safe (Schenck & Hobbs, 2004). The QuEChERS is a new technique used for the determination of pesticide residues in food analysis. This technique is based on solid-phase dispersion extraction (Matrix Solid-Phase Dispersion). QuEChers developed and validated by Anastassiades (2005) and quickly began to be used by many laboratories. Nowadays, QuEChERS is the common sample preparation technique of official laboratories of European Union. This technique was developed primarily for the analysis of products with high water content. The addition of water to the sample makes possible the use of this technique for the analysis of products like honey. The disadvantage of this technique is the need of expensive equipment (GC/MS/MS, LC/MS/MS etc.), because of insufficient "cleanup step" of the sample. QuEChERS was used in order to detect residues of 36 pesticides in honey. Honey samples were extracted with acetonitrile. The extraction step was followed by the addition of acetic acid with the simultaneous addition of magnesium sulphate and sodium acetate. A mixture of primary/secondary amine (PSA) and magnesium sulphate was added as a second purification step. This step was followed by a change of solvent with a mixture of hexane and acetone. The quantification of organophosphorus compounds carried out using a nitrogen phosphorus detector (NPD), while an electron capture detector (ECD) was used for the determination of chlorinated hydrocarbons and pyrethroids. Recovery experiments were made at three levels (from 0.02 mg kg^{-1} to 5 mg kg^{-1}) and the results ranged from 70% to 120%. Experimental repeatability was satisfactory, as the RSD ranged from 1% to 22%. Finally, the expanded uncertainty

was relatively high (30%), but within the limit of 50% provided in pesticide residues analysis (Barakat, 2007).

- Solid Phase Extraction (SPE). It is the most widely used technique of last decades, in the case of analysis for pesticide and veterinary drug residues. The solid phase extraction is the perfect choice for most researchers, since it requires a small amount of organic solvent (and thus is environmentally friendly), is easily automated and requires no expensive equipment. The disadvantages are the more expensive consumables (solid phase extraction microcolumns), the specialized staff, the differences between lots of microcolumns and the possible absorption of some substances on the polypropylene used in cartridges. Specifically, the sample is dissolved in water (Jimenez et al., 2000; Bernal et al., 1996), alcohol (Bernal et al., 2000) or mixtures of them (Karazafiris et al., 2008; Jimenez et al., 2008), followed by activation of the microcolumn with the same solvent. Subsequently, the sample is passed through a microcolumn containing a suitable solid material, which captures the analyte. The bound substances are eluted with the passage of an appropriate organic solvent. In the case of honey acetone (Bernal et al., 1996), dichloromethane (Jimenez et al., 1998), ethyl acetate (Tsigouri et al., 2001), hexane (Gomis et al., 1996), methanol (Bernal et al., 2000), a mixture of hexane- ethyl acetate (Tsigouri et al., 2001) have been occasionally used. With regard to the types of substrates used occasionally, the reverse phase C_{18} was the most appropriate and chosen by most researchers for the extraction of insecticides, acaricides, herbicides, fungicides and other pesticides (Jimenez et al., 2000; Bernal et al., 2000; Korta et al., 2001). Also microcolumn with Florisil gave good results in trials for determination of pyrethroid, OCP and OPP residues (Jimenez et al., 1998a) and C_8 in the determination of tau fluvalinate (Tsigouri et al., 2001). The pH adjustment proved particularly important for the good recovery of some active substances. For example, coumaphos is unstable in an alkaline environment, as opposed to amitraz, the recovery increases with increasing pH values up to 11 (Korta et al., 2001). A comparison of the effectiveness of SPE and SE in pesticide residue analysis was conducted in two publications by Bernal et al. (1996 & 2000). According to the results of the comparison, it should be noted that the recovery rate with both techniques was similar, but SPE proved superior to the purity of the chromatograms. The analysis of royal jelly using the technique of solid phase extraction was first mentioned, by Karazafiris et al., (2008b). The method proved efficient for the determination of acaricide and insecticide residues.

2.3.4 Determination of analytes

The isolation of analytes from the matrix is followed by the necessary step of separation. The practices used on pesticide and veterinary drug residue analysis are based on chromatographic methods. The choice of gas or liquid chromatography is mainly based on the chemical properties of analytes. The technique of gas chromatography was proved suitable for the determination of volatile and low molecular weight compounds, in contrast to the technique of liquid chromatography, which was used in less volatile and high molecular weight substances. The methods of analysis are classified according to the number of compounds detected. The two main categories are multi residue methods (MRM) and single residue methods (SRM). The majority of compounds identified with multi residue methods.

The analyst is able to detect many different compounds by preparing and analyzing a sample only once. However, there are certain compounds, which can be identified individually and only with the use of complex techniques (e.g. amitraz in honey or pear after derivatisation). In recent years, due to the significant improvement of the equipment (GC-MS-MS, LC-MS-MS, etc.) the number of identified substances has increased considerably and many laboratories can identify the majority of active ingredients. The improvement of the quality and quantity of results issued by laboratories has been particularly important. The development of methods includes the use of chromatography described below.

2.3.4.1 Gas chromatography (GC)

The gas chromatography was used more than any other method to determine pesticide and acaricide residues in bee hive products. As mentioned above, this technique is mainly used for determination of volatile and low molecular weight compounds, but there are cases where higher molecular weight compounds (e.g. amitraz), analyzed by gas chromatography after laborious and time consuming processes (e.g. derivatization). In these cases, the substances were converted into more volatile compounds and then analyzed using gas chromatographic system. Gas chromatographic systems consist of three main sections outlined below:

a. The first main section is called inlet and ensure the entrance of the sample in a gas chromatograph. The types of inlets used in gas chromatography is the Cool On Column, purged packed and split/splitless. The type of inlet may be a problem for some classes of substances that are sensitive to high temperature (e.g. methamidophos and dichlorvos gave better results in Cool on Column inlets due to the lower temperature). The injection in a Cool on Column inlet takes place at low temperatures and benefits in repeatability and stability of analytes. The problem in this case is the more frequent maintenance of the column. Instead, the split/splitless inlet advantages in the purity of the sample, since the majority of high molecular weight substances are removed by a flow of gas and do not enter the column. The result is the extension of the lifetime of the column and the less frequent maintenance. In return, the above advantages of the split/splitless inlet may indicate the low reproducibility due to the removal of a quantity of analyte during cleaning.
b. The second part consists of the oven and column at which the separation of analytes happens. The separation of substances achieved with the strictly programmed temperature and carrier gas flow within the oven and column respectively. The repeatability of retention time of an analyte depends on the repeatability of the above conditions. The column packing material is a very important factor for the separation and identification of various substances. Small to medium polarity columns are usually used for the detection of acaricides and pesticides. The use of more polar columns is necessary in some cases of single residue methods (e.g. determination of amitraz and its metabolites in honey and beeswax). Finally, a factor worth mentioning is the quality of the gases (carrier, auxiliary gas, etc.) that can substantially improve the sensitivity and lifetime of the column and the detector.
c. The inlet and the column associated with a suitable detector. Detector achieves the visualization of the result. In the case of beehive products the following detectors have been used:

- Electron Capture (ECD), to detect pyrethroid, organochlorine and organophosphorus insecticide and acaricide residues (Baltussen et al., 1999; Barakat et al., 2007; Jimenez et al., 1996; Jimenez et al., 1998a; Karazafiris et al., 2008b; Menkissoglu-Spiroudi et al., 2000; Rissato et al., 2004).
- Nitrogen-Phosphorus (NPD), to detect pyrethroid and organophosphorus insecticide and acaricide residues (Balayannis, 2001; Baltussen et al. 1999; Jimenez et al., 1998b; Menkissoglu-Spiroudi et al., 2000).
- Flame Ionization (FID), to identify residual acaricides (Bernal et al., 2000).
- Atomic Emission (AED), to detect acaricide residues (Jimenez et al., 1996).
- Mass Spectrometry (MSD), to detect pyrethroid, organophosphate, carbamate and organochlorine insecticide or acaricide residues (Albero et al., 2004; Baltussen et al., 1996; Bernal et al., 1996; Chauzat et al., 2006; Rissato et al., 2004).
- Flame Photometric Detector (FPD) and Pulsed Flame Photometric Detector (PFPD), for detection of organophosphorus insecticide and acaricide residues (Yu et al., 2004).

Each chromatographic system may include components that automate the process and provide valuable assistance to the analyst. The most important component in optimizing the analytical procedure is the autosampler. The performance of a chromatographic system is maximizing by the use of autosampler, while it improves the reproducibility of injection volume and the number of samples, which can be analyzed daily.

2.3.4.2 Liquid chromatography (LC)

Unlike gas chromatography, which is limited to determining the most volatile compounds, liquid chromatography is used for the isolation of a widespread group of compounds. These compounds may not be sufficiently volatile or heat-resistant to analysis by gas chromatography. The most common types of detectors used in liquid chromatography were Diode Array Detectors (DAD) (Atienza et al., 1993; Blasco et al., 2004; Jones & McCoy, 1997; Martel & Zeggane, 2002), Ultraviolet/Visible Detectors (UVD) (Jimenez et al., 2000) and Fluorescence Detector (FLD) (Bernal et al., 1997). The detection technique that is gaining ground is mass spectrometry (MS) (Blasco et al., 2004; Chauzat et al., 2006; Fernandez et al., 2002). In particular, the mass spectrometer with a triple quadrupole is concerned the most suitable detector for pesticide and veterinary drug residue analysis. The above technique enables determination of the majority of active substances, combined with excellent sensitivity (LOQ of about 0.001 mg kg^{-1} for most of analyzed substances) and fewer requirements for the cleaning of the sample. The mass spectrometry is the ideal detector in conjunction with the QuEChERS method referred above. In each case, the high cost of the equipment and the need of qualified scientific staff should be noted. The packing material and the size of the column play an important role in the analysis with HPLC. The most widely used column is a C18 reverse phase with an internal diameter of 4.6 mm id. There are also columns with different packing material (C8, ODS, etc.) and columns with very small internal diameter (e.g. 2.1 mm id and 0.32 mm id), which help to increase the sensitivity and reduce the quantities of solvents used (Atienza et al., 1993). The mobile phase used in liquid chromatography is solvents such as water, methanol and acetonitrile or mixtures of them. Also, the adjustment of pH of the mobile phase plays an important role in the effectiveness of the method. In most cases a value of pH=9 is ideal for analysis of pesticide residues.

2.3.4.3 Thin layer chromatography (TLC)

This technique is used primarily for detecting drugs in biological samples. However, TLC was used to determine pesticide residues in food. More generally, the TLC requires sample extraction with a solvent mixture and separation of the components into blocks with a suitable coating material (e.g. Silica gel). The next step is an elution with suitable solvents. Special equipment is necessary in order to achieve the visualization and quantification of results. The TLC was used by Rezic et al. (2005) to detect residues of herbicides atrazine and simazine in honey. The recovery rate was estimated at 92.3% and 94.2% for atrazine and simazine respectively. The TLC was used in the above study in conjunction with the use of ultrasound during the extraction.

2.3.4.4 Matrix effect

A fact that has to be mentioned is that differences in the chemical synthesis of bee products may affect the efficiency of extraction (Blasco et al., 2004; Yu et al., 2004) and the response of the chromatographic systems to analytes (Volante et al., 2001; Jimenez et al., 1998; Karazafiris et al., 2008b). This is the reason why, solutions for calibration curve and recoveries should be prepared in an extract of the same sample analyzed. Specifically, the analyst applies the chosen technique in honey or other hive products containing no detectable residues of analytes. The final extract is derived from the overall process used in the construction of standard calibration curves. If it is not possible to find sample with no residues, an analyst can use an extract of the sample that gives a response 30% over the reference value. The response may be due to the presence of the analyte or an interference eluting at the same retention time.

2.4 Methods for the determination of volatile insecticide residues in bee products

The research on the detection of volatile insecticides residues from substances that are used against *Galleria mellonella* has been started twenty years ago. Various methods of isolation and analysis have been developed which are mainly based on chromatographic separation. Table 2 summarizes all those methods with some analytical information and the corresponding references.

During the first SMPE isolation method a small amount of honey was diluted with water and transferred to the vials. The p-DCB molecules were collected on PDMS-fiber (5 cm, 100 μm) and the adsorption process took place for 45 min at 20-25 ^{o}C. Desorption was performed by raising the fibre temperature to 250 °C for 15 min and the analytes transferred to the GC column (DB-5ms: 30m x 0,25mm, 0,25μm). The detection was achieved with a MS detector at the level of 1 μg kg^{-1} (Bogdanov et al., 2004). Tananaki et al. (2005) developed a sensitive method for the simultaneous determination of p-DCB, EDB and naphthalene residues in honey, using a purge and trap - gas chromatography – mass spectrometry system (P&T-GC–MS). In this research the analytes were extracted by He purging and then they absorbed onto the Tenax resin. With thermal desorption the isolated compounds were transferred to the GC – MS system. Separation was performed on a fused silica capillary column (30m×0.25mm I.D., 0.25 μm film thickness). The limits of detection were found to be 0.8, 0.15 and 0.05 μg kg^{-1} honey, while the limits of quantification were 2.4, 0.5 and 0.125 μg kg^{-1} for EDB, p-DCB and naphthalene respectively.

Bee product	Analytes	Isolation	Determination	Analytical information	References
Honey	p-DCB	Head space sampling	GC – MS	Column: Rtx-624 LOD: 1 µg kg^{-1}	Bogdanov et al., 2004
		SPME (PDMS-fiber)	GC - MS	Column: J & W DB5ms LOD: 1 µg kg^{-1}	
	DBE, p-DCB, naphthalene	Purge & Trap (Tenax TA)	GC - MS	Column HP-5MS LOD: 0.5, 0.15, 0.05 µg kg^{-1} LOQ: 2.4, 0.5, 0.125µg kg^{-1}	Tananaki et al, 2005
	p-DCB	Acid-induced liquid-liquid phase separation of anionic surfactants	HPLC - UV	Column : LiChrospher-100 RP-18 LOD: 2.5 µg kg^{-1} LOQ: 7.5 µg kg^{-1}	Paleologos et al., 2006
	p-DCB, naphthalene	SPME (VB/carboxen/PDMS)	GC - MS	Column: J & W DB5ms LOD: 1, 0.1 µg kg^{-1} LOQ: 5, 1 µg kg-	Harizanis et. al., 2008
	DBE, p-DCB, naphthalene	HS-SPME (PDMS)	GC - MS	Column: DB5 LOD: 2, 1, 0.1 µg kg^{-1} LOQ: 5, 4, 0,3 µg kg^{-1}	Tsimeli et al., 2008
	Naphthalene		HPLC-DAD GC-MS	LOD: 0.023 µg kg^{-1} LOQ: 0.078 µg kg^{-1}	Beyoğlu and Omurtag 2007
Beeswax	p-DCB	ethanol, SPE (C18)	GC - MS	LOD: 0.7 mg kg^{-1}	Bogdanov et al., 2004
Royal jelly	p-DCB	Purge & Trap (Tenax TA)	GC - MS	Column HP-5MS LOD: 0.3 µg kg^{-1} LOQ: 0.9 µg kg^{-1}	Tananaki et al, 2009

Table 2. Methods for the determination of volatile insecticides residues

The acid-induced liquid-liquid phase separation of anionic surfactants in aqueous solutions and its applicability to cloud point extraction methodology were applied as a tool for the extraction of 1,4- dichlorobenzene (p-DCB) from aqueous honey samples. The analyte is extracted into the micelles of sodium dodecane sulfonate. For the separation of p-DCB a high-performance liquid chromatographic equipped with a UV detector system (225 nm) was used (Paleologos, et al. 2006). Dichlorobenzene and naphthalene residues in honey were investigated by solid-phase microextraction (SPME) coupled to gas-chromatographic/mass spectrometry from Harizanis et. al (2008). The equilibration time and the sampling time for the extraction of the analytes by the fibre was 30 min and 60 min respectively, while the honey solution was kept at 60 °C. The LOD and LOQ for the p-dichlorobenzene was 1 μg kg^{-1} and 5 μg $kg^{-1,}$ while for naphthalene 0.1 μg kg^{-1} and 1 μg kg^{-1} respectively.

Tsimeli et al. (2008) developed a method for the determination of DBE, p-DCB and naphthalene based on SMPE extraction. Commercially available 100 μm film thickness poly-dimethylsiloxane (PDMS) fiber was employed for the extraction. The fibre was exposed to the headspace above the sample for 30 min, while the sample was kept at 40±2 °C and stirred at 900 rpm. The separation and detection are carried out using gas chromatography–mass spectrometry (GC/MS) in selected ion monitoring mode (SIM).

For the determination of naphthalene in honey, a high-performance liquid chromatography with a diode array detector method was also used. The compound was detected at 220 nm and the limit of detection and the limit of quantification were 0.023 μg g^{-1} and 0.078 μg g^{-1} respectively (Beyoğlu & Omurtag 2007).

The p-DCB molecules were extracted from the bee wax with ethanol and the sample clean up was accomplished by solid-phase extraction (C_{18} columns), while the determination was achieved by capillary GC and FID detector. The detection limits of the method were 0.7 mg kg^{-1} while average recovery was 74.8±5.5% (Bogdanov et al., 1998; 2004). For the isolation of p-DCB from the royal jelly a Purge and Trap system was used (Tananaki et al., 2009). The molecules of this compound extracted from the aqua royal jelly solution by He purging at 40 ml min^{-1} for 40 min keeping the sample temperature at 40 ^{o}C and were absorbed on Tenax resin. For the separation a fused silica capillary column (HP-5MS) has been used, while the detection was achieved using a mass spectrometer detector. The LOD and LOQ of the method was 0.3 μg kg^{-1} and 0.9 μg kg^{-1} respectively.

3. Conclusion

To maximize the production of agricultural products, extended amount of insecticides, herbicides, fungicides and bactericides are used which eventually lead to contamination of water, soil, crops, animals, even humans. Many environmental studies are concerned with the bioavailability of these pollutants and their subsequent introduction into food chain. Pesticides, Persistent Organic Pollutants (OCs, PCBs, PBBs), toxins and heavy metals have been investigated worldwide as substances that contaminate man's food. Chemicals contaminate hive products like honey, wax, pollen, propolis and royal jelly, while residues may exceed the established MRLs, either because of the improper use of the products or the utilization of unauthorized products by the beekeepers.

Honeybees forage over a circular area, with radius more than 6 Km, visiting numerous plant species and various sources of water and are notorious for collecting materials contaminated with chemicals and bringing them back to the hive. In anyway, pollutants may reach the

hive products and this justifies consumers' concern on this subject. According to research studies, the risk for bee products contamination with pesticides from the environment is low. Concentrations of pesticide residues detected are below LOQs in most studies, while there are only few cases that high concentrations of pesticides were detected in bee products. Moreover, antibiotics used as plant protection products can contaminate bee products, but the concentrations detected are low.

Besides the above indirect method of pesticides transferring into bee's nest, the bigger risk for bee products contamination is the beekeeping practices. Diseases attack bee colonies and the beekeepers use acaricides, antibiotics, fungicide and other chemicals inside the hive to control them.

Antibiotics played an important role as effective chemotherapeutics for bee diseases and have been used until recently. However, the use of antibiotics against any bee disease is not permitted in Europe anymore, because pharmaceutical companies did not apply and support the experimental data for MRLs in bee products as required by the European Medicinal Evaluation Agency (EMEA). Despite of this forbiddance, monitoring results indicate that antibiotic residues are still present in European honeys, but the detection frequency is decreasing after the European ban. Antibiotic residues are usually detected in honey and royal jelly, while the concentrations are very low comparing to other products such as milk, eggs etc.

Another source of contamination that is caused by beekeepers is the chemicals that they use against Varroatosis, a disease caused by the parasitic mite *Varroa destructor* Anderson and Trueman. Varroas' presence causes many troubles to the bees including appearance of other diseases like sacbrood, American and European foulbrood. If it is left untreated it could destroy the whole colony within 2-3 years period. Varroatosis is actually the only disease of bees against which the use of pharmaceutical products within the hive is permitted. In European countries, there are authorized chemicals that can be used and limits (MRLs) that should not be exceeded. The contamination of bee products by acaricides can be minimized by careful use of the chemotherapeutic products. As far as we know the percentage of honey samples containing residues exceeding MRLs is low. A major problem could be the use of unauthorized products in order to control Varroatosis.

Additional problem for the quality of bee products was the volatile insecticides and other chemicals that beekeepers used in storehouses to protect bee combs from the larvae of the insect *Galleria mellonella* (wax moth). This insect attacks the honeycombs during storage and can even damage the wooden frames in which they hang. The devastating activity of these insects is known to beekeepers all over the world. To save the combs, beekeepers use several chemical fumigants that are incorporated into wax and from there they are readily translocated into bee products. Some of those like PDCB, DBE and naphthalene pose a potential health hazard. Although beekeepers stopped using these compounds, residues were still in old combs for many years and readily transferred into honey. Volatile insecticide residues detected in bee products are below LOQ in countries like Greece, which had a major contamination problem the previous decade.

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Part 2

Environmental Impacts of Pesticides

7

Ecological Effects of Pesticides

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1. Introduction

1.1 Definition of ecology

At a community level, ecology can be defined as complex interactions that exist among interdependent organisms that cohabitate the same geographical area and with their environment (Johnson and Strinchcombe, 2007). At individual level, it entails the relationships that exist between that particular individual with numerous physical and biological factors. The physical environment along with organisms (biota) inhabiting a particular space make up an ecosystem. Some typical examples of ecosystems include a farm pond, a mountain meadow and rain forest. In a natural environment, an ecosystem follows a certain sequence of processes and events through the days, seasons and years. The processes include not only the birth, growth, reproduction and death of biota in that particular ecosystem, but also the interactions between species and physical characteristics of the geological environment. From these processes and interactions, the ecosystem gains a recognizable structure and function, and matter and energy are exchanged and cycled through the ecosystem. Over time, better adapted species come to dominate; entirely new species may change, perhaps in a new or altered ecosystem.

1.2 The organisation in ecosystems

The basic level of ecological organisation is with the individual such as a single plant, insect or bird. The definition of ecology is based on the interactions of organisms with their environment. In the case of an individual, it would entail the relationships between that individual and numerous physical (rain, sun, wind, temperature, nutrients, etc.) and biological (other plants, insects, diseases, animals, etc.) factors. The next level of organization is the population. Populations are no more than a collection of individuals of the same species within an area or region. We can see populations of humans, birch trees, or sunfish in a pond. Population ecology is concerned with the interaction of the individuals with each other and with their environment.

The next, more complex, level of organization is the community. Communities are made up of different populations of interacting plants, animals, and microorganisms also within some defined geographic area. Different populations within a community interact more among themselves than with populations of the same species in other communities, therefore, there are often genetic differences between members of two different communities. The populations in a community have evolved together, so that members of that community provide resources (nutrition, shelter) for each other.

The next level of organization is the ecosystem. An ecosystem consists of different communities of organisms associated within a physically defined space. For example, a forest ecosystem consists of animal and plant communities in the soil, forest floor, and forest canopy, along the stream bank and bottom, and in the stream. A stream bottom community, for example, will have various fungi and bacteria living on dead leaves and animal wastes, protozoans and microscopic invertebrates feeding on these microbes, and larger invertebrates (worms, crayfish) and vertebrates (turtles, catfish). Each community functions somewhat separately, but are also linked to others by the forest, rainfall, and other interactions. For example, the stream community is heavily dependent upon leaves produced in the surrounding trees falling into the stream, feeding the microbes and other invertebrates.
Terrestrial ecosystems can be grouped into units of similar nature, termed biomes (such as a "deciduous forest," "grassland," "coniferous forest," etc.), or into a geographic unit, termed landscapes, containing several different types of ecosystems. Aquatic ecosystems are commonly categorized on the basis of whether the water is moving (streams, river basins) or still (ponds, lakes, large lakes) and whether the water is fresh, salty (oceans), or brackish (estuaries). Landscapes and biomes (and large lakes, river basins, and oceans) are subject to global threats of pollution (acid deposition, stratospheric ozone depletion, air pollution, the greenhouse effect) and human activities (soil erosion, deforestation, pesticides use).

1.3 Why pesticides are unique among environmental contaminants

Pesticides released into the environment may have several adverse ecological effects ranging from long-term effects to short-lived changes in the normal functioning of an ecosystem. Despite the good results of using pesticides in agriculture and public health, their use is usually accompanied with deleterious environmental and public health effects. Pesticides hold a unique position among environmental contaminants due to their high biological toxicity (acute and chronic). Pesticides by definition are toxic chemical agents. A pesticide is usually capable of harming all forms of life other than the targeted pest species. On account of this behavior then, they can best be described as biocides (capable of killing all forms of life). Although some pesticides are described to be selective in their mode of action, their range of selectivity is only limited to the test animals.

1.4 The vast potentials of pesticides distribution and fate in the environment

The term chemodynamics of pesticides refers to the study of the movement and transformation of pesticides as well as their fate in various compartments of the environment. The environment can be divided into four major compartments, namely; air, water, soil and biota (Fig.2. 1).
The widespread use and disposal of pesticides by farmers, institutions and the general public provide many possible sources of pesticides in the environment. Pesticides once released into the environment may have many different fates. Pesticides that are sprayed can move through the air and may eventually end up in other parts of the environment, such as in soil or water. Pesticides that are applied directly to the soil may be washed off the soil into nearby bodies of surface water or may percolate through the soil to lower soil layers and groundwater (Harrison, 1990). This incomplete list of possibilities suggests that the movement of pesticides in the environment is very complex with transfers occurring continually among different environmental compartments. In some cases, these exchanges occur not only between areas that are close together (such as a local pond receiving some of the herbicides applied on adjacent land) but also may involve transportation of pesticides

over long distances. The worldwide distribution of DDT and the presence of pesticides in bodies of water such as the Great Lakes far from their primary use areas are good examples of the vast potential of such movement.

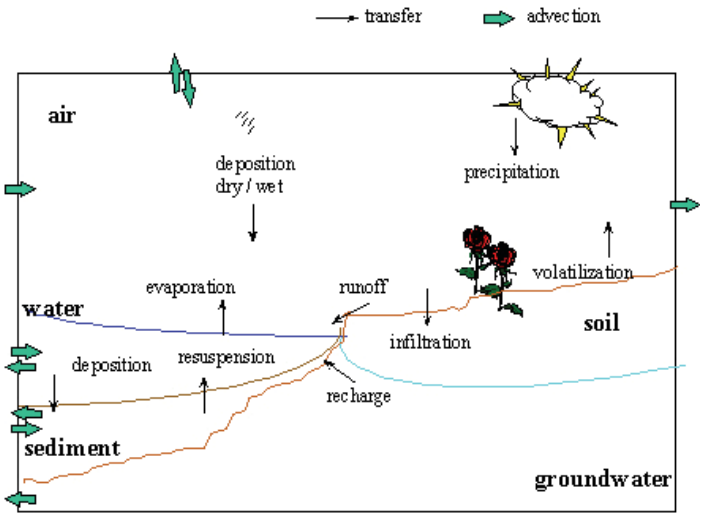

Fig. 2.1 Distribution of pesticides in different environmental compartments

In this chapter, a closer and detailed look on the major ecological effects of pesticides are described based on contemporary accumulated knowledge on the behavior of pesticides and the damage they cause to the ecosystem and the environment at large as a result of excessive use and/or injudicious use of pesticides. The effects may range from minor deviation on the normal functioning of the ecosystem to the loss of species diversity in the ecosystem. Since organisms in the ecosystem live in a complex interdependent association with each other, the loss one key species may result in the collapse of the particular ecosystem. These effects are an important reason for the current strict regulations on the judicial use of pesticides.

2. Ecological effects of pesticides

The primary objective of using pesticides in the fields and the environment in general is to achieve a control of crop pests and disease vectors. This has been a deliberate human effort in a search for increasing agricultural yields and improving public health (Helweg, 2003). Pesticides applied to the environment have shown to have long term residual effects while others have shown to have acute fatal effects when not properly handled. Organochlorine pesticides for example have shown to be persistent in the environment, the result of which find their way to contaminate ground water, surface water, food products, air, soil and may affect human being through direct contact. Pesticides exposure to humans have been well documented to be the route cause of some diseases such as cancer, respiratory diseases, skin diseases, endocrine disruption, and reproduction disorders. It is this aspect of pesticide in the environment that has raised concern among environmental scientists to study their behaviour in the environment and then come out with a sound alternative so as to rescue the human population from their adverse effects.

Fifty years (half a century) after Rachel Carson's eloquent warning to the world about the devastating effect pesticides have on birds and beneficial insects, pesticides continue to be

a pervasive and insidious threat to the world's ecosystems. A massive chemical assult on our environment is launched each year. This poisonous barrage aggravates other pressures on our ecosystems such as expanding suburbarn development and dammed rivers, threatening the survival of many birds, fish, insects, and small aquatic organisms that form the basis of the food web. More generally, pesticides reduce species diversity in the animal kingdom and contribute to population decline in animals and plants by destroying habitats, reducing food supplies and impairing reproduction (Kegley, *et al*, 1999).

2.1 Loss of species diversity among the food chains and food webs

Organisms in ecosystems exist in complex interdependent associations such that losses of one keystone species as a result of pesticides (or other causes) can have far reaching and unpredictable effects. A keystone species is a species that is disproportionately connected to more species in the food-web. The many connections that a keystone species holds means that it maintains the organization and structure of entire communities. The loss of a keystone species results in a range of dramatic cascading effects that alters trophic dynamics, other food-web connections and can cause the extinction of other species in the community. Sea otters (*Enhydra lutris*) for example, are known to be keystone species in marine ecosystems that limits the density of sea urchins (Mills, *et al*, 1993).

A pesticide may eliminate a species essential to the functioning of the entire community, or it may promote the dominance of undesired species or it may simply decrease the number and variety of species present in the community. This may disrupt the dynamics of the food webs in the community by breaking the existing dietary linkages between species. The literature on pest control lists many examples of new pest species that have developed when their natural enemies are killed by pesticides. This has created a further dependence on pesticides. Finally, the effects of pesticides on the biodiversity of plants and animals in agricultural landscapes, whether caused directly or indirectly by pesticides, constitute a major adverse environmental impact of pesticides.

2.2 Effects involving pollinators

Some natural pollinators, such as honeybees and butterflies, are very sensitive to pesticides. Pesticides can kill bees and are strongly implicated in pollinator decline, the loss of species that pollinate plants, including through the mechanism of Colony Collapse Disorder (Hackenberg, 2007), in which worker bees from a beehive or Western honey bee colony abruptly disappear. Application of pesticides to crops that are in bloom can kill honeybees, which act as pollinators. The USDA and USFWS estimate that US farmers lose at least $200 million a year from reduced crop pollination because pesticides applied to fields eliminate about a fifth of honeybee colonies in the US and harm an additional 15% (Miller, 2004).

Since these are important pollinators of both crops and native plants, reduced number of natural pollinators can therefore result into reduced seed and fruit production. This is both an ecological effect as well as economical effect. Bees are extremely important in the pollination of crops and wild plants, and although pesticides are screened for toxicity to bees, and the use of pesticides toxic to bees is permitted only under stringent conditions, many bees are killed by pesticides, resulting in the considerably reduced yield of crops dependent on bee pollination.

Fig. 2.2 A butterfly and bee as representative natural pollinating agents for plants

2.3 Effects on nutrient cycling in ecosystem

A large proportional of pesticides used in the environment ultimately reaches the soil where soil building processes and the cycling of nutrients back into living plants is accomplished. Pesticides can affect the soil organisms involved in these processes directly or indirectly hence interfering with the natural nutrient cycling in the ecosystem.

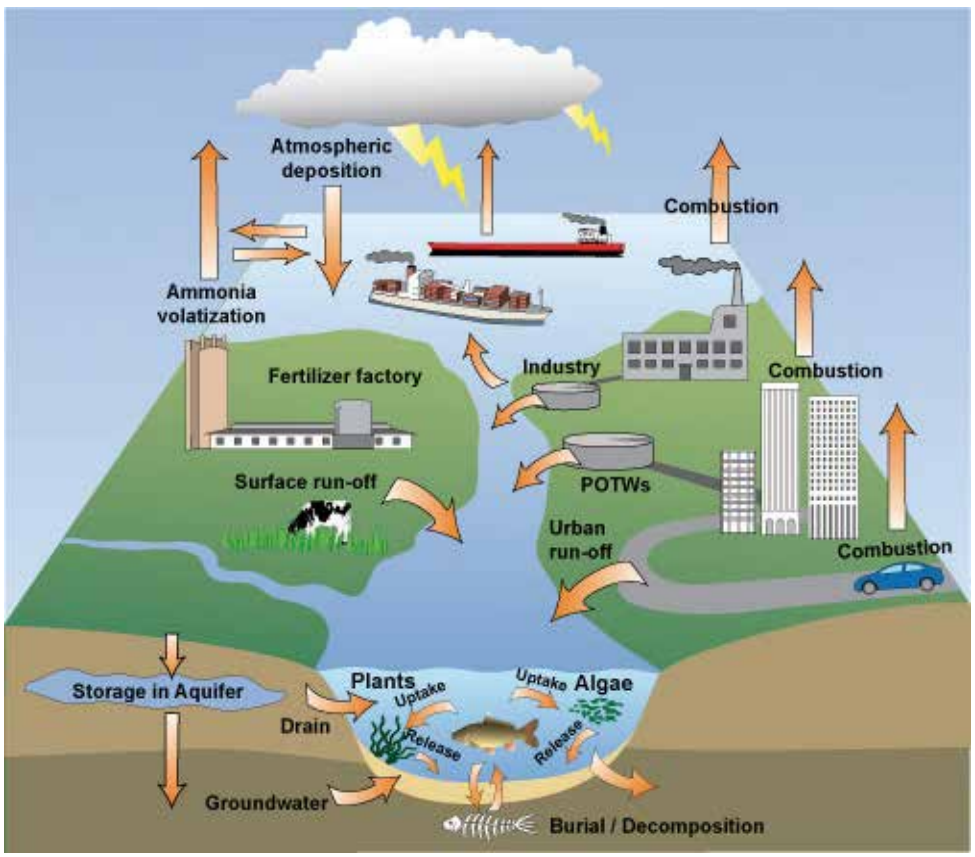

Fig. 2.3 Nutrient cycling in ecosystem

Nitrogen fixation, which is required for the growth of higher plants, is hindered by pesticides in soil. The insecticides DDT, methyl parathion, and especially pentachlorophenol have been shown to interfere with legume-rhizobium chemical signaling. Reduction of this symbiotic chemical signaling results in reduced nitrogen fixation and thus reduced crop yields (Rockets, 2007). Root nodule formation in these plants saves the world economy $10 billion in synthetic nitrogen fertilizer every year (Fox, 2007). When the natural nutrient cycling (figure 2.3) in the ecosystem is interfered in any way by pesticides or other sources of pollution, it will lead to decline in soil fertility and soil productivity.

2.4 Effects on soil erosion, structure and fertility

Many of the chemicals used in pesticides are persistent soil contaminants, whose impact may endure for decades and adversely affect soil conservation. The use of pesticides decreases the general biodiversity in the soil. Not using the chemicals results in higher soil quality (Johnson, 1986), with the additional effect that more organic matter in the soil allows for higher water retention. This helps increase yields for farms in drought years, when organic farms have had yields 20-40% higher than their conventional counterparts. A smaller content of organic matter in the soil increases the amount of pesticide that will leave the area of application, because organic matter binds to and helps break down pesticides (Lotter, *et al*, 2003).

Herbicides for example can reduce vegetative cover of the ground, thus promoting soil erosion via runoff and wind. Soil erosion deforms the soil structure and therefore creates an imbalance in soil fertility. A bare land with poor soil structure and poor soil fertility cannot support the growth of plants on it. Ecologically this land cannot support other forms of life in it hence may lead to the collapse of the particular ecosystem.

2.5 Effects on water quality

Pesticides applied in the environment can find their way into water bodies either from the air or by runoff or by percolation to groundwater. There are four major routes through which pesticides can reach the water bodies: it may drift outside of the intended area when it is sprayed, it may percolate, or leach, through the soil, it may be carried to the water as runoff, or it may be spilled, for example accidentally or through negligence. They may also be carried to water by eroding soil. Factors that affect a pesticide's ability to contaminate water include its water solubility, the distance from an application site to a water body, weather, soil type, presence of a growing crop, and the method used to apply the chemical. Once pesticides enter water bodies they have a potential to cause harmful effects on human health, aquatic organisms and can cause disruptions of the aquatic ecosystems. This may result into a loss in fish production in streams and large water bodies especially where fishing is one among the major economic activities of a particular community.

In the United States for example, pesticides were found to pollute every stream and over 90% of wells sampled in a study by the US Geological Survey (Gillion, *et al*, 2007). Pesticide residues have also been found in rain and groundwater. Studies by the UK government showed that pesticide concentrations exceeded those allowable for drinking water in some samples of river water and groundwater (Bingham, 2007).

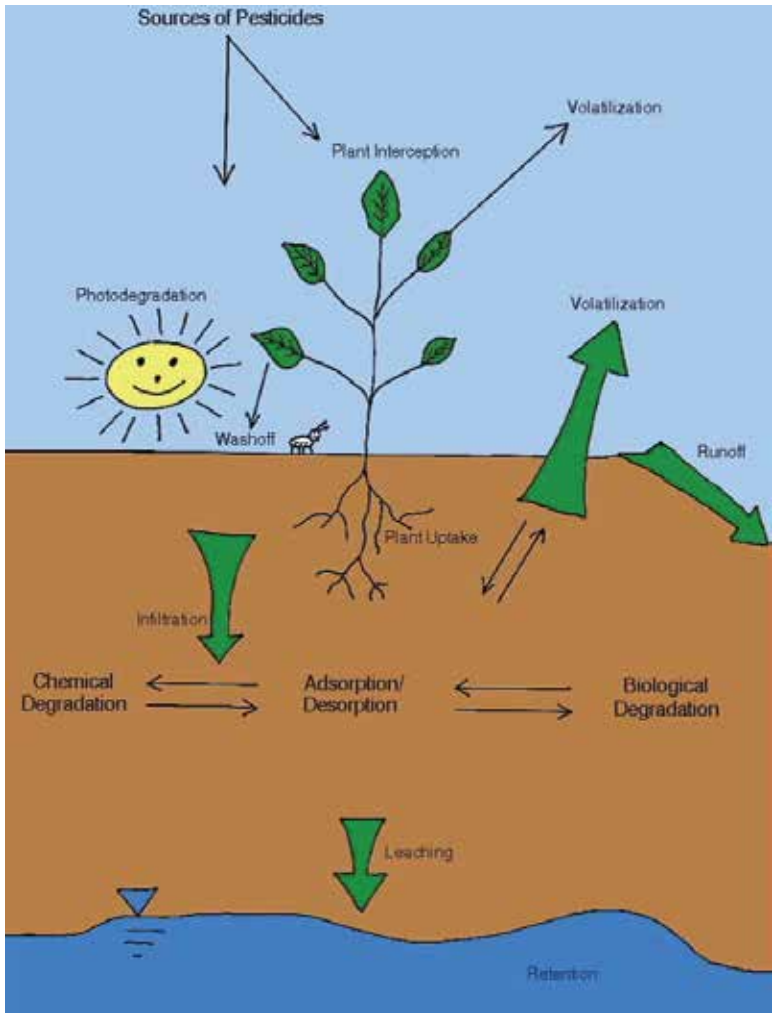

Fig. 2.4 Pesticieds pathways in contaminating water bodies (Heather, *et al*, 1997)

2.6 Effects on birds

Pesticides have had some of their most striking effects on birds, particularly those in the higher trophic levels of food chains, such as bald eagles, hawks, and owls. These birds are often rare, endangered, and susceptible to pesticide residues such as those occurring from the bioconcentration of organochlorine insecticides through terrestrial food chains. Pesticides may kill grain- and plant-feeding birds, and the elimination of many rare species of ducks and geese has been reported. Populations of insect-eating birds such as partridges, grouse, and pheasants have decreased due to the loss of their insect food in agricultural fields through the use of insecticides. The loss of even a few individuals from rare, endangered or threatened species pushes the entire species close to extinction. Some pertinent examples associated with birds' kills as a result of pesticides include the insecticides diazinon and carbofuran which are well document as causing bird kills in many parts of the world (Kegley *et al*, 1999). Organochlorine insecticides such as DDT are also well

documented to have continued impairing avian reproduction even after years of banned use. Most bird kills go undocumented, with reported kills representing only a small fraction of actual bird mortality due to pesticides.
Birds exposed to sublethal doses of pesticides are also afflicted with chronic symptoms that affect their behaviour, reproduction, and nervous system. Weight loss, increased susceptibility to predation, decreased disease resistance, lack of interest in mating and defending territory, and abandoning of nestlings have been observed as side effects of pesticides exposure.

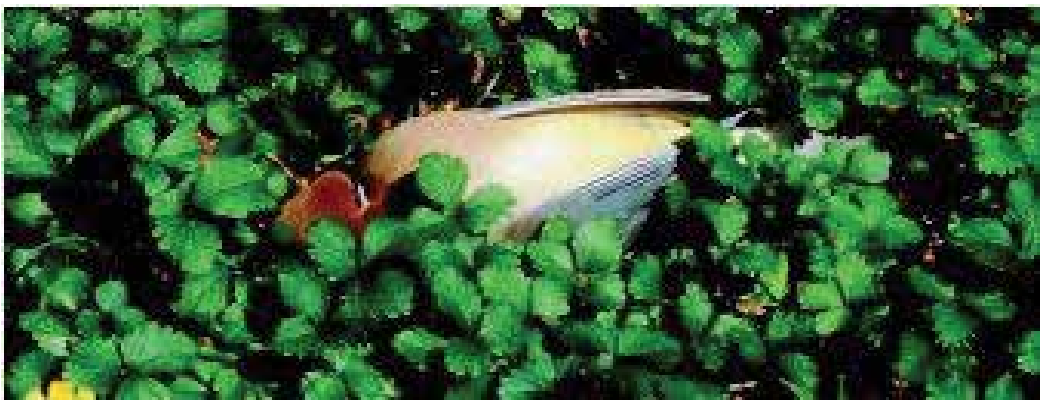

Fig. 2.5 A bird that died as a result of pesticides use (U.S. EPA)

2.7 Effects on fish and other aquatic organisms

A major environmental impact has been the widespread mortality of fish and marine invertebrates due to the contamination of aquatic systems by pesticides. This has resulted from the agricultural contamination of waterways through fallout, drainage, or runoff erosion, and from the discharge of industrial effluents containing pesticides into waterways. Historically, most of the fish in Europe's Rhine River were killed by the discharge of pesticides, and at one time fish populations in the Great Lakes in USA became very low due to pesticide contamination. Additionally, many of the organisms that provide food for fish are extremely susceptible to pesticides, so the indirect effects of pesticides on the fish food supply may have an even greater effect on fish populations. Some pesticides, such as pyrethroid insecticides, are extremely toxic to most aquatic organisms. It is evident that pesticides cause major losses in global fish production. Furthermore, recent laboratory studies of endosulfan and fenitrothion in the tilapia species from Lake Victoria in Tanzania indicated a high capacity of the species to absorb the two pesticides from water with rapid distribution in the organs each with a bioaccumulation factor of 33 and 346 L/kg fresh weight respectively (Henry, 2003).
Multiple pesticides contamination are very common in water and sediments, frequently at concentrations exceeding the lethal limits for many species of zooplankton, small species of animals eaten by fish. Because of the significant high water solubility of the insecticides diazinon and chlorpyrifos and the herbicides simazine, diron, and EPTC are found most commonly in water bodies and have been associated with fish kills and decline in zooplankton population in aquatic environment.

Fig. 2.6. Spraying an aquatic herbicide

2.8 Effects on frogs and other aquatic amphibians

Atrazine being one of the world`s most used pesticide has recently reported by laboratory studies to have a effect on changing male frogs (African clawed frog; *Xenopus laevis*). Adult frogs exposed to atrazine turn female one in ten (10%). These male frogs are missing

Fig. 2.7 Kihansi spray toads from Kihansi Gorge in Tanzania

testesterone and all things controlled by testesterone including sperm production. So their fertility is as low as 10 percent when treated in isolation, but when treated with normal males, they stand a zero chance of reproducing. Although 10 percent of these mutant females can successful mate with male frogs, their offspring are all male because they are genetically male frogs. The ultmate effect of this is that the sex ratios of frogs is badly skewed and this is very dangerous for the survival of that species (Hayes *et al*, 2010).Kihansi spray toads is one among the world`s rarest amphibian species that was close to extinction from their natural environment in Tanzania. The species was first discovered in 1996 during an environment impact study for a large new hydroelectic dam in Udzungwa mountains in Southern Tanzania. The toads lived exclusively in a five acre zone under spray of a waterfall from Udzungwa mountains, hence the name Kihansi spray toads. Among other reasons that contributed to the decline is the use of pesticides in the environment. To rescue this rare species of toads, a colony of them was taken to Bronx zoo and Toledo zoo in USA where they were reared and bread in laboratories for 10 years.

2.9 Pesticides disrupt the natural balance between pest and predator insects

Broad spectrum pesticides such as organochlorine, organophosphorus and carbamate insecticides destroy both pest and beneficial organisms indiscriminately, thus upsetting the natural balance between pests and predator insects. Beneficial organisms serve many valuable functions in an agricultural ecosystem including pollination, soil aeration, nutrient cycling, and natural pest control through pest-predator relationship. Application of insecticides indiscriminately kills both pests and beneficial organisms. Pest populations often recover rapidly because of their lager numbers and ability to develop resistance, but beneficial organisms do not, resulting in a resurgence of the target pest as well as secondary pests that reproduce rapidly without natural predator to check down their numbers. This prompts an escalation in the use of more pesticides by the farmers in an attempt to control them and boost their harvest.

Fig. 2.8 Aerial spraying of pesticides onto the crops using an aircraft

2.10 Pesticides cause pest rebound and secondary pest outbreaks

Non-target organisms, organisms that the pesticides are not intended to be killed, can be severely affected by the use of pesticides. In some cases, where a pest insect has some controls from a beneficial predator or parasite, an insecticide application can kill both pest and beneficial populations. A study comparing biological pest control and use of pyrethroid insecticide for diamondback moths, a major cabbage family insect pest, showed that, the insecticide application created a rebounded pest population due to loss of insect predators, whereas the biological control did not show the same effect (Muckenfuss, *et al* 1990). Likewise, pesticides sprayed in an effort to control adult mosquitoes, may temporarily depress mosquito populations, however they may result in a larger population in the long run by damaging the natural controlling factors. This phenomenon, wherein the population of a pest species rebounds to equal or greater numbers than it had before pesticide use, is called pest resurgence and can be linked to elimination of predators and other natural enemies of the pest (Daly, *et at*, 1998).

The loss of predator species can also lead to a related phenomenon called secondary pest outbreaks, an increase in problems from species which were not originally very damaging pests due to loss of their predators or parasites (Daly, *et at*, 1998). An estimated one-third of the 300 most damaging insects in the US were originally secondary pests and only became a major problem after the use of pesticides (Miller, 2004). In both pest resurgence and secondary pest outbreaks, the natural enemies have been found to be more susceptible to the pesticides than the pests themselves, in some cases causing the pest population to be higher than it was before the use of pesticide.

2.11 Effects on human beings

Pesticides can enter the human body through inhalation of aerosols, dust and vapor that contain pesticides; through oral exposure by consuming contaminated food and water; and through dermal exposure by direct contact of pesticides with skin (Sacramento, 2008). Pesticides are sprayed onto food, especially fruits and vegetables, they secrete into soils and groundwater which can end up in drinking water, and pesticide spray can drift and pollute the air.

The effects of pesticides on human health are more harmful based on the toxicity of the chemical and the length and magnitude of exposure (Lorenz, 2009). Farm workers and their families experience the greatest exposure to agricultural pesticides through direct contact with the chemicals. But every human contains a percentage of pesticides found in fat samples in their body. Children are most susceptible and sensitive to pesticides due to their small size and underdevelopment. The chemicals can bioaccumulate in the body over time. Exposure to pesticides can range from mild skin irritation to birth defects, tumors, genetic changes, blood and nerve disorders, endocrine disruption, and even coma or death (Miller, 2004).

2.12 Pesticides may cause pest resistance

Pests may evolve to become resistant to pesticides as a result of continued use of pesticides in a particular environment. Many pests will initially be very susceptible to pesticides, but some with slight variations in their genetic makeup they become resistant and therefore survive to reproduce. Through natural selection, the pests may eventually become very resistant to the pesticide. Pest resistance to a pesticide is commonly managed through pesticide rotation, which involves alternating among pesticide classes with different modes of action to delay the

onset of or mitigate existing pest resistance. Tank mixing pesticides is the combination of two or more pesticides with different modes of action in order to improve individual pesticide application results and delay the onset of or mitigate existing pest resistance.

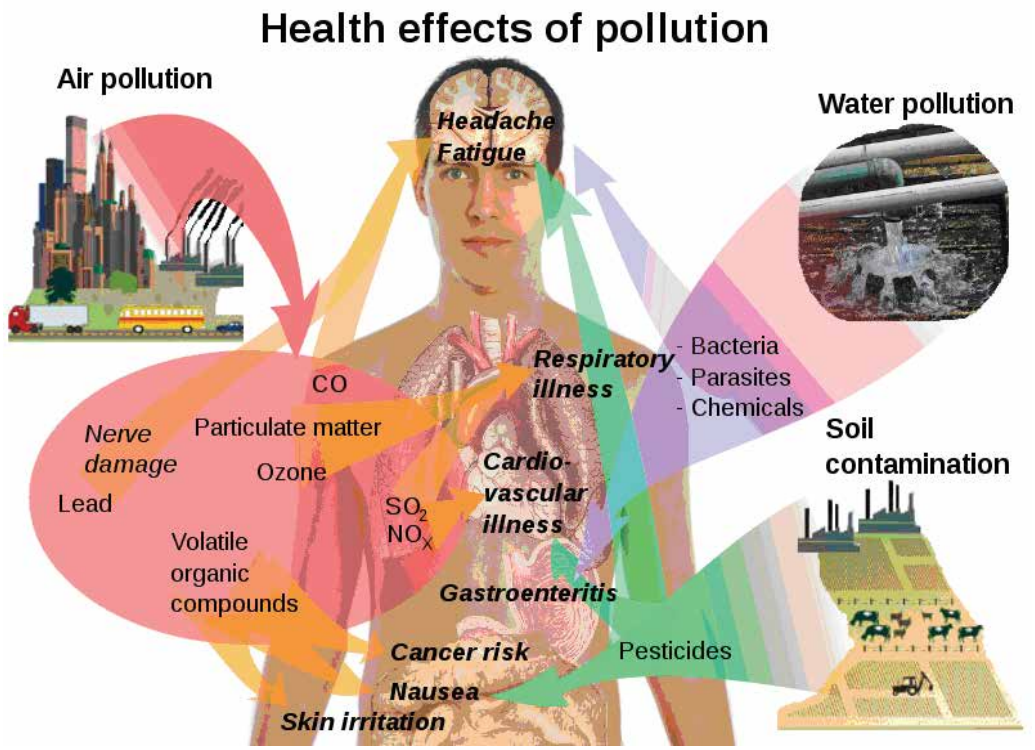

Fig. 2.9 Impacts of pesticides on human health

3. Summary and recommendations

3.1 Summary

In summary, the adverse ecological effects from pesticides occur at all levels of biological organization. The effects can be global or local, temporary or permanent, or short-lived (acute) or long-term (chronic). The most serious effects involve loss in production, changes in growth, development and/or behavior, altered diversity or community structure, changes in system processes (such as nutrient cycling), and losses of valuable species. These ecological losses in turn may be economically or socially important. Hence, ecological effects are of serious concern in regulating pesticides use and a variety of tests have been devised to help evaluate the potential for adverse ecological effects of pesticides. Developing an understanding of how these tests and other information can be used to prevent environmental problems caused by pesticides is the basis for ecological risk assessment research.

3.2 Recommendations

Pesticides destroy the delicate balance between species that characterize the functioning ecosystem. With pesticides now being found routinely in drinking water, on food and in the

air, we are all taking part in an experiment in pesticide exposure on a global scale, but without the benefit of an exposed control group for comparison. For that matter we are likely not be able to quantify the exact risk of these exposures. Because we cannot know for certain the consequences of the expanding pesticides use, the rational and most protective course of action is to take a precaution approach phasing out the use of the most dangerous pesticides, reducing our reliance on toxic chemicals for pest control and promoting ecologically based pest management.

The adverse effects of pesticides on humans and wildlife have resulted in research into ways of reducing pesticide use. The most important of these is the concept of integrated pest management (IPM), first introduced in 1959. This combines minimal use of the least harmful pesticides, integrated with biological and cultural methods of minimizing pest losses. It is linked with using pesticides only when threshold levels of pest attacks have been identified. There is also a move toward sustainable agriculture which aims to minimize use of pesticides and fertilizers based on a systems approach.

There has been a growing concern recently on the promotion of organic farming which emphasize on techniques such as crop rotation, green manure, compost and biological methods of pest control to maintain soil productivity. Organic farming strictly excludes the use of manufactured fertilizers, pesticides, plant growth regulators, livestock antibiotics, food additives, and genetically modified organisms. Organic foods resulting from organic farming are deemed free from pesticides and hence providing an alternative source of quality and safe food in the future. By promoting the use of organic foods means will push the farmers to opt for organic farming. Market forces are a powerful incentive to encourage famers to go organic.

Pesticides manufacturers should conduct long-term studies on ecosystem-wide impacts to demonstrate that a pesticide has no adverse effects before allowing it to be registered for use in the environment. The fact that present regulations view a pesticide as innocent until proved guilty is detrimental to the environment health. It is critical to know more about the long-term ecological effects of a pesticide before it is released to the environment. Using a combination of prior gained field experience with the existing pesticides and applying fundamental chemodynamic principles to newly developed compounds, we can now predict with some degree of accuracy the fate of new chemicals before they are even used in the environment.

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8

Ecological Impacts of Pesticides in Agricultural Ecosystem

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1. Introduction

Pesticides are essential tools in integrated pest management (IPM) programs which can have the great influence if they are used properly. However, the adverse impacts of these compounds on the environment and ecosystem should not be ignored. The ecological effects of pesticides can be discussed from different points of view. Some of the significant consequences of use of pesticides are side effects of the pesticides on non-target organisms, sub-lethal effects of the pesticides on target and non-target organisms, emergence of resistant populations and pesticide residue and their entry into the trophic network. Side effect of the pesticides is a controversial issue in pesticides applications. They kill natural enemies present in the field and ecosystem and destroy the natural equilibrium between the hosts and their natural enemies. In the absence of natural enemies, pest populations increase rapidly and makes more controlling efforts, usually pesticides, necessary. In spite of pests, pesticide resistance in natural enemies is not common due to lower exposure to pesticides. Sub-lethal deposits of pesticides can change some biological traits of the organisms exposed to low and highly low concentrations of the toxicants. Sublethal impacts of pesticides are mostly ignored in ecological pesticide assessment because most pesticide assessments are performed as individual-level bioassays and population-level of toxicants has not been considered. Insects (pests and natural enemies) exposed to sub-lethal concentrations of pesticides show some changes in their life history's traits. Resistant populations emerge due to the misuse of pesticides. The populations with high ecological potential are gradually selected generation by generation and subsequent populations are remarkably or completely insensitive to pesticides. Resistant populations are usually different from natural population in their fertility life table characteristics. Nowadays, the existence of pesticides residue in agricultural crops and their entrance into the trophic network has endangered human health and environment, and it has also necessitated the correct use of the pesticides. In the current chapter, the most significant ecological impacts of pesticides in agricultural ecosystems have been discussed.

2. Impacts of pesticides on natural enemies

The concept of Integrated Pest Management (IPM) was initially defined as the combined use of natural enemies and pesticides to manage pests (Stern et a1., 1959). The IPM

concept later includes coordinated use of all possible tactics to suppress pest damage (Smith et al., 1976, as cited in Ruberson et al., 1998). Use of selective pesticides or rates, temporal separation of pesticides and natural enemies, and spatial separation of pesticides and natural enemies are three main area of integrating natural enemies with pesticides in pest management programs (Ruberson et al., 1998). Conventional use of insecticides can have deleterious effects on natural enemy populations because beneficial arthropods can have greater susceptibility to low concentrations of insecticides than their prey or host (Ruberson et al., 1998; Torres & Ruberson, 2004). Pesticide compatibility with biological control agents is a major concern to practitioners of IPM, and knowledge about the activity of insecticides toward pests, non-target insects and the environment is a necessity (Stark et al., 2004).

Pesticides exert a wide range of lethal (acute and chronic) and sublethal (often chronic) impacts on natural enemies (Rezaei et al., 2007; Ruberson et al., 1998; Stark et al., 2004). Talebi et al. (2008) have published a comprehensive reviewed on the impacts of pesticides on arthropod biological control agents. Sublethal effects are expressed as some changes in the insect's life history attributes (Ruberson et al., 1998). Many studies have been performed on the evaluation of the toxicity of various pesticides to beneficial organisms (Kavousi & Talebi, 2003; Lucas et al., 2004; Medina et al., 2003; Oomen et al., 1991; Paine et al., 2011; Rezaei et al., 2007; Steiner et al., 2011; Urbaneja et al., 2008; Van de Veire et al., 2002; Van den Bosch et al., 1956; Walker et al., 1998). Some important issues including natural enemy species, life stages/sexes, routes of pesticide entry, life history parameters, plot size for field screenings and pesticide formulations and rates must be considered for designing bioassays evaluating the effects of pesticides on natural enemies (Ruberson et al., 1998). One of the commonly used methods in testing the side effects of pesticides on natural enemies, recommended by the International Organization of Biological Control (IOBC), is a tiered approach whereby initial pesticide screening is done in the laboratory, and, depending on the results obtained, semi-field or field tests may be conducted (Dohmen, 1998; Hassan, 1998). This method has been designed to evaluate the acute residual toxicity as well as sublethal effects of the pesticides on the reproductive performance (Vogt et al., 1992). In this method, dead subjects are recorded (often daily) and the total mortality is calculated. The value of mortality (M) for the treated series is determined as the corrected mortality according to Abbott (1925). The average number of progenies (R) is measured as fecundity affected by exposing to a pesticide. The total effect of a pesticide (E) is calculated by the formula $E = 100\% - (100\% - M) \times R$ proposed by Overmeer & Van Zon (1982). Based on the total effects, a pesticide is classified using IOBC evaluation categories (Sterk et al., 1999). Rezaei el al. (2007) investigated the effects of imidacloprid, propargite and pymetrozine in laboratory experiments using IOBC-system on the common green lacewing, *Chrysoperla carnea* (Stephens). All three tested pesticides produced significant adverse effects on pre-immaginal survival ($p<0.01$). Imidacloprid had no significant effect on fecundity, but propargite and pymetrozin caused significant reductions ($p<0.05$). According to IOBC classification, imidacloprid was found to be harmless (E=27.44%), propargite (E=49.78%) and pymetrozine (E=66.9%) were determined as slightly harmful.

3. Population-level impacts of pesticides

Sublethal effects of pesticides on the fitness of individuals are usually assessed using laboratory bioassays with insects (Grant, 1998) due to reduced variation among subject

insects and high validity of statistical analyses (Robertson & Preisler, 1992). On the contrary, insecticide bioassays with field-collected insect subjects reduce reliability on the real effects of insecticides because of heterogeneity among tested individuals (Robertson & Worner, 1990). In fact, the main research aim in ecotoxicological studies is predicting of insect field populations faced with sublethal concentrations/doses of pesticides (Ferson et al., 1996). However, most studies deal with the impact of insecticides on individuals or some components of individuals (Stark & Banks, 2003). In laboratory tests, individual responses to chronic toxicity may be evaluated from morphological, biochemical, physiological, molecular and ecological point of view. Some responses such as reduction of growth or fecundity or increase in mortality rates or development times (Grant, 1998) are more easily measured in most insecticide bioassays. Although, mortality is the endpoint of interest for many acute studies and it may be used as an endpoint criterion in chronic exposure bioassays, reproductive inhibition or growth retardation are generally considered more sensitive measurements, particularly for the estimation of sublethal responses (Villarroel, 1999; Stark & Banks 2003). Sublethal doses/concentrations of toxicants may change life span, development rates, fecundity, egg viability, sex ratio, consumption rate and behavior of subjects (Dempster 1968; Ruberson et al., 1998; Stark & Banks, 2003, Stark & Rangus 1994; Stark et al. 1992a, 1992b; Vinson, 1974). Individual (sub-population) level responses and population level consequences can be related using life table response experiments (Caswell, 1989). Data generated within life table response experiments give valuable information to the assessment of population-level consequences of toxicant sublethal effects (Caswell, 1989b; 1996a, 1996b; Ferson et al., 1996; Grant, 1998; Stark & Banks 2003).

3.1 Life table response experiments

Lethal dose/concentration of an insecticide that kills 50% of a population (LD_{50} or LC_{50}) is commonly used as a simplistic criterion for determining and comparing the effects of toxicants (Stark et al., 2007). This approach relies on the death of individuals and ignores many consequent impacts of a toxicant on survivors. In addition to death, exposure to a toxicant may result in simultaneous manifestation of multiple sublethal effects (Stark & Banks, 2003; Stark et al., 2004). Under the phenomenon population compensation, if sublethal effects do not occur but the population density is reduced, survivors may have more resources available and actually produce more offspring than untreated populations (Stark et al., 2007). Effective concentration/dose of a toxicant that affect x% of a population (EC_x or ED_x) is also used when sublethal effects are scored. (Kammenga & Laskowski, 2000). Demographic toxicological analyses or life table response experiments is another approach, which takes into account total effects that a toxicant might have at the levels of organization higher than the individual (Stark et al., 2004). The advantage of this approach is that a total measure of the effect is determined that incorporates lethal and sublethal effects into a single endpoint, the intrinsic rate of natural increase or r_m (Kammenga & Laskowski, 2000; Stark & Banks, 2003), which can detect subtle, individual-level effects of contaminants that alter the growth of populations at rates below the lethal concentration limits (Bechmann, 1994, as cited in Rezaei et al., 2007). The first life table response experiment was performed by Birch (1953) to study the impacts of temperature, moisture and food sources on flour beetles (as cited in Kammenga & Laskowski, 2000). The approach has been widely used in ecotoxicological studies; however few studies have been published on the use of demography and similar measures of the population growth rate for evaluating the effect of pesticides on insects, especially insect natural enemies (Kammenga & Laskowski, 2000;

Rezaei et al., 2007; Stark & Banks, 2003). Life table response experiments are being increased to measure multiple endpoints of effects and have been recommended as a superior laboratory toxicological endpoint (Stark et al., 1997). In general, the main reason to use life table response experiments in toxicological studies is revealing of total effect (lethal, sublethal and too subtle impacts) of a toxicant on an insect at the population level. In a few investigations, especially in pesticide side effect studies, total effect of a pesticide is measured using the index E which incorporates mortality and fecundity (Overmeer & Van Zon, 1982; Rezaei et al., 2007). However, the index E is not like the demographic parameters (such as r_m) which measure the impact of a toxicant at population level.

3.2 Construction of a life table

Demography has been used in a small number of toxicological studies to evaluate lethal and sublethal effects of toxicants on insect populations (Stark & Banks, 2003; Stark et al., 2007). The basic principal in insect toxicological demography is construction a fertility table. The construction of a number of life tables is an important component in the understanding of the population dynamics of a species (Carey, 1993).

A life table, for each treatment (toxicant concentration or dose), is constructed by following an insect cohort (egg, larva or adult), till the death of all individual members of a cohort, individually, and recording the age of each female (x), the probability that a new individual is alive at age x (L_x), and the number of female offspring produced by a female with attributed x (m_x) were recorded. Each individual from the initial cohort is treated according to a convenient procedure depends on test subject, toxicant and purpose. The survived individuals from the treated individuals are maintained and monitored individually to collect necessary data for construction life tables.

The precise value of the intrinsic rate of increase (r_m) is obtained by solving the Euler equation (Andrewartha & Birch, 1954):

$$\sum_{x=0}^{y} L_x m_x e^{-rx} = 1 \quad (1)$$

In this equation, y is the oldest age class, L_x is the survival of a newborn female to the midpoint of an age interval, and x is the age of each female at each age interval. In addition to r_m, the other main fertility life table parameters including net reproductive rate (R_0), generation time (T), doubling time (DT), and finite rate of increase (λ) are also computed using the following formulas, respectively:

$$R_0 = \sum L_x m_x \quad (2)$$

$$T = \sum x L_x m_x \Big/ \sum L_x m_x \quad (3)$$

$$DT = \ln(2)/r_m \quad (4)$$

$$\lambda = e^{r_m} \quad (5)$$

In fact, these parameters are estimations for a given population; therefore, the uncertainty associated with them must be estimated. Uncertainty associated with the parameters can be estimated using two techniques; jackknife and bootstrap. However, jackknife technique is

more popular and nearly all estimations are performed according to this method. The jackknife technique is used for ease of statistical comparisons among life table parameters related to each treatment and for estimating the standard errors (SE) associated with the parameters. First, the precise value of r_m is calculated for all of the raw data (r_{total}). Then, one of the insect subjects is omitted and an r_m is computed for the remaining insects (n-1). Based on the suggested equation by Meyer et al. (1986) the jackknife pseudo-values were calculated for this subset of the original data according to:

$$\tilde{r}_i = nr_{total} - (n-1)\hat{r}_i \tag{6}$$

The value of n is the number of insects needed to construct a fertility life table. This process is repeated until pseudo-values were calculated for all n possible omissions of one insect from the original data set. Finally n number of calculated $\tilde{r}_i$ are provided to calculate the mean (r_j) and its SE.

$$r_j = \frac{1}{n}\sum_{i=1}^{n}\tilde{r}_i \tag{7}$$

$$\hat{SE}(r_j) = \sqrt{s_i^2/n} \tag{8}$$

In the equation 8, s_i^2 is the variance of the n jackknife pseudo-values. This algorithm is used for estimating uncertainties associated with the four other parameters. All jackknife pseudovalues for each treatment are usually subjected to analysis of variance (ANOVA) followed by a convenient mean comparison test. The nonparametric tests are also used for some pseudovalues which are not meet ANOVA perquisites (Rezaei et al., 2007).

3.3 Life table parameters

Intrinsic rate of natural increase, r_m, is the main and the best estimator for growth rate of insect populations. When values of r_m are positive, a population is increasing exponentially; when r_m is equal to zero, a population is stable and when r_m is negative, a population is declining exponentially and headed toward extinction (Kammenga & Laskowski, 2000). In toxicological studies, values for r_m are statistically compared among different cohorts (toxicant-treated and control). Rezaei et al. (2007) in life table response experiments of *C. carnea* with some pesticides revealed that imidacloprid and propargite had no significant effects on the intrinsic rate of natural increase, while pymetrozine caused a 34% reduction in r_m value ($p<0.05$). Propargite was non-toxic to *C. carnea* under the tested conditions. The life table assay showed more adverse effects of pymetrozine than a non-life table response experiment method (IOBC method). Lashkari et al. (2007) studied the efficiency of imidacloprid and pymetrozine on population growth parameters of cabbage aphid, *Brevicoryne brassicae* L. (Homoptera: Aphididae). They revealed that r_m were lower in imidacloprid and pymetrozine treatments than in controls. In such investigations, simple statistical comparisons of r_m values among cohorts determine efficiency of toxicants. However, a more precise and complicated method is estimating of a concentration/dose of a toxicant at which r_m value is reduced by 50% (population-level EC_{50} or ED_{50}) or specific proportions (population-level EC_x or ED_x) under laboratory conditions. (Suter & Glenn, 1993; Tanaka & Nakanishi, 2001).

3.4 Age-stage two-sex life table

In construction of a fertility life table, raw data is commonly collected from survival and reproduction of female individuals. In this method, males are completely ignored and only used for fertilizing females in a cohort. Ignoring the sex of individuals can result in errors (Chi, 1988). Chi & Liu (1985) and Chi (1988) developed a new method, age-stage two-sex life table, for construction a life table with taking into consideration both female and male sexes. In a small number of investigations, two-sex life table theory have been used for construction of the life table and data analysis (Chi & Su, 2006; Kavousi et al., 2009; Refaat et al., 2005; Schneider et al., 2009; Yang et al., 2006; Yu et al., 2005). As far as the authors aware, there is only one investigation, Schneider et al. (2009), on the effect of a toxicant on an insect according to the age-stage two-sex life table theory. Schneider et al. (2009) determined the side-effects of glyphosate (a herbicide) on development, fertility and demographic parameters of *C. externa* (Neuroptera: Chrysopidae) in the laboratory. They revealed that glyphosate will decrease arthropod population performance and the major detrimental effect observed on *C. externa* was on fecundity and fertility.

3.5 Drawbacks to the use of life table response experiments

Although life table response experiments may provide the most complete data for the impacts of a pesticide on an animal subject at population-level, there are some disadvantages associated with this method. The most important one is that life table response experiments are expensive and time consuming. Construction of a life table is difficult or impossible for some species (long-lived species) when exposed to a pesticide because of the low rate of reproduction. The other major disadvantage is unrealistic conditions under which a life table is constructed. These conditions are far from the natural conditions in field (Kammenga & Laskowski, 2000; Stark & banks, 2003).

4. Resistance of pests to pesticides

Pesticides are used extensively for control of invertebrate pests, plant pathogens, weeds and rodents and other pests in a wide range of crops and for veterinary purpose. Resistant to pesticides develop in insects, mites, fungi, weeds, bacteria and rodents. Repeated applications and extensive use of the synthetic pesticides has toxicity toward natural enemies and cause resistance development in pest species against major classes of pesticides throughout the world. The repeated and extensive application of pesticides caused majority on susceptible individuals in population and only some resistant individuals survive from pesticide exposure. The offspring genotype of survival individual is homozygous or heterozygous that depends on history of pesticide application and type of pesticides. The offspring inherit the resistant genes and survival ability from the exposure to the pesticides. The surviving individuals multiply in absence of their natural enemies and finally replace the non-resistant population. The development of pesticide resistance is a Darwinian evolutionary process at a rate that rare genes conferring resistance to pesticides are selected by the high selection of pesticides. Resistance to pesticides is defined as "the development of an ability in a population of a pest to tolerate doses of pesticides that would prove lethal to the majority of individuals in a normal population of the same species" (Stenersen, 2004).
The first case of resistance occurrence in insect pests was reported in 1908. This document reported the failure in the control of *Quadraspidiotus perniciosus* (Hem.: Diaspididae) by sulphur. After this report, Melander (1914) reported resistance of three scale strains in

United State to sulphur and sulphur–lime (as inorganic pesticide) (as cited in Stenersen, 2004). The organochlorine and synthetic insecticides were commercialized for chemical control of pests in the 1940's. The first case of DDT resistance in insect was reported in *Musca domestica* few years after introduction. After that, new insecticides such as cyclodienes, pyrethroids, organophosphates (OP), carbamates, formamidines, *Bacillus thuringiensis*, avermectins, spinosyns, insect growth regulators (IGR) and neonicotinoids were introduced for pest control and the cases of resistance to these compounds appeared a few years after their application. Now, more than 504 key pest species were resistant to pesticides and the resistance to pesticides has become a major contemporary problem in pest management programs (IRM) worldwide. Stuart (2003) reported resistance of 520 insect and acari species, 150 plant pathogen species and 273 weed species to pesticides.
Pesticides resistance reduces the ability control of pesticides on pests and leads to higher application rates to achieving satisfactory pest control. Pimentel (2003) estimated the major economic and environmental losses due to the application of pesticides on crops and veterinary purpose in the USA and showed the following costs: "public health, $1.1 billion year-1; pesticide resistance in pests, $1.5 billion; crop losses caused by pesticides, $1.1 billion; bird losses due to pesticides, $2.2 billion; and ground water contamination, $2.0 billion" (Pimentel, 2005).

4.1 Detection and monitoring of resistance

Reduced pesticide selection pressure for each resistance mechanism is necessary for avoiding and delaying control failure prior to occurrence of resistance. For achieving this purpose, successful detection techniques are required for avoiding resistance developing and a control failure. "These techniques could be able to detect of resistant individual at low frequency in natural population" (Scott, 1995). Techniques for monitoring resistance to different pesticides in pest population gathering valuable information for insecticide resistance management (IRM) employer.
Detection and identification of resistance mechanisms to pesticides require monitoring approach with appropriate bioassay method. Monitoring of resistance is required in order to sustainable management of pesticide resistance and to know the status of resistance. Therefore, developing precision and reliable susceptibility test must be developed. These tests must be also accurate, chip, easy to perform in variety of conditions in laboratory and on farm site. So far, many standardized susceptibility test method were presented by Food Agriculture Organization (FAO) and World Health Organization (WHO) such as exposure to standard residue treatment on glass scintillation vial or filter paper, plastic bags, topical application, spray application of standard solutions, and resistance detection kits and strip.
Resistance frequencies can be detected and monitored by bioassays using diagnostic (discriminating) dose (LD_{99}) and estimating resistance factor (Rf= LD_{50} of resistant population/LD_{50} of susceptible population). The diagnostic dose (ie. LD_{99}) can be calculated from regression line of log dose probit-mortality data using appropriate software such as POLO-PC. This dose discriminate the tested population as susceptible and resistant and the pests that die after exposures with LD_{99} of pesticide are classified as susceptible and those individual that survive from exposure considered as resistant. The discriminating-dose assay is a chip, less time consuming approach for monitoring resistance in natural pest populations. These bioassay procedures provide valuable data for monitoring of resistance but this method is not practical for detection of resistance in low frequencies in field population of pests (Roush and Miller, 1986).

The mechanisms of resistance are behavioral, reduced penetration, metabolism of toxicant to inactive product and target site insensitivity. These mechanisms can be detected using biochemical assay techniques (spectrophotometric and fluorometric methods) and molecular assays (base on DNA diagnostic) in one individual or small number of insect. Identification of resistance mechanisms is critical for determining of the cross resistance spectrum (Brogdon and McAllister, 1998).
"Molecular methods and traditional assays (ie. bioassay) used for distinguish heterozygotes (SR), homozygous susceptible (SS) and homozygous resistant (RR) genotypes" (Scott, 1995). The environmental conditions such as temperatures, humidity, pH and light increase errors in biochemical and bioassay results but these conditions can not affect the results of molecular methods (Scott, 1995). Now, PCR-based techniques have been designed for field detection of modified acetylcholinesterase (AChE) and *knock down (Kdr)* in individual *Myzus persicae* (Field et al., 1996). "The amplified E4 or FE4 genes can be identified by restriction enzyme analysis or polymerase chain reaction (PCR)-based methods" (Field et al., 1996).

4.2 Mechanisms of resistance to pesticides

Biochemical and molecular basis of resistance mechanisms to pesticides in insects, acari, fungi, bacteria, weeds and vertebrate pests are similar. An exhaustive knowledge on biochemical and molecular resistance mechanisms in pests are useful for designing insecticide resistance management (IRM) strategies. Also, identification of resistance mechanisms is necessary for developing discriminating techniques for detecting and monitoring resistance genes and cross resistance spectrum in the field populations of pests (Hammock and Soderlund, 1986). The factors affecting pesticides effectiveness were distinguished in two classes: The first class decreases the amount of pesticide dose in action site including behavioural resistance, reduced penetration or adsorption, sequestration and detoxification. The second class is decreased target site sensitivity to pesticides that reduce the affinity of target protein toward activated pesticide (van leeuwen, et al., 2009). "In practice, probably more than 90% of all resistance cases in insects and mites are caused by a less sensitive target site and/or an enhanced pesticide detoxification" (Roush and Tabashnik, 1990 as cited in van leeuwen, et al., 2009). The relative importance of these mechanisms depends on pest species and history of chemical application.

4.2.1 Genetic mechanisms

Genetic mechanisms of pesticide resistance involve some point mutations in genes and their over expression. These mechanisms were elucidated as follow:

4.2.1.1 Gene amplification

Devonshire and Moores, 1982 showed that the gene amplification of one of two closely related carboxylesterases (E4 and FE4) in *M. persicae* were associated with resistance to OP, carbamates and pyrethroids. Carboxylesterases sequester or degrade carbamate and OP insecticides before they reach to AChE in the nervous system. E4 and EF4 overproduction in resistant strains of *M. persicae* is due to amplification of structural genes encoding these enzymes (Field et al., 1988).

4.2.1.2 Up- and down-regulation

The research showed that cytochrome P450 enzyme were over expressed in some resistance strain of *M. domestica* through the increase of gene transcription by up-regulated

mechanism. The up-regulation of a cytochrome P450 enzyme led to resistance when an insecticide is used in its toxic form on *M. domestica*. If pro-insecticide, i.e. a chemical must be converted in pest through metabolism to the active form, used against *M. doimestica*, down-regulation of cytochrome P450 or other metabolizing enzymes will increase resistance (Scott, 1995).

4.2.1.3 Structural change in insecticide- target molecules

AChE, the gamma-aminobutyric acid (GABA) receptor, Voltage-gated sodium channels, nicotinic acetylcholine receptor, octopamine receptor and the juvenile hormone (JH) receptor are known as targets of pesticides and substitution of amino acid residues in these sites led to insensitivity of structural protein toward pesticides (Kono and Tomita, 2006).

4.2.2 Behavioral resistance

"Behavioral mechanisms, defined as evolved behaviors that reduce an insect's exposure to toxic compounds or that allow an insect to survive in what would otherwise be a toxic and fatal environment" (Sparks et al.,1989). There is a little literature on behavioural resistance mechanisms in insect due to difficulties in detection (as cited in Jensen, 2000). It seems the significance of this mechanism for resistance is less than other resistance mechanisms.

4.2.3 Reduced penetration

Reduced penetration of insecticide as a resistance mechanism has been studied in few insect species such as *Leptinotarsa decemlineata*. Reduced insecticide penetration via cuticle led to decrease the amount of dose in action site. The resistance ratio by this mechanism was lower than 3-fold (Scott, 1990), but because several different mechanisms are responsible for resistance to an insecticide and multiple resistance mechanisms may co-exist in an insect and act either additively or synergistically.

Patil & Guthrie, 1979 compared the composition of the cuticular lipids of two resistant strains of *M. domestica* and their results showed that "total lipids, monoglycerides, diglycerides and sterol esters, sterols, fatty acids and phospholipid phosphorus were higher in resistant strains than in the susceptible strain".

Three methods for detecting of this mechanism include: Wash-off, diffusion cell and disk technique. In wash-off radiolabelled insecticide was topically applied to the insects and then, at fixed times after application, un-penetrated insecticide was washed off with an appropriate solvent and quantified (as cited in Jensen, 2000).

4.2.4 Metabolism of toxicants

Three enzyme groups involved in metabolic resistance to pesticides: esterases, glutathione S-transferases (GST) and mixed function oxidaes (MFO). The following technique can be used for detection of these mechanisms.

4.2.4.1 Esterase

Esterases metabolize a variety of pesticides such as OP, carbamate, pyrethroids with ester linkages. "These enzymes confer resistance to pesticides in over 50 species of insects, ticks and mitesa" (Devorshak and Roe 1998; as cited in van Leeuwen et al., 2009). Detection and investigation of esterases-based mechanism can be achieved from synergistic bioassays and biochemical assays. For synergistic bioassays, some synergists such as DEF (S,S,Stributylphosphorotrithioate), TPP (O,O,O-triphenylphosphate), and IBP (O,O-bis[1-

methylethyl] S-phenylmethylphosphorothioate) were used to inhibit esterases (Raffa & Priester, 1985 as cited in Jensen, 2000). However, the synergistic bioassays are useful to achieve valuable data, but DEF in higher concentrations inhibits MFO and esterase activity (Scott, 1990).

In biochemical assay, increased esterase activity in resistant insect can be checked with some artificial substrates such as α- and ß- naphthyl acetates, α- and ß- naphtyl butyrates, α- and ß- naphtyl propionates and p- nitrophenyl acetates. These substrates hydrolyzed by general esterases and the involvement of esterase in resistance must be checked by more than one substrate. Also, the specific elevated esterase can be detected with immunological methods and an antiserum for example antiserum of E4 carboxylesterase *M. persicae (*Devonshire et al., 1986). An affinity purified immunoglobulin G (*IgG*) fraction from this antiserum has been used in a immunoplate assay to distinguish between the different resistant strains of *M. persicae* (Devonshire et al., 1986).

4.2.4.2 GST

The GSTs are involved in the detoxification of a wide range of xenobiotics including insecticides, fungicides, acaricides and herbicides (Salinas & Wong, 1999). GSTs catalyze the conjugation of hydrophobic electrophile compounds such as pesticides and their metabolites with the thiol group of reduced glutathione (GSH) (Habig *et al*., 1974).

Elevated GST activity has been associated with resistance to the major classes of pesticides and the involvement of GSTs in resistance to insecticides is well reviewed in Enayati et al., (2005). Because GSTs can metabolize a wide variety of xenobiotics such as insecticides and plant allelochemicals, increased GST activity may be due to exposure of insect with foreign compound in environment not resistance mechanisms. Diethyl maleate (DEM) is used as synergist for inhibiting of GSTs involved in resistance in bioassays.

GST activity can be measured from direct measurement of the conjugation of reduced glutathione with non-fluorescent monochlorobimane (MCB), l-chloro-2,4-dinitrobenzene (CDNB) and 3,4-dichloronitrobenzene (DCNB) as substrates using a spectrophotometer at 340 nm.

4.2.4.3 Cytochrome P450

Cytochrome P_{450} plays important role in metabolism of pesticides and confers resistance to many classes of pesticides in mite, insect, weeds and fungi. These enzymes widely distributed in fungi, bacteria, yeast, insect, mite, weeds and invertebrate. Piperonyl butoxide (PBO) and sesamex was used in synergistic bioassays for involvement of MFO in resistance. However, PBO can inhibit both MFO and esterases-based resistance mechanisms in some insect and mite species (Gunning et al., 1999; Young et al., 2005).

There are many P_{450}-monooxygenase isoenzymes with different substrate specificity within an insect. Therefore, in measuring activity of MFO- based resistance mechanisms must use different substrates and methods (Rose et al., 1991). Different biochemical methods have been used to study P_{450}-monooxygenase activity in insects and mites. One of these methods is measuring the total amount of heme containing protein using a heme-peroxidase assay (Brogdon et al., 1997). Another method uses the aldrin as substrate to measure P_{450}-monooxygenase activity. *O*-demethylase can be detected using *p*-nitroanisole as substrate using spectrophotometer. O-deethylation activity of the artificial substrates, 7-ethoxycoumarin (7-EC) and ethoxy-4-trifluoromethylcoumarin, by MFO can be measured with fluorometric microplate assay (van Pottelberge et al., 2009).

4.3 Fitness costs associated with pesticide resistance

Genetic changes confer insecticide resistance in insects can affect their developmental time and reproductive potential. Resistance genes can alter some life table and physiology parameters of pests and thus causing a fitness cost (as cited in Gazavi et al., 2001). In some insect and mite species, genetic changes that enhance survival due to pesticides exposure reduce pest fitness in the absence of pesticides (Roff and Derose, 2001; Higginson et al., 2005). Because insecticides caused a huge selection on pest population in a short time, therefore susceptible and resistant populations are useful models for evaluating fitness trade-off (Crow, 1957; McKenzie, 1996; Higginson et al., 2005; Ghadamyari et al., 2008). Mutation associated with insecticide resistance can disturb pest physiology (MacCarroll et al., 2000).

Resistant and susceptible strains differ in some properties due to their adaptation to insecticides, such as developmental time, fecundity and fertility, overwintering success and sensitivity to alarm pheromone. Differences in the biological parameters affecting the net reproductive rate of insect populations are important for insecticide resistance management (IRM) (Haubruge and Arnaud 2001). Altered acetylcholinesterase (AChE) and esterase-associated resistance in peach aphids have life history disadvantages compared with susceptible counterpart (Ghadamyari et al., 2008). Also, altered GABA receptor, esterase, MFO and GST associated abamectin resistance in *Tetranychus urticae* showed life history disadvantages.

Field and laboratory studies on different strains of *M. persicae* have been showed that adverse selection by pesticides caused "poorer winter survival, maladaptive behaviour and reduced reproductive fitness" (Foster et al., 2000).

4.3.1 Maladaptive behavior

Some resistance mechanisms can confer more fitness disadvantages to resistant strains than others. Some strains of *M. persicae* with over expressing high levels of carboxylesterase (due to structural gene amplification) show a reduced tendency to move away from senescing leaves compared with susceptible counterpart (Foster et al. 1997, 2003). This behaviour caused higher mortality during worst winter weather conditions and so can be regarded as a deleterious pleiotropic effect of pesticide resistance. Studies have been shown that peach–potato aphids caring both gene amplification and the knock down mutation has reduced response to alarm pheromone (Foster et al., 2003).

4.3.2 Reduced reproductive success

Many experiments measured reproductive fitness in the absence of pesticides in resistant and susceptible strains. The results of these experiments showed that individuals carrying resistant genes have lower reproductive rate than susceptible. When pressure of insecticide is diminished on resistant strain, the number of resistant individual in population quickly reduce due to fitness cost (Crow, 1957; Carrière and Roff, 1995; McKenzie, 1996; as cited in Arnaud et al., 2005). Although the majority of insecticide resistance strains show fitness cost (McKenzie, 1996; Foster et al., 2003; Berticat et al., 2004; as cited in Arnaud et al., 2005; Ghadamyari et al., 2008), a few researches present species with no fitness cost (McKenzie, 1996; Oppert et al., 2000; McCant et al., 2005). For example "some resistant strains of mosquitoes in absence of pesticides showed only one quarter of the reproductive potential of susceptible strains" (Georghiou and Taylor, 1977). *Varroa destructor* has little or no reproductive fitness cost associated with pyrethroid resistance in Texas (Martin et al., 2002).

Tetranychus urticae resistance to abamectin showed reduced reproductive success compared with susceptible populations and the r_m of susceptible population was higher than r_m of resistant population (unpublished data).
In contrast, in *Tribolium castaneum* "resistant to malathion, susceptible male individuals show reduced reproductive success compared with resistant lines" (Arnaud et al., 2005). Foster et al. (2000) showed reduced reproductive success in *M. persicae* expressing the highest levels of carboxylesterase.

4.3.3 Reduced overwintering ability

Winter field trials by Foster et al., 1996 showed that UK *M. persicae* clones expressing high levels of esterase-based resistance (i.e. R2 and R3) present higher mortality than their susceptible (S) and -R1 counterparts during worst weather conditions (Foster et al., 1996). In *Heliothis virescens*, resistance to Cry1Ac is recessive and associated to cadherin gene (Morin et al., 2003), research showed fitness costs associated resistance affecting overwintering success and survival on non-Bt cotton (Carriere and Tabashnik, 2001). The frequency of pink bollworm resistance to Bt cotton has not increased in the field compared with laboratory that show fitness cost (Tabashnik et al., 2003). Monitoring *Culex pipiens* mosquitoes overwintering in a cave in southern France (in an area where OP insecticides are widely used) showed a "decrease in the frequency of insecticide-resistant mosquitoes compared with susceptible counterpart, indicating a huge fitness cost" (Gazave et al., 2001). In the pink bollworm, *Pectinophora gosypiella*, has been shown fitness costs at low temperatures associated with resistance to Bt and this can delay resistance of this pest to Bt cotton (Carrie`re et al., 2001; Tabashnik et al., 2005).

4.3.4 Why insecticide resistance caused a fitness cost?

The occurrence of fitness costs in insecticide resistant strain is reported for many pests such as *M. persicae* (Ghadamyari et al., 2008), *T. urticae* (unpublished data) and *Sitophilus zeamais* (Coleoptera: Curculionidae) (Arau´jo et al., 2008a, 2008b; Guedes et al., 2006). Populations of *T. urticae*, *S. zeamais* and *M. persicae* with different levels of resistance to different pesticides have shown to be good subjects and models for evaluating the physiological base of fitness cost associated pesticide resistance.
Recently, some attempt was done to show the relationship between the energy consumption and the energy reserves available for metabolism of pesticides. The energy consumption could be measured using the electron transport activity (at the mitochondrial level), while reserve energy for metabolism could be achieved by measuring total lipids, protein and sugar contents by spectrophotometric method. The differences between energy consumption and the energy reserves represent the energy available for growth and biomarker of fitness cost in resistant populations. Our research on fitness cost of *T. urticae* resistant to abamectin showed that no significant differences were presented between the amounts of fuel nutrients macromolecules (carbohydrate, protein and lipid) in the resistant and susceptible populations of *T. urticae*, the amount of energy consumed was higher for resistant population when compared to its susceptible counterpart. Also the susceptible population exhibits a significantly higher r_m than the resistant population. These suggested that the resistant population may be less fit than the susceptible compartment.
The following theory was presented by Guedes et al., 2006, Arau´jo et al., 2008a, 2008b and Lopes et al., 2010 and we attempt to discuss their theory with their justifying fitness cost in *S. zeamais*.

Populations of *S. zeamais* with different levels of susceptibility to insecticides were used a model for evaluating the mechanisms of fitness cost associated resistance. Demographic and competition studies carried out on different strains of *S. zeamais* susceptible and resistant to pyrethroids showed fitness costs associated with insecticide resistance in some strains and no fitness costs in other strain (Fragoso et al., 2005; Oliveira et al., 2007).

The susceptible, resistant no-cost and resistant cost strains showed some differences in some biochemical parameters as follow:

I. Some differences in the accumulation and consumption of fuel nutrients macromolecules were observed between *S. zeamais* pyrethriod-resistant and susceptible strains. These differences caused *S. zeamais* could be able detoxify insecticides without reduction in its reproductive potential. Pyrethroid- resistance cost strain has greater stored total proteins and carbohydrates compared with susceptible and resistant cost strains (Arau´jo et al., 2008a, 2008b). Finally Arau´jo et al. (2008a, 2008b) concluded that increased energy reserves may be due to increased digestive enzyme activities.

II. The pyrethroid -resistant strains showed increased serine- and cysteine-proteolytic and cellulolytic activity. Also, kinetic parameters of these enzymes were different in susceptible, resistant no-cost and resistant cost strains. These differences suggested that cysteine-proteinase and cellulase activities were more important for justifying the cost of insecticide resistance in *S. zeamais* strains (Arau´jo et al., 2008b). The activity of carbohydrases specially amylase was higher in the resistant no-cost strains suggesting that a more efficient energy storage may justify the fitness costs due to the over expression of detoxify enzymes (Lopes et al., 2010).

III. The pyrethroid-resistant no-cost strain of *S. zeamais* show higher grain loss, higher respiration rate, higher body mass, and larger energy reserve cells than the pyrethroid-resistant cost strain and susceptible strains (Guedes et al., 2006). These advantages cause resistant no-cost strain has additional reserved energy for detoxifying insecticides without any adverse effect on their life table parameters (Guedes et al., 2006).

Some resistant strains of green peach potato aphid in the UK showed various fitness costs, such as reduced overwintering ability, lower rate of movement away from senescing plant leaves at low temperatures, reduced responses to alarm pheromones and reduced reproduction (Foster et al., 2000, 2002, 2005; Ghadamyari et al., 2008). Foster et al., 2000 concluded that differences in behavior reducing *M. persicae* survival due to pleiotropic effects of the *kdr* mechanism and over expression of E4 and FE4 gene (Foster et al., 2000) Also behavioural characteristics is associated with knock down resistance in the *M. domestica* (Foster et al., 2003).

5. Pesticides residue in the environment

Chemical pesticides are used to control target pests. Extensive use of pesticides after World War II has substantially increased the agricultural production. However non target organisms including human and wildlife are affected. Pesticides are bioactive molecules that interfere with vital biochemical and physiological processes in organisms. Some are lethal to exposed organisms and many can cause disorder at sub lethal level. Extensive research is necessary to clarify the side effects of pesticides on organisms. About 3 billion kg of pesticides is applied each year with a purchase price rose to $47 billion in 2008, worldwide (Pimentel, 2009; Frabotta, 2009).

The environmental persistence is different from pesticide to pesticide. Some are persistent and remain in the environment either as a parent compound or transferred products. The fate of pesticides in soil depends on the value of Koc, carbon sorption coefficient. High values of Koc indicate a pesticide that strongly adsorbs to the soil particles and less likely to move with water. Moreover, soil composition, pH, moisture content and microbial activity affect pesticide persistence.

5.1 Insecticides

The most toxic and environmentally persistent compounds are found among insecticides; therefore, the emphasis is on groups of insecticides that have been studied in detail.

5.1.1 Chlorinated insecticides

The first synthetic insecticide was DDT with a wide spectrum of insecticidal action that was used in agriculture and against insect vectors of deadly diseases. DDT solubility in water is very low, about 0.006 mg/l, which makes it one of the most hydrophobic insecticides. DDT residues either as parent compound or its metabolites DDD and DDE are stable and have high persistence in the environment. It has a great tendency to be stored in fatty tissue of different organisms. After the introduction of DDT, HCH was marketed. HCH has eight isomeric forms of which γ-isomer is called Lindane. Lindane is a volatile insecticide and was used against agricultural and households pests. Lindane is less persistent than the other organochlorine insecticides especially under moist conditions. The cyclodiens are stable organochlorine soil applied insecticides. These included aldrin, dieldrin, endrin, chlordane, heptachlor and endosulfan. Cyclodiens are environmentally persistent compounds that have raised concern about adverse effects on human health and wildlife. Residues of DDT and its metabolites DDD and DDE, dieldrin and heptachlor epoxide were detected in high percentage of soil and water samples from agricultural areas decades after their use were banned. Extensive studies on organochlorine pesticides has shown the environmental persistence of these Compounds (LeaMond et al. 1992; Reiser & O'Brien, 1999).

5.1.2 Organophosphorus Insecticides

Organophosphorus insecticides replaced persistent organochlorine compounds. Utilization of these insecticides increased rapidly and for several decades comprised high proportion of total insecticide use. Organophosphates are unstable compounds, however some of these insecticides are more acutely toxic to invertebrate than chlorinated insecticides. Parathion was the first marketed product that was effective against a wide variety of pests. Some organophosphates caused severe toxicity associated with many deaths especially in developing country, whereas a few compounds such as malathion are relatively safe to mammals and degrade fairly rapidly in the environment. Most organophosphates are harmful to beneficial arthropods, though few compounds such as phosalone and dimethoate are considered as harmless compounds.

The occurrence and movement of some organophosphate pesticides are reported in rivers and streams. Several studies conducted to find out the presence of organophosphate residues in California rivers during 1993-1994. Diazinon, methidathion, dimethoate and chlorpyrifos residues were detected in water samples. The detection occurred mostly during rainy season, showing how run off influences the presence of pesticide residues in rivers and streams (Ganapathy et al. 1997).

5.1.3 Carbamate Insecticides

Carbamate insecticides are a group of synthetic compounds derived from carbamic acid. The first carbamate carbaryl was an N-methyl carbamate with high insecticidal activity against many insect pests and ectoparasites of animals. Carbamates especially N-mathyl carbamates are extremely toxic to hymenoptera and are lethal to exposed foraging bees.
Carbamates biodegradation in environment is relatively rapid.
Oxime carbamates are a group of carbamate with systemic action. Aldicarb, an oxime carbamate is the most potent toxic substance (LD_{50}=0.9 mg/kg) ever used in crop protection. Because of high toxicity it is used as granular formulation. Aldicarb sulfoxide is its oxidative metabolite that may undergo further oxidation to the sulfone. Oxidative residues and its parent compound (Tatal aldicarb) are toxic and highly mobile in the environment. Total aldicarb is detected especially in shallow ground water since 1979. Ground water quality monitoring has shown that many samples contain aldicarb residues and some of them exceeded maximum acceptable concentration (Priddle et al. 1989; Marade & Weaver 1994).

5.1.4 Pyrethroids

Pyrethroids are synthesized based on the model of naturally occurring pyrethrins with more stability to light and air. Pyrethroids are used in agriculture, homes, restaurants and hospitals. These compounds are readily metabolized by man but they are effective against insects. Most pyrethroids are esters however non-ester pyrethroids are discovered with good insecticidal activity and low mammalian toxicity. These readily penetrate insects and paralyze their nervous system (Reigart et al., 1999). Since pyrethroids are highly toxic to insects, both the beneficial and pest insects are affected.
Sunlight, microbial activity, heat, and moisture accelerate pyrethroids break down, hence in areas with limited sunlight, pyrethroids persist for a long time. After treatment in the home, cypermethrin persist for about three months (Wright et al, 1993). Pyrethroids are lipophilic compound that strongly absorb to colloids of soil. Dissipation of cypermethrin, fenvalerate, and deltamethrin, were investigated in yellow red soils. The half-life of theses compounds were 17, 19, 18 days in unsterilized, compared to 76, 92 and 80 days in sterilized soil (Gu et al, 2008). This experiment shows the effects of biodegradation in pyrethroids life span in soil.

5.1.5 Neonicotinoids

Neonicotinoids are similar to nicotine with the same mode of action. These insecticides have been used worldwide. Most neonicotinoids are absorbed and translocated to the tips of the plants. Imidacloprid is the first widely used insecticide of this group with relatively low mammalian toxicity. However, it is harmful to beneficial arthropods including bees (LD_{50}=0.008 μg /bee). Imidacloprid and clothianidin are more toxic to bees as spray than as seed dressing (Tennekes, 2010). Most neonicotinoids are moderately soluble and so they are mobile in the environment. In ground water 18 feet below sandy loam soil concentrations of imidacloprid ranged from < 0.1 ppb to 1 Ppb (Bacey, J. 2000). This observation shows the potential of imidacloprid to leach downward into shallow groundwater. Imidacloprid has a moderate binding affinity to soil colloids. Half-life in soil varies under different conditions. The half-life of imidacloprid in soil was 48-90 days, depending on the ground cover (Scholz & Spiteller, 1992). Laboratory experiments showed that persistence of another neonicotinoid, thiamethoxam is highly depending on moisture and the half-life varied from

45 to 300 days (Gupta et al. 2008). The half-life of neonicotinoids increases with increasing soil colloids. Overall, neonicotinoids have a low potential to persist in soil and accumulate in the environment.

5.2 Herbicides

Herbicides are the major class of pesticides to control weeds. Little attention is paid to herbicides as a source of pollutants; mainly because with a few exception; most herbicides have not appreciable mammalian toxicity. Among toxic herbicides are paraquat (LD_{50}=125 mg/kg) and dinoseb (LD_{50}=58 mg/kg); however widely used herbicides including 2,4-D and glyphosate are not highly toxic to mammals. On the other hand groups of herbicides that have potential to persist in soil and enter surface water include triazines, sulfonylureas, phenylureas and uracils. Laboratory experiments have shown that among four triazines; prometryn and terbutylazine half-lives were 263 and 366 days in ground water respectively. The half lives of simazine and atrazine were shorter than prometryn and terbutylazine (Navarro et al. 2004).

Sulfonylureas are high potent herbicides group effective at very low dose (10-15 g/ha), for that reason persistent herbicides from previously sprayed farms may damage the next crop. These herbicides are able to penetrate into deeper layers of the soil profile, where they have a relatively high persistence. A number of sulfonylureas were detected in wetland sediments. Etametsulforun methyl, sulfosulforun and metsulforun-methyl were determined in wetland sediments with mean concentration ranging from 1.2 to 10.0 μg kg^{-1} (Degenhardt et al. 2010). According to Cessna et al (2006) a half-life of 84 days was observed for metsulforun-mathyl in farm dugouts. Residues of 10 herbicides were detected in prairie farm dugouts. 2,4-D was the most frequent with median concentration 0.05 μg L^{-1}(Cessna & Elliot, 2004).

Based on these studies, herbicides have different tendency for binding to soil colloids and so have different movement ability.

5.3 Fungicides

Fungicides are substances that destroy or inhibit the growth of fungi. Fungicides are used in agriculture and industry. Early fungicides were organic derivatives of metals such as mercury. Organomercury fungicides were widely used as seed dressing to control diseases of cereals. Although mercury content of these fungicides formulation were less than 5%, the main concern is the side effects of residues remaining in the environment long enough to enter soil and water. Both inorganic and organic compounds of mercury are toxic, however organic compounds are more lipophilic than inorganic and are liable to adsorption by soil colloids and storage in fat depot. Bioconcentration factor up to 100000 times is reported for the methyl mercury content in fish (USEPA, 1980). Dithiocarbamates (e.g. mancozeb, thiram, zineb and maneb) are the first synthetic organic fungicides. Some fungicides are toxic to aquatic organisms. Maneb is highly toxic to fish and triadimefon is highly toxic to crustaceans. Dithiocarbamate fungicides have low persistence. Among high persistent group of fungicides are triazoles (penconazole, myclobutanil and flusilazole), carboximides (boscalid) and pirimidines (fenarimol) (Wightwick et al., 2010).

6. Conclusion

The use of pesticides is essential for protecting agricultural products from pest damages; however their adverse effects are inevitable almost on all habitats. From the preceding

information it is clear that side effects of pesticides on natural enemies, emergence of resistant populations and entrance of pesticides into the environment are the main issues that have been considering for a long time. More precise methods should be considered to evaluate these adverse impacts at the population-level and ecosystem as well as laboratory-based and individual-level assessment. Life table response experiments reveal total effects of any pesticides on an individual (target or non-target) at the population level. However, most publications in the field of insect toxicology are based on individual-level bioassays. Meanwhile, population genetics and resistance inheritance have mostly been ignored in insect toxicology which can provide great information on the ecological impacts of pesticides in agricultural ecosystems. It is believed that modern sciences such as insect biotechnology and nanotechnology facilitate designing novel and effective pesticides with less adverse effects in the environment.

7. References

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9

Environmental Impact and Remediation of Residual Lead and Arsenic Pesticides in Soil

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1. Introduction

Chemical control of insects is considered one of the most beneficial developments of civilization (Klassen & Schwartz, 1983). As long ago as 1000 B.C., sulfur compounds were used to control insects in Asia Minor (National Academy of Science, 1969). However, the extensive use of chemicals to control pests has developed only in the last 150 years. The first example of large-scale effective chemical control of an insect pest occurred in 1867, with the use of Paris green (copper acetoarsenate) to control Colorado potato beetle, *Leptinotarsa deecemlineata* (Say). Paris green was later used to control codling moth, *Laspeyresia pomonella* (Linnaeus), on fruit trees (Klassen & Schwartz, 1983). Due to its effectiveness in controlling gypsy moth, *Porthetria dispar* (Linnaeus), lead arsenate replaced Paris green in New England in 1892. Lead arsenate was later used to control codling moth in apple, plum, and peach orchards (Klassen and Schwartz, 1983; Peryea, 1998a).

This chapter will focus on the inorganic pesticide lead arsenate ($PbHAsO_4$) and its effects on the environment. Both lead (Pb) and arsenic (As) have been used to produce a large number of chemical and manufactured products. Some of these products have been used in agriculture as defoliants, insecticides, and fungicides to control pests in apple, plum, and peach orchards, turf, vegetable crops, and on cattle. From the late 1800s to about 1947, lead arsenate was the most commonly used insecticide for control of codling moth in deciduous tree fruit orchards in countries throughout the world, including the USA, Australia, Canada, New Zealand, England, and France, because of its low cost, high efficiency, and low phytotoxicity (Focus, 2006; Peryea and Kammereck, 1997 and Shepard, 1951). The wide use of lead arsenate significantly increased its annual production during the early 1900s. Worldwide, lead arsenate production increased from 2,268 metric tons in 1908 to more than 41,000 metric tons in 1944. Even though the total amount of lead arsenate used on orchards is not known, this pesticide was applied frequently and at high application rates. Annual application rates as high as 215 kg Pb ha^{-1} and 80 kg As ha^{-1} were recommended for apple orchards (Peryea and Creger, 1994**).** Such high application rates helped minimize the development of resistant insects, a problem that farmers were facing with other insecticides. Moreover, the fact that lead arsenate has multiple sites of action made it unlikely that insect resistance could be achieved with single mutations (Georghiou, 1983). Lead arsenate was used as an insecticide until the introduction of the organochlorine dichlorodiphenyltrichloroethane (DDT) in the 1940s (Peryea 1998a; Wolz et al., 2003). However, lead arsenate continued to be used in some locations into the 1970s and was not officially banned until 1988 (Focus, 2006).

2. Environmental effects of lead arsenate pesticides

2.1 Occurrence of lead arsenate in the environment

In the early 1900s, fear and concern arose about the potential for retention of excessive pesticide residues on fruits and vegetables treated with lead arsenate. This concern became a reality in 1919, when western pears were condemned in Boston because of excessive arsenic residues (Klassen & Schwartz, 1983). Arsenate residue was also observed in fruits grown on lead arsenate contaminated soils in Canada. Arsenic concentrations in fruit juice and juice concentrate were higher when prepared from skin and cores. Arsenic concentrations in fruit and fruit products were influenced by the size of fruits at spraying and by spraying frequency (Bishop & Chisholn, 1966).

As a result of repeated lead arsenate application, lead and arsenic concentrations increased significantly in orchard soils. In one orchard soil studied, lead concentration ranged from 500 to 1500 mg kg^{-1} and arsenic concentration ranged from 200 to 500 mg kg^{-1}, whereas the concentrations found in uncontaminated soil normally range from 2 to 300 mg kg^{-1} for lead and 0.1 to 20 mg kg^{-1} for arsenic (Alloway, 1995). In an apple orchard with 70 years of lead arsenate use, Frank et al. (1976) reported lead concentrations in the range of 6.4 to 774 mg kg^{-1} and arsenic concentrations from 7.4 to 121 mg kg^{-1}. The extent of contamination is considerable in former fruit growing areas. It has been estimated that approximately five percent of the soils in New Jersey are affected with lead arsenate, for example, while about 188,000 acres in Washington State and 50,000 acres in Wisconsin are contaminated (Focus, 2006).

Lead and arsenic concentrations in orchards soils vary depending on the type of orchard (peach, plum, or apple), soil type, organic matter content, rate and frequency of pesticide application, and management practices after old trees are removed. Lower soil lead and arsenic concentrations were observed in peach orchards and vineyards than in apple orchards, due to the infrequency and lower rate of lead arsenate application in the former (Frank et al., 1976). When replanting orchards, some farmers, after removing the old trees, shift the rows when replanting new trees in order to protect them from lead and arsenic toxicity. Under this management practice, there is little disturbance of the surface, and lead and arsenic concentrations will be much higher in the surface soil compared to their levels under management practices in which fields are plowed after removal of old trees and then planted with agronomic crops such as corn, wheat, and soybean for two or three years before being replanted with new trees. Lead and arsenic concentrations in the surface soil under this management will be lower due to the mixing of the surface and subsurface soil. This practice will increase lead and arsenic in subsurface soils, which, in turn, will increase the potential for vertical movement of lead and arsenic to ground water, especially if these soils are sandy.

Because lead and arsenic generally do not dissolve, biodegrade, or decay, are not rapidly absorbed by plants, and do not readily move through the soil profile, they remain in the soil long after their use (Wu et al., 2010). When lead arsenate reaches the soil, it undergoes hydrolysis, separating into lead and arsenic, which are bound to soil particles and organic matter and become immobilized. Lead is only slightly soluble and therefore accumulates in the surface soil (0-15 cm depth). Arsenic is slightly more soluble and will move through the soil profile (Focus, 2006). Arsenic mobility is enhanced by addition of phosphorus (Peryea & Kammereck, 1997). Arsenic is more mobile compared to lead regardless of the soil type and texture (Eflving et al., 1994). When lead is applied to soil, it will react with sulfate,

phosphate, and carbonate to formed complexes such as lead sulfate ($PbSO_4$), lead carbonate (Pb_3 $(OH)_2$ CO_3), and chloropyromorphite ($Pb_5(PO_4)_3Cl$). These compounds vary in their solubility in the soil. Lead is also adsorbed directly to clay minerals and indirectly by forming complexes with organic matter (such as humic and fulvic acids) that is adsorbed onto soil solids (Harrison & Laxen 1984). In some cases, lead has accumulated in orchard soils in amounts equal to the application rate. In a survey of soils from 31 apple orchards, soil lead concentration averaged 821 mg kg^{-1}, almost equal to the 817 mg kg^{-1} applied, while mean soil arsenic concentration was 188 mg kg^{-1}, considerably lower than the 245 mg kg^{-1} that was applied (Frank et al., 1976).

2.2 Lead and arsenic toxicity

Neurological impairment in children and hypertension in adults are the main health problems associated with chronic high lead levels in the blood. Lead toxicity in humans also affects red blood cells and their stem cells, the kidney, heme biosynthesis, vitamin D metabolism, and the neurobehavioral development of newborns, infants, and children (Carrington & Bolger 1992; Dudka & Miller 1999; Needlemann et al., 1990; Wolz et al., 2003). It has been suggested that blood Pb levels should be no higher than 6 µg dl^{-1} in children to avoid neurological symptoms and no higher than 25 µg dl^{-1} in adults to prevent hypertensive symptoms. Blood Pb levels above 10 µg dl^{-1} could result in spontaneous abortion and potential damage to the fetus in women who are pregnant. Dietary exposure that results in these blood levels of concern was estimated to be 60 µg Pb per day for children age 6 and younger, 150 µg Pb per day for children 7 years and older, 250 µg Pb per day for pregnant mothers and 750 µg Pb per day for adults (Carrington & Bolger, 1992).

In humans, circulatory disorders, skin cancer, and internal cancer are the main hazards related to arsenic exposure (Chaney & Ryan 1994; Dudka & Miller, 1999). Although arsenic is readily absorbed by humans, 40 to 70 percent of As intake is absorbed, metabolized, and excreted within 48 hours. The minimal risk level (MRL) for chronic oral ingestion of arsenic has been estimated to be 0.3 µg arsenic kg^{-1} day^{-1} (ATSDR, 1998; Wolz et al., 2003). Arsenate (V) and arsenite (III) are the dominant inorganic species of arsenic in soils (Chaturvedi, 2006). Arsenate is the predominant species in aerobic soils and arsenite is dominant under anaerobic conditions (Chaturvedi, 2006; Smith, 1998). Inorganic arsenic is very toxic to plants because phosphate and arsenate are analogs and are therefore absorbed by the same transport system (Meharg, et al., 1994; Meharg, 1992). When arsenic is absorbed into the plant it interferes with plant metabolic processes, uncouples phosphorylation, and inhibits phosphate uptake, resulting in purpling of lower leaves, symptoms that are similar to those of phosphorus deficiency (Cox & Kovar, 2001; Gang et al., 2006).

2.3 Risk assessment of lead arsenate exposure

2.3.1 Introduction

It has been reported that the health risk of living on old orchard land contaminated with lead arsenate is very low (Focus, 2006). Nevertheless, there is concern that when such contaminated land is converted to other uses such as vegetable crop production and residential development, (homes, schools, child care facilities, and parks) lead and arsenic will enter the food chain. It has been estimated that that over 6 million acres of farm land have been converted to non-agricultural uses (Focus, 2006). Because excess consumption of non-essential metals such as lead and arsenic can result in neurological, bone, and

cardiovascular diseases, impaired renal function, and various cancers even at low levels (Calderon, 2000; Jarup, 2002; Khan et al., 2003, Miller et al., 2004; Watt et al., 2000), there is a need for the development of risk assessment of these soils. The development of any risk assessment for these soils must consider both toxicity and exposure (Carrington & Bolger 1992; Pocock et al., 1984). Presently, each state has its own guidelines on the utilization of lead and arsenic contaminated orchard soils. However, with the increasing conversion of old orchard land to residential development or to other agricultural uses, a national effort is needed to prevent excessive human exposure to lead and arsenic.

2.3.2 Pathways of lead and arsenic uptake

Lead and arsenic can enter the human or animal body through direct or indirect pathways. A direct pathway is the unintentional consumption of lead and arsenic via the drinking of contaminated water or the inhalation and/or ingestion of contaminated soil and dust. An indirect exposure pathway is consumption of plants that have taken up Pb and/or As from the soil. The direct ingestion of lead and arsenic contaminated soils, especially by children, is considered the most important exposure route of arsenic and lead to the human body (Dudka & Miller. 1999). The segment of our population most vulnerable to lead poisoning is children below the age of 5 years, due to their unintentional ingestion of contaminated soil through hand to mouth activity or by breathing house dust brought inside the house on shoes (Chaney & Ryan. 1994). A recent risk analysis which is considered conservative stated that a soil arsenic concentration of 40 mg kg^{-1} and a soil lead concentration of 300 mg kg^{-1} do not result in excessive intake of arsenic and lead by humans as evaluated by the direct ingestion exposure mode (Dudka & Miller. 1999; US Environmental Protection Agency [USEPA], 1993).

2.3.3 Plant uptake of lead and arsenic

Even though plants do not readily take up lead and arsenic in large quantities, research has shown that some crops will remove lead and arsenic from lead arsenate contaminated soils. Plant lead concentration increased in some crops grown on lead arsenate contaminated soils, exceeding the 2.0 mg kg^{-1} Canadian residue tolerance level (Chisholm, 1972). Miller et al, (2004) observed that arsenic concentrations in agricultural produce grown on lead and arsenic contaminated soils near a Columbia mining area were below existing Canadian guidelines for arsenic content in commercially sold vegetables, but that lead levels in carrots, lettuce, and beetroots from some locations exceeded the recommended guideline of 2.0 mg kg^{-1} Pb. They concluded that the greatest risk was from consumption of contaminated soil particles adhering to vegetables, and that this risk can be reduced by washing and peeling vegetable crops before eating them. Carrot lead and arsenic concentrations increased when grown on lead arsenate contaminated soils (Zandstra & Dekryger, 2007).

In a growth chamber study, Codling et al. (2011, in press) measured lead and arsenic uptake by carrots grown on five orchard soils with history of lead arsenate use, with total soil arsenic and lead ranging from 93 to 291 and 350 to 961 mg kg^{-1}, respectively. Arsenic concentration in peeled carrots ranged from 0.38 to 1.64 mg kg^{-1} dry weight compared to 0.05 mg kg^{-1} in the control. Lead concentration in peeled carrot ranged from 2.67 to 7.32 mg kg^{-1}, compared to 0.19 mg kg^{-1} in the control. Lead concentration was higher in peeled roots compared to the peel and shoot, while arsenic concentration was higher in the shoot and peel than in peeled roots. This study demonstrated that carrots will accumulate lead in

edible tissue. Lead in food is less well absorbed by humans than lead in water, and further studies are needed to determine what fraction of lead and arsenic in such carrots are bioavailable to humans.

There is a possibility that lead arsenate contaminated lands may be used for rice production. A greenhouse study was conducted to determine arsenic uptake by rice from two lead arsenate contaminated soils under flooded conditions (Codling, 2009). Flooding reduced grain yield and increased grain arsenic concentration in both soils. Lead concentration in the grain decreased with flooding for one of the soils but increased for the other. Lead and arsenic concentrations observed in the rice grain would not be expected to become a human health risk. However, the bioavailabilities of lead and arsenic in this rice grain need to be determined. Arsenic and lead concentrations in the straw and husk were much higher than in the grain. Straw from rice grown on these soils under flooded conditions could indirectly become a human health risk because rice straw is used for livestock feed and bedding.

2.3.4 Animal uptake of lead and arsenic

Herbivores and their predators that live in old orchards with history of lead arsenate use are at risk of lead and arsenic contamination (Elfving et al., 1978). Earthworms, for example, have been shown to concentrate lead from these soils (Ash & Lee, 1980; Morgan & Morgan, 1999. Worm eating birds will accumulate lead from consuming these worms. Animals such as meadow voles have been shown to accumulate high levels of lead and arsenic in their liver, kidney, and bones compared to control animals (Haschck, 1979). Predators of these animals such as owls, hawks, and foxes potentially will accumulate lead and arsenic in their tissue. Animals grazing on these contaminated soils unintentionally consume large amounts of soil containing lead and arsenic (McGrath et al., 1982). Even though there is no evidence of human lead and arsenic toxicity from eating animal tissue that was grazed on lead arsenate contaminated soil, there is a potential for lead and arsenic entering the human food chain via this route.

3. Remediation of lead arsenate contaminated soil

3.1 Introduction

Several remediation methods have been proposed and used for remediating lead arsenate contaminated soils, including removal and replacement of surface soil, chemical treatment *in situ*, establishment of a grass cover to prevent erosion and direct contact with humans, and phytoremediation (Peryea 1998b; Codling & Ritchie 2005). Physical removal of contaminated soil by excavation is acceptable and has been used. The cost of excavation, however, is quite expensive, with cost ranging from US$ 25,000 to US$ 1 million per acre, depending on the depth of soil removed, availability of a disposal site, and cost and availability of replacement uncontaminated soil (Peryea 1998a**).** This method would not be applicable for the large areas that are contaminated with lead arsenate. Remediation by dilution, such as by mixing contaminated surface soil with uncontaminated subsurface soil, may not be acceptable because arsenic in the subsoil may leach to the ground water.

3.2 In situ remediation of lead and arsenic

In situ inactivation methods reduce the hazards associated with contaminated soils through the use of chemicals that change the ionic and/or molecular species of metals to stabilize the

metal chemically and physically in place (Berti & Cunningham, 1997; Brown et al., 2004). This method has been used effectively to sequester either lead or arsenic in soils contaminated from metal smelters, leaded gasoline, lead paint, lead batteries, cattle dips, and arsenic treated lumber. Remediation of lead arsenate contaminated soils, however, is more challenging because of (1) the vast amount of lead arsenate contaminated soil throughout the world (Peryea & Kammereck 1997), and (2) some of the common *in situ* remediation methods that have been proven effective for the remediation of lead contaminated soils result in the release of arsenic from lead arsenate contaminated soil, thereby creating a new environmental problem (Codling, 2007; Peryea 1991b). Phosphate, for example, has been shown to be very effective in sequestering lead in contaminated soils (Chaney & Ryan 1994; Ruby et al., 1994), but this remediation method is not suited for lead arsenate contaminated soil; because arsenate and phosphate exhibit similar physicochemical behavior in soil and compete directly for sorption sites on soil particles, the use of phosphate on a lead arsenate contaminated soil will promote arsenic release from the soil into the soil solution phase, threatening the ground water (Dupanport & Peryea 1991b; Eflving et al., 1994**;** Peryea & Kammereck 1997; Peryea, 1991). Increasing the number of adsorption sites via the addition of high oxide minerals such as iron and manganese might allow for the resorption of arsenate after its release caused by phosphate competition during lead sequestration.

Another *in situ* method that has been considered for the remediation of lead arsenate contaminated soils is biomethylation, in which the soil is flooded after application of a carbon source such as apple pomace. Under the high carbon and flooded condition, biomethylation of arsenic will occur (Peryea, 1991b). However, the quantity of lead arsenate contaminated land and the topography of these sites would make biomethylation difficult and expensive (Focus, 2006).

3.2.1 Long term study of *in situ* remediation of lead arsenate contaminated soil

Codling (2007) determined the effect of amendment with calcium carbonate, iron, and phosphate on water-extractable lead and arsenic in two orchard soils with history of lead arsenate use. A soil from Maryland (Thurmont sandy loam) had total lead and arsenic concentrations of 677 and 133 mg kg^{-1}, respectively, and a soil from Washington State (Burch) had total lead and arsenic concentrations of 482 and 93 mg kg^{-1}, respectively. Calcium carbonate, iron oxide, and phosphorus (as potassium phosphate) were mixed with these soils as individual treatments and as combinations. Soils and amendments were mixed and allowed to incubate for 60 weeks. Each treatment was sampled at 2, 4, 6, 8, 10, 16, and 60 weeks for water-extractable lead and arsenic. Iron oxide treatment without calcium carbonate did not change water-extractable arsenic concentration for both soils, compared to the control. The phosphate and iron+phosphate treatments increased water-extractable arsenic compared to the control (Figure 1, only Thurmont data shown). In these treatments, water-extractable arsenic concentrations were higher than the recommended drinking water limit of 10 μg L^{-1}. Application of phosphorus and iron plus phosphorus increased water-extractable lead concentration for both soils with and without calcium carbonate, compared to the control and the iron alone treatment (Figure 2, only Thurmont data with calcium carbonate shown). The higher water-extractable lead concentrations observed with the iron plus phosphorus treatment suggests that iron reacted with phosphorus making it less available for lead precipitation. Because of these observed increases in water-extractable lead and arsenic caused by application of iron and phosphorus to lead arsenate

contaminated soils, this *in situ* remediation method should not be use without further studies to determine the appropriate ratio of iron to phosphorus needed to sequester lead and arsenic in these soils.

3.3 Phytoremediation of lead arsenate contaminated soil

Using plants to remove toxic metals from soils, a process known as phytoremediation, is an inexpensive alternative to conventional methods (Lim et al 2004). In order for a plant to be considered a hyperaccumulator, it must accumulate at least 1000 mg kg^{-1} of the metal in the above ground tissue. Chaney et al. (1994) demonstrated that *Alyssum murale* Waldst. & Kit. will accumulate nickel from high nickel serpentine soil. Francesconi et al. (2002) and Ma et al. (2001) demonstrated that Chinese brake fern (*Pteris vittata* L) and silver fern *Pityrogramma calomelanos* (L) can hyperaccumulate arsenic. Some researchers do not believe phytoremediation of lead arsenate contaminated soil holds much promise because it is slow, potentially taking decades or longer to effectively remove the contaminant. While other researchers believe that phytoremediation is a cost effective, non-intrusive technology that needs improvement (Alkorta, et al., 2004). Alkorta, et al. (2004) stated that improvement of the capacity of plants to tolerate and accumulate metals by genetic engineering should open up new possibility for phytoremediation. They also suggested that a better understanding of metal uptake and translocation mechanisms and the external effects of phytoremediation should also increase its application. Research is ongoing to identify plants that could potentially be used as accumulators of lead and/or arsenic on lead arsenate contaminated soils. In a greenhouse study, Codling & Ritchie (2005) tested Eastern gamagrass [*Tipsacum dactyloides* (L.)] for lead and/or arsenic accumulation from two lead arsenate contaminated orchard soils. This species was chosen because the plant has an extensive root system and is used to reduce soil erosion on disturbed soils. Eastern gamagrass did not remove substantial amount of arsenic from these soils, making this species a poor candidate for phytoremediation of lead arsenate contaminated soils.

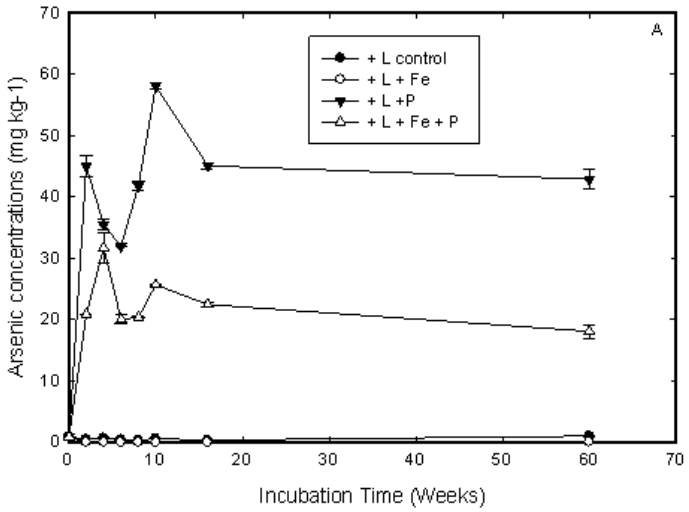

Fig. 1. Water-extractable arsenic in limed iron- and phosphorus-amended lead arsenate contaminated Thurmont soil during incubation at 26 °C. Values are mean and standard deviation (n=3).

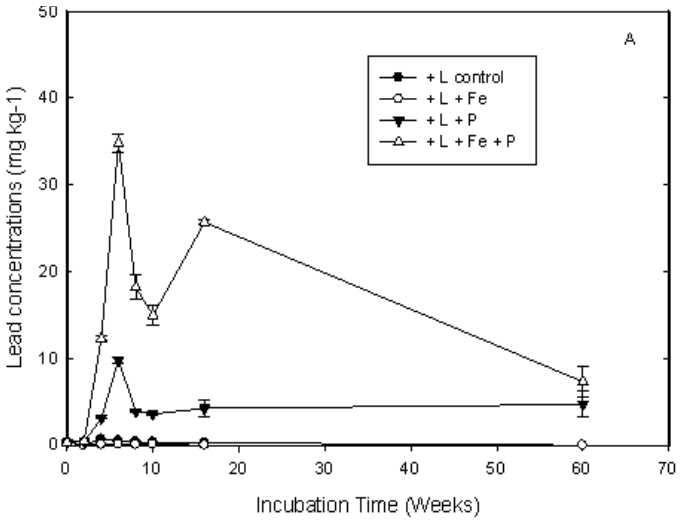

Fig. 2. Water-extractable lead in limed iron- and phosphorus-amended lead arsenate contaminated Thurmont soil during incubation at 26 °C. Values are mean and standard deviation (n=3).

4. Conclusions

The existence of large areas of lead arsenate contaminated orchard soils and their increasing conversion to vegetable crop production and to residential development has created a potential risk to public health. Young children are especially at risk because of their unintentional consumption of soil. Children exposed to lead may develop neurobehavioral impairment, while arsenic is a human carcinogen. Humans can also be exposed indirectly to lead and arsenic through the consumption of vegetable crops grown on contaminated soils, although the bioavailability of lead and arsenic in vegetable crops consumed by human and animals is not known. Because hundreds of thousands of acres have been contaminated with lead arsenate, removal of contaminated soil and replacement with clean surface soil is not economically feasible. Chemical *in situ* treatment with phosphorus, although effective in sequestering soil lead from other sources, has been shown to increase the leaching of arsenic to ground water in lead arsenate contaminated soils. Application of iron oxide has been shown to be effective in sequestering arsenic in lead arsenate contaminated orchard soils. Using plants to remove metals from contaminated soil (phytoremediation) is a method that is being considered for removing lead and arsenic from soils, but even if lead and arsenic accumulating plants are identified, this method may be too slow to be practical. Further research needs to be done on remediation of lead arsenate contaminated soils. Presently, each state has its own guidelines on the utilization of lead and arsenic contaminated orchard soils. However, with the increasing conversion of old orchard land to residential development or to other agricultural uses, a national effort is needed to prevent excessive human exposure to lead and arsenic.

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10

Arsenic – Pesticides with an Ambivalent Character

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1. Introduction

Arsenic (As) is a naturally occurring ubiquitous element. It is found in the environment in the earth crust and quantities in media such as soil, water, rock and air. It is present in the environment naturally and due to human activities and other industrial processes such as mining and coal-fired power plants. Arsenic has also been used as a pesticide to protect animals, wood, fruit and vegetables from insects. Because of its therapeutic properties, arsenic has also been used as a medicinal agent. The dark side of the medicine was the reputation as an attractive poison

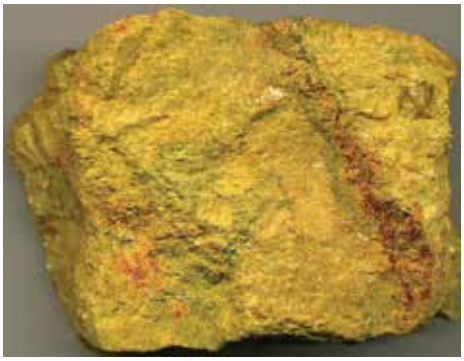
Arsenic is mainly transported in the environment by food, except in areas with high levels of arsenic in the drinking water e.g. India, Taiwan and Bangladesh (2004; Singh, Kumar, and Sahu 2007). The outbreak of As was triggered by deep drilled wells and the desire to obtain microorganism-free safety drinking water. Arsenic contamination of drinking-water is a hazard to human health. Because of the toxicities and side effects of arsenic compounds it is known as a major environmental pollutant. The IARC classified arsenic and arsenic compounds as a human carcinogen (Group 1) (2004). But although arsenic compounds have been known and used for centuries, their mechanisms of interaction in humans are not fully elucidated.

The paradox of arsenic compounds is that, on the one hand, they are considered extremely dangerous for human health with acute and chronic adverse health effects. Long-term arsenic exposure can lead to several types of cancer. The exposure to As has been associated also with non-carcinogenic effects e.g. such diabetes and cardiovascular diseases. On the other hand arsenic compounds are regarded as potential drugs against cancer and ranging from the use as poisons to applications in semiconductors and pesticides. Especially the discovery of organoarsenicals for the treatment of hematological malignancies and solid tumor has awakened interest.

2. Background and basics

Arsenic is a chemical element in the period table that has the symbol "As", the atomic number 33 and an atomic mass of 74,92159 g/mol. Arsenic exhibits both metallic and non-metallic properties. Arsenic exists as unstable oxides and sulfides or as arsenites or arsenates of sodium, calcium and potassium. Arsenic has two biologically important oxidation states: arsenite (the trivalent form, As III) and arsenate (the pentavalent form, As V). As III is 60 times

more toxic than As V (Yousef, El-Demerdash, and Radwan 2008). From biological and toxicological view, arsenic compounds can be classified into three major groups: Inorganic arsenic compounds, organic arsenic compounds and arsine gas (Hardman et al. 1996). The metalloid is found mostly as yellow complex sulfides. Organic arsenic is non-toxic whereas inorganic arsenic is toxic. The inorganic forms of arsenic are yellow (AS_2S_3, orpiment), red (AS_2S_2 realgar) and grey to silver white (FeAsS, arsenopyrite) (Waxman and Anderson 2001).



a) Orpiment

b) Realgar

c) Arsenopyrite

Fig. 1. Orpiment, realgar and arsenopyrite - from left to right- (Photographs from the Ohio State University Newark)

Arsenic compounds have no smell or taste, but heat can cause As to sublimate to gas with a distinctive garlic odor (Jones 2007).
Arsenic naturally occures in the earth´s surface, mostly in inorganic form (Hine, Pinto, and Nelson 1977). It exists in low concentrations in many rock types but is frequently associated with metal ore deposits e.g. Au (gold) , Ag (silver), Cu (copper) and Fe (iron) (Gochfeld 1997). The most important natural sources of arsenic in the environment are volcanoes. The organic form result when arsenic combines with carbon and hydrogen.

Compound	CAS No.	Molecular formula
Arsenic	7440-38-2	As
Arsenic trioxide	1327-53-3	As_2O_3
Arsenic pentoxide	1303-28-2	As_2O_5
Arsenic sulphide	1303-33-9	As_2S_3
Dimethylarsinic acid (DMA)	75-60-5	$(CH_3)_2AsO(OH)$
Potassium arsenate	7784-41-0	KH_2AsO_4
Potassium arsenite	10124-50-2	$KasO_2HAsO_2$

Table 1. Physiochemical properties (1980)

3. History

Arsenic and arsenic compounds are known since the ancient times. As early as 500 B.C. the ancients knew about arsenic, whose name comes from the Greek word "arsenikon" for potent or bold, which means orpiment form Latin auripigmentum. In the 16th an 17th centuries, red and white arsenic were put into amulets that were worn around the neck and close to the heart to ward off the plaques (Cullen 2008). Most arsenic is found in conjunction with sulfur in minerals such as arsenopyrite (AsFeS). Because of the association with ore and the stability of As in form like AsFeS, As was used as a "pathfinder element" in geochemical exploration for gold (Jones 2007).

Fig. 2. Acidum Arsenicosum Anhydricum bottle, Global Antiques

Through the centuries Arsenic was a common method of homicide. The death of the French emperor Napoleon Bonaparte, on 5 May 1821 was believed to be a victim after drinking arsenic-tainted wine that was served him (Leslie and Smith 1978; Lin, Alber, and Henkelmann 2004). Arsenic was a popular murder weapon because of the odorless and tasteless properties and the poisoning result in symptoms that can be confused with other natural disorders. In the Middle ages arsenic was a favorite poison and has been called the Poison of Kings and the King of Poison (Vahidnia, van, V, and de Wolff 2007).
Arsenic was also used as healing agents. The Greek physicians such as Hippocrates and Galen popularized it use for treating skin ulcers and tumors such as superficial epitheliomas. Arsenic has been used as topical pastes, as vapor inhalation, intravenous injection, orally in liquid or in solid form. A paste of the sulfides were used for treatment of neuralgia, rheumatism, arthritis and skin disease (Shen et al. 1997). Also Fowler´s solution, a 1% arsenic trioxide preparation, was widely used during the 19th century. Fowler´s original recipe was described as "64 grains arsenic oxide, 64 grains purest vegetable alkali, distilled water half pound. Heat until clear. Cool. Add half pound spirit of lavender and make up to 15 oz with water." (Cullen 2008).

Fig. 3. Fowler´s Solution made by Wisconsin Pharmacal Co

It was used to treat diseases like leukemia, Hodkin´s disease and pernicious anemia.
The first organic arsenical used therapeutically was Salvarsan, which was developed by Paul Ehrlich 1907. It was used to treat syphilis, until penicillin became available in the 1940s. A model representation from Salvarsan and a picture of Ehrlich adorned the 200 Deutschmark banknote.

Fig. 4. German 200 Deutschmark banknote with hologram and Paul Ehrlich

For centuries arsenic has been used for different purposes. Arsenic was an ingredient of a lot of consumer products e.g. wallpapers, toys, food wrappers, cosmetics, pigments in paints – known as "Paris green". William Withering, an english doctor, who discovered 1775 digitalis was a proponent of therapies with arsenic. He argued:" Poison in small doses are the best medicines; and the best medicines in too large doses are poisonous (Aronson 1994).
Arsenic-containing compounds have been used for cancer-treatment in both tradition Western and Chinese medicine. The first use of arsenic in the treatment of leukemia was in 1865 by Lissauer (Lissauer and H. 1865). With the development of modern medicine against cancer the use of arsenic in the western world diminished.

4. Epidemiology

Arsenic exposure occurs from inhalation, absorption through the skin and by ingestion. Arsenic is mainly transported in the environment by food, which contains both organic and inorganic As, but mostly accrue as relatively no-toxic organic compounds (arsenobentaine and arsenocholine). Seafood, fish and algae are the richest organic sources (Edmonds and Francesconi 1987). The following table shows an overview of the arsenic content of various foods.

Food	**Estimates of daily intake (µg/d)**
Milk	1,39
Fruits and vegetable	0,46
Meat	2,14
Cereals and bakery wares	6,57
Fish	34,9
Eggs	0,13
Sweeteners	0,2
Beverages	4,67

Table 2. Estimated of daily arsenic intake from diet (Sorvari et al. 2007)

Concentrations of arsenic vary in the environment, e.g. 0,03-025 ppm in soil, 0,023-0,35 ppm in plants, up to 55 ppm in groundwater, 0,0001-0,08 ppm in seawater, 4-170 ppm in fish, 0,008-0,85 ppm in wine and up to 0,00049 or 0,63 mg/m³ in urban air (2004; Jones 2007; Rahman 2006; Basu et al. 2001). Contamination of arsenic in ground water is a global problem and millions of people are at a risk of arsenicosis. People from countries in Asia (Taiwan, Bangladesh, West Bengal (India) and South America (Chile and Córdoba) get presented to inorganic arsenic in ground water with very high concentration. The arsenic poisoning from drinking As-contaminated underground water was often triggered by the introduction of deep tube-pump wells to replace surface water. The World health organization (WHO) and US environment protection agency (EPA) had set up the standard for drinking water known as maximum contamination level (MCL) which is 10 µg/l (Effelsberg 1992). The WHO recommended 0.01 mg/l of arsenic in drinking water as an allowable ranger for human consumption. Millions of people are compelled to use the drinking water higher arsenic level than MCL worldwide. In addition there are industrial exposures for workers, e.g. semiconductor workers and famers handle with arsenical herbicides. Arsenic has been used as feed additives e.g. poultry feeds.
It was found an increase in the prevalence of skin lesions at 0,005 mg As/l in the drinking water, which is a lower level than the drinking water quality standard of WHO (Yoshida,

Yamauchi, and Fan 2004). The skin is very sensitive to As and skin lesions, which are As-induced, are the early effects to chronic As exposure.
Arsenic is released to the atmosphere from both natural and anthropogenic sources.
Tobacco smoke may contain arsenic, especially when the plants have been treated with arsenate insecticide.

Country	Daily dietary intake of total arsenic from diet (μg/d)
Bangladesh	515
Japan	182
USA	20-130
Spain	245
French	62
Germany	52
UK	66
Denmark	64

Table 3. Estimated daily intake of arsenic by the general population ((Devesa et al. 2001; Jelinek and Corneliussen 1977; Leblanc et al. 2005; Mohri, Hisanaga, and Ishinishi 1990; Saipan and Ruangwises 2009; Sorvari et al., 2007; Watanabe et al. 2004)

The principal natural source is volcanic activity, with minor contribution by exudates from vegetation and wind-blow dust. Man-made emissions to air arise from the smelting of metals, the combustion of fuels, especially of low-grade brown coal, and the use of pesticides. Because of the use of numerous arsenical pesticides the arsenic concentration raised in the soil.

5. Effects on human

The biological activity of arsenic in the body covers a broad spectrum from toxic to therapeutic agent. Not to forget- Arsenic is a human carcinogen (IARC 2004). A number of studies show that arsenic is an essential element for humans. Other studies have attempted to show that arsenic has not been demonstrated to be essential to humans (Ohtake 2000).
The major routes of arsenic absorption in the general population are ingestion and inhalation. Meat, fish and poultry account for 80 % of dietary arsenic intake (Edmonds and Francesconi 1987). Arsenic is absorbed in the small intestine by an electrogenic process involving a proton gradient. The absorbed arsenic undergoes hepatic biomethylation. The products are less toxic but not completely innocuous. About 50 % of the ingested dose may be eliminated in the urine in 3-5 days. Metabolism of As involves reduction of As V to a trivalent state and subsequent oxidative methylation.

5.1 Acute effects

After acute poisoning studies show that the highest concentration of arsenic is in the kidney and liver (Benramdane et al. 1999). Most cases of acute arsenic poisoning occur from accidental ingestion of insecticides or pesticides. Acute exposure to arsenic arise symptoms like abdominal pain, vomiting, diarrhea. The abdominal pain may mimic an acute abdomen (Mueller and Benowitz 1989). Other clinical features are muscular weakness and cramping, erythematous skin eruptions like diffuse skin rash and swelling of acrals. A progressive deterioration in the motor and sensory responses and toxic cardiomyopathy may also result

leading to shock and death. Depending on the quantity of arsenic, death usually occurs within 1-5 days. In acute poisoning the best indicator of recent ingestion (1-2 days) is urinary arsenic concentration. Dimethylarsinic acid is the dominant urinary metabolite compared with monomethylarsoonic acid (Hopenhayn-Rich, Smith, and Goeden 1993).

5.2 Chronic effects

Chronic ingestion of inorganic arsenic causes multisystem adverse health effects. The clinical features of chronic arsenic toxicity vary between individuals, population groups and geographic areas. In chronic arsenic ingestion, arsenic accumulates in the liver, kidneys, heart and lungs and smaller amounts in the gastrointestinal tract, spleen and muscles (Benramdane et al., 1999). High doses of arsenic cause characteristic skin manifestation, vascular, renal and neurological diseases, cardiovascular and chronic lung diseases and cancer of skin, lungs, liver, kidney and bladder. After about two weeks arsenic is deposited in the hair and nails. Levels between 0,1 and 0,5 mg/kg on a hair sample indicate chronic poisoning. Various epidemiological studies have reported that arsenic exposure is associated with hypertension, atherosclerosis and endothelial dysfunction (Yang et al. 2007) (Chen et al. 2007) (Kwok et al. 2007). Increasing exposure of arsenic is also associated with non insulin dependent diabetes mellitus (Wang et al. 2003). Studies reported that arsenic is associated with the growth retardation in children (Wang et al. 2007).

5.3 Skin symptoms

Skin manifestation is the most common and initial sign of chronic arsenic exposure. Chronic ingestion of arsenic causes characteristic melanosis, keratosis, basal cell carcinoma and squamous cell carcinoma (Maloney 1996). Melanosis includes hyperpigmentation, spotted pigmentation, depigmentation and leucomelanosis. Keratosis is a late feature of arsenical-dermatosis and appears especially on palm as a uniform thickening or as discrete nodules (Wong, Tan, and Goh 1998b). Both palmar and solar keratosis are significant diagnostic criterion. Bowen´s disease is a precancerous lesion and predisposed to an increased incidence of the squamous cell carcinoma. Chronic ingestion of arsenic lead to accumulate in keratin rich areas of body and appears as white lines in the nails, called Mee´s lines (Fincher and Koerker 1987). The latency period of skin lesions of arsenic after first exposure varies

Fig. 5. Patient with plantar keratosis (2004; Wong, Tan, and Goh 1998c).

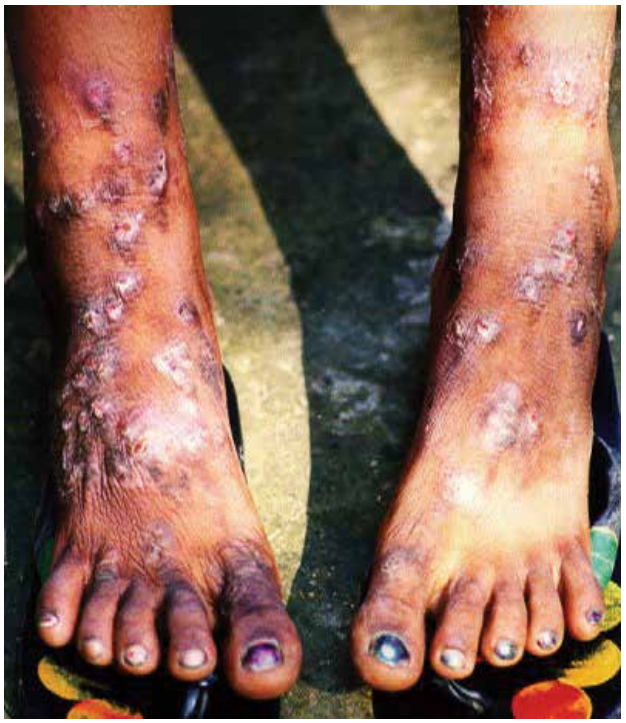


Fig. 6. Blackfoot diseases after chronic arsenic exposure (Better Life Laboratories, USA)

from 20 to 50 years (Haque et al. 2003). It is described that the latent period after exposure can be as long as 60 years, which has been reported in patients treated with Fowler´s solution, in vineyard workers using arsenical pesticides and from drinking contaminated wine (Everall and Dowd 1978).

Many different systems within the body are affected by chronic exposure. Some of these systems and their associated toxic effects from chronic arsenic exposure are listed in the following table.

System	**Effect and symptoms**
Skin	Skin lesions (melanosis, keratosis)
Cardiovascular	Blackfoot disease, atherosclerosis, hypertension
Hepatic	Hepatomegaly, fibrosis, cirrhosis, altered heme metabolism
Hematological	Bone marrow depression (anemia, leucopenia, thrombocytopenia)
Endocrine	Diabetes
Renal	Tubule degeneration, papillary and cortical necrosis
Nervous	Peripher and central neuropathy, encephalopathy
respiratory	Pulmonary insufficiency, emphysem
Gastrointestinal	Hemorrhage

Table 4. Human effects after chronic arsenic exposure (Singh, Kumar, and Sahu 2007; Schuhmacher-Wolz et al. 2009; Hughes 2002; Balakumar and Kaur 2009; Rahman, Ng, and Naidu 2009).

6. Toxicity

Arsenic compounds or arsenic-containing compounds vary in toxicity to mammalian cells. Arsenic does not directly react with DNA or cause gene mutations, except to a small extent at high dose. As can cause gene amplification and chromosomal damage at lower doses and can enhance mutagenesis by other agents, apparently by inhibiting DNA repair. The following table gives an overview over the modes of carcinogenic action of arsenic.

Modes of carcinogenic action of arsenic
Genotoxicity
Oxidative damage
Modification of cell signalling
Influence on DNA repair
Influence on DNA methylation
Changes in cell proliferation
Co-mutagenesis and transformation
Tumor promotion

Table 5. Modes of carcinogenic action of arsenic (Schuhmacher-Wolz, Dieter, Klein, and Schneider 2009; Hughes 2002).

The binding with sulfhydryl groups by arsenite compounds has the potential to influence a wide range of metabolic activities. Arsenic toxicity inactivates up to 200 enzymes. The effects of As occur through indirect alteration of gene expression via disruption of DNA methylation, inhibition of DNA repair, oxidative stress, or altered modulation of signal transduction pathways. Another indirect mechanism is the influence of growth-stimulating chemicals or cytokinesed generated in response to arsenic exposure. Biotranformation is the major metabolic pathway for inorganic arsenic in humans. Toxic inorganic arsenic species can be biomethylated by bacteria, algae, fungi and humans. The high affinity of arsenic for sulphydryl groups makes keratin-rich cells a target for arsenic.
The order of toxicity of arsenicals is:
Monomethylarsonic acid (MMA III) > Arsenite (III) > Arsenate (V) > MMA(V) (Singh, Kumar, and Sahu 2007).
In arsenic biotransformation the intermediate product MMA III is highly toxic than other arsenical, which might be responsible for the arsenic-induced carcinogenesis and other effects (Styblo et al. 2000). As III binds thiol or sulfhydryl groups in tissue proteins of the liver, lungs, kidney, spleen, gastrointestinal mucosa and keratin-rich-issues (skin, hair, nails). By binding a wide range of metabolic activities are influenced including cellular glucose uptake, gluconeogenesis and fatty acid oxidation (Jones 2007). Many other toxic effects of arsenic compounds are detailed by Abernathy et al in 1999 (Abernathy et al. 1999).
The acute toxicity is related to its chemical form and oxidation state. In the human adult the lethal range of inorganic arsenic is estimated at a dose of 1-3 mg As / kg (Schoolmeester and White 1980). The characteristics of acute arsenic toxicity in humans include gastrointestinal discomfort, vomiting, diarrhea, bloody urine, anuria, shock, convulsions, coma and death.

7. Pharmaceutical use

Arsenic has been used therapeutically for over 2000 years. During the 18^{th}- 20^{th} centuries arsenic compounds have been used as medicines, including arsphenamine and arsenic

trioxide. In 1910, Paul Ehrlich introduced the arsenic-based drug Salvarsan (arsenobenzol) as a remedy for syphilis in all stages, a sexually transmitted disease. It was efficient in various similar diseases such as relapsing fever, Vincent´s angina.
Arsenic trioxide is also known as an anti-bacterial and anti-cancer agent (Bardos, tta-Gupta, and Hebborn 1966). Inorganic As has been also used pharmacologically for the treatment of eczema, pemphigus and psoriasis under the name of Fowler´s solution. It was a 1 % solution of potassium arsenite, colored with a tincture of lavender-which contained a very high concentration of arsenic (Rahman 2006). Some arsenic containing drugs are still presently used to treat diseases like asthma rheumatism, cough, pruritus and itching (Ko 1999; Wong, Tan, and Goh 1998a).
In 2000, the US Food and Drug Administration approved the use of arsenic trioxide for treatment of relapsed or refractory acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia (AML) (Antman 2001). It is based on its mechanism as an inducer of apoptosis (programmed cell death) (Soignet et al. 1998).

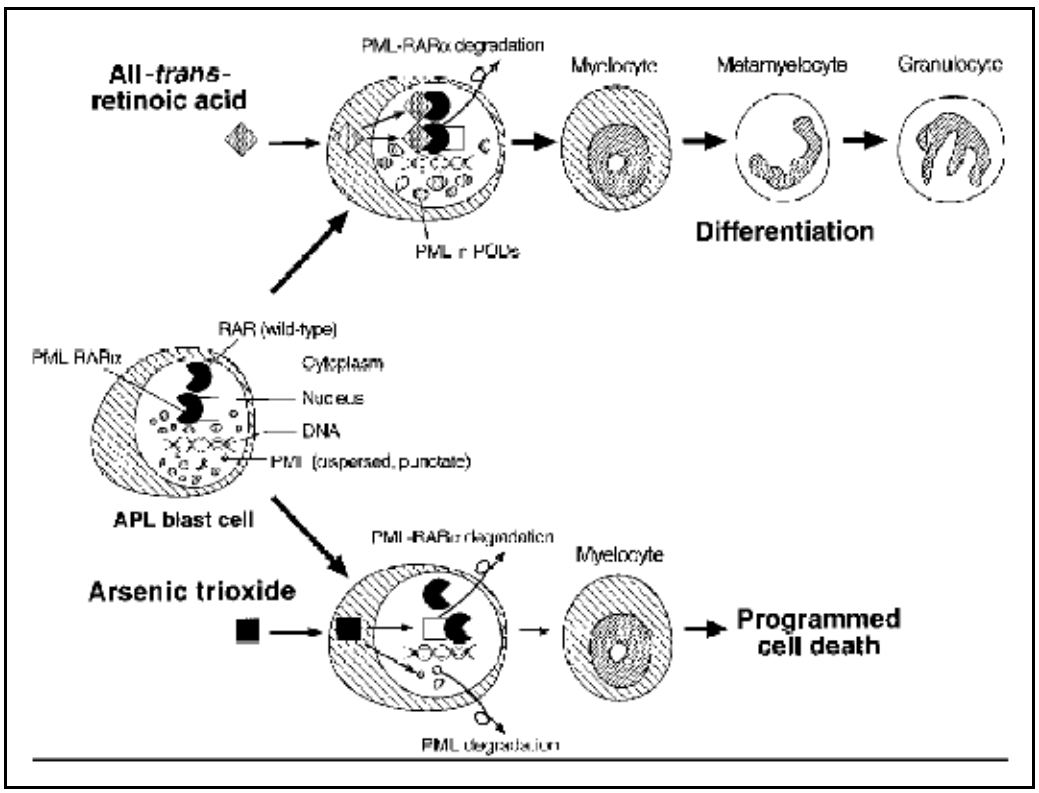


Fig. 7. Effects of all-trans-retinoic acid and arsenic trioxide in the blast cells of acute promyelocytic leukemia (APL) (Look 1998).

Traditional medicine products contain arsenic sulfides (realgar) and are available as pills and tablets. They are still used for psoriasis, syphilis, asthma, rheumatism, hemorrhoids, cough and pruritus and are rescribed as a health tonic, an analgesic, anti-inflammatory agent (Ko 1999; Wong, Tan, and Goh 1998d). In Korea arsenic is prescribed in herbal medicine for anal suffering such haemorrhoids (Mitchell-Heggs, Conway, and Cassar 1990).

8. Industrial use

In industry, arsenic is used to manufacture polants, fungicides, insecticides, pesticides, herbicides, wood preservatives, and cotton desiccants.
In most local hardware stores arsenic-containing herbicides are readily available, the most common are e.g. Disodium methylarsonate (DSMO), monosodium methylarsonate (MSMA), monomethyl arsenic acid (MMA(V)). These compounds can kill crabgrass and other unwanted grass types. Arsenic trioxide (AS_2O_3) is commonly used as an antisecticide. Arsenic acid and arsenous acid are common rodenticides. The major use for arsenic is in the form of chromated copper arsenate, which reduces termites and ants from wood.
Arsenic is used industrially as an additive to glass to reduce coloring, in semiconductors, in pigments such as Paris green (CuHAsO3) and in pesticides. Paris Green is a common name for copper (II) acetoarsenite, which is a toxic emerald-green crystalline powder. Other names for the chemical are Emerald Green, Vienna Green, Schweinfurt Green and Parrot Green. The use has been abandoned around 1960. The III-V semiconductors are very important in the fabrication of LED´s, tunnel diodes, infrared emitters, laser window and Hall-effect devices.

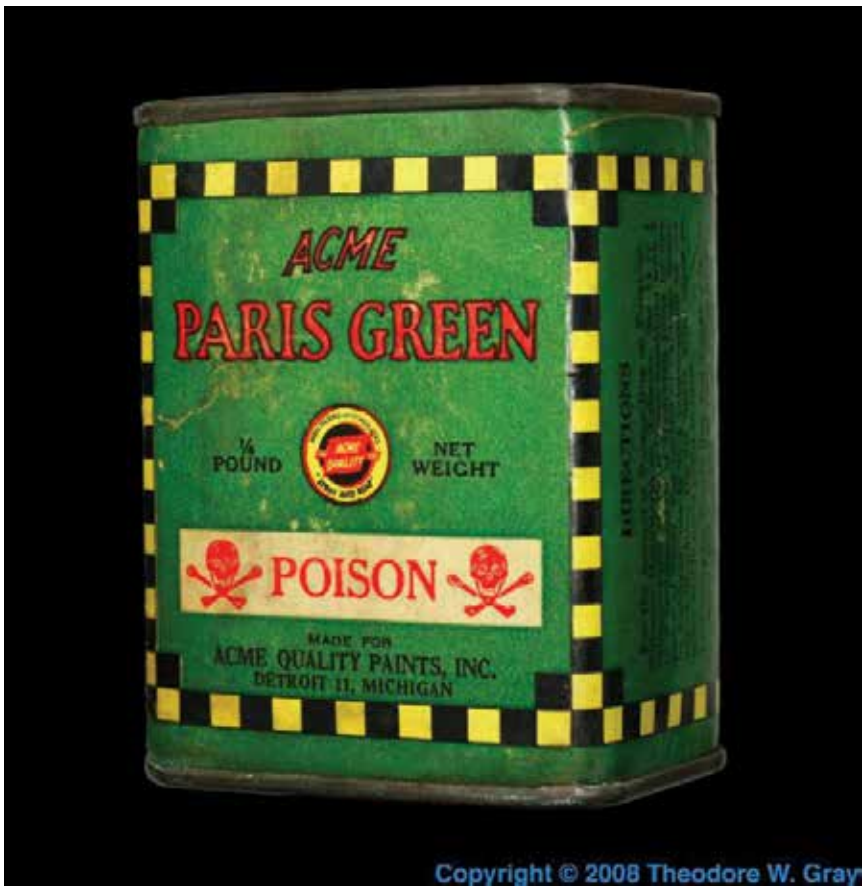


Fig. 8. Paris Green bottle
(http://theodoregray.com/periodictable/Elements/033/index.s7.html).

9. Arsenic, wine and profession

Arsenic was used in vineyards for only some years as a pesticide. It was officially introduced as a pesticide in viniculture in 1925. Its purpose was to protect the wine plants. But it was banned in 1942. It was used in Germany until the mid 1950ties (Shab, 2009). Consumption of the so-called wine-grower´s house drink led to severe symptoms and illnesses, especially liver damage. This homemade wine was produced by watering down the wine obtained from a second pressing of the grape skins. It was consumed in large quantities, which had a low alcohol content, from 3-5 %, but high arsenic content (Kunz and Kunz 2008). Exposure to arsenic has been reported to lead to cirrhosis and to angiosarcoma among famers exposed to arsenical insecticides. Chronic liver disease which can be caused by arsenic toxicity includes also steatosis and noncirrhotic portal hypertension (Von Hyman J.Zimmerman. 1999).

Not only at the vineyards there was an occupational exposure to arsenic. Other important occupational exposure opportunities exist for processing of metal ores, roasting of pyrites in the chemical industry, the production and use of arsenic colors and tints for glass, porcelain and ceramics industry, pesticides and wood preservatives as well as at the battery and semiconductor. Occupational diseases caused by arsenic and its compound can be recognized as an occupational disease (BK-Nr. 1108 in Germany) (1964).

Fig. 9. Wine glass with wine yard (Markus Ebert - photographer Heidelberg/Potsdam)

10. Conclusion

From history to the present, the story of arsenic is double-edged: a poisonous edge and a medicinal edge. Arsenic has been mentioned mainly as a poison and public health problem

than as an effective anticancer drug. Arsenic is one of the most toxic metals derived from the natural environment. Inorganic Arsenic is a human carcinogen, but nowadays also acts as a beneficial chemotherapeutic agent. The major cause of human arsenic toxicity is from contamination of drinking water and from As-contaminated food through fertilization. Current uses of arsenic compounds are in the glass industry, as a wood preservative and in the production of semiconductor. Over the centuries, arsenic has been used for a variety of purposes. In industry arsenic is used as a potential weapon against insecticides concerning humans as a modern weapon. Arsenic compounds became available e.g. in Fowler´s solution as indication for skin conditions and treatment for acute and chronic diseases. Arsenic affects many cellular and physiological pathways, which is useful in treating malignancies like hematological cancer and solid tumors. The ability of arsenic trioxide to treat APL has changed the point of view.
Still today moderately elevated concentrations of inorganic arsenic in drinking water is a major public health concern as well as arsenic exposure from food, especially rice products (Sun et al. 2008).
Chronic arsenicism may lead to multiple benign skin diseases as well as potentially fatal skin and visceral malignancies e.g. lungs, bladder, liver kidneys. Pigmentation changes and hyperkeratosis are the earliest signs of toxicity from chronic exposure. People with chronic arsenicism should undergo regular skin and systemic examination. There are no evidence based treatments to reduce chronic arsenic poisoning, but antioxidants have been advocated: Pharmacological interventions such as vitamin C, folic acid, vitamin b12 have been identified to halt the development of arsenic-induced toxicity. More studies are needed. The essential and basic efforts for the reduction of chronic arsenic toxicity are prevention. Although current exposure to arsenic is decreasing, continual surveillance programs to detect unrestricted and unsupervised manufacture and sale of drugs that may contain inorganic arsenic must be implemented to prevent a potentially fatal disorder.

11. Acknowledgment

Arsenic is a fascinating element. We were inspired by treating patients having contact to arsenic. Quod vide:
Shab, C. Crößmann und C. Bayerl, „Multiple Basalzellkarzinome und aktinische Keratosen bei einem landwirtschaftlichen Arbeiter nach Arsen-Exposition: Immer nur BK 1108?“ Dermatologie in Beruf und Umwelt (Vol. 57,No. 4/2009(4. Quartal)).

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11

Freshwater Decapods and Pesticides: An Unavoidable Relation in the Modern World

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1. Introduction

1.1 Levels of organisation in biological systems and their relationships

Scales in nature can be difficult to define and understand because several ecological factors can interact. The study of different biological scales contributes to information that varies in its quality and significance for humans. Observations at the ecosystem scale are of great ecological significance but can be of low quality or provide little information about causes; at the other extreme, molecular studies that provide exact determinations of causes can have very little relevance to effects at a larger scale (Figure 1). In the middle of these extremes are observations that provide more or less significant and relevant information. Increasing the level of biological complexity in our observations can lead to an unexpected increase in the number of variables to be considered, requiring the consideration of n-adimensional conditions.

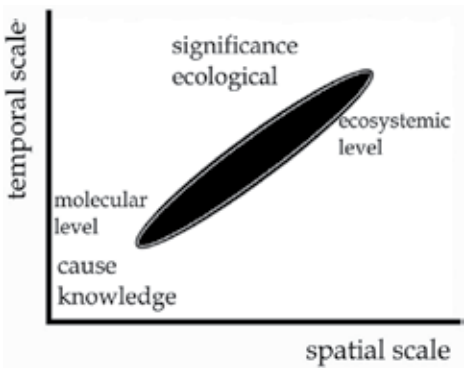

Fig. 1. Relationship between spatial and temporal scales with the quality information obtained

Each level of study is influenced by the level below it, and each level affects the level above it, which is mediated by interspecific relationships that influence ecosystem structure, and this can vary according to the heterogeneity of an ecosystem. When biological complexity

increases, it is important to consider that temporal and spatial dimensions are interconnected, e.g., molecular reactions occur in spaces smaller than one-hundredth of a millimetre and at reaction times of less than one second. At the same time, the effects of the predation of one species on the populations of other species may play out in spaces at the scale of kilometres and at timescales that can exceed a year. According to the heterogeneity of a system, its component species and the processes involved, variations in the time and space involved in a given process can be very important (Figure 1).

1.2 Fauna in aquatic systems

Year after year, the quality of aquatic environments is recognised as a priority for humanity, with particular emphasis on the quantity and quality of freshwater.

Among the faunal components of aquatic environments, decapods, an order of crustaceans, are an interesting group that possesses biological characteristics useful in assessing the quality of inland aquatic systems. In addition, some species of decapods may be used as food by humans and are part of the food chain of other species used by humans as food, mainly fish and birds.

Five decapod families occur in southern South America and east of the Andes. Some of these decapods are endemic at the family level, others at the genus level and still others at the species level. These families include prawns and shrimp (Palaemonidae and Sergestidae), crabs (Trichodactylidae), pseudocrabs (Aeglidae) and crayfish (Parastacidae) (Collins et al., 2007).

Some of these families live in burrows constructed of fine sediment (some Trichodactylidae and Parastacidae). Others live in the background using clasts, rocks or tree trunks for hide under this cover (Aeglidae). Some decapod families live among aquatic vegetation (some Trichodactylidae and Palaemonidae), while others live all or part of their lives in the water column (some Palaemonidae and Sergestidae). Thus, the habitats used by this group are very diverse, and different taxa have different relationships to the land environment. The densities of decapods can be very high at certain times of year and may exceed 500 animals per square meter (e.g., Palaemonidae). Their diets are varied and may include plant matter (e.g., aquatic plants and phytoplankton debris), microinvertebrates (e.g., protozoa, cladocerans, rotifers, and copepods), macroinvertebrates (Palaemonidae insect larvae, oligochaetes, molluscs) and vertebrates (fish). The trophic resource used by decapods is mainly composed of live animals, but dead animals are also commonly fed upon. Consumption intensities are very high, transferring energy and material from various bottom levels (e.g., oligochaetes, chironomid larvae, zooplankton, and vegetal remains) to the top trophic levels (e.g., fish, mammals, reptiles, birds) (Collins & Paggi, 1998; Collins 1999; Williner & Collins, 2002; Collins 2005; Collins et al., 2006).

Since the industrial revolution, the human population has been growing rapidly and has therefore required more intensive management of natural environments. This need for intensive management has included the use of more land for growing food, causing the conversion of forests, jungles, and grasslands, among other ecosystems, into farmland. Subsequently, different poisons (e.g., herbicides, insecticides, fungicides) have been employed with the aim of eliminating those plants and animals that could use the crop resources (cereal or other crops), which humans call "pests". The use of these chemicals grew during the last century in an unprecedented manner in both volume of use and in the

different formulations available. In addition, the creation of new compounds, the genetic modification of plants to withstand pesticides, and the improved effectiveness of the methods of pesticide application have had substantial economic support for research and development. Unfortunately, studies of the damage caused by these chemicals have not had similar financial support.

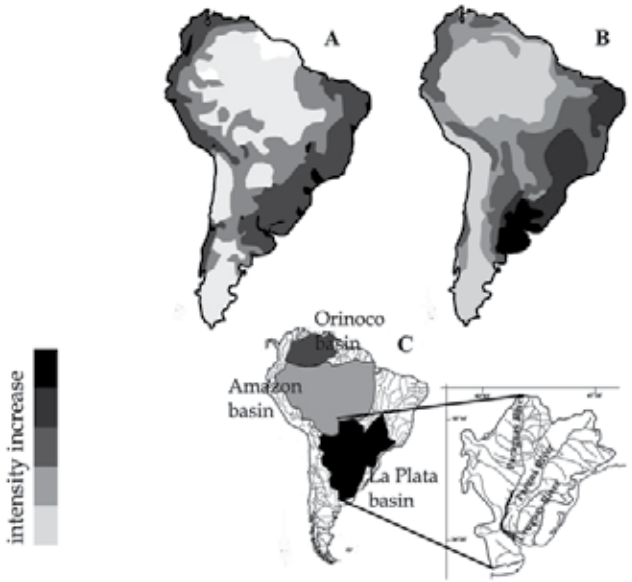

Fig. 2. Land use in South America indicating the surface of Orinoco, Amazon and La Plata basins, approximately. A) urban development and level (A) farm and industrial activities (B). Scale indicates differences in the intensities of the activities (modifies of Collins et al., in press).

Thus, the man invades and uses natural systems, intensively modifying them, and the fauna suffers extreme stress. Aquatic systems, although generally not targeted by direct application of pesticides, are impacted as a result of runoff after rain. This water, with all the elements that may be associated with it, goes into rivers or depressed areas. Groundwater may also be contaminated by percolation after rainfall. This mobility of elements occurs more intensively when the fields have no vegetative cover.

Moreover, the higher populations in cities have caused an increase in the urban area required to accommodate people and their families. This, together with increased biocide use and the increased area of impermeable surfaces in cities has meant that household chemicals and waste products are transported rapidly to aquatic systems during rains (José de Paggi et al., 2008).

Watersheds are continually being impacted, and care must be taken to ensure their quality control because these watersheds provide people with water to live. In South America, there are three major basins with water flows ranging from 18.000 $m^{-3}s^{-1}$ to approximately 220.000 $m^{-3}s^{-1}$ (Bonetto & Waiss, 1995; Lewis et al., 1995). These basins are the Amazon, Orinoco and La Plata. Of these three, the most densely populated watershed, with the greatest number of agricultural enterprises and the largest number of factories, is La Plata Basin (Figure 2) (Collins et al., in press).

1.3 Biocides

The variety of active ingredients used as biocides and their commercial formulations, solvents and coadjuvants or related chemicals is immense. All of them are used by application with agricultural aircraft, sprayers, hand-held units, or trucks that carry the spraying equipment, according to the extension land, application protocols, crop types and soil characteristics. Studies on native fauna are scarce, and only for very few taxa have the biological effects of biocides been studied. Studies on the interrelationships among the fauna components in relation to pesticide use have also been scarce. The actions of each biocide cause different biological responses, e.g., cypermethrin provokes an increase in metabolic activity and glyphosate a decrease (Collins et al., in press). The action of each pesticide is different, and the scarce information in their effects makes it very difficult to recognise the magnitude of the harm caused by these biocides on non-target species and on aquatic environments. The studies that have been conducted have focused on assays involving the active ingredient; however, it is not only the active ingredients that cause damage to the environment but also those compounds that are in the formulation and are considered inert. These compounds can increase the toxicity of the active ingredient, facilitating its ingression in biological systems, or may be toxic by themselves. It is therefore necessary for studies not to ignore commercial formulations, because they may include several compounds that can affect aquatic systems.

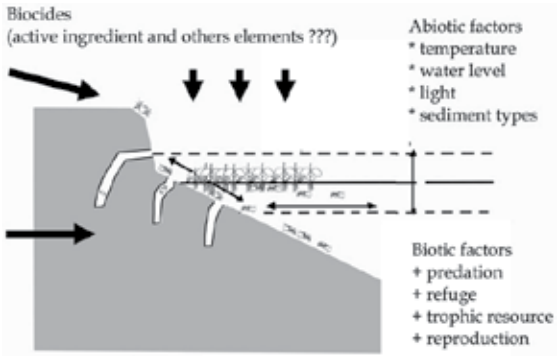

Fig. 3. Structure of typical area more affect by biocides through of sprayer with airplane, runoff after rain or groundwater potentially contaminated

2. Biocides in organisms: internal actions

2.1 Uptake at the tissue and cellular level

The toxicants direct absorption from the water by the integument and gills of crustaceans and/or through the ingestion of contaminated food via the gastrointestinal tract can cause serious toxicity to normal biological functions at the tissue, cellular, and molecular levels. However, the permeability of biological barriers and the rate of transport of chemicals into an organism are affected by the metabolic activity of the animal and, indirectly, by factors influencing this activity (water temperature, pH, hardness, the presence of other chemicals). The metabolic activity of the animal is influenced by its body size, growth rate, physical activity, and physiological state (juvenile or mature, moulting, feeding) (Zitko, 1980).

As the mechanisms of the toxic action of many pesticides usually occur on the surface of or inside the cells (Fent, 2004), the movement of these xenobiotics across membranes depends

on the chemical nature of the pesticide involved. Matsumura (1977) summarised the specific properties that influence uptake into aquatic organisms: lipid and water solubility, chemical stability against degradative action by biological systems (biotransformation), and the molecular weight of the chemical. These physicochemical properties determine the affinities of toxic compounds for the materials comprising the arthropod cuticle and plasma membrane of the cell (Hartley & Graham-Bryce, 1980).

Because lipids constitute a substantial part of the plasma membrane, lipid solubility is a very significant factor determining the rate of penetration of many toxic compounds (such as organochlorine pesticides) by passive diffusion through the non-polar portion of the membranes. Lipid solubility is usually characterised by the octanol/water partition coefficient (K_{ow}). In other cases, both facilitated diffusion and active transport are required for the passage of toxic into the cell through channel proteins and via their association with carrier proteins, respectively (Newman & Unger, 2003). The passage through a protein channel occurs down a concentration gradient that may be subject to saturation kinetics, and it is influenced by the size of the molecule, which determines a lower permeability of the membrane with increasing molecular size (Zitko, 1980). Moreover, the uptake of several pesticide compounds requires an active process with an expenditure of metabolic energy in living tissue. Through these pathways, toxicants enter cells and cause alterations in the physicochemical properties of the cytoplasm and the pH of the medium, destruction of the membranes of the organelles, disruption of the normal functioning of the cell proteins, and inhibition of the actions of the enzymes (Sohna et al., 2004; Collins, 2010).

Because in multicellular organisms the distribution of toxicants occurs in more than one compartment, within the crustacean body, haemolymph circulation may be involved in the transport of these chemicals to their sites of action and even more so if it is an open system that flows around the organs. In other arthropods, such as insects, Brooks (1974) reported that phosphoric acid penetrates the cuticle more rapidly than organochlorine insecticides, and having passed this barrier, the toxicant enters the haemolymph and may be transported to all parts of the organism in solution, if water soluble, or bound to proteins or dissolved in lipid particles, if lipophilic. The relatively hydrophilic molecules are much more likely to remain in this circulatory fluid than small, hydrophobic molecules, which are rapidly distributed in several organs and stored in lipid tissue (Hartley & Graham-Bryce, 1980).

2.2 Toxicity and biotransformation

The adverse effects of toxic products on crustaceans depend on its concentration and affinity, activity (intrinsic toxicity, which is function of molecular structure) and chemical biotransformations (James, 1987) and the acclimation responses of the individual (Klerks, 1999). For biocides, such as organophosphates and carbamate anticholinesterases (anti-ChEs), intrinsic toxicity can be judged by measuring the inhibition of cholinesterase and propagation of action potentials on synaptic transmission (see biomarkers section).

While some organic compounds are sufficiently water-soluble (hydrophilic) for excretion and can be eliminated rapidly, many lipophilic components cannot be directly excreted and would accumulate if not processed to more polar derivatives. Because the unaltered toxicant and any of its transformation products (metabolites) may be excreted, excretion represents a possible protective mechanism against the toxicant (Newman & Unger, 2003). Usually, organic pesticides are subject to modifications through enzyme-catalysed biotransformations leading to *detoxification* or *activation* (Figure 4). Chemical

biotransformation in animals occurs via Phase I (functionalisation) and Phase II (conjugation) reactions, which are more readily excreted than the parent compound (Brooks, 1974; Oesch & Arand, 1999).

1. **Phase I reactions.** In this phase, several enzymes introduce a polar reactive group to the molecule, making it more water soluble while also increasing the possibility of further metabolism by Phase II enzymes. Two major groups of enzymes involved in Phase I metabolism include oxidoreductases and hydrolases that are located in the endoplasmic reticulum of the cell in many organs and tissues (James, 1987).
    1. The oxidoreductases include the quantitatively most important superfamily of xenobiotic-metabolising enzymes, the cytochrome P450-dependent monooxygenases (CYP), flavin-containing monooxygenases (FMO), monoamine oxidases (MAO), and cyclooxygenases (COX), all of which introduce oxygen into or remove electrons from their substrates, with a few exceptions.
    2. The dehydrogenases and reductases, such as alcohol dehydrogenases, aldehyde dehydrogenases, and carbonyl reductases, add or remove hydrogen atoms to or from the target molecule. The hydrolases comprise families of enzymes specialised in the hydrolysis of esters, amides, epoxides, or glucuronides (Oesch & Arand, 2005).

The predominant functions of Phase I reactions are the conversion of polar, lipophilic compounds into more polar, more hydrophilic compounds and the introduction or liberation of functional groups that can be used for conjugation in the subsequent Phase II of xenobiotic metabolism.

2. **Phase II reactions.** Phase II enzymes often conjugate the polar groups produced by Phase I enzymes to introduce more bulky hydrophilic substituents, such as sugars, sulphates, or amino acids, into the molecule. This conjugation substantially increases the water solubility of a chemical, making it more easily excreted. The conjugation of the xenobiotic metabolism is carried out by transferases.
    1. Electrophilic substrates are taken over by the glutathione S-transferases (GSH S-transferase).
    2. Nucleophilic substrates (i.e., those with hydroxyl, sulfhydryl, amino, or carboxyl groups) are metabolised by UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), acetyltransferases (AT), acyl-CoA amino acid N-acyltransferases, and methyltransferases.

Phase II involves reactions such as glycosylation, sulfation, mercapturic acid formation, amino acid conjugation, and acetylation. Carboxylic acid groups in xenobiotics can be conjugated with amino acids prior to excretion (Tang et al., 2005). Metabolites formed by conjugation reactions are usually less toxic than the unconjugated compound, although there are notable exceptions to this rule (James, 1987). In addition, the metabolic events that increase the water solubility of a chemical usually cause a significant reduction in its biological half-life by making it more readily excreted (Brooks, 1974).

However, the patterns of activity of key enzymes involved in the detoxification of pesticides can be modified by the same toxic effect of xenobiotics. An elevation in glutathione S-transferase (GSH S-transferase) levels in the hepatopancreas and gills was reported for freshwater prawns (*Macrobrachium malcolmsonii*) and crabs (*Paratelphusa hydrodromus*) exposed to endosulfan, reflecting the formation of glutathione (GSH) and endosulfan complexes as a means of detoxification/elimination (Yadwad, 1989; Saravana Bhavan &

Geraldine, 2001). Conjugation of xenobiotics with reduced glutathione (GSH), catalysed by glutathione S-transferase (GSH S-transferase), is an important physiological process in the elimination of toxic substances from the body. These authors suggest that the activation of such a mechanism probably confers cytoprotection against endosulfan-induced cellular stress.

There are some toxicants in which biotransformation through either Phase I or Phase II can produce a highly reactive chemical, for example, the organophosphorus compounds. Although many of the insecticides in other chemical classes are toxic in their original parent forms, this is not true for many of the organophosphorus insecticides, especially those of the phosphorothioate configuration (such as parathion, chlorpyrifos, and diazinon), characterised by a P=S group. The insecticides possessing a P--S group are usually not very potent anti-ChEs, and they require bioactivation of their P=O metabolites, called oxons, to display appreciable anti-ChE potency (Tang et al., 2005). This bioactivation (reaction of desulfuration) is mediated by cytochrome P450-dependent monooxygenases through an attack on the sulphur by oxygen to create an unstable phosphooxythiiran intermediate (a three-membered ring composed of P, O, and S) that subsequently decomposes to the oxon (P---O) metabolite plus an active form of S (S:). In addition, the S is reactive in the tissues and is capable of damaging some proteins, including the cytochrome P450-dependent monooxygenases.

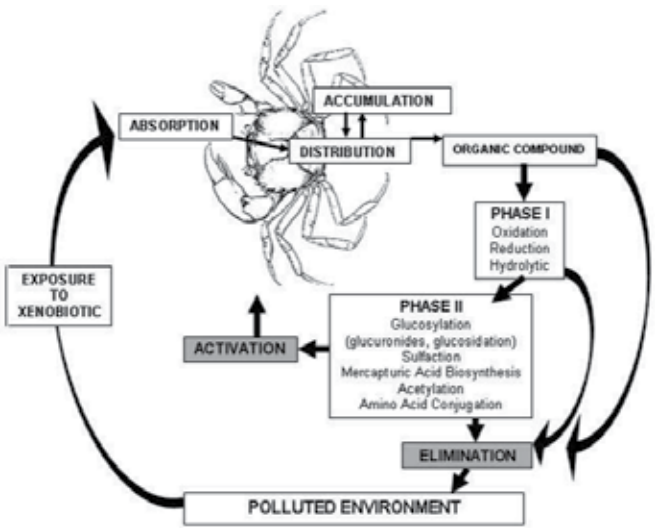

Fig. 4. Different process that can occur in decapods when the animals are affected by some biocide.

Other modifications to the toxic action of xenobiotics in crustaceans may occur via the phenomenon of physiological acclimation. In this case, an individual organism that becomes exposed to a specific contaminant may be less severely affected by this contaminant if it had been previously exposed to it. This effect is generally the result of the induction of a detoxification mechanism, as cytochrome P450, in response to the initial exposure (Tang & Garside, 1987; Stuhlbacher et al., 1992). Klerks (1999) observed (in the shrimp, *Palaemonetes pugio*) that acclimation results in an increased resistance at only a limited range of concentrations, with generally no change in resistance at lower pre-exposure levels and a

decreased resistance at higher pre-exposure concentrations that are stressful or result in a significant increase in contaminant body burdens. Such resistance occurs for some contaminants but not for others, and a lack of acclimation to complex mixtures occurs because positive responses to one contaminant are offset by negative responses to another contaminant. According to this observation, this can be explained by the fact that the energetic costs resulting from exposure to one contaminant (either for damage-repair functions or for detoxification processes, such as the production of P450 oxygenases) would compete with the energetic requirements associated with exposure to the other contaminant.

2.3 Biomarkers

To evaluate effects of pollutants on animal populations, communities and ecosystems, various methods have been developed, ranging from the (sub)cellular to the ecosystem level of biological responses. However, the predictive ability of measurements at higher levels of biological organisation is limited because ecologically important effects (e.g., death or impaired organismal function) have already occurred before they can be detected at population and community levels. In recent decades, biomarkers at suborganismal levels of organisation (biochemical components or processes, physiological functions, and histological structures) have been considered to be viable measures of responses to stressors (Hansen, 2003). These indicators of stress responses are useful in assessing the short-term well-being or long-term health status of an animal (Paterson & Spanoghe, 1997).

Metabolic changes observed in crustaceans exposed to pesticide pollution create widespread disturbances in general physiological processes, such as enzymatic activities, oxygen consumption, and changing energetic requirements. Some of the standardised types of biomarkers are those linked to disturbance to osmoregulation and water balance/ion-homeostasis, cholinesterase inhibition activity, protein stress, oxidative stress, and endocrine disruption.

2.3.1 Haematological parameters

Alterations in the haemolymph protein, haemocyanin, osmolality, ion compositions, total haemocyte counts, differential haemocyte counts, total free amino acid, nucleic acids (concentrations of DNA and RNA), phenoloxidase (PO) activity, and superoxide anion (O_2^-) may occur in crustaceans as a result of toxicant expositions. Yeh et al. (2005) reported a significant depression in haemolymph osmolality that mainly resulted from a decrease in the haemolymph chloride concentration (Cl^{-1}) in the prawn, *Macrobrachium rosenbergii,* after 8 days of exposure to sublethal concentrations of trichlorfon. However, a decrease in haemolymph pO_2 was found among these prawns, which may be related to decreased ventilation and impeded respiratory gas exchange, leading to respiratory disturbances via the inhibition of respiratory mechanisms and damage to respiratory organ epithelial cells. Similarity, a decrease in the pH and HCO_3^- of the haemolymph induced an increase in the pCO_2 level, benefiting the excretion of CO_2 in the haemolymph and resulting in a decrease in TCO_2, suggesting that trichlorfon disturbs the extracellular acid–base balance of prawns.

In crustaceans, gill lamellae and epipodites are involved in osmoregulation, and the histopathological changes in these structures (haemocytic congestion, gill lamellae necrosis, and the accumulation of particles surrounding the gill lamellae) were observed with lethal concentrations of fenitrothion (Lignot et al., 1997). According to these authors, the presence of particles surrounding the gill lamellae may have been a consequence of a lack of

ventilation in the branchial cavity due to the inhibitory action of the pesticide on the nervous system. In contrast, Saravana Bhavan & Geraldine (2001) observed in the prawn, *M. malcolmsonii,* an increase in the content of total free amino acid in the haemolymph as a result of protein degradation. In addition, the accumulation of soluble protein suggests that this was necessary to serve as a compensatory pool to restore enzymes lost to tissue necrosis and to provide prawns with the energy required to cope with the stress of exposure to endosulfan.

2.3.2 Cholinesterase (ChE) activity (inhibition)

Cholinesterases are serine hydrolase enzymes and degrade the neurotransmitters in cholinergic synapses. The toxicity of some pesticides, such as organophosphates and carbamate insecticides, is mainly caused by the inhibition of ChE activity of vertebrates and invertebrates. This inhibition leads to the accumulation of acetylcholine in the synaptic terminals and therefore to a change in the normal transmission of the nervous impulse. This interference may result in neurological manifestations, such as irritability, restlessness, muscular twitching, and convulsions, that may end in the respiratory failure and death of the animal (WHO, 1986). Consequently, most studies describe the use of ChE levels as a biomarker of exposure and/or the effect of several pesticide compounds in aquatic species. However, distinct enzyme isoforms with different sensitivities towards anticholinergic contaminants may exist, depending on the species. These isoforms are usually divided into two broad classes: acetylcholinesterases (AChE) and butyrylcholinesterases (BChE), which are distinguished primarily based on substrate specificity (Sultatos, 2005).

In crustaceans, published studies have also shown mixed results with regard to substrate preference. Fulton & Key (2001) reported that AChE in *Palaemonetes pugio* hydrolyses acetylcholine iodide (ACTH) and acetyl-b-methylthiocholine iodide (AMTH) much faster than other choline esters (such as propionylcholine) and is inactive on butyrylcholine. In contrast, BChE not only hydrolyses butyrylcholine but may also hydrolyse acetylcholine. The two enzyme isoforms may also be distinguished by their susceptibility to selective inhibitors; 1,5-bis-(4-allydimethyl-aminoniumphenyl)-pentan-3-one dibromide (BW284c51) and tetraisopropyl pyrophosphoramide (*iso*-OMPA) are selective inhibitors for AChE and BChE, respectively (Sultatos, 2005).

Organophosphates are generally irreversible inhibitors because the dephosphorylation rate of the bound enzyme proceeds at an insignificant rate. Therefore, the inhibitory effects of organophosphate exposure may be long lasting, with recovery depending on new enzyme synthesis (Habig & Di Giulio, 1991). Several studies with prawn, crab, and lobster species have shown that AChE inhibition in the animals still occurred days after exposure had ended (Reddy & Rao, 1988; McHenery et al., 1991; Abdullah et al., 1994; Key & Fulton, 2002). A slow time course for recovery of depressed AChE levels may cause exposed organisms to be susceptible to other anthropogenic or natural hazards or to exhibit behaviours not conducive to maintaining the population.

2.3.3 Stress proteins

The most abundant and widely studied group of stress proteins is the hsp70 (heat shock protein 70) protein family. The cellular functions of these proteins include the stabilisation of unfolded protein precursors before assembly, translocation of proteins into organelles, rearrangement of protein oligomers, dissolution of protein aggregates, and refolding or

degradation of denatured proteins (Feige & Polla, 1995). Induction of stress protein synthesis by pesticides is reported to be highly tissue-specific in aquatic animals. Among the tissues analysed (gill, skeletal muscle and hepatopancreas) by Selvakumar et al. (2005) in *Macrobrachium malcolmsonii*, induction of hsp70 synthesis was recorded only in the gill tissue of prawns that had been exposed to sublethal concentrations of endosulfan. In contrast, exposure of prawns to sublethal concentrations of carbaryl failed to elicit hsp70 synthesis in any of the three tissues analysed.

2.3.4 Oxidative stress

Under normal conditions, equilibrium exists between the amounts of free radicals generated and antioxidants available to quench or scavenge them, thereby protecting the organism against the deleterious effects of pollutants. However, oxidative stress occurs when the critical balance between oxidants and antioxidants is disrupted as a result of the depletion of antioxidants or excessive accumulation of the reactive oxygen species (ROS), or both, leading to damage to macromolecular components (Scandalios, 2005). Many xenobiotics, such as pesticides, may cause oxidative stress, leading to the generation of ROS and alterations in antioxidants or free oxygen radicals scavenging enzyme systems in aquatic animals (Dettbarn et al., 2005). However, the cells of crustaceans possess a variety of chemical and enzymatic mechanisms to protect them from oxidative damage. These mechanisms include an enzymatic antioxidant defence system comprising enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione S-transferase (GSH S-transferase) and non-enzymatic antioxidants like glutathione (GSH), ascorbic acid (vitamin C) and a-tocopherol (vitamin E), which are capable of neutralising or scavenging the reactive oxygen species (Vijayavel & Balasubramanian, 2009). These authors showed that the toxicity of fenvalerate to the prawn, *Penaeus monodon,* led to a significant induction of lipid peroxidation and GSH S-transferase activity in the hepatopancreas, muscle and gills. On the contrary, the activities of SOD, CAT, glutathione peroxidase, vitamin C, vitamin E and GSH were reduced in prawns exposed to sublethal concentrations of fenvalerate.

2.3.5 Neuroendocrine systems

Toxicity induced by a pesticide is the result of interaction of the compound or one of its metabolites with the biochemical events involved in the homeostatic control of a physiological process (Newman & Unger, 2003). Physiological processes are mostly coordinated by hormones. Therefore, the effects of organic compounds on functions regulated by hormones in crustaceans could be used as biomarkers of environmental pollutants.

According to Rodríguez et al. (2007), endocrine disruption can take place at different physiological levels: 1) altering (inhibiting or stimulating) the secretion of hormones; this possible effect is related to mechanisms that control both the release of hormones from endocrine cells and the synthesis of these hormones; 2) interfering with hormone-receptor interaction; in this sense, endocrine-disrupting compounds (EDCs) can act as agonists or antagonists by directly binding to a hormone receptor. Indirectly, however, an EDC could interfere via several mechanisms at any step of the transductional pathway of a hormone, therefore altering its final effect; 3) modifying the metabolism of circulating hormones, that

is, by increasing or decreasing their excretion rates and/or biotransformation in the liver, hepatopancreas or other organs.

Neurosecretory structures (X-organ–sinus gland) in the eyestalk are the most important components of the neuroendocrine system of the stalk-eyed crustaceans. The main hormones secreted by the sinus gland are the following: MIH (moult-inhibiting hormone), GIH (gonad-inhibiting hormone), MOIH (mandibular-organ-inhibiting hormone), CHH (crustacean hyperglycaemic hormone), several colour change hormones (controlling pigment migration) and NDH (neurodepressing hormone). Some of these hormones have a second endocrine gland as their target (MIH, GIH, MOIH), while the others have somatic tissues as targets. MIH, GIH, MOIH and CHH belong to a single family of peptides (Fingerman et al., 1998; Chang, 2001). These neuropeptides, synthesised in the XO (X-organ), a cluster of neuron perikarya located in the medulla terminalis of the eyestalk, are transported to and stored in the axon terminals, forming a neurohaemal organ named SG (sinus gland) and released by exocytosis into the haemolymph (Lorenzon, 2005).

The CHH have been shown to regulate carbohydrate metabolism in the shore crab, *Carcinus maenas*; the kumuran prawn, *Penaeus japonicus*; the lobster, *Homarus americanus*; the freshwater crab, *Oziotelphusa senex senex*; and the fiddler crab, *Uca triangularis* (Kegel et al., 1989; Lorenzon 2005; Purna Chandra Nagaraju et al., 2005). The neurotransmitter, 5-HT (serotonin), plays a fundamental role in hormone (CHH) modulation, and at the same time, pollutants can alter their level and function. Therefore, 5-HT has been known to have a potent hyperglycaemic effect with increases in the glucose haemolymphatic concentration resulting mainly from the stimulation of glycogen breakdown in the hepatopancreas (Fingerman et al., 1998). Hyperglycaemia is a typical response of several crustacean species to chemical stressors, including some pesticides, hydrocarbons and heavy metals. However, several reports have shown that an increased haemolymphatic level of glucose alone does not necessarily prove that there was a disruptive effect on the endocrine system. Because CHH is released to raise glycaemia as an adaptive response to several stimuli (such as emersion, starvation, critical temperatures and others), this hormone has been proposed as functioning as a crustacean stress hormone (Chang, 2001).

2.4 Histological effects

Crustaceans are considered as carrying a simple and primitive immune system (Fig. 5). The hepatopancreas is known as the detoxification site and also as a sensitive organ to stress, as it quickly responds to exposure to noxious compounds.

The hepatopancreas is essentially composed of branched tubules and of 4 types of epithelial cells: embryonic cells (E-cells), fibrillenzellen cells (F-cells), restzellen cells (R-cells) and blasenzellen cells (B-cells). E-cells are the only ones showing mitotic activity, being important in dead cell replacement. R-cells have absorptive functions supported by the presence of lipid droplets in the cytoplasm. These cells are involved in the delivery of nutrients to other organs via the haemolymph; the nutrient reserves are mobilised through R-cells to provide energy to the rest of the body. In addition, R-cells are interpreted as sites of intracellular waste deposition characterised by autophagosomes and residual bodies. These cells detoxify heavy metals and other lipophilic compounds by their accumulation in a soluble form in the cytoplasm, followed by excretion. F-cells are where protein synthesis and enzyme production occurs (Sousa et al., 2005).

Exposure to pesticides causes an imbalance in epithelial cells. Among the effects found, biocides cause an increase in R- and F-cells and an inhibition in E-cells. An R-cell increase in response to noxious compounds may be related to two different strategies. More R-cells may increase the detoxification rate because a higher number of cells increases detoxification. However, noxious compounds cause effects not only in the hepatopancreas but also in gills, gonads, and other organs. As other body parts require energy to recover from deleterious effects, the R-cell number increases for transporting energetic resources, i.e., lipids. When submitted to pesticides, F-cells increase for the production of more enzymes as a way of deactivating toxic compounds. R- and F-cells increase because both cellular types play roles in detoxification, and each one develops a different action for the same purpose. A decrease in E-cells becomes important if we consider that pesticides cause necrosis and increase cellular apoptosis. These cells replace dead cells with new ones, trying to mitigate cell loss. Exposure to pesticides also causes haemocytic infiltration in the interstitial sinus, abnormal lumen of the tubules, separation of necrotic cells from basal laminae, thickened basal laminae, necrotic tubules containing tissue debris, melanisation and coagulation in the thickened basal laminae and walling off of the tubules by haemocytes around the thickened basal laminae. All these effects combined may cause deficiencies in hepatopancreas function, with in turn may cause death (Saravana Bhavan & Geraldine, 2000, Bianchini & Monserrat 2007, Collins 2010).

In gills, one of the most important intake sites, biocide exposure also causes several histological damages, which in turn may cause functional deficiencies. Haemocytic infiltration in the haemocoelic space, swelling of the gill lamellae, lifting of lamellar epithelium, fusion of lamellae, abnormalities in the histoarchitecture, necrosis and other malformations are some the effects produced by pesticides in freshwater prawns and crabs.

Gills are related to the transport of respiratory gases, their obvious function, and also with ammonia excretion, as the majority of waste nitrogenous compound excretion occurs through the gill epithelium. Gill damage may also cause difficulties in oxygen intake, eventually asphyxia, and disrupt osmoregulatory function. A decrease in oxygen consumption may cause progressive internal hypoxia, with several effects such as metabolism shifts and locomotive difficulties. Crustaceans generally maintain an aerobic metabolism as a way of obtaining energy from food reserves. Aerobic metabolism, through the Krebs cycle, provides more energy than anaerobic metabolism. However, this kind of cellular "respiration" requires enough oxygen to be developed. When the amount of oxygen needed for the maintenance of aerobic metabolism in not achieved, crustaceans obtain energy by glycolysis, an anaerobic metabolism of carbohydrates. This type of metabolism, although it allows individuals to obtain energy for vital actions, has two serious effects: lactic acid release and an underutilisation of the energy accumulated. While in aerobic metabolism, animals obtain 36 mol of ATP from 1 mol of glucose, in anaerobic metabolism, they obtain only 2 mol of ATP from 1 mol of glucose, with the production of 2 mol of lactic acid (Schmidt-Nielsen, 1997). In animals with sporadic hypoxia, lactic acid is used as a substrate for further oxidation, completing the Krebs cycle and gaining the full energy value of the original carbohydrate substrate. However, in animals with oxygen intake decreased by histological damage, hypoxia may be not temporary; if they continue to be exposed to the aggressor agent, gills are not able to reconstitute itselves, or recuperation time is not quick enough to supply the oxygen demand. The continuous internal hypoxia may provoke a constant release of lactic acid as metabolic waste, with the consequent acid imbalance. This

imbalance and its histological effects may eventually cause the death of the affected individual because of acidosis or progressive asphyxia (Vonk, 1960, Schmidt Nielsen, 1997).

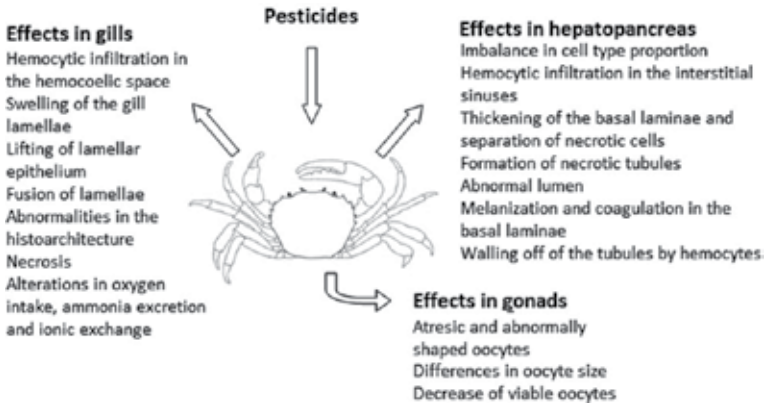

Fig. 5. Histological pesticide effects in crustacean organs and its results. Crab image modified from Collins et al. (2004).

Another effect of hypoxia is a decrease in locomotion. Crustaceans regulate their oxygen consumption within a range of dissolved oxygen concentrations. The minimal dissolved oxygen concentration within this range is called the critical oxygen concentration, below which crustaceans are not able to regulate their oxygen consumption. Given this situation of hypoxia, many crustaceans reduce their movements as a way of reducing the oxygen consumed by muscles, using the available oxygen instead of using it for metabolism (Zou et al., 1992, Zou & Stueben, 2006). The same response occurs if an animal has a deficiency in oxygen uptake, as in both situations the oxygen concentration in haemolymph is unsaturated. The oxygen deficiency causes a decrease in locomotive activities, with several effects. Animals are unable to escape from the contaminated area, exacerbating the effects of the contaminants. At the same time, a decrease in movement, as mentioned above makes animals more susceptible to predators.

In addition to their respiratory function, gills play an important role in nitrogen compound excretion. Crustaceans are ammoniotelic animals, i.e., their nitrogenous metabolic end products are mainly excreted in the form of ammonia. The antennal gland plays the key role in body water and divalent cation regulation, but it plays a minor role in ammonia excretion, as in some cases less than 2% of the total ammonia is excreted in the urine via the antennal gland system (Parry, 1960; Cameron & Batterton, 2004).

The high lipid solubility of ammonia makes it more diffusible through phospholipid bilayers. The mechanism supporting ammonia excretion in crustaceans is the simple diffusion of the non-ionic NH_3 along a concentration gradient and the partial excretion of the ionised form NH_4^+, whose release through diffusion is facilitated because of its hydrophobicity (Weihrauch et al., 1999, 2004).

Several aquatic crab species possess an excretion system based on the ionised form of ammonia, NH_4^+, a water soluble compound which effluxes through the gill epithelium. Freshwater crabs have tighter gill epithelia than their marine relatives, developed to avoid ionic efflux and tolerate a hyposmotic environment. This epithelium is much less permeable by NH_4^+, and freshwater crabs release their nitrogen compounds mainly as ammonia (Weihrauch et al., 1999).

Concentrations of NH_3 in the environment are kept low as a result of bacterial nitrification of ammonia to nitrite and nitrate, followed by the absorption of autotrophic organisms. This kind of environment favours ammonia excretion as a passive process driven by diffusion along a gradient. This process applies only to pelagic animals, generally prawns, colonising the water column, where the dilution and nitrification processes of aquatic biota keep ammonia concentrations really low. Benthic animals, such as crabs and crayfishes, are often faced with higher ambient concentrations of ammonia, present especially in anoxic, deep, stagnant water. Some species take refuge by hiding in riparian rocks and vegetation. Other species bury themselves in mud or, in the case of burrowing species, build extensive caves, in some cases more than 1 metre deep, with aerial or aquatic entry holes. They live in the bottom, where they find protection from predators and where the water is stagnant for hours. The very low water exchange rate, along with the fact that animals produce and excrete metabolic ammonia, increases the ammonia concentration and difficult simple diffusion.

The process that these crustaceans uses to eliminate ammonia is active excretion. Ammonia excretion rates are correlated with Na^+ absorption (Pressley et al., 1981, Harris et al., 2001). NH_4^+ substitutes for K+ in the activation of the ouabain-sensitive Na^+/K^+-ATPase, which is located in the basolateral membranes of the gill epithelium cells (Towle et al., 1981; Towle & Kays, 1986). This Na^+/K^+-ATPase is synergistically stimulated by NH_4^+ and K^+. In freshwater decapods, at high NH_4^+ concentrations, the pump exposes a new binding site for NH_4^+ that modulates the activity of the Na^+/K^+-ATPase independently of K^+ ions (Romano & Zeng, 2007, 2010).

Histological damage in gills, and the mucus segregation observed in prawns exposed to pesticides, may hinder ammonia excretion. These effects are especially relevant in freshwater benthic crustaceans, mainly crabs and crayfishes. As mentioned above, the passive efflux of ammonium (NH_4^+) is difficult because of the thickened gill epithelium, while ammonia excretion (NH_3) is difficult because of the environmental concentration. Nitrogenous waste compounds are eliminated by active efflux, and histological damage provoked by pesticides hinders this excretion process. When decapods are not able to eliminate the ammonia produced by nitrogen compound metabolism, it accumulates in the haemolymph, with several effects on individuals. Ammonia modifies the release of cytokines and increases the activity of lysosomal hydrolases. Ammonia toxicity is mediated by the excessive activation of *N*-methyl-*D*-aspartate (NMDA)-type glutamate receptors in the brain. As a consequence, cerebral ATP is depleted, while intracellular Ca^{2+} increases, with subsequent increases in intracellular K^+ and, finally, cell death (Weihrauch et al., 1999, 2004).

The intensity of the observed effects is related to pesticide concentration and animal resistance. Nevertheless, many of the described effects were achieved at concentrations that usually occur in the environment after aerial or terrestrial pesticide applications. The constant aggression provoked by biocides induces malfunctions in this vital organ, which eventually may cause the death of an individual.

2.4.1 Histopathological effects on female gonads

Freshwater decapods modified their reproductive strategy when they conquered freshwater environments. Larval stages were abbreviated or suppressed, and females invest their energy in fewer but more expensive progeny, which hatch at a more advanced stage.

Gonads are characterised by fewer but bigger oocytes, with more energetic reserves for the extended embryonic stage. In subtropical regions, gonad development occurs during late winter, spring and summer, the same period when pesticide applications. The drift and runoff provoke the migration of biocides to aquatic environments, causing a continuous contact with females during gonad maturation.

Ovary growth in crustaceans has two different periods: endogenous vitellogenesis (vitellogenesis I) and exogenous vitellogenesis (vitellogenesis II). The first period is characterised by an autosynthesis of lipovitellin and slow oocyte growth. The second period is characterised by the input of exogenous vitellogenin (a vitellin precursor) from outside of the ovary, mainly from the hepatopancreas, and rapid oocytic growth. Along with all the compounds provided by the hepatopancreas, lipophilic pesticides migrate to the ovaries (Lubzens et al., 1995). The effects of these biocides include abnormalities in shape, as the loss of the typical spherical shape of ovarian follicles; abnormal oocyte area increase or decrease, depending on pesticide type; and oocyte atresia (Rodriguez et al., 1994, Lee et al., 1996). The abnormal development of the ovaries causes a reduction in the available oocytes for fecundation, with the consequent reduction in eggs and the future brood, decreasing the population over the short and medium term.

Once fecundation occurs, females carry their eggs in their pleon until juveniles or mysis hatch. If these females live in contaminated areas, the exposure to biocides causes different effects in eggs and embryos. The easiest observable effect is death, but embryo death may occur at relatively high pesticide concentrations. Eggs are surrounded by the chorion, which isolates them from the environment. In the case of freshwater decapods, the chorion is thicker than that present in marine decapods because it has to protect the embryo from the osmotic stress caused by the environment. This thicker chorion also isolates the embryos from biocides and other compounds (Lindley et al., 1999; Varó et al., 2006). This protective effect makes embryos more resistant to toxicants, in some cases more resistant than juveniles, with a median lethal concentration similar to adults in several cases (Key et al., 2003; Li et al., 2006). Furthermore, embryos are more sensitive to pesticides when they are close to hatching because of the thinning of the chorion, which allows more pesticide to enter into the egg. This effect is also observable in prawns exposed to different salinity levels, as embryos are more sensitive to osmotic stress when they are close to hatching (Ituarte et al., 2005).

In addition to lethality, constant exposure to pesticides may cause differences in incubation periods and several abnormalities in embryos. Among these abnormalities, biocides may cause hydropsy, abnormal eye spots and several atrophies in the eyes, the pleon and the dorsal spine (Rodriguez & Pisanó, 1993; Lee & Oshima, 1998). All these abnormalities provoke the death of the juvenile, either from internal malformations of organs or from the incapability to moult successfully. Additionally, abnormalities in pleopods and pereiopods cause the inability to eat, find food or avoid predators.

Constant exposure to pesticides causes a reduction in functional oocytes, resulting in fewer eggs, a reduction in surviving embryos and a decrease in juveniles that will reach the adult stage, which in turn provokes effects on populations, the community and the ecosystem.

3. Reproduction effects

Freshwater environments impose a severe osmotic stress to the animals living there. Marine crustacean reproduction is characterised by a large brood, which hatches as larvae and

undergoes several stages up to the juvenile stage. Freshwater environments impose a severe osmotic stress on unprotected eggs and the free larvae stage. In the same way, developing embryos must be protected against this stress. When they conquered these environments, decapods developed different strategies to protect eggs and embryos. The primitive pelagic larval phases were suppressed; larval stages occur inside the egg, and the offspring hatch as mysis or juveniles. This internal development (i.e., inside the egg) imposes a greater protection to embryos against environmental pressures, especially in the susceptible larval stages. To support these internal stages, eggs increased in size and energy resources, mainly lipoproteins, because embryos grow inside the eggs and use their internal energy resources. Freshwater decapod females carry their eggs in the pleon, protecting them until the larvae or juveniles hatch. Because of their increased size, the number of eggs that a female can carry decreased, resulting in a concomitant decrease in the number of offspring (Ruppert & Barnes, 1994; Lee & Bell, 1999).

Several pesticides are highly lipophilic and are accumulated mainly in lipid reserves. During ovary development, oocytes accumulate lipids and lipoproteins, mainly lipovitellin, forming the vellum, which in turn will be used by the embryo as an energetic resource (the embryo "feeds" on the vellum). Attached to the lipovitellins, pesticides enter to the oocytes and accumulate on them. One explanation for the relatively greater resistance of females to organic pollutants is the distribution of these toxicants in the ovary, decreasing their concentrations in vital organs such as the hepatopancreas and delaying death (Sheridan, 1975; Menone et al., 2000; Wirth et al., 2001; Menone et al., 2004, 2006; Santos de Souza et al., 2008). The presence of pesticides in the oocytes implies that the embryo, beginning with fertilisation, is exposed to pesticides. Embryos grow and feed on the lipid reserves present on the vellum, with the consequent intake of pesticides. This may provoke not only the death of the embryos, with the release of dead eggs by the female, but also sublethal effects, such as abnormal size in eggs; deformation of embryos, such as tissue dropsy, atrophy, abnormal or depigmented eyes; and abnormalities in the pleon, telson, and spine, pereiopods and pleopods (Rodriguez et al., 1994; Saravana Bhavan & Geraldine, 2001). Deformation may cause difficulties in hatching or in brood survival, as activities such as swimming and searching for prey or escaping from predators may be hampered, and even moulting may not be successfully completed (Fig. 6).

In the case of freshwater decapods, the amount of vittelins and the time that embryos spend inside the eggs are greater than found in their marine relatives. This provokes an extended exposure time to different concentrations of pesticides, which depends on the exposure of females during gonad development and pesticide concentration in the ovaries.

Because of the osmotic stress that freshwater environments present, freshwater decapods possess a thicker chorion for protecting embryos from external aggressions. This chorion also protects them from biocides, making eggs as resistant as adults in some freshwater prawns and crabs, leaving juveniles as the most vulnerable (Key et al., 2003; Li et al., 2006). When the embryo is close to hatching, the chorion narrows to allow embryos to hatch, also allowing external agents to come into contact with embryos, making them more vulnerable to external agents, as observed in the prawn, *Palaemonetes argentinus* (Ituarte et al., 2005).

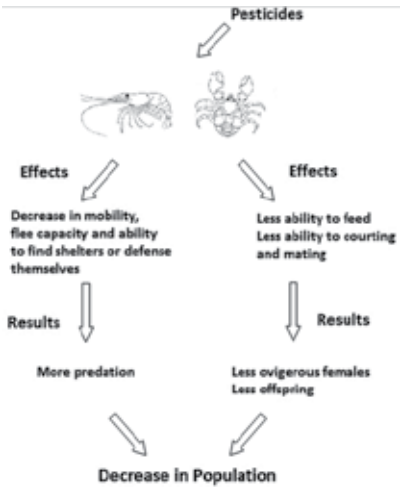

Fig.6. Pesticide effects in crustaceans' behavior and its results in population. Crustacean image modified from Collins et al. 2004.

The eggs of ovigerous females that live in contaminated areas may be resistant to pollutants, but toxicants may cause the death of juveniles after hatching, when they are not protected by a chorion. Moreover, moult events are a critical period for crustaceans, as their exoskeletons become softer and they are more vulnerable to external contaminants such as pesticides. In juveniles, the intermoult period is short, and the lethal effects of pesticides are increased during that critical period.

4. Growth

Growth is an interesting aspect in decapods in that it includes both internal and external factors. The intermoult period and increase in size are affected by different factors, such as diet (mainly protein, and lipid level variation), interspecific interactions (searching for agonistic behaviour and hierarchical conditions), temperature, biocides (or xenobiotic elements). Moreover, the growth in many species shows isometry and/or allometry variations in the ontogeny, and thus growth pattern can be affected. The study methods are different according to a study's objectives. In some cases, the animals are evaluated in groups, e.g., with diets; in other cases, a study is conducted with isolated animals to observe the xenobiotic effects on growth through chronic assays.

The capacity of an organism for survival, growth, and reproduction involves competition for energy resources at the individual level (Schmidt-Nielsen, 1997). Toxicant-induced shifts in energy allocations to these life-history activities will have important consequences on population. For example, higher respiration rates of estuarine crustaceans sublethally exposed to a variety of pesticides reduced juvenile growth by lowering growth efficiency rates, suggesting that increased metabolic demands lowered the amount of assimilated energy available for production of new tissue (McKenney & Hamaker, 1984; McKenney & Matthews, 1990). The assessment of changes in growth and energy stores of toxicant-sensitive life stages have a direct link to ecological consequences of environmental stress and can be useful as biomarkers to diagnose early damage in aquatic populations (Newman & Unger, 2003).

Crustaceans do not grow continuously but by periodically shedding the hard exoskeleton in a process called moult or ecdysis. Moulting is a very important physiological process because it not only allows for growth and development of these animals, which possess a rigid, confining exoskeleton but is also tied to metamorphosis during the early stages of the life cycle and reproduction during the adult stage (Passano, 1960). The process of ecdysis of decapod crustaceans is an antagonistic interaction by ecdysone and the MIH (moult inhibiting hormone), which originates from the Y-organ and X-organ/sinus gland (XO/SG) complex, respectively. The X-organ/sinus gland complex is located within the eyestalks. A reduction in MIH in the haemolymph is believed to induce moulting and stimulate the Y-organs to synthesise and secrete ecdysone, which will be converted to the active moulting hormone 20-HE (20-Hydroxyecdysone). Moreover, a significantly lower level of 20-HE was recorded in the haemolymph during the interval of moulting (Chang, 1995).
Limb regeneration is also an aspect of moulting. In this case, the regenerate first develops as a limb bud folded within a layer of cuticle and becomes free to unfold when the individual undergoes ecdysis as part of the moulting process (Fingerman et al., 1998). However, low levels of pollutants (such as chlorinated compounds) had an inhibitory effect on moulting and limb regeneration in some decapods (Fingerman ,1985).
Growth rate is usually described in terms of independent moult periods. These consist of a description of the size increment for each individual moult (moult increment) and a description of the time increment between moults (intermoult period) (Hartnoll, 1982). In many decapod species, growth alterations by toxicant may be caused by variations in the moult increment, but principally by changes in the intermoult duration. A reduction in growth by the lengthening of the intermoult period was observed in juvenile prawns, *Palaemonetes argentinus,* during the first moult cycles exposed to cypermethrin (Collins & Cappello, 2006) and to chlorpyrifos and endosulfan insecticides (Montagna & Collins, 2007). In contrast, this same freshwater prawn showed a shortening in the intermoult period with a reduction in the moult increment at the highest concentration of glyphosate tested (0.070 ml l^{-1}) (Montagna & Collins, 2005). These changes may involve perturbations to the X-organ and the sinus gland, which affect the production and storage of the inhibitory moult hormone or, more integrally, the neurohormonal system located in the eyestalks. Snyder & Mulder (2001) reported a delay in the onset of moulting of larvae of the lobster, *Homarus americanus,* exposed to heptachlor. This delay was correlated with both reduced levels of circulating ecdysteroids and increases of some P450-dependent detoxifying enzymes. Although it is known that 20-hydroxyecdysone itself can induce the expression of these enzymes, it is quite possible that this induction can also be produced by some toxicants.

5. Biocide effects on behaviour

Among the movements made by an animal, there are vital movements, such as breathing and cardiac movements; locomotive movements for prey finding and predator escaping; and behavioural movements, such as courtship and copulation. Every movement, even the simplest, depends on the harmony of every single movement to complete a desired action, i.e., for swimming, a prawn needs each pleopod to move in the right direction at the right time and with the right intensity to accomplish the final desired movement. Movements are transmitted through the nervous system and the synaptic gap by neurotransmitters, such as acetylcholine, while they are inhibited by enzymes, such as acetylcholinesterase, which stops the nerve impulse.

Some pesticides are acetylcholinesterase inhibitors in crustaceans and other animals (Saravana Bhavan & Geraldine, 2001; Braga da Fonseca et al., 2008). The inhibition of this enzyme enhances the contraction of skeletal muscles and impairs movement. When exposed to biocides that provoke an acetylcholinesterase inhibition, decapods are affected in their vital, locomotive and behavioural actions, with several different implications for the individual and the community. A prawn, crab or crayfish that is not able to swim or run correctly will be more susceptible to predation. Freshwater prawns and crabs exposed to acetylcholinesterase inhibitor biocides had a stimulus that improves appendage movements. Nevertheless, these movements are not synchronised, and the total movement efficiency is lower than that of normal locomotion. Prawn jumps are uncontrolled, and they keep jumping in the same place, without escaping from the area; crabs walking becomes frenetic, and they jump and walk, but move more slowly than normal. The increasing of impaired movements, which provokes a greater demand on muscle activity, more rapidly tires the affected animals. After the initial excitation, animals become quiet because of this tiredness, with slower movements and even immobility, making them more susceptible to predation (Williner & Collins, 2003; Collins et al., 2004; Collins & Cappello, 2006; Montagna & Collins, 2008). In a natural environment, escape from natural predators will be more difficult if crustaceans are affected by this kind of biocide, enhancing predation and decreasing the population.

Moreover, impaired movements not only affect locomotion as a way of escaping from the risk area but also affect the capacity of crustaceans to quickly locate refuges. Some freshwater crabs are pleustonic; they live between the roots of aquatic plants. As these roots act as filters for suspended organic matter and planktonic organisms, crabs go to the periphery for feeding. When detecting predators, they quickly migrate to the inside of the roots or stay still as a way of camouflage. Prawns and crabs also use rocks or burrows as refuges, either made by themselves or by other animals, and they swim or run to these refuges or bury themselves when they detect predators. Some crabs, especially the bigger species, use their chelipeds to attack their predators as a way of intimidating them and allowing themselves to flee (Collins et al. 2006, Collins et al., 2007). All these actions require a complex sequence of movements. If these decapods are affected by biocides, uncoordinated movements or tiredness will hinder their ability to find refuges, leading to increased predation and decreasing the population (Fig. 7).

Coordination of movements is not only necessary for escaping predators but also for finding food resources. Freshwater crabs and prawns are omnivorous animals. Some groups are specialised to filter sand and clay, feeding on the microbiota inhabiting these sediments. Other groups eat algae, macrophytes and animals tissues. Animal food may come from carrion or from hunting live prey. Decapod prey includes insect larvae, cladocerans, copepods, benthic organisms such as annelids and molluscs, fishes, other crustaceans and even eggs, juveniles and adults of the same species (Collins et al., 2006, Collins et al., 2007).

The hunting of mobile prey, such as fishes and crustaceans, and the manipulation of molluscs, which enclose themselves in their shells, requires both coordination in movements and strength. These actions become more difficult if decapods are subjected to acetylcholinesterase inhibitors or narcotic pesticides, decreasing the feeding capacity. Combining this decreased feed capacity with the increase in the energetic expenditure provoked by the impaired movements, biocide exposure eventually causes a depletion in

energetic resources, with several detrimental results for survival, growth, gonad development and reproduction (Saravana Bhavan & Geraldine, 1997).
The coordination of movements is also important in behaviours, such as territorial defence, courtship, mating and copulation. Decapod crustaceans, like many others animal species, have a courtship routine that is more or less complex, depending on the species. Mate selection is related to size and previous learning, and some crab species have a kind of "aggressive" courtship during which the male subjugates to the female (Fig. 7).
Agonistic behaviour is common in decapods, especially in crabs, and it is characterised by a series of coordinated movements that lead disputes in which the animals involved are at risk of serious injuries, loss of pereiopods and/or chelae or death of during combat. The more common resources involved in the disputes include shelters, mates and/or food. This behaviour may be affected by side effects of biocides; Williner & Collins (2003) and Collins & Cappello (2006), observed hyperactivity in freshwater crabs and prawns treated with cypermethrin. This hyperactivity capped oxygen consumption, resulting in an obligated hypoactivity during which there was a recovery state with reduced metabolism and lower oxygen consumption. This finding may show that decapods affected by biocides are acerbating the agonistic behaviour in the beginning, with a subsequent negative effect on recuperation. Reproductive behaviour may also be affected. Palaemonid males court females by swimming, chasing after them until they successfully place a piece of spermatophore on the female's abdomen, and cypermethrin produces erratic movements in *Palaemonetes argentinus* (Collins & Cappello, 2006). This could affect both courtship and reproduction itself, especially in regard to "freezing" of the spermatophore, as this requires coordination and precision. It is also possible that a female would find "defective" males under the influence of toxic and remove their sperm packages to obtain offspring with higher fitness or more viable eggs. It has been found that the effect of stress on egg masses affects the viability of these eggs (Siegel & Wenner, 1984). Stress may also disrupt or alter the chemical communication of these animals, as studies show that in many crustaceans, this type of communication occurs, permitting these animals to determine states of dominance. It is also known that during courtship, the chemical perception needed to recognise the state of female receptivity may also be disrupted by the action of biocides. In addition, in relation to energy, oxygen consumption increase as a result of biocide action causing hyperactivity, reduces the energy available for reproduction, either reducing the number of eggs or the effectiveness of the fertility of eggs (Siegel & Wenner, 1984), or in relation to the behaviour during parental care (Fig. 7). Moreover, shrimp in estuaries, such as penaeid shrimp, when exposed to biocides, exhibit decreases in the percentages of proteins as energy resources (Galindo Reyes et al., 1996). This alteration in energy storage could affect animals not only directly but the energy available for reproduction. Huang & Chen (2004) show that endocrine abnormalities were related to levels of testosterone and vitellogenin in *Neocaridina denticulata* treated with toxic. These abnormalities could affect the reproductive behaviour and gonadal development of these shrimp. It is known that female crabs, particularly freshwater crabs, incubate the eggs in their abdomens until hatching, and in some cases keep their offspring alive for some time after hatching, requiring sufficient energy to do so (Senkman, unpublished data). The effects of biocides may provoke the death of eggs and juveniles and the development of abnormal juvenile behaviour caused by stress. During embryonic development, many crustacean females move their eggs with opening and closing movements of the abdomen in rhythm, and their pleopods are used to remove

bad eggs or foreign particles or microorganisms entering via the same motions for the abdomen.

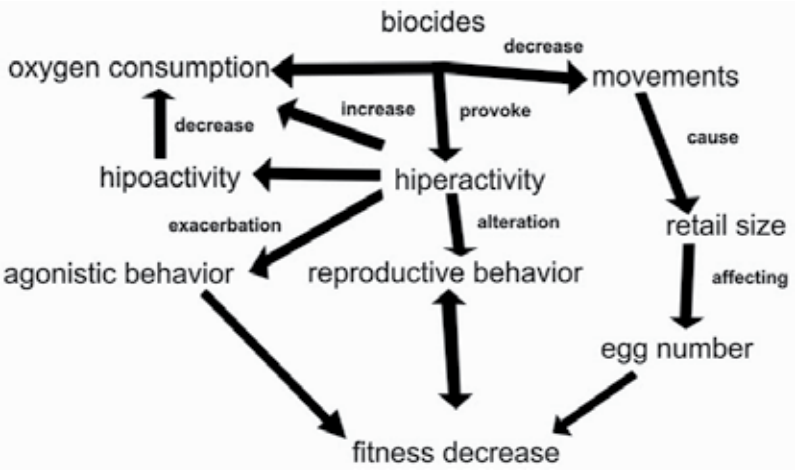

Fig. 7. Different effects that can occur when the animals are expose to biocide in relationship to oxygen consumption, activities, movements, and reproduction. All these affect the fitness of the species.

Biocides are known to affect moulting events in some decapod crustaceans, affecting their growth and keeping many adult individuals in sizes below the average body size of conspecifics. It is estimated that the size of individuals is an important factor in mate choice, as there is a direct relationship in many crustaceans between adult size and the number of eggs a female is capable of carrying, so the effect of biocides may include the number of eggs, or indirectly the reduction of average adult size.

6. Relationships between the external medium and an animal's body

In assessing organisational levels, it is necessary to analyse those relationships beyond the physical dimension of the animal's body and that provoke the defined interactions. Among these relationships are the connections between the various components of a community, i.e., trophic webs. According to the environment, the trophic web may be more simple or complex, e.g., with more connections and interactions between components or with greater or lesser possibility of prey choice by top members.

In these communities, whether they are subjected to fumigation or the biocides that enter the physical environment with runoff caused by rain, there will be species that are more sensitive than others, and these pollutants can make these species disappear or decrease their numbers extensively. This alteration will also be reflected in species that use this directly affected species as food, leading to increased competition among predators for fewer prey species. In this way, a decrease in diversity and a simplification of the system occurs.

In addition, all community members are in contact with the biocide, which may accumulate in organisms. When predators eat contaminated prey the toxic conditions of the biocides from the lower elements of the food chain are transferred to the other trophic levels of the chain, magnifying their effects.

Thus, the direction of flow of energy and matter through the food web can be affected, changing both direction and intensity, affecting the ability of each species and population to persist.

The movement of populations occurs and can be induced by abiotic, biotic and human factors. In the last case, this movement can be induced after rainfall, when biocides accompany rainwater. The xenobiotics in sediment, suspended in colloids or dissolved in rainwater are trapped. These biocides can cause changes in abiotic conditions (pH, conductivity, nutrients) and water and sediment qualities. The different factors make it difficult to identify a cause and/or cause-effect relationship, but these factors increase stress and impair various species' activities under various conditions. According to the timing of a rainfall event, the population may or may not be in its most vulnerable condition, based on its endogenous cycles (e.g., moult, reproduction).

7. Conclusions

In the modern world with the farmland activities, the aquatic communities are affected by different pesticides used in these agro-ecosystems. Herbicides, insecticides, fungicides are the most common elements used, and in some cases with several millions of liter what are used in the systems nearby to aquatic environments. This occurs due to the input of toxic compounds to water bodies by several ways, such as drift, and runoff provoking a risk to the fauna, and thus, creates the need of a constantly updates. The biological communities in rivers with floodplain and their tributaries are abundant, and with very high diversity. An interesting group is the decapods crustaceans by their abundance. These included prawns, crabs, pseudo-crabs and crayfish of South America. Populations of decapods could be reduced by lethal effects on the individual, and chronic alterations modify their fitness. The pesticide exposures cause damage in several tissues as hepatopancreas, gills, muscles and gonads, affecting the aquatic fauna. Among physiological functions, growth and reproduction could decrease by alterations in growth, gonad tissues, genetical material, and eggs development. Changes in metabolism, cell composition of the hepatopancreas, neuro-hormones have effect on behavior, growth rate, reproduction efficiency and survival due to the exposition of pesticide. Even more, these variations could affect the trophic web, and alter the transfers of material and energy into the aquatic systems. The proposed focus gives a snapshot from the macroscopic view of the ecosystem - community together to the molecular view. The different levels of organization with their temporal and spatial scale are necessary to achieve a better idea of the problem facing modern society with pesticides.

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12

Effects of Pesticides on Marine Bivalves: What Do We Know and What Do We Need to Know?

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1. Introduction

Estuaries are among the most productive environments in the world, by serving as feeding grounds, as nurseries for juvenile economically important fish and invertebrate larvae, and by providing shelter for many types of benthic organisms. However, they also rank among the most contaminated areas.

Among pollutants, pesticides have become more common in estuarine areas. They are mainly introduced into rivers via run-off and then may enter marine areas, particularly estuarine and coastal zones. These pollutants may have major ecological consequences and could endanger organismal growth, reproduction or survival (Banerjee et al., 1996).

Among important organisms inhabiting estuarine zones, bivalves are sessile and filter-feeder species, able to accumulate contaminants in their tissues. Moreover, bivalve farming is an ancestral activity all around the world. It has been expanded and intensified in the last century and represents a major economic activity in various countries. In the majority of cases, bivalve species are reared in estuaryine zones, continually impacted by pollutants including pesticides. Natural and man-made toxicants enter marine ecosystems by various routes, including direct discharge, land run-off, atmospheric deposition, *in situ* production, abiotic and biotic movements and food-chain transfer.

Pollutant run-off into the ocean represents a potential threat to marine organisms, especially bivalves living in coastal environments. In this context, bivalve molluscs such as mussels and oysters have been postulated as ideal indicator organisms because of their wide geographical distribution, and sensitivity to environmental pollutants. They filter large volumes of seawater and may therefore accumulate and concentrate contaminants within their tissues (Ramu et al., 2007; Bernal-Hernandez et al., 2010). As an example, the level and extent of organic contaminants along the Korean coast has been estimated through a mussel watch program (Choi et al., 2010). Moreover, development of techniques allowing effect analysis of pollutant on bivalve biology may lead to the development of diagnosis tools adapted to analyze pollutant transfer towards estuarine areas.

A pesticide is defined as a chemical substance used for killing pests, as insects, weeds or rodents. Pesticids are often classified by the type of organism: fungicides, herbicides, insecticides, nematocides and rodenticides. They are used especially in agriculture and around areas where humans live. Some are harmful to humans, either from direct contact or

as residue on food, or are harmful to the environment because of their high toxicity, such as DDT (which is now banned in many countries). All pesticides act by interfering with the target species normal metabolism. Some inadvertently may affect other organisms in the environment, either directly by their toxic effects or via elimination of the target organism.
By World War II, only about 30 pesticides existed. Dichloro-Diphenyl-Trichloro-ethane (DDT) was recognized as an insecticide until 1942. Other pesticides soon followed, such as chlordane and endrin. Poison gas research in Germany yielded the organophosphorus compounds, the best known of which is parathion. Further research yielded hundreds of organophosphorus compounds including malathion. The Environmental Protection Agency (EPA) estimates that the use of pesticides doubled between 1960 and 1980 with over 1.8 billion kilograms a year used today worldwide. In most countries, pesticides must be approved for sale and use by a government agency. However pesticide regulations differ from country to country. To deal with inconsistencies in regulations among countries, an International Code of Conduct on the Distribution and Use of Pesticides has been adopted in 1985 under the umbrella of the United Nations Food and Agriculture Organization and then updated several times.
Bivalves in culture may be affected by the presence of pesticides, potentially increasing their susceptibility to a wide range of infectious diseases. The effects of environmental contaminants may result from direct toxic actions on tissues or cells or from alterations of the homeostatic mechanisms including the immune system (Coles and Pipe, 1994; Carajaville et al., 1996). It has been suggested that bivalves may be weakened in relation to the presence of these pollutants. It has been shown in several vertebrates and invertebrates that pesticides are capable of diminishing immune defenses and/or of modifying genomes. They may render animals more vulnerable to infectious diseases (Ross et al., 1996; Gagnaire et al., 2007).
Although pesticide effects on marine bivalves have been already studied in bivalves, a few of reviews summarizing their different effects are available. In this context, one of the major objectives of this chapter relies on summarizing existing body of data on pesticide detection in marine environments and their effects on bivalve physiology including genotoxicity and immunotoxicity. Moreover, another aim of the present chapter is to identify the topics on which scientific data are needed in order to better understand the complex interactions between pesticides, environment, marine bivalves and their infectious agents.

2. Pesticides in the marine environment

Aquatic habitats are particularly subjected to contamination by pesticides, via run-off, leaching, spray drift or accidental spills. Pestices contamination of the marine environment has been and is monitored worldwide through analysis of water, sediment and marine species samples in order to elucidate the contamination status, distribution and possible pollution sources and to assess the risks on aquatic organisms and human.
Levels of pesticides measured in superficial waters generally range below lethal concentrations for aquatic species. However, sublethal adverse effects may result from exposure to these products at environmentally relevant concentrations.
Buisson et al. (2008) reported recently results about the monitoring of contamination levels in the Pacific cupped oyster, *Crassostrea gigas*, reared in Normandy (France). Six herbicides were detected in seawater for a total of 15 herbicides. Although the most estuarine sites showed

relatively high values in sea water samples, no pesticides were detected in the flesh of collected oysters (Buisson et al. 2008). At the contrary, Monirith et al. (2003) reported that all samples collected from all the sampling sites demonstrated the detection of organochlorines (OCs) with considerable residue levels of p,p(')-DDT and alpha-hexachlorocyclohexane (HCH) in mussels collected from coastal waters in the Asia-Pacific region.

Pandit et al. (2006) conducted a multi-compartment monitoring (sediment, water and marine species) of residue levels of pesticides in coastal marine environment of Mumbai in India. The total HCH concentration in sediment samples varied from 3.8 to 16.2 ng g^{-1} lindane (gamma-HCH) contributing almost 55% to the total HCH. The concentration of total HCHs in seawater ranged from 0.16 to 15.92 ng L^{-1} and concentrations of total DDT varied from 3.01 to 33.21 ng L^{-1}.

The presence of herbicides, such as diuron, has been also detected in many aquatic ecosystems worldwide. For instance, in France, diuron has been detected in surface waters with concentrations ranging from 0.05 µg L^{-1} to 20.3 µg L^{-1} (Léonard, 2002). In Atlantic bays and estuaries, concentrations up to 0.7 and 1 µg L^{-1} have been reported (Munaron et al., 2006).

Due to its toxicity, the use of diuron has been forbidden by French policies since 2008. Diuron and isoproturon are also included in the list of priority to contaminants of the EU Water Framework Directive (European Comission, 2000). However, it is well-known that some herbicides may persist in the environment even if their use has been banned, e.g. atrazine (EEA, 2000). A recent study reported the presence of diuron on French aquatic environments, confirming its persistence despite restriction policies (Pesce et al., 2010).

Diuron metabolites such as DCPU (N-(3,4 dichlorophenyl)-urea), DCPMU (N-(3,4 dichlorophenyl)-N-(methyl)-urea) and DCA (3,4-dichloroaniline) have also been detected in aquatic environments (Munaron et al., 2006). Studies on biofilms have reported DCPMU to be more toxic than DCA (Pesce et al., 2010). However, the principal product of degradation of diuron reported in the literature is DCA, which has shown to be more toxic for various organisms of higher trophic levels, such as crustacean, insects and fish (Giacomazzi & Cochet, 2004). This product exhibits higher toxic effects than the parent diuron, and can affect organisms, such as crustacean with low concentrations (1 $\mu g.^{-1}$, Giacomazzi and Cochet, 2004).

Different compounds including herbicides and their metabolites (Lanyi & Dinya, 2003; Sorensen et al., 2003; Vargha et al., 2005) are detected simultaneously in aquatic environments, suggesting that experimental approaches with toxicant mixtures are needed. Studies with diuron and its metabolites have shown additive, enhanced, antagonistic or independent effects (Knauert et al., 2008; Pesce et al., 2010; Neuwoehner et al., 2010). Thus, there is still a lack of data concerning the toxicity and effects of pesticide metabolites on bivalves, whether individually or in mixture with their parent compounds.

3. Lethal effect of pesticides on marine bivalves

Several studies have been conducted in various marine bivalve species in order to define LC_{50} for different pesticides including DDT, diuron, atrazine or lindane.

Chung et al. (2007) evaluated the sensitivity of the juvenile hard clam, *Mercenaria mercenaria*, to DDT (organochlorine pesticide) by exposure to contaminated sediments (10 day) and seawater (24-h). The aqueous LC_{50} (24h) value was defined at 0.61 mg L^{-1} DDT. and the LC_{50} (10 day) value for sediment toxicity tests was 5.8 mg kg^{-1} DDT. The authors concluded that

based on comparisons to toxicity data for other marine species, the hard clam, *Mercenaria mercenaria*, is one of the more sensitive species to contaminants.
No significant mortalities were reported after two months of exposition to 100 mg L^{-1} of diuron while with 100 µg $L^{-1}$of isoproturon, 60% of mortalitites were observed (Moraga & Tanguy, 2000). Isoproturon has shown to be present at lower concentrations than diuron on aquatic environments (Munaron, 2006).
Lawton et al. (2010) studied the effects of atrazine on the hard clam, *Mercenaria mercenaria,* in aqueous and sediment laboratory assays. Through an acute aqueous bio-assay, these authors determined a 96h LC_{50} for the juvenile clams at 5608 µg L^{-1}. They conducted also a chronic aqueous bio-assay at low atrazine concentrations and a chronic sediment bioassay over a 10 day exposure period to examine both lethal and sublethal (dry mass, shell size, and condition index) endpoints (Lawton et al., 2010). On the basis of their results, the authors suggested that atrazine is not directly toxic to *M. mercenaria* at environmentally relevant concentrations.
Bouilly et al. (2003) reported similar results for the Pacific cupped oyster, *Crassostrea gigas*:, in adult and juvenile animals subjected to 2 different concentrations of atrazine (46.5 nM and 465 nM). These authors did not observed any effect on mortality.
In vivo in laboratory assays testing 10 different concentrations (0 to 10 mg L^{-1}) of lindane (gamma-hexachlorocyclohexane [gamma-HCH]) allowed to define the median lethal concentration (LC_{50}) after a 12 day period as 2.22 mg L^{-1} in the Pacific cupped oyster, *Crassostrea gigas* (Anguiano et al., 2006). Lindane and isoproturon tested at concentrations of up to 10 mg L^{-1} for a 9 day esposure period showed negative effects on survival and growth of Pacific cupped oyster, *Crassostrea gigas,* larvae (Hiss & Seaman, 1993).
Domart-Coulon et al. (2002) assessed the acute cytotoxicity of an organic molluscicide, Mexel-432, used in antibiofouling treatments in industrial cooling water systems on primary cell cultures derived from 2 marine bivalve species, the Pacific cupped oyster, *Crassostrea gigas,* and the carpet clam, *Ruditapes decussatus*.

4. Genotoxicity in marine bivalves

Results reported by Jha et al. (2002) suggested that tributyltin oxide is both cytotoxic (proliferation rate index) and genotoxic (sister chromatid exchanges and chromosomal aberrations) to embryo-larval stages in the blue mussel, *Mytilus edulis*.
Bouilly et al. (2003) researched potential genotoxic effects of atrazine in the Pacific cupped oyster, *Crassostrea gigas*. Adult and juvenile oysters were subjected to 2 concentrations of atrazine: 46.5 nM, representing a realistic potential exposure (peak value found in polluted environment) and 465 nM. These authors reported significant differences in aneuploidy after atrazine treatments in comparion to control: 9% in control oysters, 16% at 46.5 nM and 20% at 465 nM atrazine. Similar aneuploidy levels were observed in adults and juveniles.
Bouilly et al. (2007) showed that the herbicide diuron induced also aneuploidy in adult Pacific cupped oysters after a 11 week exposure period at 300 ng L^{-1} and 3 µg L^{-1}. The induced aneuploidy observed appeared to be transmitted to the next generation as offspring exhibited significantly higher aneuploidy levels when their parents had been exposed to diuron (Bouilly et al., 2007).
Genotoxicity induced by lindane at 0.7 mg L^{-1} was also demonstrated in Pacific oyster, *Crassostrea gigas*, hemocytes after a 12 day contamination period (Anguiano et al., 2006).

Wessel et al. (2007) investigatied embryotoxic and genotoxic effects of the organochlorine pesticide, endosulfan, on *Crassostrea gigas* embryos. Embryotoxicity and genotoxicity in terms of DNA strand breaks were observed for 300 nM and 150 nM.
Siu et al. (2008) used green-lipped mussels (*Perna viridis*) in order to study the bioaccumulation of organic pollutants, including organochlorine pesticides. Micronuclei and DNA strand breaks were observed in mussels transplanted in different sites and collected after 4, 8, 12, 16 and 30 days.
Revankar and Shyama (2009) explored genotoxic effects of monocrotophos, an organophosphorous pesticide, at different time periods, 2, 3, 7 and 14 days. A significant increase of micronuclei in a dose dependant manner was observed indicaring possible chromosomal damages induced by monocrotophos.

5. Immunotoxicity in marine bivalves and susceptibilty to infectious diseases

The impact of contaminants and other environmental factors on the immune system of bivalves is an issue of ecological and economical concern, because it may result in clinical pathology and disease, by increasing the susceptibility of affected organisms to pathogens.
Contaminants known to induce alterations of immune functions including pesticides (Vial et al., 1996; Banerjee et al., 1996; Banerjee et al., 2001) are present in almost all coastal areas. Among physiological processes possibly disturbed by pollutants, the immune system is likely to be one of the more sensitive (Baier-Anderson & Anderson, 2000; Fournier et al., 2000).
In contrast to the vertebrate immune system which consists of innate and acquired mechanisms, invertebrate immunity relies only on innate defence mechanisms. The fact that invertebrates represent more than 90% of the total number of species living on earth demonstrates the efficiency of their «primitive» host defence systems. It becomes more and more obvious that some of these innate mechanisms are conserved in invertebrates and vertebrates (Medzhitov et al., 1997; Means et al., 2000). Thus, the fundamental importance of the toxically-induced modulation of non-specific immune functions has increasingly been perceived.
Bivalve immunity is mainly supported by hemocytes and participate directly in eliminating pathogens by phagocytosis (Cheng, 1981; Feng, 1988). In addition, hemocytes produce compounds including lysosomal enzymes and antimicrobial molecules which contribute to the destruction of pathogens (Coles & Pipe, 1994).
Investigating the effects of pesticides on hemocyte functions and immunity in bivalves has been based on the monitoring of several biomarkers (Pipe & Coles, 1995). As an example, Gagnaire et al. (2006) tested the effect of 23 pollutants on Pacific cupped oyster haemocytes by flow cytometry monitoring different cell parameters and demonstrated that 3 pesticides (2,4D, paraoxon, and chlorothalonil) induced a modulation of hemocyte activities. However, biomarkers used differ very often between published studies.
Triforine, a fungicide, induced decreased hemocyte viability in the eastern oyster, *Crassostrea virginica* (Alvarez & Friedl, 1992). Cytotoxic effects were also observed in adult Pacific cupped oyster, *C. gigas*, hemocytes: the mean cell viability was significantly decreased at 1.0 mg L^{-1} of lindane (gamma-hexachlorocyclohexane) after 12 day exposure period (Anguiano et al., 2006). Alteration in cell viability was also reported in the blue mussel, *Mytilus edulis*, exposed to 0.1 mg L^{-1} azamethiphos, an organophosphate pesticide

(Cantry et al., 2007). Moreover, a mix of herbicides containing atrazine, diuron and isoproturon showed an effect on *C. gigas* hemocyte aggregation (Auffret et Oubella., 1997).
Chlordan, an insecticide, demonstrated effects on *C. virginica* hemocyte phagocytosis at 250 µM *in vitro* (Larson et al., 1989). A decreased phagocytosis activity was observed after a triforine exposure in the eastern oyster, *C. virginica* (Alvarez and Friedl, 1992). A pesticide mixture (alachor, metolachlor, terbutylazine, glyphosate, diuron, atrazine, carbaryl and fosteyl aluminium) representative for surface waters of the Marennes-Oleron Basin (Charente Maritime, France, 0.25 nM to 4 nM) induced a decrease of phagocytic activity (Gagnaire et al., 2007). Moreover, Cantry et al. (2007) reported a decrease in phagocytic index in the blue mussel, *Mytilus edulis*, after a short exposure to 0.1 mg L^{-1} azamethiphos. This result suggests that azamethiphos can modulate haemocyte function in mussels at environmentally relevant concentrations.
At the contrary, Gagnaire et al. (2003) reported no effect on cell viability, cell cycle and cellular activities except for peroxidase activity for Pacific cupped oyster haemocytes exposed to atrazine in *in vitro* and *in vivo* assays.
Pentachlorophenol decreased the production of ROS by the inhibition of NADPH production in the eastern oyster, *Crassostrea virginica* (Baier-Anderson & Anderson, 1996). Dieldrin, tested *in vitro* on *C. virginica* hemocytes induced a decrease of chemiluminescence at concentrations ranging from 3 to 300 µM (Larson et al., 1989). Hemocytes of *C. virginica* exposed to chlorothalonil (fungicide) for 20 h at concentrations between 4 nM and 2 µM showed no modification of cell mortality and phagocytosis, but a decrease of ROS production (Baier-Anderson & Anderson, 2000).
In the past decades, the emergence of infectious diseases has been reported in marine species and disease outbreaks have also increased (Harvell et al., 1999). According to Snieszko (Snieszko, 1974), the development of an infectious disease results from an unbalance between the host and the pathogen due to external factors (including pollutants) and/or internal factors of both protagonists (virulence of the pathogen, susceptibility of the host). Animals presenting impaired defence mechanisms may be more susceptible to infectious diseases.
Demonstration of the relationship between pollution and increase of susceptibility to infectious diseases exist in vertebrates (Fournier et al., 1988; Van Levoren et al., 2000; Jepson et al., 2005), a few of studies was carried out in invertebrates (Galloway & Depledge, 2001). Rare studies have attempted to link contaminant presence and susceptibility to infectious diseases in marine molluscs and demonstrated harmful effects of pollutants in bivalves.
Contamination of the eastern oyster, *Crassostrea virginica*, by polluted sediment and tributyltin increased the intensity of *Perkinsus marinus* infection, but no cellular or humoral parameters were modulated (Anderson et al., 1996; Chu et al., 2002). Anderson et al. (1981) demonstrated previously that the hard clam, *Mercenaria mercenaria,* exposed to PCP were unable to kill injected bacteria. Kim et al. (2008) studied the relationship of parasite detection to contaminant body burden in sentinel bivalves through a 'Mussel Watch' Program. These authors showed that correlations between parasites/pathologies and pesticides were frequent in mussels and oysters (Kim et al., 2008).
The Pacific cupped oyster, *Crassostrea gigas*, has been also used to evaluate the impact of a pesticide mixture (atrazine, glyphosate, alachlor, metolachlor, fosetyl-alumimium, terbuthylazine, diuron and carbaryl) on some immune-related parameters and to demonstrate a relationship between infectious diseases, defence capacities and pollutants.

Indeed, a mixture of 8 pesticides reduced phagocytosis on hemocytes and enhanced susceptibility to *Vibrio splendidus* (Gagnaire et al., 2007). Pacific cupped ysters were exposed over a 7 day period to the mixture of pesticides. The pesticides were selected on the basis of spread amounts in the Marennes-Oleron Basin (Charente-Maritime, France), one of the most important oyster producing areas in France (Léonard, 2002; Munaron et al., 2006). Moreover, a down-regulation of the LBPB/BPI, TIMP and lysozyme genes were reported in Pacific oysters exposed to the mixture of 8 pesticides (Gagnaire et al., 2007).

6. Other effects of pesticides on marine bivalves

The evaluation of acetylcholinesterase activity in marine organisms has been and is at present time extensively used as a biomarker of exposure to neurotoxic agents such as organophosphorus and carbamate pesticides. Indeed, organophosphorous compounds and carbamates including paraoxon and carbaryl are known to inhibit acetylcholinesterase (AChE) and carboxylesterase (CE) (Cooreman et al., 1993).

Paraoxon inhibited the activity of AChE in the hepatopancreas of the blue mussel, *Mytilus edulis,* in vitro at concentrations ranging from 1 µM to 1 mM (Ozretic and Krajnovic-Ozretic, 1992). Inhibition by carbaryl was less distinct. AChE from *M. edulis* hemocytes was inhibited in vitro by 0.1-3 mM paraoxon, eserine and DFP (Galloway et al., 2002). Cantry et al. (2007) showed that exposure of the blue mussel, *M. edulis*, to 0.1 mg L^{-1} azamethiphos, an organophosphate pesticide used to combat sea lice infestations in farmed salmonids, for periods of up to 24h caused a significant reduction in acetylcholinesterase activity in both the haemolymph and the gill. However, cholinesterases found in the Pacific cupped oyster, *Crassostrea gigas,* appeared to be insensitive to organophosphorous insecticides (Bocquene et al., 1997).

Anguiano et al. (2006) showed that after a 4 h exposure to lindane (gamma-hexachlorocyclohexane), filtration rates of adult Pacific cupped oysters, *Crassostrea gigas*, were significantly reduced compared with controls at concentrations of 0.3 and 0.7 mg L^{-1}. However, a short term exposure of the blue mussel, *Mytilus edulis*, to azamethiphos did not change the feeding rate (Chantry et al., 2007). Studies carried out in adult Pacific cupped oysters revealed that diuron induces partial spawning and atrophy of the digestive epithelium after 1 week of exposure at 1 µg L^{-1} (Buisson et al., 2008).

Greco et al. (2010) investigated effects of a mixture of herbicides on the physiological status of the soft clam, *Mya arenaria*. Clams were exposed for 28 days to 0.01 mg L^{-1} of a pesticide mixture: dichlorophenoxyacetic acid (2,4-D), 2-(2-methyl-4-chlorophenoxy) propionic acid (mecoprop), and 3,6-dichloro-2-methoxybenzoic acid (dicamba). Although a gradual sexual maturation was reported in both sexes during the course of the experiment, females demonstrated a higher sensitivity to pesticides compared to males.

Favret and Lynn (2010) during the course of a study monitoring sperm viability by flow cytometry in the eastern oyster, *Crassostrea virginica*, after exposure to a pesticide (Bayluscide) reported effects on mitochondrial membrane potential and plasma membrane in the sperm. Buisson et al. (2008) studied impact of pesticides in the cupped Pacific oyster, *C. gigas,* and reported partial spawning and atrophy of the digestive tubule epithelium in relation to pesticides.

A study with a mix of herbicides containing atrazine, diuron and isoproturon revealed effects on gene expression in the Pacific cupped oyster, *Crassostrea gigas* (Tanguy et al., 2005). Gagnaire et al. (2007) studied also the impact of pesticides on *C. gigas*, monitoring

gene expression in hemocytes by real-time PCR. The expression of genes involved in *C. gigas* hemocyte functions was up-regulated in pesticide-treated oysters compared to untreated oysters after a bacterial challenge. The authors hypothesized that gene over-expression could lead to an injury of host tissues, resulting in higher mortality rates.
Collin et al. (2011) explored under experimental conditions the effects of a cocktail of three pesticides (lindane, metolachlor and carbofuran) on physiological functions of the Pacific cupped oyster, *C. gigas,* using the suppression subtractive hybridisation technique. The authors reported a site and organ-specific response to the pesticides. Effects of imidacloprid and thiacloprid, 2 neonicotinoid insecticides, at transcriptomic and proteomic levels in the marine mussel, *Mytilus galloprovincialis*, were also reported by Dondero et al. (2010).
Tlili et al. (2010) compared the size-distribution of the intra-sedimentary bivalve *Donax trunculus* collected in two sites in Tunisia, a polluted site and a comparatively reference site The auhors showed that the size-distribution from the polluted site consisted of 4 cohorts.whereras 5 cohorts were observed in the comparatively reference site. Moreover, the mean total length size and the growth rate of cohorts were significantly reduced in the impacted site compared to the reference site. These results suggest effects of pollutants on marine bivalves at a population level with an ecological relevance.
Pariseau et al. (2010) studied haemic neoplasia in the soft-shell clam *Mya arenaria,* in relation to exposure to fungicides, chlorothalonil and mancozeb, without demonstrating a link.

7. Conclusions and perspectives

The results obtained through the cited studies alerts to the negative effects of pesticides in bivalves and may be important to initiate and implement programs to protect the bivalve estuaries. These studies bring scientific evidence regarding the biological effects of pesticides on the animals inhabiting contaminated estuaries and the potential effect of the contaminates on shellfish and on human health if the seafood is consumed.
The great variability of response (depending on duration of exposure, toxicant concentration, test species or experimental conditions) is a reminder that the effects of pollutants on the marine environment cannot be assessed by simple methods (e.g. short-term bioassays with one or two test species). As an example, Greco et al. (2010) investigating effects of a mixture of herbicides on the physiological status of the soft clam, *Mya arenaria*, showed that in clams kept at 18°C, pesticides appeared to induce minor effects compared with animals kept at 7°C. They concluded that increased temperature may modify the response of *Mya arenaria* to pesticides.
It is recognized that bivalve habitats may differ in environmental parameters. Thus, animals may be exposed to numerous variables that include other pollutants, different temperatures, salinities, amounts of dissolved oxygen, and changes in pH. In this context, a better understanding of the possible interactions between pesticides and other abiotic environmental factors (temperature, salinity,) and biotic factors associated with the physiological status of bivalves is necessary.
As it is possible to evaluate only a limited number of environmental factors in laboratory assays, it appears difficult to investigate all of the potential environmental factors that also may affect bivalve physiology.
A lot of studies concerning effects of pesticides in bivalves have been carried out using high pollutant concentrations. However, levels of pesticides measured in superficial waters generally range below lethal concentrations for aquatic species. In this context, sub-lethal

adverse effects need to be more documented through experiments carried out using pesticides at environmentally relevant concentrations.

Among studies focused on pesticides, most of them have been carried out by exposing animals to relatively long periods of time (Auffret et Oubella., 1997; Tanguy et al., 2005; Bouilly et al., 2007, Buisson et al., 2008) giving an insight on the effects of chronic exposures on physiological functions of the organism.

Nevertheless, it is well known that in natural waters, uneven concentrations of pesticides are found in the water mass because of different factors such as seasonal agricultural practices, weathering processes and peak concentrations are often found in the aquatic environment for short periods of time (Munaron, 2006; Hyne et al., 2008). Thus, long-term studies may be not so predictive of what could happen on a natural environment. Short-term exposures of herbicides under laboratory controlled conditions have shown to exert an effect on aquatic organisms (Bretaud et al., 2000; Saglio et al., 2002). They can give an insight of the potential effect of contaminants in organisms in the natural environment.

In order to assess the impact of persistent pollutants on the marine ecosystem a suite of biomarkers are being extensively used worldwide (Ozretic & Krajnovic-Ozretic, 1992; Lowe & Fossato, 2000). These biomarkers are being used to evaluate exposure of various species of sentinel marine organisms (e.g. mussels, clams, oysters.) to and the effect of various pesticides using different molecular approaches (Wong et al., 1992; Cajaraville et al., 1996; Galloway et al., 2002).

As an example, Matozzo et al. (2010) developed a multibiomarker approach in order to assess effects of environmental contaminants in the Manila clam, *Ruditapes philippinarum*, collected in 8 sites of the Lagoon of Venice (Italy). The authors used several biomarkers includig total haemocyte count and lysozyme activity, acetylcholinesterase activity in gills, vitellogenin-like protein levels in both digestive gland and cell-free haemolymph, and survival-in-air widely used to evaluate general stress conditions. In addition, different pollutants were also measured in collected animals. Results showed that the selected integrated approach between biomarkers and chemical analyses is a useful tool in biomonitoring (Matozzo et al., 2010).

Different compounds including different pesticides have been found simultaneously in aquatic environments, underlining that experimental approaches with toxicant mixtures are needed. Most of the studies evoked before, have been carried out in adults, but juvenile organisms are known to be generally more sensitive to environmental stress than adults (Perdue et al., 1981). Additional residue-effects data on sublethal endpoints, early life stages, and a wider range of legacy and emergent contaminants will be needed.

Finally, research in ecotoxicology needs also to fill the gap existing between sub-organismal responses to toxicants and effects occurring at higher levels of biological organisation (e.g. population) (Tlili et al., 2010).

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Immunotoxicological Effects of Environmental Contaminants in Teleost Fish Reared for Aquaculture

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1. Introduction

Contamination is one of the major problems associated with the environmental sciences. Many of the environmental pollutants affect to the different aquatic animals to certain degree depending on the toxic substance, concentration, self-life and animal behaviour and biology. Direct ingestion of environmental contaminants and bioaccumulation of toxic substances in bivalves, crustaceans, molluscs or fish for human supply is a serious task to consider in human nutrition. Furthermore, it is known that to provide the necessary proteins that need and will need the world's population must intensify efforts in production of both proteins of plant origin and animal origin. Among the latter is predicted that aquaculture will be one of the fields over the coming years will increase. In this regard, aquaculture is trying for some decades to compensate this negative balance for human consumption. Among the important issues to consider in the aquaculture business the impact of the environmental contaminants in the species produced for humans need to be controlled by the farmer. In this specific field, most of studies have evaluated the toxic effects in terms of fish viability or induction of tumors using different fish models. However, relevant fish species for aquaculture are less used in these experiments. Moreover, the impact of the environmental contaminants in the immune response of these fish, and consequently in the disease resistance, have received much less attention.

2. Overview of the teleost fish immune response

Fish are the first group of vertebrate animals with both innate and adaptive immune responses and are essential for proper understanding of the immune system and its evolution. The fish adaptive immune responses are less effective than in mammals because they are poikilotherms and completely dependent on the environmental temperature. Therefore, the importance of the innate immune response is more relevant, but not exclusive, in the fish disease resistance to pathogens. Overall, the mechanisms and molecules involved in the immune response are quite well conserved during the immune system evolution. However, there are major differences in terms of haematopoietic organs structure and function as well as in leucocyte distribution and function (Figure 1).

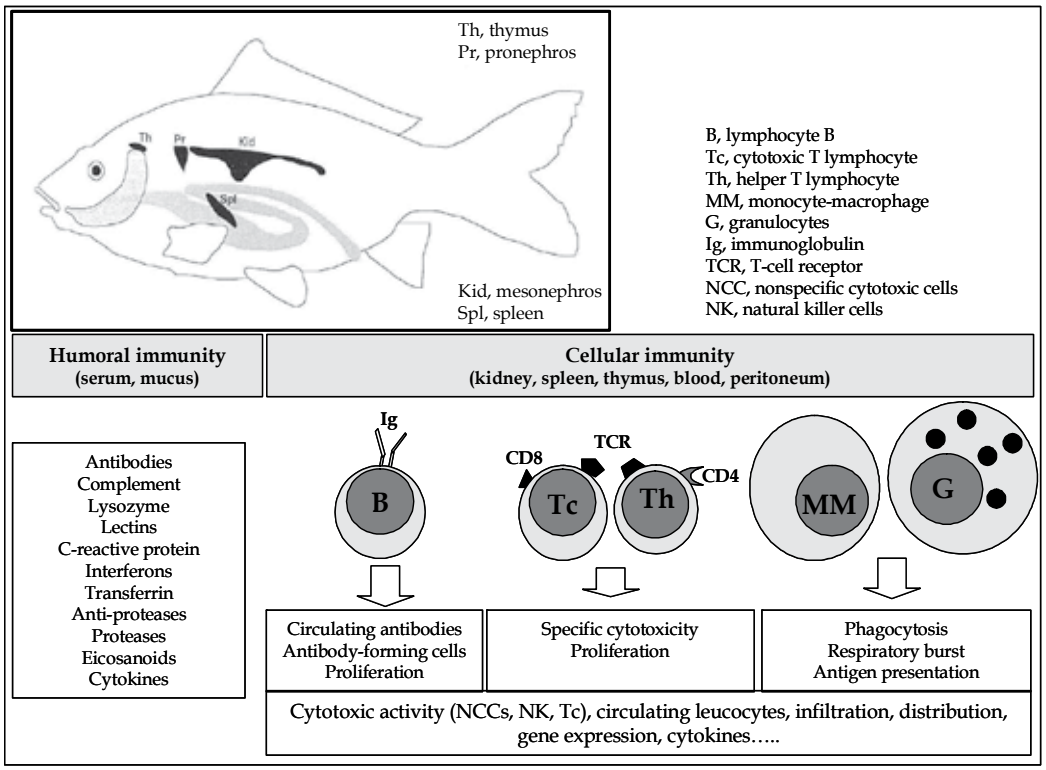

Fig. 1. Fish immune system organization (from Manning, 1998) and representative humoral and cellular immune responses used in immunotoxicological studies.

Firstly, the immune tissues are quite different since fish lack the bone marrow and lymphatic nodules (Manning, 1998). Thus, pronephros (anterior/head-kidney) is the main lympho-haematopoietic tissue in fish, whilst the posterior part or mesonephros is mainly excretory and the first site for development and B cells production. Thymus is the main tissue for T cells development and maturation whilst spleen is the main secondary lymphoid tissue in fish. Other important site for the immune response is the mucosal associated-lymphoid tissue (MALT), disperse in the skin, gill and gut. The leucocyte-types present in fish are quite similar between vertebrates but with some specific differences (Meseguer et al., 1994; Secombes et al., 2005; Miller et al., 1998; Rombout et al., 2005). Thus, fish lymphocytes are responsible for the production of antibodies (B cells) and the specific cellular immune response (T cells). B lymphocytes express and secrete immunoglobulin M (IgM), respond to the mitogen lipopolysaccharide (LPS) and constitute about 30% of the circulating lymphocytes. T lymphocytes are mainly detected in the thymus, express the T-cell receptor (TCR) and proliferate with the mitogens concanavalin A and phytohemagglutinin (PHA). They are responsible for the humoral and cellular immune response against T-dependent antigens by the different populations of CD4+ (Th or helper) and CD8+ (Tc or cytotoxic). Moreover, there are also subpopulations of fish lymphocytes lacking proper cell markers, Ig or TCR, and constitute the natural killer (NK) cells (Shen et al., 2002). By other side, monocyte-macrophages are the leucocytes displaying similar characteristics to both mammalian circulating monocytes and tissular macrophages.

Moreover, they are mainly localized in kidney and spleen where they concentrate the ingested particles and aggregate in melano-macrophage (MM) centres. Granulocytes can be divided in neutrophils, eosinophils and basophils according to their staining properties but in the case of fish the distribution and functions do not fit well with their mammalian counterparts. Monocyte-macrophages and some granulocytes form the phagocytic cells involved in phagocytosis of particulated antigens and in production of a machinery of lytic enzymes and the respiratory burst reaction, in which very toxic reactive oxygen species (ROS) and nitrogen intermediates (RNI) are produced. Finally, nonspecific cytotoxic cells (NCCs) are involved in the lysis of tumor cells, virus-infected cells and parasites in a similar fashion than the mammalian NK cells (Evans et al., 1984). However, they are a heterogeneous population (lymphocytes, granulocytes and/or monocyte-macrophages) and therefore some authors talk of nonspecific cytotoxic activity more than a cellular type or population (Cuesta et al., 1999).

The humoral immune response is a compilation of proteins and glycoproteins with defense functions found in the fish plasma and other body fluids such as mucus or sexual products (Kaattari & Piganelli, 1997). The complement system, in plasma and mucus, shows classical, alternative and lectin activation pathways with levels 5-10 times higher than in mammalian species with most of its components detected and characterized (Holland & Lambris, 2002). Direct lytic activity against bacteria, virus and parasites is the most relevant and studied function but it also acts as opsonin, chemotactic and neutralize endotoxins (Boshra & Sunyer, 2006). An important bacteriolytic enzyme is the lysozyme, mainly found in eggs, mucus, plasma and leucocytes (Magnadottir, 2006). There are also other innate immune factors such as acute phase proteins (C-reactive protein CRP), antimicrobial peptides, interferon (IFN), lectins, proteases, protease inhibitors or eicosanoids (Secombes, 1996; Aranishi, 1999; Bayne & Gerwick, 2001; Robertsen, 2006; Cammarata et al., 2007; Cuesta et al., 2008a). Finally, and the most interesting in fish, Ig are the major component of the adaptive humoral immune response. Fish were thought to have only one immunoglobulin isoform, the IgM. The fish IgM is tetrameric instead of pentameric as it occurs in mammals. Both membrane and soluble forms are observed by alternative processing of the mRNA (Wilson et al., 1990). Igs are found in the membrane of the B lymphocytes and this can be used to separate Ig+ and Ig- cells. The Ig functions are antigen neutralization, precipitation, opsonization and activation of the classical pathway of the complement system. In the last years, the presence of other Ig isoforms (IgD, IgZ or IgT) is throwing some light into the repertoire of fish immunoglobulins and their evolution in vertebrates (Hsu et al., 2006; Hikima et al., 2011).

3. Immunotoxicological effects of environmental contaminants

Environmental contaminants are widely distributed in aquatic environments. Although many of them are prohibited or restricted most of them are very persistent in the nature. Field and semi-field experiments are good to have suspicions about the contaminant presence but the setup of laboratory experiments with controlled parameters and precise and pure compounds are strictly necessary to understand the impact on fish immune response and their potential mechanisms. In line with the immunotoxicological studies in mammals, most of fish studies have evaluated the immune response (Figure 1) by measuring the macrophage functions (i.e. phagocytosis and ROS production), lymphoproliferative responses, host disease resistance, antibodies (circulating antibody

levels or antibody-forming cell numbers), number of circulating leucocytes, lymphoid organ cellularity and weights (Luebke et al., 1997; Bols et al., 2001).

3.1 Heavy metals

Heavy metals in aquatic environments are receiving more and more attention. Among the adverse effects, they can produce mortality, alteration of sexual maturation or immunodeficiency. Some heavy metals may transform into the persistent metallic compounds with higher toxicity, which can be bioaccumulated in the organisms and magnified in the food chain, thus threatening human health (Zhou et al., 2008).

Chromium (Cr) is a naturally occurring element found in rocks, animals, plants, and soil, predominantly in its insoluble trivalent form [Cr(III)]. Unfortunately, excessive industrialization and other anthropogenic activities have led to the global occurrence of soluble Cr (VI) in concentrations above permissible levels (Velma et al., 2009). The very scarce data *in vitro* have demonstrated that incubation of common carp (*Cyprinus carpio*) leucocytes with 2-200 μM hexavalent chromium showed depressed lymphocyte proliferation upon mitogen induction, as well as phagocytic functions, at much lower concentrations that produced cytotoxicity or cell death (Steinhagen et al., 2004). Moreover, neutrophils changed their morphology and reduced the amount of ROS and RNI. *In vivo* studies are more abundant and diverse and have also demonstrated the direct negative effects on fish leucocyte function and viability. Thus, tilapia (*Oreochromis mossambicus*) specimens exposed to sublethal doses of Cr-containing tannery effluents suffered a decreased antibody production, serum lysozyme activity and production of ROS and RNI by peripheral blood leucocytes (Sudhan & Michael, 1995; Prabakaran et al., 2007). Tilapia specimens exposed for 28 days with 0.5 and 5 mg Cr (VI)/L also decreased the disease resistance to bacterial infection and non-specific and specific immune response whilst the exposure with 0.05 mg Cr (VI)/L produced the opposite effects (Prabakaran et al., 2006). In another study, the spleen weight and the lymphocyte and leucocyte counts were significantly reduced by chronic exposure to Cr (III) and Cr (VI), producing the hexavalent form the greatest inhibitions (Arunkumar et al., 2000). In *Tilapia sparrmanii,* acute or chronic water exposures to potassium dichromate (0.098 mg/L) produced general haematological disorders including thrombocytopenia (Gey van Pittius et al., 1992). Moreover, and depending on the pH, fish showed leucocytosis and leucopenia at acidic and basic pH values, respectively (Wepener et al., 1992). In another more extensive study, the freshwater fish *Saccobranchus fossilis* were exposed for 28 days to 0.1-3.2 mg Cr (IV)/L and showed important changes in humoral and cellular immune responses and disease resistance (Khangarot et al., 1999). Concretely, they found a significant increase in the spleen size accompanied by an increment of spleenic lymphocytes. However, the number of plaque-forming cells and the phagocytic activity was reduced in spleen and head-kidney leucocytes. On the other hand, at blood level, the number of lymphocytes was decreased, but neutrophils and thrombocytes were increased, as well as the level of circulating antibodies and resistance to *Aeromonas hydrophila* infections. Otherwise, in plaice (*Pleuronectes platessa*), Cr-treatment increased the number of melano-macrophage centres but reduced their size (Kranz & Gercken, 1987). In the case of common carp and brown trout (*Salmo trutta* L.), 38 weeks of exposure with potassium dichromate diminished the primary and secondary humoral responses being the carp more susceptible to the heavy metal (O'Neill, 1981). In other kind of studies, the chromium exposure was carried out by dietary

intake and resembling the food chain bioaccumulation. In this case, rainbow trout (*Oncorhynchus mykiss*) fed diets containing 1540 to 4110 ppb Cr showed increased serum lysozyme activity as well as respiratory burst and phagocytic activity of macrophages in a dose- and time-dependent manner (Gatta et al., 2001).

Mercury (Hg), and derivatives such as methylmercury, are also important contaminants in aquatic environments inducing organ lesions, neurological, haematological and immunological disorders (Sweet & Zelikoff, 2001). First evidences, in rainbow trout, described a decrease in the number of mucous-producing cells and mucus production after exposure to mercury and methylmercury, which can be associated to impaired immunity (Lock & Overbeek, 1981). Afterwards, serum C-reactive protein was increased in freshwater murrel (*Chana punctatus*) (Ghosh & Bhaattacharya, 1992) and major carp (*Catla catla*) (Paul et al., 1998) by exposure to mercury. However, plasmatic lysozyme of plaice was decreased after exposure to sublethal doses of mercury (Fletcher, 1986). In sharp contrast, blue gourami (*Trichogaster trichopterus*) showed increased kidney and plasma lysozyme activity, but at the same time reduced the production of agglutinating specific antibodies after chronic exposure to 0.045 or 0.09 mg $Hg2^{+}$/L (Low & Sin, 1998). Further evidences have been obtained *in vitro*. Blue gourami lymphocytes incubated with mercury showed increased proliferation at low dosages, which was reversed by higher levels (>0.045 mg/L) (Low & Sin, 1998). In the marine fish *Sciaenops ocellatus*, mercury treatment (≤10 μM) produced a high-dose inhibition and a low-dose activation of leukocytes as determined by Ca-mobilization and tyrosyne phosphorilation of proteins (MacDougal et al., 1996). More recently, in the European sea bass (*Dicentracrchus labrax*), *in vitro* treatment with $HgCl_2$ induced apoptosis in head-kidney macrophages as well as reduced the ROS production and the benefits of macrophage-activating factors (MAF) (Sarmento et al., 2004).

Cadmium (Cd) is a nonessential heavy metal causing great toxicity. Among the first observations, Robohm (1986) found that Cd treatment inhibited the antibody levels in cunners (*Tautogolabrus adspersus*) and enhanced the antibody levels and chemotactic activity of peritoneal exudate cells in striped bass (*Morone saxatilis*). In rainbow trout exposed to 2 ppb of Cd, the same level found in some contaminated waters, the lysozyme activity was unaffected while the macrophage functions, phagocytosis and production of ROS, were significantly impaired (Zelikoff et al., 1995). These authors also demonstrated that Japanese medaka (*Oryzias latipes*) leucocytes increased their production of ROS and phagocytic functions without any change in many haematological parameters or antibody levels (Zelikoff et al., 1996). In the European sea bass, while *in vivo* exposure had a similar inhibitory effect on phagocytic functions the *in vitro* treatment produced an increment (Bennani et al., 1996). In the case of juvenile common carp experimentally infected with the blood parasite, *Sanguinicola inermis* (Trematoda: *Sanguinicolidae*) there were tissue changes and while the counts of neutrophils, eosinophils and thrombocytes increased in the thymus the number of neutrophils in the pronephros was reduced due to $Cd2^{+}$ treatment (0.1 mg/L) (Schuwerack et al., 2003). More recently, the Cd exposure has been related to the increase of melano-macrophage centres on several fish tissues (Suresh, 2009). In the hybrid tilapia (*Oreochromis niloticus* × *O. aureus*), the Cd exposure increased the lysozyme activity but greatly reduced the alternative complement activity (Wu et al., 2007).

Copper (Cu) is an essential nutrient but intensive use against fungal infections has shown to become a contaminant in some aquatic environments with immunosuppressive effects in general. *S. fossilis* fish exposed to sublethal Cu concentrations (0.056 to 0.32 mg/L) adversely

affected the humoral and cell-mediated immune system parameters (Khangarot et al. 1988; Khangarot & Tripathi, 1991) and reduced the fish resistance to *A. hydrophila* infections (Khangarot et al., 1999). European sea bass exposed to copper also showed an inhibited phagocytosis and ROS production both *in vivo* and *in vitro* (Bennani et al., 1996). Similar findings were also recorded in other experimental fish such as rainbow trout, goldfish (*Carassius auratus*), *Puntius gonionotus* or *Colossoma macropomum* (Hetrick et al. 1979; Knittel, 1981; Muhvich et al., 1995; Shariff et al., 2001; Lugo et al., 2006). Both *in vitro* and *in vivo* data have also demonstrated a decrease in the NCC activity and phagocytic responses in zebrafish (*Danio rerio*) (Rougier et al., 1994). Strikingly, further studies in common carp have shown increased humoral and cellular immune responses after Cu treatment (0.1-2.5 mg/L) (Dautremepuits et al., 2004a, 2004b). Very recently, Cu-incubation of trout macrophages up-regulated the expression of immune-relevant genes (interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNFα), serum amyloid A (SAA) and trout C-polysaccharide binding protein (TCPBP)) trying to understand the mechanisms and regulation of the immune response by heavy metals (Teles et al., 2011).

The immunotoxic impact of other heavy metals in fish has received less attention. Thus, zinc (Zn) was able to induce lymphoproliferation and NK-cell activity against tumor cells in common carp pronephros (Ghanmi et al., 1989, 1990). In zebrafish kidney leucocytes, Zn treatment increased the NCC activity and reduced the phagocytic responses both *in vitro* and *in vivo* (Rougier et al., 1994). $MnCl_2$ treatment also increased lymphoproliferation and NK cell activity in carp (Ghanmi et al., 1989, 1990). By contrast, Ni exposure reduced the lymphoproliferative response in medaka and deeper analysis led to the authors to suggest that the targets were the T-cells since neither the LPS-induced B-cell proliferation and antibody-forming cells were unaffected (Luebke et al., 1997). Arsenic (As) reduced the leucocyte respiratory burst, expression of some immune-relevant genes and disease resistance in zebrafish (Hermann & Kim, 2005; Nayak et al., 2007) in a similar fashion than in the catfish *Clarias batrachus* (Ghosh et al., 2007; Datta et al., 2009).

3.2 Polycyclic aromatic hydrocarbons (PAHs)

Aquatic environments are usually contaminated by PAHs derived form industry or petroleum, which produce external abnormalities, somatic mutations, cancer and immunodepression (Skupinska et al., 2004). The most toxic and the best studied are 7,12-dimethylbenz[a]anthracene (DMBA), benzo[a]pyrene (BaP) and 3-methylcholanthrene (3-MC) (Davila et al., 1995). In fish, as in mammals, the immunotoxicological effects are somehow contradictory and depend on the dose and time of exposition.

Liquid creosote (3-10 µl/L), containing PAHs, exposure of rainbow trout produced decreased respiratory burst of head-kidney leucocytes but increased phagocytic activity and percentage of Ig+ cells at short exposition times (Karrow et al., 2001). However, after 28 days, respiratory burst and phagocytic activity returned to control levels while the count of B cells remained decreased. The use of the heavily polluted Elizabeth River (Virginia, USA) has been extensively used for immunotoxicological evaluations. In the case of mummichogs (*Fundulus heteroclitus*), contamination produced a decrease in the levels of circulating IgM, both total and specific, and NCC activity while the plasmatic lysozyme was increased (Faisal et al., 1991a; Frederick et al., 2007). Moreover, lymphoid cells expressed higher levels of lysozyme and COX-2 (cyclooxygenase-2), the last as indicator of macrophage activation. Native fish (*Leiostomus xanthurus* and *Trinectes maculates*) from this river also showed lower

chemotactic and phagocytic activities that those kept in clean waters, and this suppression was reversed by maintenance in clean waters for several weeks (Weeks & Warinner, 1984; Weeks et al., 1986). Treatment of rainbow trout with 10-70% sewage plant effluents (containing PAHs among other contaminants) also reduced the number of circulating lymphocytes but increased their *in vitro* proliferation capacity (Hoeger et al., 2004). Strikingly, this effluent failed to alter any other immune functions such as respiratory burst, phagocytosis, lysozyme activity, leucocyte populations other than lymphocytes and *A. salmonicida*-specific IgM production. Intraperitoneal (ip) injection of diesel oil-based drilling mud extracts produced no effect on IgM levels and complement activity, suppression of serum lysozyme and elevated head-kidney lymphocyte proliferation in response to phytohemagglutinin (Tahir & Secombes, 1995). Petroleum-containing sediments also affected the immune response of flounder (*Pseudopleuronectes americanus*) since the number of melano-macrophage centres were diminished (Payne & Fancey, 1989). Deeper studies have evaluated the effects of heavy oil contamination (3.8 g/L for 3 days) in the Japanese flounder (*Paralichthys olivaceus*) using cDNA microarrays (Nakayama et al., 2008). They have found an alteration of expression in immune-related genes including down-regulation of immunoglobulin light chain, CD45, major histocompatibility complex class II antigens and macrophage colony-stimulating factor precursor, and up-regulation of interleukin-8 and lysozyme. Moreover, *in vitro* incubation with oils, pure and single PAHs, of European sea bass plasma produced significant changes in lysozyme and alternative complement activities indicating that these contaminants caused changes in the production of them by the leucocytes but also directly affects the enzymatic activity (Bado-Nilles et al., 2009). Similarly, PAHs mixture spiked-sediments (10 mg/kg dry wt) failed to change the serum lysozyme but reduced the ROS activity of kidney leucocytes of dab (*Limanda limanda*) (Hutchinson et al., 2003) while decreased the number of circulating lymphocytes (Khan, 2003). In the marine fish spot, *L. xanthurus*, exposed to PAH-contaminated sediments the T-lymphocyte proliferation was suppressed but the B-cell proliferation was greatly increased (Faisal et al., 1991b). Rainbow trout fed diets containing 0.66 or 7.82 μg PAH mixtures/g bw/day resulted in suppressed disease resistance against bacteria (Bravo et al., 2011).

Regarding the effects of single and pure PAHs, injections of DMBA (0.6 or 12.7 mg/kg body weight-bw) depressed the number of plaque-forming cells in head-kidney and spleen to T-independent antigens in Chinook salmon (*Oncorhynchus tshawytscha*) (Arkoosh et al., 1994). Injection of tilapia (*Oreochromis niloticus*) with DMBA (25 or 75 mg/kg bw) produced hipocellularity in spleen and head-kidney whilst phagocytosis and respiratory burst activity were not altered unless mortality occurred (Hart et al., 1998) similarly to the unaffected trout phagocytosis (Spitsbergen et al., 1986). By contrast, i.p. injection of 1-100 mg DMBA/kg bw to oyster toadfish (*Opsanus tau*) resulted in a peritoneal macrophage activity suppression in essentially a linear fashion, whereas NCC activity was virtually obliterated at all dosages (Seeley & Weeks-Perkins, 1997). BaP suppressed B cell immunity in tilapia at 15 mg/kg while increased at 25 mg/kg (Smith et al., 1999). Injection of 5-50 mg/kg also produced important histological changes in pronephros (reduction of lymphoid elements and augmentation of immune cells in apoptosis) and while the phagocytic activity was unaltered the respiratory burst was reduced (Holladay et al., 1998). In Japanese medaka, BaP injection (2–200 mg/kg bw) greatly reduced lymphocyte proliferation and number of antibody-forming cells (Carlson et al., 2002, 2004). In European sea bass, ip injection of BaP (20 mg/kg bw) significantly depressed the leucocyte phagocytosis and completely abrogated the ROS

production (Lemaire-Gony et al., 1995). In rainbow trout, BaP and BaA (benzo(a)anthracene) injection failed to significantly change the phagocytic activity (Walczak et al., 1987). Finally, 3-MC injection (40 mg/kg bw) into common carp increased the proliferative ability of resting circulating lymphocytes, rainbow but reduced their proliferative activity with the B- and T- mitogens, as well as the macrophage respiratory burst (Reynaud et al., 2002, 2003; Reynaud & Deschaux, 2005). Similarly, trout exposed to 3-MC increased the serum C-reactive protein 10-20-fold but not affected the IFN activity of leucocytes, measured as the resistance to bluetongue virus (Winkelhake et al., 1983).

3.3 Organochlorinated (OCs) contaminants

This group of contaminants comprises many of the most toxic and persistent compounds for aquatic environments such as DDT and relatives, lindane, polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs or dioxins) or polychlorinated dibenzofurans (PCDFs or furans). These are common contaminants in water ecosystems and their residues still have toxic consequences including immunotoxicity, reproductive deficits, teratogenicity, endocrine toxicity and carcinogenicity (Ahlborg et al., 1994). Unfortunately, although OC levels detected in fish worldwide seems to be declining they still should be lowered to decrease risk for human consumers (Gómara et al., 2005).

DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane), and its metabolites DDE (p,p'-DDE and o,p-DDE), are among the most important OCs in agricultural and aquatic environments. However, though no information exists regarding the direct effect of DTT on fish immunology some data are available about its derivatives. Thus, o,p-DDE treatment (10 ppm) of Chinook salmon, at fertilisation and hatch stages, failed to affect viability and growth but these fish still suffered immunosuppression one year later as consequence of the contamination (Milston et al., 2003). *In vitro*, p,p'-DDE (0-15 mg/L) produced a reduction in lymphocyte-granulocyte viability, by increasing the percentage of apoptotic cells, and in lymphocyte proliferation, in both spleen and head-kidney that was also observed *in vivo* (59 ppm exposure) (Misumi et al., 2005). By contrast, marine gilthead seabream leucocytes incubated with p,p'-DDE (5 ng to 50 mg/ml) failed to change their viability and main innate cellular immune parameters but up-regulated the expression of some immune genes (IL-1beta, TNFalpha, MHCIalpha, MHCIIalpha, Mx, TLR9, IgM and TCRalpha) indicating only effects at genetic level but not in function (Cuesta et al., 2008b).

Lindane (gamma-hexachlorocyclohexane) is another OC that have focused much of the attention. Dietary intake of lindane (10-1000 ppm) failed to affect the spleen weight, serum and mucus antibody levels and phagocytosis in the common carp though most of the tissues reflected great contamination (Cossarini-Dunier, 1987; Cossarini-Dunier et al., 1987). In rainbow trout, intraperitoneal injection of lindane (10-100 mg/kg bw) greatly depressed the number of antibody-secreting cells, serum lysozyme levels, respiratory burst activity and myeloperoxidase (contributes together with ROS and RNI to pathogen killing), proliferating capacity of B cells, but not of T cells, and its percentage in the head-kidney but at the same time increased the plasmatic ceruloplasmin, an acute phase protein (Dunier & Siwicki, 1994; Dunier et al., 1994). The same group also demonstrated that oral administration of lindane (1 mg/kg) for 30 days significantly decreased the respiratory burst activity of head-kidney leucocytes but unaffected the lymphocyte proliferation and number of circulating B lymphocytes in a similar way to the previous data in carp (Cossarini-Dunier et al., 1987; Dunier et al., 1994). Moreover, they have also demonstrated that these negative effects can be reversed by the *in vitro* addition of nitrogranulogen (Siwicki & Dunier, 1994) or dietary

intake of vitamin C (Dunier et al., 1995). Lindane bath of Nile tilapia also reduced the counts of circulating leucocytes, phagocytic activity and antibody levels (Khalaf-Allah, 1999). *In vitro*, lindane (2.5-100 μM) treatment was able to increase ROS production in rainbow trout head-kidney phagocytes and MAF (macrophage activating factors) production by peripheral blood leucocytes, in both cases depending on the dose and with contradictory results (Betoulle et al., 2000; Duchiron et al., 2002a, 2002b). These studies also demonstrated that low lindane concentrations increase the cytoplasmatic cAMP but high doses increase the intracellular Ca2+, and these two factors contribute to the dual effects of induction/reduction of the leucocyte immune functions produced by lindane treatment in leucocytes (Betoulle et al., 2000; Duchiron et al., 2002a, 2002b). In gilthead seabream, head-kidney leucocyte incubation (5 ng to 50 μg/ml) with lindane failed to significantly change the leucocyte viability (by necrosis and apoptosis) and innate cellular immune functions (phagocytosis, respiratory burst and cell-mediated cytotoxicity) but strikingly increased the expression of many immune-related genes (IL-1beta, TNFalpha, MHCIalpha, MHCIIalpha, Mx, TLR9, IgML and TCRalpha) (Cuesta et al., 2008b).

PCBs, with theoretically 209 distinct congeners, may be divided into those with coplanar geometry, the most toxic as they bind and activate AhR (hydrocarbon receptors) and CYP1A (cytochrome P4501A) expression, while noncoplanar congeners can interfere with AhR signalling but also affect cells via AhR-independent pathways (Duffy & Zelikoff, 2006). Immunotoxicological effects of PCB mixtures, such as Arochlor, have been evaluated in fish. Thus, Aroclor 1254 depressed plaque-forming cells in head-kidney and spleen to a T-independent antigen in Chinook salmon after ip injection (Arkoosh et al., 1994). However, it failed to modulate the innate disease resistance and antibody production by oral administration of environmental doses in the same fish (Powell et al., 2003). In Artic charr (*Salvelinus alpinus*), diets containing 100 mg Aroclor 1254/kg diet resulted in increased disease susceptibility to furunculosis (Maule et al., 2005). In Atlantic salmon (*Salmo salar*), by contrast, water exposure with 1-10 μg/L produced increased T lymphocyte proliferation at short and long-term (Iwanowicz et al., 2005). In rainbow trout, while the C-reactive protein levels in serum were increased the leucocyte IFN and NCC activities were unchanged (Winkelhake et al., 1983; Cleland & Sonstegard, 1987). Another study using Aroclor 1248, in the brown bullhead (*Ameiurus nebulosus*), have provoked a decrease in the bactericidal activity and antibody titers (Iwanowicz et al., 2009). PCBs mixture (Aroclor 1242, 1254 and 1260) failed to modify lysozyme and ROS activity in *L. limanda* (Hutchinson et al., 2003). Regarding the effects of pure PCBs, the congener 126 has been the most studied. PCB 126 injection (0.01-1 μg/g bw) to Japanese medaka reduced the antibody forming cell numbers (Duffy et al., 2002) but either reduced or increased the phagocyte-mediated ROS production at 3 or 14 days post-treatment, respectively (Duffy et al., 2003). Dietary administration (100 ng/g bw) to European eel (*Anguilla anguilla*) completely abrogated the production of specific antibodies against a parasite (Sures & Knopf, 2004). PCB 126 also produced a reduction of phagocyte respiratory burst and NCC activities in channel catfish (*Ictalurus punctatus*) at (0.01–1 mg/kg bw) (Rice & Schlenk, 1995). In the bluegill sunfish (*Lepomis macrochirus*), the coplanar PCB 126 (0.01 or 1.0 μg/g bw) also slightly affected the B-lymphocyte proliferation while the noncoplanar PCB 153 (5.0 or 50.0 μg/g bw) significantly reduced the phagocyte-mediated respiratory burst activity and the B- and T- lymphocyte proliferation (Duffy & Zelikoff, 2006). Strikingly, short incubation of rainbow trout head-kidney leucocytes with PCB 126 (1 μM) increased the expression of IL-1β gene and failed to abrogate the LPS effects on gene regulation (Quabius et al., 2005). The PCB Clophen A50 (0.4-2 μg/egg) injected into the eggs of rainbow trout with

pathogenic bacteria resulted in a higher disease resistance than those injected with the bacteria suggesting a direct effect on the immune response (Ekman et al., 2004).
Chlorinated dioxins, as typified by the most potent isomer TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), are also very toxic for fish. Injection of 0.1-10 μg TCDD/kg bw to rainbow trout resulted in very little changes in humoral and cellular immune responses (Spitsbergen et al., 1986). However, while the C-reactive protein levels in serum were increased the leucocyte production of IFN was unchanged (Winkelhake et al., 1983). In common carp, TCDD injection produced histological alterations including increase of melano-macrophage centres and reduction of lymphocyte numbers (van der Weiden et al., 1994). Further studies have also evaluated fish tissue alterations and CYP1A staining patterns have been described in European flounder (*Platichthys flesus*) and gilthead seabream (Grinwis et al., 2000; Ortiz-Delgado & Sarasquete, 2004).
Some studies have also evaluated the immunotoxicological effect of other OCs. In the case of furans (PCDF), most authors have focused on other fish toxicity tests rather than in immunotoxicology. Endosulfan exposure produces developmental and neurological disorders and acts as endocrine disruptor. Rainbow trout leucocyte treatment with endosulfan inhibited the lymphoproliferative activity where the B-cells were more sensitive than the T lymphocytes (O'Halloran et al., 1996). In another study, crimson-spotted rainbowfish (*Melanotaenia fluviatilis*), golden perch (*Macquaria ambigua*) and Murray cod (*Maccullochella peelii*), but not silver perch (*Bidyanus bidyanus*), leucocytes showed decreased phagocytosis after endosulfan treatment (10 mg/L) (Harford et al., 2005). *In vivo* treatment of Nile tilapia for 96 h at 7 ppb produced an increased phagocytosis and ROS production by spleen leucocytes, IgM levels and production of IL-2-like, but at the same time reduced the spleen viability and relative weight (Tellez-Bañuelos et al., 2009, 2010).

3.4 Organophosphorous pesticides (OPs)

OPs are insecticides used world-wide as an alternative to the persistent and more bioaccumulative OCs. They are potent neurotoxic and immunotoxic since are irreversible acetylcholinesterase inhibitors (Galloway and Handy, 2003). Malathion exposure (0.2-0.8 mg/L) of medaka resulted in reduced number of antibody-forming cells but unchanged circulating leucocyte numbers and T-cell proliferation (Beaman et al., 1999). Vaccinated Nile tilapia exposed to malathion or diazinon presented lower blood cell counts, phagocytosis and antibody levels than those unexposed (Khalaf-Allah, 1999). Diazinon exposure of bluegill had biphasic effects with immune response increases at low concentrations and depressions at high dosages (Dutta et al., 1997). In Nile tilapia, Girón-Pérez et al., (2007, 2008, 2009) have showed that diazinon altered the spleen counts and lymphocyte proliferation, serum IgM and lysozyme levels, phagocytic activity and respiratory burst depending on the exposure dose and time. Chlorpyrifos displayed little immunotoxicity, although there was a dose-dependent reduction in Murray cod lymphocytes (Harford et al., 2005). Nile tilapia exposed to the LC50 failed to change blood parameters but the phagocytic activity was significantly reduced (Girón-Pérez et al., 2006). Chlorpyrifos exposure produced an up-regulation of hsp60, hsp70 and hsp90 genes, related to the cellular stress response in Chinook salmon. Moreover, the cytokine (IL-1b, TGF-beta, Mx and insulin growth factor (IGF)-I) gene expression was unaltered or down-regulated but not affected the virus susceptibility of the fish (Eder et al., 2008, 2009). Dichlorvos and trichlorfon insecticides have been used in aquaculture against ectoparasites in the past. Trichlorfon exposure decreased the serum lysozyme, lymphocyte proliferation, respiratory burst and

phagocytosis of common carp leucocytes (Siwicki et al., 1990; Dunier et al., 1991) but unchanged the production of specific antibodies (Cossarini-Dunier et al., 1990). Water exposure to dichlorvos failed to change the specific IgM production but altered other serum innate immune parameters (Dunier et al., 1991). Edifenphos and glyphosate exposure reduced the lymphocyte proliferation, antibody-producing cells and circulating IgM levels in Nile tilapia (el-Gendy et al., 1998). Glyphosate exposure of silver catfish (*Rhamdia quelen*) resulted in decreased phagocytosis and resistance to disease (Kreutz et al., 2010).

3.5 Pyrethorids

Pyrethroids are extensively used insecticides since they are very stable and produce low mammalian toxicity but this is very high for aquatic animals (Bradbury & Coats, 1989). Among them, deltamethrin injection to *Ancistrus multispinis* increased peritoneal leucocyte numbers and production of RNI by macrophages (Pimpão et al., 2008). Short exposure to deltamethrin (30 min., 1-4 μg/L) of rainbow trout resulted in decreased serum lysozyme and IgM levels (Siwicki et al., 2010). Water exposure of rohu (*Labeo rohita*) to alpha-permethrin produced a reduction in lysozyme activity and resistance to bacteria (Nayak et al., 2004). Rainbow trout exposure to cypermethrin failed to alter any of the immune parameters (Shelley et al., 2009). Esfenvalerate exposure produced an up-regulation of hsp60, hsp70 and hsp90 stress genes, down- or non-regulated cytokines and unaffected the virus susceptibility of the Chinook salmon (Eder et al., 2008, 2009). Using microarrays, delta smelt (*Hypomesus transpacificus*) exposure to esfenvalerate produced alterations in the expression of genes associated with immune responses, along with apoptosis, redox, osmotic stress, detoxification, growth and development (Connon et al., 2009).

3.6 Organotins

Organotin compounds or stannanes are chemical compounds based on tin (Sn) with hydrocarbon substituents showing different toxic effects. TBT (triorganotins) is specially important since it has been widely used as marine anti-biofouling agent. Injection of 0.01-1 mg TBT (tributyltin)/kg bw of channel catfish altered leucocyte counts, NCC, phagocytic and respiratory burst activities, production of specifc antibodies and number of antibody-produceing cells (Rice et al., 1995). TBT treatment signinficantly reduced the lymphocyte numbers in spleen, the thymus volume and the leucocyte NCC activity in European flounder (*P. flesus*) (Grinwis et al., 2000). In rainbow trout, *in vitro* incubation with 2.5-500 ppb TBT and DBT (dibutyltin) reduced the lymphoproliferation activity in pronephros and spleen but failed to affect the NCC activity showing DBT higher toxicity than TBT (O'Halloran et al., 1998). *In vitro* incubation of several Australian fish head-kidney leucocytes with TBT or DBT depressed the phagocytic activity and reduced the numbers of lymphocytes and granulocytes (Harford et al., 2005).

3.7 Other chemicals

Herbicides are still widely used and end in aquatic environments producing many physiological alterations but little studies have focused on their immunotoxicological effects in fish. Herbicides mixture, containing atrazine, simazine, diuron and isoproturon, exposition of goldfish increased spleen and head-kidney ROS production and serum lysozyme but reduced the specific antibodies and resistance to bacterial infections (Fatima et al., 2007). Atrazine exposure of silver catfish resulted in decreased phagocytosis and

resistance to disease (Kreutz et al., 2010) whilst failed to do so in common carp (Cossarini-Dunier et al., 1987; Cossarini-Dunier & Hattenberger, 1988). Phenols are another group of toxics. Phenol, pyrocatechol and hydroquinone decreased the cell-mediated cytotoxic activity of spleen lymphocytes in common carp (Taysse et al., 1995), pentachlorophenol reduced macrophage production of cytokines in goldfish (Chen et al., 2005) but activated phagocytosis and unaltered other immune functions and disease resistance in rainbow trout (Shelley et al., 2009). Endocrine disrupting chemicals produce population decline, an increasing incidence of cancer, inhibition of reproductive function, and developing disruption of the immune and nervous systems. However, there are very limited data concerning the role of endocrine disrupting chemicals on aquatic organism, including the fish immune response. Zebrafish embryos exposed for 3 days to 17α-ethynyestradiol, permethrin, atrazine and nonylphenol (0.1-12.5 μg/L) altered the expression of immune-relevant genes (TNFα, IFN, IL-1β, IL-8, CXCL-Clc, CC-chemokines, iNOS, etc.) indicating their single and combined effects upon fish immune response (Jin et al., 2010).

4. Conclusion

As described above, most of the aquatic contaminants have shown either activations or suppressions in the immune response that greatly varied with the exposure route, time, dosage and fish specie with many similarities to immunotoxicological data in mammals. Therefore, although researchers do not have precise contamination biomarkers in aquatic animals some conclusions may rise: i) heavy metals contamination is usually followed by metallothionein overexpression (Misra et al., 1989; Hansen et al., 2007; Costa et al., 2009); ii) OCs exposure is concomitant to decreased number and size of melano-macrophage centres (Schmitt et al., 2005; Hinck et al., 2007); iii) immunotoxicological effects due to PHAs and PCBs are generally parallel to an increase in the activity of the detoxification proteins cytochrome P4501A (CYP1A), through the involvement of aryl hydrocarbon receptors (AhR), and/or EROD (ethoxyresorufin-O-deethylase) (Lee & Anderson, 2005; Duffy & Zelikoff, 2006; Reynaud & Deschaux, 2006; Bravo et al., 2011); and iv) further and deeper studies are needed to understand the real effect of environmental contaminants in fish and the mechanisms for toxicity. Moreover, looking at the fish species studied and those subjected to aquaculture, most of the data come from wild fish, salmonids and cyprinids but other major species are almost ignored. Even further, most of the studies focus on freshwater fish and very little is known for marine species. These aspects should be covered by future works to progress in the understanding of the immunotoxicological effects and mechanisms and the consequences and risks they may have on human consumers as consequence of the bioaccumulation.

5. Acknowledgments

This work has been funded by national (IDI-20091041, AGL2008-05119-C02-01 and AGL2010-20801-C02-02) and regional projects (04538/GERM/06). A. Cuesta thanks to the *Ministerio de Ciencia e Innovación* for the *Ramón y Cajal* contract.

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Using Zooplankton, *Moina Micrura* Kurz to Evaluate the Ecotoxicology of Pesticides Used in Paddy Fields of Thailand

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1. Introduction

Thailand is an agricultural country where agriculture is a very important part of the economy. Thailand expanded exports of agricultural products and also imports fertilizers and pesticides intensively. Pesticides are used widely in agriculture and trade of agricultural products to increase agricultural yield and to protect plant from diseases, weeds and insect damage (Department of Agricultural, 2010). Since pesticides were first imported into Thailand under the "Green Revolution Policy" as part of the 1st National Economic and Social Development Plan in 1966, the total amount of imported pesticides has dramatically increased year by year. Most pesticides used in the country are imported (Department of Pollution Control, 2005), and the quantities of imported agricultural pesticides have increased 3 times from 1994 to 2005, reaching more than 80 thousand tonnes in 2004. Pesticides are applied in the highest quantity in vegetable and fruit farming, where market pressure for appearance is higher. In 2000, organophosphates contributed the majority of imported pesticides followed by carbonates and organochlorines; most were herbicides, followed by insecticides, disease control agents and plant growth regulators (Department of Pollution Control, 2002).

The result from increasing pesticides uses has resulted in significant increased crop contamination and human health hazard (Office of Epidemiological, 2009). The risk of pesticide contamination in fruits and vegetables in Thai market often occurs.

Rice is the major crop and food source for most Asian countries including Thailand. Rice production from paddy fields faces variety of pests that require a range of pesticides and herbicides to manage the presence of insects and weeds, as well as fungal and bacterial pathogens. Indeed, losses of the total world rice crop due to insects have been estimated to occur at a rate of 28% per annum, which is four times greater than the average for other world cereal crops. More than 90% of the global end-user market in pesticides for rice production is applied in Asia (Abdullah, 1995). In Thailand, pesticides play an important part and widely use on rice production because its benefits in pest control and increased rice production. Therefore, pesticide contamination in draining water from paddy field has been one of non-point source pollution in aquatic ecosystem (Sanchez et al., 2006).

This is attributed to be relatively large amounts pesticides applied in paddy field, in addition to common practice of draining the paddy water in draining canals (Tejada, 1995). Around 95 % of freshwater in Thailand is withdrawn to irrigate the more than 5 million hectares of irrigated agriculture. Waste water from this activity may pose significant environmental hazards for aquatic ecosystem in particularly aquatic biota. Furthermore, this contaminate affect wildlife species ether by direct exposure or through bioaccumulation in food web.

Pesticide contamination sites associated with paddy field activities may pose significant environmental hazards for terrestrial and aquatic ecosystems. They are important sources of agro-sourced pollution and may result in ecotoxicological effects, particularly following transfer of irrigation waters following use. Ecotoxicological effects occur at all trophic levels, from the molecular to the ecosystem level and effects may be observed via biomonitoring with both individual organisms and the ecosystem function and structure.

Pesticide monitoring is traditionally based on evaluations of individual pesticides identified through chemical analyses. A variety of techniques may permit an examination of actual pesticides, herbicides and their metabolites that are present (Iwai et al., 2007). These techniques are based on sampling approaches that use concentration following collection or during collection. Although these techniques still are not able to show the direct response that ecotoxicity gives, they do give an indication of what is inducing the response of the organism. However, chemical analyses obviously do not reveal complex interaction phenomena and polar degradation products are often missed. In contrast to the use of chemical analyses, the ecotoxicity bioassay approach integrates the biological effects of all compounds present and factors such as bioavailability, synergism, or antagonism are reflected directly in the bioassay results.

Ecotoxicological assessment of pesticides in paddy field are therefore expected to give a more comprehensive indication of environmental effects. The use of ecotoxicological assessment to evaluate the impact of pesticide residues in the paddy field is strongly recommended in order to have a more direct and integrated estimate of environmental impact. In fact, biological response to a complex mixture of chemicals integrates different factors such as pH and solubility, antagonism or synergism, and the bioavailability of substances.

Pesticides contamination associated with paddy field has been increased a big concern in Thailand. For risk assessment study on the impact of pesticides on aquatic environments that surrounding area, information about effect of pesticides on local species were limited, especially the ecotoxicological data on aquatic organism in Thailand, and it unknown, whether ecological effects test guideline developed elsewhere in the world (US. EPA, ATSM etc) may be use in Thailand. Countries located in the tropical zone rely, mostly, on data from temperate countries about ecotoxicity data. However, this data may be not suitable for tropical countries. Due to the difference organisms species, temperature, rainfall, and agriculture practices that might greatly influence pesticides behavior (Abdullah et al., 1997) and toxicity of pesticides on organisms. Considering the climate adaption of tropical species, assessment of effects of pesticide use on local ecosystem should be performed with local species since their sensitive to toxicants may differ considerably form temperate organism (Domingues et al., 2007). Differential response of organism representing diverse physiological capabilities and niches in aquatic system can help focus field studies where nontarget effect due to off - site movement of pesticides are suspected.

Therefore, Thailand need ecological effects test guideline, this guideline typically derived data on toxicological response of local organism to environmental contaminant. The toxicity test is procedure that involves the exposure of organism to complex environmental sample under controled condition to determine if adverse effects have occurred (Edmondson, 1959).

The objective of this study selected the fresh water cladoceran *Moina micrura* Kurz order *Cladocera*, family *Moinidea*. In Thailand, this zooplankton is very common in pond, muddy pool and paddy field and it can be mass culture by some local fish farmer as a high quality fish food. *M. micrura* is an ideal animal for ecological relevance, wide occurrence, short life cycle, genetic uniformity, relative ease of culture in the laboratory and more sensitive to toxicants (Wang, 1994; Wongrat, 2001). The present study was determine the acute and chronic toxicity of pesticides on *M. micrura*. The result would be useful as an input to developing a biomonitoring tool and using local species test for evaluation pesticide contamination in Thailand aquatic ecosystem.

2. Materials and methods

2.1 Test organism culture

The Moina micrura obtained from Fisheries Research Institute, Khon Khaen (Khon Khaen, Thailand) and have been maintained in cultured under control laboratory conditions in Ecotoxicology and Environmental Sciences Laboratory, Faculty of Agriculture, Khon Kaen University, Thailand. The culture was incubated at 25± 2 ^{0}C with 16:8 h light:dark photoperiod. *M. Micrura* were cultured using moderately hardwater and fed on single-celled green alga, *Chlorella vulagaris* from axenic culture. The medium, used for zooplankton, as well as for experiments, was tap water at the Faculty of Agriculture, Khon Kaen University, Thailand. Water was filtered by using 0.45µm polymembrane filter. Dissolved oxygen concentration was between 5-7 mg/L and pH was 7-8. The culturing period for one generation was 2 weeks before testing.

2.2 Test chemicals

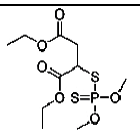
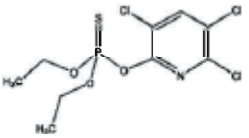
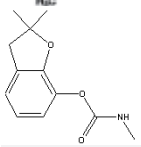
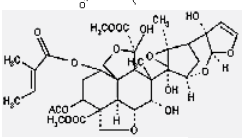
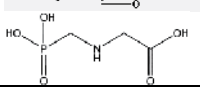
The pesticides test were selected from the common pesticides used in paddy filed of Thailand. Five selected pesticides were malathion 58% w/v (CAS: 121-75-5), chlorpyrifos 40% w/v (CAS:2921-88-2), carbofuran 3 % GR (CAS:1563-66-2), neem extract 40% w/v (CAS:1141-17-6) and glyphosate 36% w/v (CAS:1071-83-6) (Table 1.). Stock solutions were prepared by dissolving the pesticide directly in distill water immediately before to each experiment. Stock solution were added to each of three replication test beakers (50 ml total volume) to obtain nominal exposure concentration. Rang in nominal aqueous exposure concentration of chlorpyrifos,malathaion, glyphosate, carbofuran and neem extract on *M. micrura*, were arranged in geometic series between 0.5 -0.0005, 1-50, 500-2500, 3-15 and 50-250µg/L respectively.

2.3 Experiment design

2.3.1 Acute toxicity test

Preliminary acute toxicity tests were conducted in order to calculate malathion, chlorpyrifos, carbofuran, neem extract and gyphosate LC_{50} data. All experiments were performed according to the US.EPA document OPPTS 850.1010 (1996) for determining 48 h LC_{50} values for *M. micrura*. Three replication of 10 neonates (<24 h) per treatment and control laboratory well – wate were used. The neonates were exposed in a 150 ml glass beaker containing 50 ml for each test concentration and control were static bioassay under laboratory. Test organisms were not fed during the testing period. Observation motality was made at 24 and

48 h, and results recorded. For water quality, temperature, pH, conductivity and dissolved oxygen were measured according to APHA (1992).

Pesticide	Group of pesticide	Chemical name and number (Chemical Abstract Service)	Structure
Malathion	Insecticide (organophosphate)	O,O-dimethyl phosphorodithioate of diethyl mercapto- succinate ; CAS no. 121-75-5	
Chlorpyrifos	Insecticide (organophosphate)	Phosphorodithioic acid, O,O-diethyl O- (3,5,6-trichloro-2-pyridyl) ester; CAS no:2921-88-2	
Carbofuran	Insecticide (carbamate)	3,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate CAS no. 1563-66-2	
Neem extract	Insecticide (biopesticide)	Azadirachin; CAS no. 1141-17-6	
Glyphosate	Herbicide (amine)	N - (Phosphonomethyl) glycine; CAS no. 1071-83-6	

Source: Extoxnet (1996); Chemical Book (2007); Compendium of Pesticide common name (2008 a, 2008 b, 2008 c)

Table 1. Chemical formulation of pesticides tested with *M. micrura* .

2.3.2 Chronic toxicity test

Chronic toxicity of pesticides to *M. micrura* followed the procedure recommend by US.EPA document 6004-91/002 (1994). Based on acute toxicity result, *M. micrura* were exposure to control and concentration test malathion concentration of 0.05, 0.25 and 0.50 µg/L, chlorpyrifos concentration of 0.00005, 0.00025 and 0.00045 µg/L, carbofuran concentration of 0.25,1.00 and 2.50 µg/L, neem extract concentration of 15, 40 and 65 µg/L and glyphosate concentration of 50, 250 and 325 µg/L. In the chronic tests, three replication of 10 neonates <(24 h)per treatment and control laboratory well – water were used. The neonates were exposed in a 50 ml glass beaker containing 30 ml for each test concentration and control. Test organism were fed with a concentrated suspension of the green algae, *Chlorella* sp. Test solution and food were renewed completely every day. The measurement of water quality at the beginning and end of the test on control and treatments. The number of offspring was noted each day used to evaluate the effect of pesticide on reproduction of test organism.

2.4 Statistic analysis

The values of lethal concentration 24 and 48 h LC_{50} and 95 % confidence limit were caculated by appropriate statistical method intervals by probit analysis.Data from chronic

test were analyzed using ANOVA with SPSS version 12 statistical software to detected variation significances ($P<0.05$) between treatment group and control.

3. Results and discussion

Acute toxicity

Table 2. show the estimated 48-h LC_{50} for pesticides, with were calculated from standard toxicity test with *M. micrura*.

Malathion.: Our 48-h *M. Micrura* LC50 of 10.44 µg/L was compareable to the 48-h LC_{50} to other Cladocera species. This results shown 48-h- LC_{50} *M. Micrura* were nealy repoted the 5-10 µg/L for *M. marcocopa* by Wang et al. (1994) and the 8 and 13 µg/L that reported by Khan et al. (1993) and Siefirt (1987) for *D. Magna.* Differnce in LC_{50} value were observed wih *Cerodahnia dubia* have been reported between 1.14 -3.35 µg/L(Hernadez et al.,2004; Maul et al., 2006; Nelson et al.,1997,1998; Ankley et al., 1991).

Chlorpyrifos: Of the five pesticides tested in this study, chlorpyrifos was the most toxic to *M. Micrura*. The 48-h LC_{50} value for *M. micrura* was 0.08 µg/L. Other values in literature were higher between 0.13-3.7 µg/L using *D. ambigue, D. Magnaand and D. Duplex* (Caceres et al.,2007; Van Wijngaarden et al., 1993; Barata et al.,2004; Kersting et al.,1997; Van der Hoeven and Gerrisen, 1997).

Carbofuran: The 48-h LC_{50} for *M. micrura* obtain in this study 6.96 µg/L is compareable to the 48-h LC50Of 2.69 µg/L obtain with *C. Dubia* (Nerberg et al.,1997) for carbofuran in *D. magna* were higher than concentration tested (6.96 µg/L). In comparison, Poirer (1990); DBR (2000) and Dopsikova (2003) found an acute 48-h LC_{50} were 86.1, 38.6 and 18.7 µg/L respectively.

Neem extract: The present study found that the 48-h LC_{50} for neem extract in *M. micrura* was 196.3 µg/L. The acute toxicity data for *D.magna* with 48-h LC_{50} were 570- 1,250 µg/L (John , 2001; Stark ,2001; Scott and Kaushik, 2001), and were <6000 – 380,000 µg/L for *D. Duplex* (Goktepe and Plhak, 2002,2003)**.**

Glyphosate: Glyphosate was the lowest toxic (LC50 was 3042 µg/L) to *M. micrura*. Other values in reports were higher between 1150 – 107,000 µg/L and 30000 µg/L for *C. Dubia and D. Magnaa,* respectively. The LC50 value of pesticides showed that toxicity of chlorpyrifos > carbofuran > malathion > neem extract > glyphosate. *M micrura* were susceptible to pesticides from µg/L to mg/L, with chlorpyrifos was the most toxic (LC50 = 0.08 µg/L) and glyphosate was the lowest toxic (LC50 = 3042 µg/L) to *M. micrura*.

Pesticides	48 h-L50 (µg/L)
Malathion	10.44 (9.10 -11.85)
Chlorpyrifos	0.08 (0.03 - 0.20)
Carbofuran	6.96 (5.97 – 7.63)
Neem extract	196.3 (161.5 -263.9)
Glyphosate	3043 (1974 – 1778)

Table 2. Acute toxicity (Medium lethal concentration [LC_{50}]) of pesticides on *M. micrura* at 48 h.

In this studied were founded that toxicity of the insecticide group (chlorpyrifos, carbofuran, malathion and neem extract) were more toxic to *M. micrura* than the herbicide group (glyphosate), because insecticide had mode of action that affect on organism directly but herbicide acted in indirect way. US. EPA (1998) reported chlorpyrifos had very high toxicity to freshwater fish and aquatic invertebrates, carbofuran and neem extract had higher toxicity but glyphosate had less toxicity on zooplankton (Henry et al.,1994; ENTOXNET, 1996; PMRA, 2002; Dopsikova, 2003; Saglam and Saler, 2005). On the basis LC_{50} value, *M. micrura* of this study were sensitive to pesticides nearly Ceriodapnia species but were more sensitive to pesticides than Dahpnia species indicated by other studies in Table 3. Due to Daphnia species were bigger than Moina species and Ceriodapnia species thus its tolerant than M. Micrura and Ceriodapnia species. The result were found similar to Scott and Kaushik (1998); Liane (2002) and Grant and Schmutter (1987) reported were size, age, species, life-cycle of zooplankton and environment such as temparature, pH and harness have influent to chemical toxicity on zooplankton.

The observation of *M micrura*, after treated with pesticides especially in high concentration, the swimming activity of *M. micrura* was changed. They moved faster then normal conditions, after a time later, the movement on antenna and limbs become slowly and death after that. Concentration of pesticides had disrupt respiratory membrane of *M. micrura*, their swimming behavior changes in high concentration and *M. micrura* were loosed their original colored. The similar results were reported in Rassolzadegan (2000); Saglam and Saler (2005)John et al. (2007)

Chronic toxicity

Effect of sublethal pesticides concentration on the number of offspring per female of *M. micrura* is shown in (Table 4). Number of offspring per female of *M. micrura* was significant reduced ($P<0.05$) at malathion concentration 0.50 µg/L, chlorpyrifos concentation greater than 0.00025 µg/L, at carbofuran concentration at 2.50 µg/L and at glyphasate concentration 325 µg/L. For neem extract concentration had no effect on the number of offspring per female significantly ($P>0.05$). Sublethal effects for each pesticide, were founded similar to other reports (Wong et al, 1995; Alberdi et al,1996; US EPA 2006). An estimate of no observed effect concentration (NOEC) and lowest observed concentration (LOEC) were 0.25 and 0.50 µg/L for malathion, 0.00005 and 0.00025 µg/L for chlorpyrifos, 1.00 and 2.50 µg/L for carbofuran, 250 and 325 for glyphosate and LOEC 65 µg/L for neem extract. Cladocerans contribute an important component of aquatic ecosystem especially, for fish food source. If the number of clardocerans were down, it may affect fish and another organisms.

The number of offspring per female is one endpoint used to determine the maximum acceptable - toxicant concentration (MATC). The 16 % reproduction impairment have been used as the endpoint for many aquatic ecotoxicology (Biesinger and Chistensen, 1972). Therefore, this studies used 16 % reproduction impairment estimate the chronic values MATCs for pesticides (Table 4). According to the obtained results the calculated values of MATCs and 48-h LC_{50} were for estimate application factor (AF) of pesticides on *M. micrura* (Table 5).

This value was used to predict the safe concentration (SC)applies for pollutant prevention in aquatic ecosystem. However, the application factor will vary with type of pesticide and organism (Mounth and Stephan, 1967).

Pesticide	Species	48-h LC_{50}	References
Malathion	*M. micrura*	10.44	This studies
	M. marcocapa	5-10	Wang et al. (1994)
	C. dubia	3.18	Hernadez et al. (2004)
		3.35	Maul et al. (2006)
		1.14	Nelson et al. (1997, 1998)
		2.12	Ankley et al. (1991)
	D. magna	0.90	Ren et al. (2007)
		8.0	Khan et al. (1993)
		13	Siefirt. 1987)
Clorpyrifos	*M. micrura*	0.08	This studies
	C. dubia	0.117	Bailey. (1997)
		0.056	Harmon et al. (2003)
	D. ambigue	0.050	El- Merhibi et al. (2004)
		0.035	Harmon et al. (2003)
	D. magna	0.30 - 0.80	Caceres et al. (2007)
		1.28	Van Wijngaarden et al. (1993)
	D. duplex	1.0 - 3.7	Barata et al. (2004)
		0.13	Kersting et al. (1997)
		> 1.6	Van der Hoeven and Gerrisen. (1997)
		0.17-0.49	Hooftmant et al. (1993)
Carbofuran	*M. micrura*	6.96	This studies
	C. dubia	2.69	Nerberg et al. (1997)
		> 20	Poirer (1990)
		> 162	DBR (2000)
	D. magna	86.1	Dopsikova (2003)
		38.6	
		18.7	
Neem extract	*M. micrura*	196.3	This studies
	D. magna	1250	John. (2001)
	D. duplex	570 - 680	Stark. (2001)
		570	Scott and Kaushik. (2001)
		<6000 - 243000	Goktepe and Plhak. (2002)
		30000 - 380000	Goktepe and Plhak. (2003)
Glyphosate	*M. micrura*	3043	This studies
	C. dubia	1150	Hensen et al. (1994)
		5890 - 107000	Tsui et al. (2004)
	D. spinulata	30000	Lutufu et al. (2001)
	D. magna	20000 - 21880	Al –Omar et al. (2000)
	D. duplex	218000	Henry et al. (1994)
		7900	Office of pesticide program. (2000)

Table 3. Comparison of 48-h LC_{50} (µg/L) Value of *Moina micrura* and another clardocerans species.

Aquatic ecosystems in tropical regions differ from those in temperate regions. The biodiversity in tropical zones is higher than that in temperate zones, which means that in tropic regions there are potentially more species that can be exposed to certain pollutants. However, many countries in the tropics are developing countries, in which pollution control

is not carried out due to a lack of funds and other resources. Furthermore environmental quality criteria for some pollutants are often obtained by extrapolating toxicity data derived for a reduced number of species mainly distributed in temperate regions (e.g. Europe or the US) (Kim *et al.*, 2001 in Kwok *et al.,* 2007). Kwok *et al.* (2007) investigated to which extent the sensitivity distributions of temperate species to toxic substances were similar to those of tropical species. They found that the temperate species seemed to be more sensitive to metals than the tropical species (Kwok *et al.,* 2007). However, it should be noted that these differences might be due to the different species composition included in the species sensitivity distributions (SSD). Kwok *et* al (2007) used mainly fish species, which could be less sensitive to pollutants than the invertebrate species that are predominantly used in the temperate species sensitivity distributions. A better comparison can be made when using similar taxonomic groups for the distribution.

In Thailand ecotoxicological research is quite new and has many limitations. Although ecotoxicological issues arise in this country and there is a need for water quality management and ecological risk assessment tools, there is a lack of ecotoxicological data on aquatic organisms from Thailand. Until now, like other developing countries, they have relied on over sea data to develop ecotoxicological test guidelines. However, these guidelines may be unsuitable for Thailand. The Thai indigenous aquatic organisms might be more or less sensitive to contaminants than their temperate surrogate species (Iwai, 2004; Iwai and Noller, 2010; Somparn et al., 2010). Moreover, there are differences in physicochemical and biological characteristics of aquatic habitats between tropical and temperate regions (Kwok et al., 2007). The characteristic of the sediment and water in Thai rivers may differ from those in other countries (Iwai and Noller, 2010; Somparn et al., 2010), influencing the concentration, availability and accumulation of pollutants and therefore their toxicity. An example of this is given by Jeon et al. (2010). They found that clay and food content in the water influence the toxicity of pollutants on aquatic biota.

Tirado et al. (2008) report that the main rivers in Thailand were monitored from 1993 to 1999 for the presence of pesticide residues; most water samples contained insecticide and herbicide residues in levels above advisable limits, whereas less contamination was observed in sediment samples. In river water, organochlorine pesticides were detected in 40.62% of the samples (in concentration ranging from 0.01 to 1.21 μg/L), organophosphate pesticides were detected in 20.62% of samples (in concentration ranging from 0.01 to 5.74 μg/L). The safety limit established by the European Union is 0.1 μg/L for any single pesticide and 0.5 μg/l for the sum of all pesticides detected. Both organochlorine and organophosphate pesticide residues were found above those safety limits. Additional compounds, like carbamate pesticides were detected in 12.39% of samples (in concentration ranging from 0.01 to 13.67 μg/l), triazines were detected in 20.0% of samples (in concentration ranging from 0.01 to 6.63 μg/L), and paraquat was detected in 21.36% of samples (in concentration ranging from 0.14 to 87.0 μg/L) (Chulintorn et al., 2002). An earlier study has also found residues of the pesticides DDT and dieldrin in five Thai rivers (Upper Ping, Lower Ping, Wang, Yom, Nan, Chee), in concentrations above acceptable standard levels (Sombatsiri, 1997). The Division of Agricultural Toxic Substances in the Department of Agriculture (Ministry of Agriculture and Cooperatives) has also monitored the presence of pesticide residues in rivers and canals around agricultural areas in the country. The contamination of pesticides in water and sediments was generally low in water resources used for domestic consumption like ponds and reservoirs that have no connection to agricultural plantations. However, the water resources in certain agricultural areas, like orchid and ornamental plantations, were contaminated with organophosphate and

carbamate insecticides. From 1999 to 2001, a survey of three major rivers along paddy field areas (Thachin river in Suphanburi and Nakornpathom, the Chao Phraya river in Pathumthani and Nonthaburi, and the Bangpakong river in Chachengsao), found the highest residues of the insecticide endosulfan in the Thachin River, followed by the Chao Phraya and Bangpakong Rivers. In all cases, the levels of pesticide residues were above the safety limit set by the European Union (0.1 µg/L) (Chatsantiprapha, et. al., 2002).
In 2001, groundwater in the lower Central and the lower Northeastern region of Thailand was contaminated with pesticides residues, in many cases in concentration above the safety limit set by the EU (0.1 µg/l). In the lower Central region during the rainy season in 2001, 68% of 15 GRL-TN-03-2008 the total groundwater samples were contaminated with endosulfan and other insecticides, in concentration ranging from 0.02 to 3.2 µg/l, and paraquat, 2,4-D, butachlor, atrazine and metribuzin herbicide residues ranging from 0.02 to 18.9 µg/l. In lower Northeastern region during the dry season in 2001, 71.2% of the total groundwater samples were contaminated with endosulfan and other insecticides, in concentrations from 0.01 to 0.33 µg/l, and atrazine and paraquat herbicide residues at the level of 0.5-4.0 µg/l (Sakultiangtrong, et.al., 2002). In 1993, the Department of Agriculture investigated shallow groundwater wells from Rayong Province. From 160 samples collected from wells, 67% were contaminated with organochlorine and organophosphate pesticides, but in concentration below the safety limits (Pollution Control Department, 2004).

Pesticide	Concentration (µg/L)	Number of offspring per female	% Reproductive impairment
Malathion	0.00	55.13±0.45a	0.00
	0.05	53.13±0.50a	3.68
	0.25	49.03±0.70a	11.06
	0.50	36.50±0.46b	33.79
Chlorpyrifos	0.00	53.37±0.35a	0.00
	0.00005	53.37±0.35a	6.741
	0.00025	49.77±0.41b	9.43
	0.00045	43.00±0.52c	28.23
Carbofuran	0.00	53.37±0.35a	0.00
	0.00005	53.37±0.35a	6.74
	0.00025	49.77±0.41b	19.43
	0.00045	43.00±0.52c	28.23
Neem extrat	0.00	56.33±3.15a	0.00
	15.00	55.10±2.12a	2.18
	40.00	53.76±1.72a	4.56
	65.00	52.33±1.99a	7.10
Glyphosate	0.00	56.06±1.62a	0.00
	50.00	55.17±0.95a	1.59
	250.00	47.66±2.12a	14.19
	325.00	42.43±3.74b	24.31

*Note:Value are maen + standard deviation. Mean with the same letter in the column are not significantly different (P>0.05).

Table 4. Chronic toxicity of malathion, chlorpyrifos, carbofuran, neem extract and gyphosate on the number of offspring per female and % reproductive impairment of M. micrura.

Pesticides	48 h-LC_{50} (μg/L)	MATC	AF
Malathion	10.44	0.36	0.03
Chlorpyrifos	0.08	0.0001	0.001
Carbofuran	6.96	2.41	0.35
Neem extract	196.3	281.89	0.09
Glyphosate	3043	172.04	0.88

Table 5. The maximum acceptable - toxicant concentration (MATC) and Application factor (AF) for each pesticide.

4. Conclusion

The aim of this study was using zooplankton, *Moina micrura* Kurz. which is an important species in aquatic ecosystem of Thailand to evaluated ecotoxicity of main pesticide used in paddy field (malathion, chlorpyrifos, carbofuran, neem extract (azadirachtin) and glyphosate). The acute toxicity (48-h LC_{50}) of malathion, chlorpyrifos, carbofuran, neem extract and glyphosate on *M. micrura* were 10.44, 0.08, 6.96, 196.3 and 3043 μg/L, respectively. Chlorpyrifos had highest toxicity followed by carbofuran, malathion, neem extract and glyphosate, respectively. Chronic toxicity test, the effect of pesticides to *M. micrura* on reproduction was studies by observing the number of offspring per female. Reproduction have significant reduced ($P<0.05$), with concentration of malathion at 0.50 μg/L, chlorpyrifos greater than 0.0025 μg/L, carbofuran at 2.50 μg/L and the concentration of glyphosate at 325 μg/L affected on reducing the number of offspring per female significantly ($P<0.05$). The neem extract had no significantly ($P>0.05$) effect on the number of offspring per female. The maximum acceptable - toxicant concentration (MATCs) of malathion, chlorpyrifos, carbofuran, neem extract and glyphosate were 0.36, 0.0001, 2.41, 172 and 281.9 μg/L, respectively. The result would be useful as an input to developing a biomonitoring tool for evaluation pesticide contamination in Thailand aquatic ecosystem.

Effect of experimental condition including duration test organism and end point on observed toxicity of pesticide to *M. micrura* were evaluated. Relative sensitivities of test varies with pesticide type. Among five pesticides toxicity test, chlorpyrifos had highest acute toxicity on *M. micrura* followed by carbofuran, malathion, neem extract and gyphosate, respectively. The significant reducing effect on number of offspring per female of *M. micrura* were observed in the present of malathion, chlorpyrifos carbofuran and glyphosate. For neem extract had no effect on the number of offspring per female. The results indicate that reproductivity parameters are very important interm of pesticide impact on aqutic population such as *M. micrura*. However, in the natural environment aquatic organism are often exposure to multiple pesticides simultaneously. Therefore under natural condition, there is the potential of pesticides may act in additive or synergistic manner, although the sensitivity of aquatic biota to multiple pesticides cannot be predicted by the individual pesticide sensitivities generate in this study.

The results showed *M. micrura* to be sensitive test organism, Thus its a good bioindicator and useful to developing a biomonitoring tool for evaluation pesticide contamination in Thailand aquatic ecosystem. However, in order to obtain more precise and conclusive toxicology data on application of these pesticide in paddy field and evaluation toxicity of pesticides on organism, similar study using another local freshwater in Thailand.

5. Acknowledgment

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission. The authors wish to express their sincere thanks to Khon Kaen University for the research funding, Groundwater research centre (GWRC) and Graduate School Khon Kaen University.

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15

Application of Some Herbal Extracts and Calcium as an Antidote to Counteract the Toxic Effects of Cypermethrin and Carbofuran in Indian Major Carp, Labeo Rohita

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1. Introduction

Pesticides that are transported to the aquatic environment are primarily of agricultural origin. Sometimes, pesticides are applied to the fish ponds to control fish diseases. In the process, the residues that reach the hydrosphere are concentrated in certain parts of the aquatic ecosystem or remain in solution for extended periods or adsorbed to the particulate matter and thereby deposited in the sediments. Thus, pesticides could be accumulated in the body of the aquatic animals. Most of the pesticides act on the respiratory process and cholinergic nervous system and hamper the cell metabolism in addition to other disturbances. Thus, a formulation of antidotes to counteract pesticides is an important aspect of pollution research and work in this direction is in the initial stages. Zamfir (1979) worked on the possibilities of removal of pesticide polluted water in treatment stations and described some methodologies, i.e., flocculation and filtration that can partially removed DOT, 2,4,5-T, Endrin, Parathion and Lindane. Chlorine oxidation can remove parathion; diuron etc., ozone and potassium permanganate appear to extract effects similar to those of chlorination. Activated charcoal has positive effects in the removal of absorption of most pesticides and U-V rays also can remove a certain amount of pesticides.

Some indirect approaches have also been employed by some scientists and their methods were environmental or nutritional manipulation. Sado et. al. (1992) reported that increased temperature and optimum levels of dissolved oxygen (by aerator) can decrease the pesticidal action. The application of lime to increase the pH for counteraction of the toxic effects of pesticides is also documented. Ghazaly (1994) and Mukherjee (1996) evaluated efficacy of ascorbic acid (vitamin C) for the intoxication of different pollutants including pesticides. Application of different herbal extracts for this purpose could play a very important role to mitigate the toxic effect of pesticides.

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2. Aim of the study

To investigate some herbal extracts and water calcium as antidotes to counteract the pesticidal effects on Indian Major Carp, *Labeo rohita* (rohu).

3. Materials and methods

Three herbal extracts and calcium as possible antidotes were tried for their efficacy to counteract Cypermethrin,a synthetic pyrethroid (trade name-Cypermethrin, chemical name-Cyano methyl,2,2-dimethyl,cyclopropane-carboxylate) and Carbofuran, a carbamate pesticide (trade name-Furadan 3G, chemical name- 2,3 dihydro,2-2 dimethyl,7 benzo furymethyl carbamate) toxicosis in fish. Antidotes study was conducted in 2 steps (1) Preparation of Crude extracts and (2) Experimental trials.

Datura (*Datura ripens*), Kalka (*Nerium indicum*) and Neem (*Azadiricta indica*) were the plants selected for antidote study and fruits of datura and kalka, and leaves of neem were the specific materials. These plant materials were minced, then grounded and finally extracted using acetone (50%) solution. The ratio of plant material and acetone solution was maintained at 1:10. The paste was collected in a fine meshed cloth and the filtrate was collected for the study. Everytime fresh extracts were prepared before trials. The doses of datura extract were 2.5 and 5.0 ml/l, for both the pesticides. The doses of kalka extract were 2.5 and 5.0 ml/l, for the Cypermethrin experiment while the same were 0.75 and 1.5 ml/l, for the Carbofuran experiment. The doses of neem extract were 10 and 25 ml/l for the Cypermethrin experiment while the same were 5.0 and 10 ml/l for the Carbofuran study.

For the application of calcium solution in water, calcium chloride was chosen as reagent. Fresh solutions were prepared before experiment and applied with different doses (50, 100, 200, 300 and 400 mg/l for both the pesticides under study) to the experiments.

Rohu fingerlings (2.25±0.16g) were used for the antidote study. The fishes were collected from the institute's pond and acclimatized at laboratory condition for 15 days after treating the fish with 0.1% potassium permanganate solution. Probable lethal doses for both cypermethrin and carbofuran were detected by error and trial method in 40 litre plastic tubs containing 20 litres of water. The antidote study was also performed for both the pesticides in the same type of plastic tubs with 20 L of freshwater. The water used for this study was 7.5 pH and 80 mg/l total alkalinity as $CaCO_3$. Four treatments, viz., control (only fish, no pesticides, no antidote), fishes treated with only pesticide (lethal dose), fishes treated with only antidote and fishes treated with pesticide (lethal dose) and antidote solution, were maintained. In every case, antidotes were applied half an hour after the pesticide treatment. Two different doses of each antidote were tried for each of the pesticides. Three replications were maintained for all the treatments. For each case, sampling was done after 1, 2, 4, 6, 12, 24, 48, 72 and 96 hours, respectively(if any mortality occurs), and observations were made and final data were calculated on the basis of comparison between the different experimental observations. The results were expressed as percentage of fish survivability.

For significance of antidote study, one way ANOVA (Duncan Multiple Range Test) were done (Zar, 1974). Test of significance was examined at 5% level.

4. Results and discussion

Among the four doses of calcium, both at 100 and 200 mg/l of water calcium levels, 67 percent of fish survived up to 96 hours compared to 100 percent fish mortality at 50,300 and 400 mg/l concentration of water calcium against the lethal concentration of cypermethrin.

There was no significant difference in fish survivability between 100 and 200 mg/l levels of water calcium (Table 1). Fifty percent of fish survivability after 96 hours of exposure with lethal concentration of carbofuran was recorded both at 100 and 200 mg/l levels of water calcium while 100 percent of fish mortality was obtained with 50,300 and 400 mg/l levels of calcium against lethal concentration of carbofuran up to 96 hours (Table 3).

Observation at different time intervals (hrs.)	Survivability percentage of control	Survivability percentage in Cypermethrin (37 ml a.i./ha/m)	Survivability percentage in cypermethrin and different doses of antidote calcium(mg/l)*				
			50	100	200	300	400
1	100	100	100a	100a	100a	100a	100a
2	100	100	100a	100a	100a	100a	100a
4	100	100	100a	100a	100a	100a	100a
6	100	25	50b	100a	100a	100a	100a
12	100	0	50b	100a	100a	50b	25b
24	100	0	25c	67b	67b	50b	25b
48	100	0	0	67b	67b	25c	0
72	100	0	0	67b	67b	0	0
96	100	0	0	67b	67b	0	0

*Values followed by the same superscript were not significantly different at the 0.05 level.

Table 1. Effect of water calcium on the toxicity of cypermethrin to *Labeo rohita* under laboratory conditions

The effects of neem, datura and kalka extracts as probable antidotes against the cypermethrin toxicity using fish survivability as indicator have shown in Table 2. Datura extract (10%w/v) at the levels of 2.5 and 5.0 ml/l exhibited 37 and 50% fish survivability after 96 hours of exposure with lethal concentration of cypermethrin. The fish survivability between these two levels of datura extract showed a significant ($P<0.05$) difference. Hundred percent of fish mortality recorded both at neem and kalka extract after 96 hours of exposure with lethal level of cypermethrin. It may be mentioned here that at neem and kalka extracts, the fish died within 12 hours.

Observation at different time intervals (hrs.)	Survivability percentage of control	Survivability percentage in cypermethrin (37 ml a.i./ha/m	Survivability percentage in cypermethrin and antidotes				
			Neem (10%w/v)@ 10 ml/1	Neem (10% w/v)@ 25 ml/1	Datura@ 2.5 ml/1	Datura@ 5.0 ml/1	Kalka@ 2.5 ml/1	Kalka@ 5 ml/1
1	100	100	100^a	100^a	100^a	100^a	100^a	100^a
2	100	100	100^a	100^a	100^a	100^a	100^a	100^a
4	100	100	100^a	100^a	100^a	100^a	100^a	100^a
6	100	25	100^a	100^a	100^a	100^a	100^a	0
12	100	0	0	12^b	62^b	75^b	12^b	0
24	100	0	0	0	37^c	62^b	0	0
48	100	0	0	0	37^c	50^c	0	0
72	100	0	0	0	37^c	50^c	0	0
96	100	0	0	0	37^c	50^c	0	0

*In control experiment of Kalka (i.e. only kalka), the fish died within 12 hours.
*Values followed by the superscript were not significantly different at the 0.05 level.

Table 2. Efficacy of some antidotes to cypermethrin on survivability (%) of Labeo rohita under laboratory conditions.

Observation at different time intervals (hrs.)	Survivability percentage of control	Survivability percentage in Carbofuran (15 mg a.i./l)	Survivability percentage in carbofuran and different doses of antidote calcium(mg/l)*				
			50	100	200	300	400
1	100	37	100[a]	100[a]	100[a]	100[a]	100[a]
2	100	37	100[a]	100[a]	100[a]	100[a]	100[a]
4	100	37	100[a]	100[a]	100[a]	100[a]	100[a]
6	100	25	50[b]	50[b]	50[b]	100[a]	100[a]
12	100	0	50[b]	50[b]	50[b]	50[b]	50[b]
24	100	0	25[c]	50[b]	50[b]	0	0
48	100	0	0	50[b]	50[b]	0	0
72	100	0	0	50[b]	50[b]	0	0
96	100	0	0	50[b]	50[b]	0	0

*Values followed by the same superscript were not significantly different at the 0.05 level.

Table 3. Effect of water calcium on the toxicity of carbofuran to *Labeo rohita* under laboratory conditions

The effects of datura, neem and kalka extracts as probable antidotes against carbofuran toxicity using fish survivability as indicator are presented in Table 4. Datura extract (10% w/v) at the concentrations of 2.5 and 5.0 ml/l showed 50 and 37 percent fish survivability after 96 hours of exposure with lethal concentration of carbofuran. The fish survivability at these two levels of datura extract exhibited a significant ($P<0.05$) difference. Hundred per cent of fish mortality was observed at all the concentrations of neem and kalka extracts with the exposure of lethal level of carbofuran. It is important to note that the fish died within an hour at neem extracts while at kalka extracts, the fish died within 4 hours against the lethal level of carbofuran.

The toxic effects of both cypermethrin and carbofuran decreased at medium level (100-200 mg/l) of calcium of water. The results indicate that the degradation of these insecticides take place at medium calcium level of water. The decreased toxicity at this level of calcium could be associated with accumulation of insecticides in excess amount which may be metabolized and stored in different tissues. The accumulated insecticides are eliminated through urine of faeces or both with the help of liver, intestine and kidney (Subbiah et al., 1985). Metz and

Observation at different time intervals (hrs.)	Survivability percentage of control	Survivability percentage in carbofuran (15 mg a.i./l)	Survivability percentage in carbofuran and antidotes					
			Neem (10% w/v)@ 5ml/l	Neem (10% w/v)@ 10ml/l	Datura@ 2.5ml/l	Datura@ 5.0ml/l	Kalka@ 0.75ml/l	Kalka@ 1.5ml/l
1	100	100	0	0	75^{a}	75^{a}	25^{a}	12^{a}
2	100	100	0	0	75^{a}	75^{a}	25^{a}	12^{a}
4	100	100	0	0	75^{a}	62^{a}	25^{a}	0
6	100	25	0	0	62^{a}	50^{b}	0	0
12	100	0	0	0	50^{b}	50^{b}	0	0
24	100	0	0	0	50^{b}	37^{c}	0	0
48	100	0	0	0	50^{b}	37^{c}	0	0
72	100	0	0	0	50^{b}	37^{c}	0	0
96	100	0	0	0	50^{b}	37^{c}	0	0

*Values followed by the superscript were not significantly different at the 0.05 level.

Table 4. Efficacy of some antidotes to carbofuran on survivability (%) of Labeo rohita under laboratory conditions.

Branacin (1975) reported that the degradation of endosulfan took place at high pH and hardness of water but in case of triazophos, its toxicity was increased in similar conditions

while Khalid (1985) reported that water hardness had no significant affect on endosulfan toxicity to four species of fish. Synthetic pyrethroids and natural pyrethrums were more toxic in hard water (Mauck et al., 1976).

From the present study, it is clear that both the neem and kalka extracts had no antitoxic effects against cypermethrin and carbofuran poisoning, while acetone extracts of datura served as anti-toxicant against cypermethrin and carbofuran poisoning. The medicinal and therapeutic roles of datura fruit and its seeds have already been established. The young fruits are sedative and slightly intoxicating. The seeds are antispasmodic, narcotic, febrifuge, anthelmintic, works well in inflammation, alexiteric, emetic and useful in leucoderma, ulcers, itching, etc. The seeds contain both hysocyamine and scopolamine (Kirtikar and Basu, 1935). These medicinal qualities have a positive role for the survivability of pesticide intoxicated fish but the specific causes behind it is unknown and its need further investigation. However, it may be due to narcotic and intoxicated properties of datura which may have a neutralizing action against pesticide contamination.

It can be mentioned here that vitamins are also having antitoxic effects against insecticide poisoning. For example, vitamins like Macraberin forte (a mixture of vitamins) served as antitoxicant against malathion poisoning to *C. punctatus* (Vaid and Mishra, 1977). When Channel catfish were exposed to various amounts of toxaphene (an organochlorine insecticide) in the diet for 150 days, a dose dependent depression of backbone collagen and spiral deformities were occurred but it recovered effectively when ascorbate (vitamin C) was added to the diet. Vitamin C supplements also reduced the whole body residues of toxaphene (Mayer et al., 1978).

5. Conclusion

From the present investigation, it is evident that though, pesticides induced changes of the fish could be improved/protected to a great extent by the application of acetone extract of datura and liming (calcium) up to a certain extent, further investigations are necessary in this regard. Therefore, judicious application of carbofuran in the paddy field to control rice pest and cypermethrin in the pond to control mosquito eggs and Argulus of fish are highly essential for sustainable growth of aquaculture.

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16

Semi Aquatic Top-Predators as Sentinels of Diversity and Dynamics of Pesticides in Aquatic Food Webs: The Case of Eurasian Otter (*Lutra lutra*) and Osprey (*Pandion haliaetus*) in Loire River Catchment, France

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1. Introduction

The Eurasian otter (*Lutra lutra, Lutrinae*, figure 1) and the osprey (*Pandion haliaetus, Pandionidae*, figure 2), formerly widespread in Europe and in France, have strongly declined during the 20th Century, following direct persecutions, habitat alteration and pollution, and consecutively decline of main prey. Direct persecutions were perpetrated because both species were considered as active competitors for fishing activity: otters were massively trapped for fur, and osprey populations were dismantled by direct shot or egg destruction in nests. Otter and osprey are both semi-aquatic top-predators species: diet is highly dominated by fish, which constitute at least 80 % and almost 98-100 % of the averaged prey biomass consumed by otter and osprey, respectively. However, diet studies of otter and osprey never showed any strong predation impact on fish diversity or biomass in rivers (reviews in Poole, 1989; Thibault et al. 2001; Clavero et al. 2003; Britton et al. 2005; Kruuk, 2006; Dennis, 2008). This diet specificity influence otter physiological characteristics: by comparison with other mammalian carnivores, a specific diversity and accumulation pattern of essential fatty acids of aquatic origin from food to otter tissues was recently shown (Koussoroplis et al. 2008). Another diet characteristic of both species is the diversity of prey and their opportunistic hunting behaviour: almost all fish species available in otter or osprey local habitat are able to be consumed, depending on hunting conditions, with important diet variations between seasons, during life cycle or between populations. This is particularly true concerning osprey, a migrating species present in northern and western Europe during reproductive season, and wintering from southwestern Europe to sub-Saharan Africa, but was also observed concerning sedentary otter. Because of their high trophic level, habitat requirements and main ecological characteristics, otter and osprey can be considered as good sentinels and indicator species of global contamination and biomagnification of toxic contaminants in aquatic food webs of large rivers, estuaries, reservoirs and lakes.

Fig. 1. Eurasian Otter (*Lutra lutra*, adult male, photo R. Rosoux).

Indeed, after direct destructions, contamination by persistent pollutants (*e.g.* pesticides, polychlorinated biphenyls (PCBs) or heavy metals) is blamed to be the causative agent of the decline of otter and osprey populations, throughout Europe as elsewhere in the world. On seldom occasions, *acute poisoning* after direct exposure or secondary poisoning by ingestion of highly contaminated prey were observed on otters in France, especially after oil spills, other industrial accidents or following heavy treatments with insecticides, avicides or rodenticides (Fournier-Chambrillon et al. 2004; Lafontaine et al. 2005; Berny, 2007; Lemarchand et al. 2010). Elsewhere in the world, acute poisoning of many marine otters (*Enhydra lutris*) were reported after Exxon Valdez oil spill in 1989 in Alaska (Garshelis and Jonhson, 2001). On the other side cases of acute poisoning were very rarely observed on ospreys. Potential long-term effects of trophic originating toxic compounds (*i.e. chronic poisoning*) on otters and ospreys were often investigated through reports of contamination or intoxication cases on monitored populations. Pesticides, and particularly organochlorine (OC) pesticides were the most commonly analyzed elements (but also heavy metals and PCBs, these latter generally associated with OC pesticides to assess the total OC contamination). Pesticides uses in the European Union are clearly specified by Directive EC 91/414, but illegal poisoning of wild, game and domestic animals still occurs, and often results from pesticides abuse or illegal use (Berny, 2007; Berny and Gaillet, 2008). Among OC pesticides, dichorodiphenyltrichloroethane (DDT) and its main metabolites, particularly dichlorodiphenyldichloroethylene (DDE), were shown to accumulate in otter and osprey, causing body condition alteration, direct reproductive failure (like eggshell thinning observed on osprey) and consecutively population decline (Spitzer et al. 1978; Wiemeyer et al. 1988; Mason and Macdonald 1993a, b; Ewins et al. 1999; Elliott et al. 2000; Ruiz-Olmo et al. 2000; Henny et al. 2008). As these deleterious effects were documented on a very large spectrum of wild and domestic species after insect pests control, DDT uses were severely controlled or banned from the 1970's in developed countries (ban from 1973 in France). Nevertheless, DDT is still used in developing countries, particularly in India or Africa by indoor spraying during anti-malaria campaigns. The risk of DDT flow into agricultural and

aquatic systems or wild environment of these countries was recently underlined (UNEP, 2008). Due to environmental stability and persistence of DDT and metabolites, and probably following post-ban use of old stocks, these compounds are still present in environment and were recently detected in otters or ospreys (Kannan et al. 2004; Lemarchand et al. 2007, 2010; Henny et al. 2008). For this latter species, continuing of DDT use in some developing countries is an additional threat during the wintering period. Lindane, and to a lesser extent, Aldrin, Endosulfan and Methoxychlor were the main other OC pesticides quantified in otter or osprey during previous studies. However, most of studies reported low concentrations of these compounds when compared to DDT residues, and therefore a limited contribution to total OC contamination (Wiemeyer et al. 1988; Mason and Macdonald, 1993a,b, 1994; Elliott et al. 2000; Rattner et al. 2004; Lemarchand et al. 2010).

Cholinesterase inhibitors as organophosphate (OP) and carbamate (CA) pesticides were widely used worldwide as insecticides for the protection of cultivated plants or livestock. Direct and indirect toxicity of cholinesterase inhibitors (*e.g.* Carbofuran, Mevinphos) were underlined on insect-consumer birds, birds of prey and scavengers like white-tailed sea eagle (*Haliaetus albicilla*) or red kite (*Milvus milvus*) (Hart et al. 1993; Elliot et al. 1996; Berny and Gaillet, 2008). These insecticides are highly toxic to birds of prey, nevertheless intoxication cases following OP and CA pesticides contamination remained rare when compared to total reported deaths (Fleischli et al. 2004). Pyrethroids insecticides were recently preferred to OP and CA pesticides uses. Indeed, pyrethroids pesticides are considered as a safer method of pest control because of their lower direct toxicity on mammals and birds (Martin et al. 1998; Chu et al. 2005). Nevertheless, data on pyrethroids insecticides diversity, persistence or toxicity in wild fauna are very poor in literature concerning top predators species.

Fig. 2. Osprey (*Pandion haliaetus*, adult female; photo C. Lemarchand)

Related to their plant-specific metabolic action, water solubility and poorly lipophilic characters, herbicides are documented as less toxic pesticides to vertebrates than insecticides

(Berny, 2007). Data on herbicides diversity and toxicity on vertebrates or predators, especially otters or ospreys, are particularly rare. Nevertheless, some studies demonstrated a direct effect of herbicides on herbivorous mammals or bird diversity or abundance during land use modifications (Santillo et al. 1989a,b). Bioaccumulation potential of herbicides to a top predator was recently confirmed by a study in Washington State (USA) on sediments, fish and ospreys (Chu et al. 2007). Furthermore, recent studies underlined a direct impact of herbicides, particularly triazines, on fish and amphibians' reproduction or survival (Langlois et al. 2009; Tillitt et al. 2010). Direct impact of herbicides on fish or amphibians' populations would indirectly affect otters and ospreys by a reduction in food resource. Therefore toxicity of some persistent herbicides on vertebrates could be underestimated by an insufficient risk evaluation.
At the beginning of the 1980's in France, otters only survived in two distinct populations: in the Massif Central mountains (centre), and along Atlantic Ocean and western wetlands of the country (Bouchardy, 1986). At the same period, osprey had disappeared of continental France as a nesting species. Legal protection of the otter and the osprey was decided from 1976. First signs of species recovery or return were recorded soon after. From 1985 increase and expanding of otter populations were proved and monitored in the whole repartition area of the species in France (Bouchardy, 1986; Rosoux and Bouchardy, 2002). In spring 1984, one pair of osprey stopped its migration towards northern Europe and built a nest along Loire River. Species is nesting again in continental France since 1985 (Coll., 1996). As osprey is a semi-colonial and philopatric species, other pairs quickly mated close to the first one, starting a new expanding population. European directives and national action plans allowed the protection and / or the restoration of both species habitat (Rosoux et al, 1999; Nadal and Tariel, 2008; Kuhn, 2009). The main characteristic of these species recoveries is their entirely natural process. Indeed, otter and osprey were never been reintroduced or reinforced in France, in order to establish habitats requirements, main natural and anthropogenic limits to populations, to locate colonization corridors and major sites for reproduction and breeding.
After about three decades of protection, otter population in France is still increasing, formerly isolated populations met from the beginning of the 2000's and the repartition area of the species covers the whole Massif Central related to the western third of the country (Bouchardy et al. 2001; Kuhn, 2009; Lemarchand and Bouchardy 2011). 37 reproductive pairs of ospreys wee noted in 2010 in continental France, mainly distributed along the medium part of the Loire River, but a geographical expansion of the species towards other river systems was recently noted (Nadal and Tariel, 2008). Increase of otter and osprey populations particularly concerns Loire River catchment, a major dispersal corridor that should be decisive for species conservation and dispersion in the whole country. As many predators, otter and osprey suffered from a bad reputation, but are now associated with preserved habitats and food resource (Chanin, 2003; Whitfield et al. 2003; Grove et al. 2009). However, otter and osprey remain sparse in France and are listed on UICN National Red Lists as "Minor preoccupation (LC)" and "Vulnerable (VU)", respectively (UICN France et al. 2008, 2009).
Objectives of this study were to evaluate the contamination of two flagship species (European otter and osprey) by a wide spectrum of pesticides, using a standard protocol of pesticides analyses in wild or domestic fauna and a non-invasive animal approach during a natural recolonization process in Loire River catchment. Since 2004 for the otter and 2007 for the osprey, a large toxicological program was launched during the "Plan Loire Grandeur Nature" program in France. 45 pesticides, including herbicides, organochlorine, organophosphate, carbamate and pyrethroids pesticides and a few main metabolites were systematically analyzed in otters and ospreys (but also in great cormorants, freshwater fish and invertebrates) from Loire River catchment (Lemarchand et al. 2007, 2009, 2010).

2. Materials and methods

2.1 Study area

The study area corresponded to the whole Loire River and main tributaries catchment in France (Fig. 3). Loire River catchment (117000 km², total length of rivers and tributaries of about 40000 km) is characterized by an important diversity of habitats and species, and is considered as one of the most preserved large hydrosystems in Western Europe. A national and European action plan, "Plan Loire Grandeur Nature", is running since 1994 to study and conserve this diversity, but also to protect inhabitants from floods and to maintain economic activity.

Fig. 3. Map of the Loire River (bold) catchment in mainland France

2.2 Animals monitoring and sampling

Concerning such rare species, it is particularly difficult to obtain sufficient sample material from enough individuals to support analysis and statistics. For ethical reasons it was not imaginable to trap or kill otters or ospreys for analyses. To avoid any vital risk related to handling, capture and bleed of animals were not considered. Furthermore, otter and osprey are fully protected by national and international laws, and listed as species of interest by the European Community (Habitats Directive 92/43/EC, Birds Directive 79/409/CEE). All operations were therefore entirely conducted under appropriate authorizations by a non-invasive approach. A large network, constituted by people in charge of otter and osprey studying and monitoring in mainland France was built to organize and enhance sampling under the coordination of the Muséum d'Orléans. The national agency for game and wildlife (ONCFS), hunting federations (FDC), the national agency for water and aquatic environments (ONEMA), health centres of the national union (UFCS) and of the birds protection league (LPO – French representative of Bird Life International), the national research centre on birds population biology (CRBPO, associated with the French national museum of natural history MNHN and Mr Rolf Wahl, in charge of osprey ringing program in France), the French Ministry of Environment (MEEDDM and DREAL Centre), the national agency for forests (ONF), private land owners and companies, museums, associations ("Loiret Nature Environnement") and regional naturalists were contributors for this study.

Concerning otters, only road-traffic killed individuals and those found dead in the wild in study area were collected. Based on visual observation, carcasses found more than 24h (during summer) or 48h (during winter) after road collision were considered too degraded

and not taken into account for post-mortem examination and toxicological analyses. Concerning ospreys, non-hatched eggs and dead young in nests were collected during chicks ringing operations. As scientists and birdwatchers monitor a majority of osprey nests in continental France, non-hatched eggs and dead young in nests were reported and sampled as soon as possible. France is also a major crossing area for migrating osprey from different populations (Hake et al. 2001; Dennis, 2008; Strandberg et al. 2009). Due, in one way, to the extreme rarity of this species in continental France (less than one hundred reproductive individuals), and in an other way that "foreigners" individuals (*i.e.* born in neighbour countries, but potentially breeders in France) are able to be found dead within the national territory (naturally or after illegal shots, electrocution on power cables, or drown in fish farms), migrating individuals flying towards reproduction areas elsewhere in Europe (Germany, Great-Britain, Scandinavia) completed sampling.

All samples were deep-frozen (-40 °C) prior to analyses. For each otter or osprey carcass, a necropsy was performed, and about 20 g of liver was sampled. This organ was preferred to fat because some otters and a lot of ospreys have very little fat, particularly at the end of spring migration concerning these latter. Otter sex and weight were determined; animals were measured (total and head, body, foot and tail lengths). Age was defined as "juvenile" (milk teeth, little size and weight), "subadult" (adult size and weight, teeth without wear and tartar) and "adult" or "old" (worn teeth with tartar). Body condition index *K* was determined according to Kruuk and Conroy (1991). Osprey sex and weight were determined; animals were measured (wing, body, foot and tail lengths). Non-hatched osprey eggs were drilled and emptied; eggshell was conserved for future studies on shell thickness. Age was defined as "egg" (non-hatched), "juvenile" (non-flying hatched individual), "subadult" (emancipated individual with the characteristic creamy fringe on feathers) and "adult" (adult size and plumage) (Dennis, 2008). Each animal (otter or osprey) is characterised by a specific case-record gathering discovery circumstances, clinical and biometrical data. After necropsies, carcasses were conserved for further showing or collection in museums or, if too degraded, systematically destroyed according to law.

2.3 Choice of compounds

Pesticides uses in France are one of the biggest in the world. Various compounds have been used for wood, vineyards, orchard, crops or ornamental plant protection, human or livestock health, and roads, railways or boat maintenance. Origins and flow of compounds are complex and aquatic habitats are exposed to both direct and indirect contamination.

In such a generalist approach, the choice of analyzed compounds is crucial and has to be representative:

- Of the diversity of uses (agrochemicals, industrials or domestics) in study area,
- Of the available analytical techniques and limits,
- Of accumulation and transfer patterns from trophic webs components to studied species.

A specific detection and quantification methodology was developed for pesticides in the toxicology laboratory of the college of veterinary medicine (VetAgro Sup, Lyon, France) during routine analyses on wild, game or domestic fauna. Compounds were chosen according to their toxicity on fauna, persistence in soil and water and accumulation in food webs. Regular complements and upgrades were added, as a function of new compounds or new detection techniques. Detected compounds are listed in Table 1 below.

Pesticides	Pesticides family and main use	Molecular formula	Date of ban in France (or current status)
Lindane (*gamma*-HCH)	Organochlorine insecticide	$C_6H_6Cl_6$	1998
Endosulfan	Organochlorine insecticide	$C_9H_6Cl_60_3S$	2007
DDT	Organochlorine insecticide	$C_{14}H_9Cl_5$	1972
Heptachlor	Organochlorine insecticide	$C_{10}H_5Cl_7$	1973
Aldrin	Organochlorine insecticide	$C_{12}H_8Cl_6$	1992
Methoxychlor	Organochlorine insecticide	$C_{16}H_{15}Cl_3O_2$	2002
Methiocarb	Carbamate insecticide, molluscicide	$C_{11}H_{15}NO_2S$	Still in use
Carbofuran	Carbamate insecticide	$C_{12}H_{15}NO_3$	2008
Mevinphos	Organophosphate insecticide	$C_7H_{13}O_6P$	2004
Phorate	Organophosphate insecticide	$C_7H_{17}0_2PS_3$	2004
Dichlorvos	Organophosphate insecticide	$C_4H_7C_{l2}O_4P$	2007
Terbufos	Organophosphate insecticide	$C_9H_{21}0_2PS_3$	2004
Diazinon	Organophosphate insecticide	$C_{12}H_{21}N_2O_3PS$	Still in use
Disulfoton sulfone	Organophosphate insecticide	$C_8H_{19}0_2PS_3$	2004
Chlorpyriphos ethyl	Organophosphate insecticide	$C_9H_{11}C_{l3}NO_3PS$	Still in use
Fenitrothion	Organophosphate insecticide	$C_9H_{12}NO_5PS$	Still in use
Pyrimiphos methyl	Organophosphate insecticide	$C_{11}H_{20}N_3O_3PS$	Still in use
Malathion	Organophosphate insecticide	$C_{10}H_{19}0_6PS_2$	2008
Fenthion	Organophosphate insecticide	$C_{10}H_{15}0_3PS_2$	2005
Parathion	Organophosphate insecticide	$C_{10}H_{14}NO_5PS$	2002
Methidathion	Organophosphate insecticide	$C_6H_{11}N_2O_4PS_3$	2004
Triazophos	Organophosphate insecticide	$C_{12}H_{16}N_30_3PS$	1992
Trifluraline	Anilide herbicide	$C_{13}H_{16}F_3N_3O_4$	2008
Atrazine	Triazine herbicide	$C_8H_{14}ClN_5$	2003
Simazine	Triazine herbicide	$C_7H_{12}CLN_5$	2003
Terbuthylazine	Triazine herbicide	$C_9H_{16}ClN_5$	2003
Cyanazine	Triazine herbicide	$C_9H_{13}ClN_6$	2004
Alachlor	Chloroacetanilide herbicide	$C_{14}H_{20}ClNO_2$	2008
Metolachlor	Organochlorine herbicide	$C_{15}H_{33}ClNO_2$	2003
Diuron	Substituted phenylurea herbicide	$C_9H_{10}Cl_2N_2O$	2008
Epoxyconazole	Fongicide	$C_{17}H_{13}ClFN_3O$	Still in use
Tefluthrine	Pyrethroid insecticide	$C_{17}H_{14}ClF_7O_2$	Still in use
Cyhalothrine Lambda	Pyrethroid insecticide	$C_{23}H_{19}ClF_3NO_3$	Still in use
Permethrine Cis	Pyrethroid insecticide	$C_{21}H_{20}Cl_2O_3$	Still in use
Cyfluthrine 2	Pyrethroid insecticide	$C_{22}H_{18}Cl_2FNO_3$	Still in use
Cypermethrine 2	Pyrethroid insecticide	$C_{22}H_{19}CL_2NO_3$	Still in use
Fenvalerate Cis	Pyrethroid insecticide	$C_{25}H_{22}ClNO_3$	Still in use
Deltamethrine	Pyrethroid insecticide	$C_{22}H_{19}Br_2NO_3$	Still in use

Table 1. List of families and uses, molecular formulae, current status of the compounds analyzed in this study.

2.4 Pesticides quantification methods

2.4.1 Organochlorine pesticides

2.0-8.0 g of tissue were sampled and 30 ml of hexane/acetone 75/25 mix was added. Each sample was blended twice with an Ultraturrax® (Ika, Werke, Germany) and filtered trough a phase separator membrane. The extract was evaporated at 60 °C in a rotary evaporator. The dry extract was dissolved in 10 ml hexane.

Two ml of fuming sulphuric acid (SO_3 7%) were added, and after centrifugation at 4x *g*, 1 ml of the supernatant was used for OC pesticides quantification by gas chromatography with electron capture detection material. Temperature program and injection conditions are described in Lemarchand et al. (2007; 2010). Each sample was run in duplicate. Organochlorine pesticides concentrations were calculated by using different mix standards. Recovery level on standard mixtures was always greater than 92%. All standards were purchased from CIL (St Foy la Grande, France), and purity was > 99%. Linearity was determined between 5 and 100 $ng.g^{-1}$ ($r^2 > 0.99$ on standards and spiked samples, 5-point calibration curves). Limits of detection were between 0.5 and 1.0 $ng.g^{-1}$ lipids for individual PCB congeners. Cod liver oil (BCR349) certified material was used as a regular quality control.

2.4.2 Organophosphate pesticides analyses

5 g of muscle sample was shaken with 60 ml dichloromethane and 10 g anhydrous sulfate. Mix was then filtered trough a Whatman 1 PS membrane, and evaporated under vacuum at 40°C. Dry samples were diluted in 3 ml ethanol, and underwent an ultrasonic step. Extract was then purified with a Sep pack R 300 (Silica Waters, 020810; 500 mg) column conditioned with 2 ml methanol and 2 ml ethanol. 2 ml dichloromethane were used for column elution. Purified samples were dried and diluted with 3 ml dichloromethane. Organophosphate (OP) and 2 carbamates (CA) pesticides (Dichlorvos, Carbofuran, Mevinphos, Phorate, Phorate oxon, Phorate sulfone, Methiocarbe, Terbufos, Diazinon, Disulfoton, Chlorpyriphos methyl, Chlorpyriphos ethyl, Fenitrothion, Pyrimiphos methyl, Malathion, Fenthion, Parathion, Methidathion, Disulfoton sulfone, Triazophos) concentrations were determined by GC/MS in SIM mode (OP + carbofuran and methiocarbe). A 5973N MS coupled with a 6890 GC (Agilent®) was used, with a 30m HP5-MS column (0.25 mm ID, 0.25µm thickness). For each samples standard and spiked sample, 2 µL were injected. The temperature program was 100°C (2 min), 55°C/min up to 200 °C (held for 5 min), 50 °C up to 220 °C (held for 3 minutes), followed by 60 °C/min up to 300°C. A final, post-run time of 2 min at 300°C was maintained. Total run time was 13.55 min. Injector was set at 250°C and the He flow was set at 2.5 ml/min. Each OP or CA was identified based on the following criteria: retention time and 3-4 fragmentation ions with pre-defined relative amounts and 20% variability acceptance for each ion. Linearity was confirmed between 25 and 500 $ng.g^{-1}$ with 5 point calibration curves and r^2 >0.99. Recovery was determined between 76% and 104% for all spiked samples and repeatability was considered acceptable with coefficients of variation <15%.

2.4.3 Pyrethroids pesticides analyses

5 g of tissue (liver or muscle) sample was shaken in 60 ml ethanol and 10 g anhydrous sulfate, and then filtered trough a Whatman 1 PS membrane. Extract was dissolved in 5 ml methanol and underwent a second filtration procedure. Concentrations were determined by GC / ECD and confirmed by GC/MS according to a modified method of the French Food Safety Authority (Anses Met AFSSA). An Agilent GC-ECD 6850 with a 30m HP1 column

(0.32 mm ID, 0.25µm film) was used. For each samples standard and spiked sample, 2 µL were injected. The temperature program was common to OCs', PCBs and pyrethroids (initial temp: 100°C, first ramp 6°C/min up to 220 °C held for 10 min, 2nd ramp 7 °C/min up to 285°C, held for 1 min, total run time 42.29 min) Injector was at 230°C, detector at 300°C. Total He flow was 9 ml/min. Pyrethroids were identified according to their retention times. Linearity was confirmed between 10 and 100 ng.g^{-1} with 5 point calibration curves and r^2 >0.99. Recovery was determined between 82% and 94% for all spiked samples and repeatability was considered acceptable with coefficients of variation <15%. For all positive samples, a confirmatory analysis was performed with GC/MS in SIM mode. Identification was based on retention times and 3 or 4 ions.

2.4.4 Herbicides analyses

2 g of muscle sample was shaken during 5 minutes in 8 ml acetone, and then centrifuged at 4x g; supernatant was placed in separate tubes, and this extraction was performed twice. Samples were evaporated under nitrogen, and dry extract was dissolved in 1 ml aceton/methanol (50:50) solution. Extract was then purified with a SPE C18 500 mg column conditioned with 2 ml acetone and 2 ml methanol. Column was vacuum dried and purified samples were diluted in 3 ml acetone. After drying under nitrogen, samples were diluted in 1 ml methanol. Herbicides (Trifluraline, Atrazine, Simazine, Terbuthylazine, Diuron, Alachlor, Metolachlor, Cyanazine, Epoxyconazloe) concentrations were determined by GC/MS spectrometry. A 5973N MS coupled with a 6890 GC (Agilent®) was used, with a 30m HP5-MS column (0.25 mm ID, 0.25µm thickness). For each samples standard and spiked sample, 2 µL were injected. The temperature program was 85°C held 1 min, followed by 6°C/min up to 170°C (held for 12 min), then followed by 20°C/min up to 280°C, held for 4.33 min (total run time 37 min). Injector was at 250°C and in the splitless mode. Each herbicide was identified based on the following criteria: retention time and 3-4 fragmentation ions with pre-defined relative amounts and 20% variability acceptance for each ion. Linearity was confirmed between 100 and 500 ng.g^{-1} with 5 point calibration curves and r^2 >0.99. Recovery was determined between 67% and 98% for all spiked samples and repeatability was considered acceptable with coefficients of variation <15%.

2.5 Calculation methods and statistical analysis

Geometric means of *p,p'*-DDE, *p,p'*-DDD and *p,p'*-DDT were added to calculate the sum of DDTs (Σ DDTs). Geometric means of lindane, endosulfan, DDE, DDD, DDT, heptachlor, heptachlor epoxyde, aldrin and metoxychlor were summed to provide the sum of pesticide concentrations (Σ Pesticides). All these were chosen by the National Veterinary School of Lyon (VetAgro Sup, France) standard protocol (Mazet et al. 2005; Lemarchand et al. 2007, 2010). The Mann-Whitney test was used to compare two independent samples, Kruskall-Wallis for *k* comparisons, Spearman correlation rank test to quantify associations between two variables. Statistics were performed using *R.* (Ihaka and Gentleman 1996).

3. Results

3.1 General characteristics of sampled material

3.1.1 Otters

Otters have been systematically collected since the beginning of the toxicological program along Loire River and tributaries catchment (2004). This program allowed an increase in the

scientific use of previously collected and stocked individuals, for toxicological analyses first, but also for genetic study of otter recolonization, diet, biometry or causes of mortality approaches (Mucci et al. 2010). Main characteristics of otters analyzed in this study are summarized in table 2.

Otter	Sex	Age	Body index K	Catchment Origin	Cause of death	Otter	Sex	Age	Body index K	Catchment Origin	Cause of death
LF 01	Female	Juvenile	1,21	Upper part	Collision	LM 123	Male	Subadult	1,07	Lower part	Collision
LM 02	Male	Adult	0,91	Upper part	Collision	LM 124	Male	Subadult	1,00	Lower part	Collision
LM 05	Male	Adult	1,08	Upper part	Collision	LM 125	Male	Adult	1,04	Lower part	Collision
LM 09	Male	Subadult	1,11	Upper part	Collision	LM 126	Male	Adult	1,04	Lower part	Collision
LM 12	Male	Subadult	0,71	Upper part	Collision	LM 127	Male	Subadult	1,10	Lower part	Collision
LM 13	Male	Adult	0,91	Upper part	Collision	LM 129	Male	Adult	1,16	Lower part	Collision
LM 14	Male	Adult	1,07	Upper part	Collision	LM 130	Male	Juvenile	1,17	Lower part	Collision
LM 16	Male	Juvenile	0,62	Upper part	Natural	LM 141	Male	Juvenile	0,85	Lower part	Collision
YL	Male	Juvenile	0,82	Upper part	Starvation	LM 144	Male	Adult	0,89	Lower part	Collision
LF 62	Female	Juvenile	0,95	Lower part	Collision	LM 148	Male	Adult	1,20	Lower part	Collision
LF 64	Female	Adult	1,01	Lower part	Collision	LM 153	Male	Adult	1,36	Lower part	Collision
LF 68	Female	Juvenile	0,64	Lower part	Collision	LM 154	Male	Subadult	1,17	Lower part	Collision
LM 71	Male	Adult	1,28	Lower part	Collision	LM 156	Male	Juvenile	1,12	Lower part	Collision
LF 72	Female	Adult	1,06	Lower part	Collision	LF 157	Female	Subadult	0,82	Lower part	Collision
LF 74	Female	Subadult	0,97	Lower part	Collision	LF 158	Female	Subadult	0,58	Lower part	Collision
LF 77	Female	Old	0,67	Lower part	Collision	LF 159	Female	Juvenile	1,03	Lower part	Collision
LF 78	Female	Adult	0,71	Lower part	Collision	LM 160	Male	Adult	1,20	Lower part	Collision
LM 83	Male	Adult	0,68	Lower part	Collision	LF 161	Female	Adult	0,95	Lower part	Collision
LF 85	Female	Subadult	0,96	Lower part	Collision	LF 162	Female	Adult	0,98	Lower part	Collision
LM 86	Male	Adult	1,39	Lower part	Collision	LF 163	Female	Adult	0,83	Lower part	Collision
LF 88	Female	Adult	1,02	Lower part	Collision	LF 164	Female	Adult	0,91	Lower part	Collision
LF 89	Female	Subadult	0,96	Lower part	Collision	LF 165	Female	Adult	0,95	Lower part	Collision
LM 90	Male	Adult	0,89	Lower part	Collision	LF 167	Female	Adult	0,92	Lower part	Collision
LF 91	Female	Adult	1,03	Lower part	Collision	LF 168	Female	Adult	1,06	Lower part	Collision
LF 93	Female	Adult	0,82	Lower part	Collision	LF 169	Female	Adult	1,05	Lower part	Collision
LM 94	Male	Adult	1,38	Lower part	Collision						

Table 2. Main characteristics and causes of death of otters in this study.

Fifty-one otters were necropsied and analyzed for this study, with 24 females (47%) and 27 males (53%). Sex-ratio of the sample was very close to the equilibrium but characterized by a slight over-representation of males. This was noted in previous studies, and was generally

attributed to the larger territory of males compared to females, with associated higher risk of vehicular collision during food or new habitat foraging, particularly in the case of natural recolonization of unknown habitats (Foster-Turley et al. 1990; Rosoux and Tournebize, 1993; Kruuk, 2006; Lemarchand, 2007). Most of dead otters (30 out of 51, *i.e.* 59%) were adults, 11 were subadults, 9 were juvenile and only one was an old individual. This mortality picture is different from those observed in previous studies, where most of discovered otters were juvenile or subadults, with an linear increasing in probability of death with age, considering the rareness of very old individuals in nature (Kruuk and Conroy, 1991; Kruuk, 2006; Lemarchand, 2007).

With the exception of only two individuals found in the wild during the study, all otters died after a vehicular collision. These results tend to confirm that road casualties seem to be one of the main causes of mortality of otters, but, as suggested by Kruuk and Conroy (1991), and Kruuk (2006), it is the easiest way to find dead otters, those dying of other causes in the wild having far less probability of being found. This bias of carcasses collect was difficult to overlap, because of the huge human and financial costs of systematic search on riverbanks, ponds and lakes in such a study area. Considering this bias of collect, assessing the real hierarchy of causes of mortality remains hard for such a species. Among those found in the wild, one was an adult found dead without any clinical sign or injury, the other was a very little otter, only a few days aged, died of starvation after the dead or the abandon by its mother. This finding was surprising: as young otters live all their time in den, the probabilities of discover them if they die is very low (Kruuk, 2006). With the exception of various injuries caused by road collisions, all otters were in good physical conditions, with no apparent organ damage due to intoxication, like hemorrhages, organ abnormality or wound. Body condition index was systematically comprised between 0,5 and 1,4: it can be assumed that none otter collected in this study was in poor health condition or particularly fat (index < 0,5 or > 1,4, respectively; Kruuk, 2006). Medium body condition index of all otters was 0,99, very close to the value of reference (=1; Kruuk, 2006). Differences between body condition indexes K, total length or weight of otters from upper or lower part of Loire River catchment were not significant. Post-mortem examinations of otters never showed any clinical sign of severe intoxication, like organ or tissue abnormality, secretions, hemorrhages or anemia. Lead pellets were found on two occasions in carcasses but were not a death causal agent. According to Bo Madsen et al. (2000) or Simpson et al. (2005), otters are generally few concerned by natural intoxication (*e.g.* botulism), viral or bacterial diseases. Individuals examined here never showed any strong disease or natural intoxication signs.

3.1.2 Ospreys

Osprey population in mainland France is monitored since the natural come back of the species as breeding one in 1984 (synthesised in Nadal and Tariel, 2008). From only one in 1984, population of breeding ospreys in the study area increased to 35 active nests in 2010, the overall breeding success during 1985-2006 periods was 2.0 fledglings per active nest. This value is higher than the stable population threshold (=0,8), and, associated with the recorded survival rate of adults of 0.97, suggests a very good reproduction dynamics of osprey in the study area during this period (Poole, 1989; Rattner et al. 2004; Wahl and Tariel, 2006; Dennis, 2008; Nadal and Tariel, 2008). The large, favourable and non-fully occupied potential habitats along the Loire River, associated with an important and diversified food resource were the main factors of this reproductive success. Ospreys have been

systematically collected in Mainland France from the end of 2007, when toxicological program on otter was extended to this top-predator. Main characteristics of ospreys analyzed in this study are listed in table 3.

17 osprey samples collected since 2007 in France were used. As some of the birds were ringed, or came from known nests, information about age and origin was established for 12 ospreys (70%). 7 osprey samples (3 non hatched eggs, 3 dead *pulli* in nests and one adult) came from the breeding population along Loire River. The other birds were subadults or adults collected during spring or autumn migration: 4 from Germany, 1 from Norway, and 5 non-ringed birds were from unknown origin. 3 ospreys died after electrocution on power cables, 3 after illegal shots in spite of the full protection of the species by law. Drawing of ospreys in fishponds with inadequate protection nets is another cause of osprey mortality currently emerging: 4 individuals died in the same structure during spring 2009 (March 23rd, 24th, 26th and 28th) in eastern France. To minimize this drawing risk, protection nets were recently modified in several fishponds situated along osprey migration corridors. As observed concerning otters, post-mortem examination never showed any showed any clinical sign of severe intoxication, like organ or tissue abnormality, secretions, hemorrhages or anemia. Three *pulli* from the same nest died of starvation during a long period of bad weather conditions. Three cases of feather pitching syndrome were observed, but these specimen were not collected early enough to support toxicological analyses. The rest of the examined individuals were in good physical conditions (normal size and weigh) and did not show any intoxication or disease sign.

Osprey	Sex	Age	Origin	Cause of death
bbz 4	female	adult	Germany	electrocution
Bbz 3	male	adult	Loire River	electrocution
Bbz 7	unknown	egg	Loire River	non hatched egg
Bbz 8	unknown	egg	Loire River	non hatched egg
Bbz 9-11	male	juvenile	Loire River	pullus dead in nest
Bbz 12	unknown	juvenile	Loire River	pullus dead in nest
Bbz 13	unknown	egg	Loire River	non hatched egg
Bbz 14	female	subadult	Norway	illegal shot
Bbz 17	male	subadult	unknown	electrocution
Bbz 19	male	juvenile	Loire River	pullus dead in nest
Bbz 20	female	adult	unknown	illegal shot
Bbz 21	male	subadult	Germany	illegal shot
Bbz 23	female	adult	Germany	drawn in fish farm
Bbz 24	female	subadult	unknown	drawn in fish farm
Bbz 25	male	subadult	unknown	drawn in fish farm
Bbz 28	male	subadult	Germany	dead in health center
Bbz 31	male	adult	unknown	drawn in fish farm

Table 3. Ospreys' characteristics and causes of death analyzed in this study. Osprey numbers corresponds to the chronological sampling order.

3.2 Contamination by organochlorine pesticides

Results concerning contamination of otters by OC pesticides are represented in table 4. OC pesticides and especially DDT metabolites were detected in all (100%) of the analyzed otters, confirming the widespread exposure of otter habitat in France to OC pesticides (Colas et al. 2006; Lemarchand et al. 2007, 2010). Mean concentrations of total OC pesticides in otter liver of the whole Loire River catchment reached 2,2 mg.kg^{-1} lipid weight, without any statistical variations with the geographical origin of the individuals: the increase in concentrations by going downstream observed in the upper part of the catchment (Lemarchand et al. 2007) was not significant at the whole catchment scale. Differences of various OC pesticides with otter age or sex were not significant. DDT was detected in 17 individuals (33%), confirming quite recent uses of this insecticide, banned in 1973 in France. 15 of these 17 DDT-contaminated otters were coming from the lower part of Loire River catchment. However, DDE was the most abundant of the analyzed DDTs metabolites, confirming the general decrease of otter's exposure to DDT and OC compounds in Europe (Mason, 1998). Lindane constituted the most abundant OC pesticide after DDTs, with quite low concentrations. Aldrin, Dieldrin, Heptachlor and Heptachlor epoxide were very low, often close to the detection limits. Methoxychlor and Endosulfan were never detected in otters. Measured OC pesticides concentrations remained below the available thresholds concerning otter survival (Mason and Macdonald 1993 a,b; 1994). Considering the actual population dynamic in France and elsewhere in Europe, OC compounds are not supposed to constitute an immediate threat to otter conservation.

	Otters (n= 51)					Ospreys (n= 17)					
OC pesticides	Males	Females	Juveniles	Sub adults	Adults & Old	Males	Females	Eggs	Juveniles	Sub adults	Adults
DDT	0,02	0,01	-	0,01	0,02	-	-	-	-	-	-
DDE	1,12	1,85	0,1	1,45	2,01	10,7	0,55	3,40	-	-	1,86
DDD	0,54	0,35	-	0,41	0,60	-	-	-	-	-	-
Lindane	0,11	0,08	0,05	0,15	0,12	-	-	-	-	-	-
Methoxychlor	-	-	-	-	-			-	-	0,01	0,3
Aldrin	0,05	0,04	-	0,04	0,11	-	-	-	-	-	-
Heptachlor	0,01	0,01	0,01	0,18	0,14	-	-	-	-	-	-
Hepta. epox.	0,01	0,01	0,01	0,01	0,02	-	-	-	-	-	-
Endosulfan	-	-	-	-	-	-	-	-	-	-	-

Table 4. Contamination of otters and ospreys from the Loire River catchment by organochlorine pesticides (mg.kg^{-1}).

Results concerning contamination of ospreys by OC pesticides are presented in table 4. OC pesticides were detected in all but 5 of the sampled ospreys, and maximum Σ OC pesticides concentrations in liver reached 10,7 mg.kg^{-1} lipid weight. Only DDTs residues (mainly *p,p'*-DDE) and Methoxychlor were found in samples. DDT by itself was never found in ospreys. These two compounds were never found simultaneously in the same samples. Lindane, Aldrin, Heptachlor, Heptachlor epoxide and Endosulfan were never found in samples. Endosulfan is the only OC pesticide never found in otter or osprey samples. Nevertheless, these compounds were noted in previous studies concerning ospreys, particularly concerning Lindane, Aldrin and Heptachlor epoxide (Ewins *et al.* 1999; Henny *et al.* 2003,

2008; Toschik *et al.* 2005). This difference could be related to a different exposure of American ospreys to OC pesticides when compared to European ones, resulting in a higher OC pesticides accumulation pattern in the whole American population. Indeed, American ospreys were exposed to OC pesticides without interruption from the beginning of industrial uses until legal ban. In France, DDT and other OC pesticides were banned before the return of the osprey or at the beginning of population expanding, resulting in a lower and decreasing exposure to contaminants. DDE was detected in 4 individuals (24 %), including 2 eggs from 2 different nests along Loire River and 2 adults, one coming from Loire River. We did not observe any significant variations in OC pesticides concentrations with osprey age, sex or origin. DDE concentrations remained quite low (range 0.0 – 10,7 $mg.kg^{-1}$ lipid weight). These values were comparable to those noted by Rattner et al. (2004) or Henny et al. (2008), and should not be of concern for osprey direct conservation. DDE concentration in available osprey eggs (n=3) reached 0.0, 4,6 and 5,9 $mg.kg^{-1}$ lipid weight, respectively. Concerning the latter, the measured values were slightly higher than the 4.2 $mg.kg^{-1}$ (measured in wet weight) eggshell thinning threshold cited in the literature (Wiemeyer *et al.* 1988; Henny *et al.* 2008), but these eggs did not show any shell breakage and were not damaged. Methoxychlor was detected in 8 individuals (47%), with low values (range 0.0 – 0.93 $mg.kg^{-1}$ lipid weight, see table 1). General Methoxychlor mean reached 0.01 $mg.kg^{-1}$ ww, far less than noted by Weber et al. (2003) in Germany, where 100% of the sampled ospreys were contaminated by Methoxychlor. Following these authors, we assume this compound is not a direct threat to ospreys.

3.3 Contamination by organophosphate, carbamate and pyrethroids pesticides

To a general point of view, contamination of otters and ospreys by the 16 highly toxic cholinesterase inhibitors appeared low and scattered, with only few individuals concerned. Only two otters (4%) and 8 ospreys (47%) were characterized by detectable cholinesterase inhibitors concentrations. Among the OP pesticides analyzed, 7 (Mevinphos, Phorate, Malathion, Parathion, Methidathion, Disulfoton sulfone and Triazophos) were quantified in ospreys and are presented in table 5. Only two OP pesticides (Parathion and Methidathion) were detected in otters, with low concentrations (between 0,02 and 0,03 $mg.kg^{-1}$ ww, data not shown). No statistical comparison could have been made concerning otter contamination by OP pesticides. Other OP pesticides analyzed in otter and osprey tissues were never been detected. Carbamates pesticides (Methiocarb and Carbofuran) were not quantified in otters and ospreys during this study, however they were recently noted in intoxicated red kites (*Milvus milvus*) in France (Berny and Gaillet, 2008). It can be assumed that diet of otters and ospreys (based on fish) is less exposed to carbamates pesticides accumulation that other diet types of some terrestrial predators like red kite.

OP pesticides were only measured in subadult and adult ospreys, and never found in eggs or *pulli* during this study. None of the individuals coming from the nesting population showed any OP pesticides contamination. OP pesticides variations with osprey age, sex or origin were not significant. Triazophos, Disulfoton sulfone and Mevinphos were the most frequently detected compounds in ospreys (n=4, 3 and 3, respectively). Phorate and Malathion were detected in only one individual, characterized by the highest diversity of compounds (4 compounds with also Parathion and Methidathion) and by the highest concentrations of total OP pesticides (0,9 $mg.kg^{-1}$ ww, see table 5).

Individuals	bbz 19	bbz 3	bbz 7	bbz 8	bbz 9-11	bbz 12	bbz 13	bbz 14	bbz 28	bbz 21	bbz 23	bbz 4	bbz 20	bbz 24	bbz 25	bbz 17	bbz 31
Mevinphos	-	-	-	-	-	-	-	-	-	-	0,03	-	0,05	-	0,3	-	-
Phorate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0,02	-
Malathion	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0,03	-
Parathion	-	-	-	-	-	-	-	0,4	-	-	-	-	-	-	-	0,8	-
Methidathion	-	-	-	-	-	-	-	-	-	-	-	-	0,02	-	-	0,02	-
Disulfoton sulfone	-	-	-	-	-	-	-	-	-	-	-	-	0,3	-	0,3	-	0,04
Triazophos	-	-	-	-	-	-	-	0,02	0,02	-	-	-	-	0,03	0,03	-	-

Table 5. Contamination of ospreys by OP and CA pesticides (mg.kg^{-1} wet weight). Data are organized according to geographic origin of individuals for a better comparison.

As described above, ospreys were generally in good physical conditions (adequate mass and total body fat) and did not show any OP pesticides poisoning sign (*e.g.* diarrhea, pulmonary oedema, tightened claws, Berny and Gaillet, 2008) during post-mortem examination. Furthermore, some of them were collected during migration flows, and none bird was found with apparent sign of exhaust potentially brought about by contamination consequences. Measured concentrations remained well below toxic doses of cholinesterase inhibitors (documented as about 10 mg.kg^{-1} ww) and were not death causal agent of these individuals. Low level of concentrations and of contamination cases frequency should not constitute a threat to the population level, taking into account recent restrictions on OP and CA pesticides uses.

Pyrethroids pesticides residues were never found in any otter or osprey samples (data not shown). The good quality and abundance of samples and the efficiency of the method used avoided methodological bias in pyrethroids pesticides detection.

These results lead to several hypotheses:

- Pyrethroids pesticides may be quickly degraded or metabolized by animals;
- Pyrethroids pesticides may be little concerned by bioaccumulation in aquatic food chains;
- Global accumulation and transfer of recently used pyrethroids is very low for the moment, but is able to raise in the future with increasing uses.

Metabolite of pyrethroids (3-phenoxybenzoic acid 3-PBA) was investigated in osprey eggs of the Washington State, USA (Chu et al. 2007) without being found. Complementary studies are needed to precisely evaluate general contamination of fauna by pyrethroids pesticides. Insect-consumers birds in treated areas (*e.g.* Eurasian skylark *Alauda arvensis*, common quail *Coturnix c.*) and their bird-eating predators (*e.g.* Montagu's harrier *Circus pygargus* or western marsh harrier *C. aeruginosus*) could be used as sentinels for an evaluation of direct transfer of pyrethroids through terrestrial systems first, before generalization of analyses to other systems, like aquatic systems and associate predators.

3.4 Contamination by herbicides

As observed for OP and CA pesticides, contamination of otters and ospreys by the 8 analyzed herbicides was generally low and few diversified. Only two otters (4%) and 7 ospreys (41%) showed detectable herbicides concentrations. Of the 10 herbicides analyzed, Metholachlor was the only herbicide detected in otters, on two occasions and with low concentrations (0,02 and 0,05 mg.kg^{-1} ww respectively, data not shown). Two herbicides

(Terbuthylazine and Alachlor for 5 individuals each) and fungicide Epoxyconazole (in only one case) were quantified in ospreys (see table 6). None of the ospreys from the nesting population in France showed any herbicide or fungicide contamination. Herbicides variations with osprey age, sex or origin were not significant. Herbicides were not found in osprey eggs during this study. It can be underlined a unique case of contamination by fungicide Epoxyconazole (5,64 mg.kg-1 ww, see table 6), detected in an osprey from Germany. This individual did not show any particular intoxication sign. As for OP and CA pesticides, concentrations of herbicides measured in tissues and low frequency of herbicide detection leads to a probable weak impact of these compounds on species' conservation. Nevertheless, herbicides were very rarely searched in ospreys and very few data are available in literature for comparison. Chu et al. (2007) reported contamination of osprey eggs by a Dacthal structural isomer, indicating that some herbicides could be accumulated in ospreys with a potential reproductive impact on populations.

Individuals	bbz 19	bbz 3	bbz 7	bbz 8	bbz 9-11	bbz 12	bbz 13	bbz 14	bbz 28	bbz 21	bbz 23	bbz 4	bbz 20	bbz 24	bbz 25	bbz 17	bbz 31
Terbuthylazine	-	-	-	-	-	-	-	0,09	0,83	0,3	-	-	-	0,54	-	0,88	-
Alachlor	-	-	-	-	-	-	-	0,01	-	0,01	-	-	0,01	0,08	-	0,04	-
Epoxyconazole	-	-	-	-	-	-	-	-	-	-	5,64	-	-	-	-	-	-

Table 6. Contamination of ospreys by herbicides and fungicides (mg.kg^{-1} wet weight).

4. Conclusion

A large non-invasive program allowed an important sampling of European otter and osprey tissues for various pesticides contamination study. Results showed that otter and osprey could be used as good sentinels of organochlorine and, to a lesser extent, organophosphate pesticides and some herbicides accumulation in aquatic food chains. Carbamates and pyrethroids pesticides were not detected in those top-predators fish-eating species. Organochlorine, organophosphate pesticides and herbicides concentrations remained low and under values of concern for species direct short-term conservation. Regular increase in populations observed since three decades in France seemed to confirm a low impact of global contamination on otter and osprey. Nevertheless, long-term consequences of global contamination on otter and osprey behaviour (*e.g.* prey or habitat foraging, hunting, mating or territory defence), synergies or antagonisms between compounds or potential long-term endocrine disruptors effects of low-concentrated contaminants remains unknown and should be elucidated during future standard monitoring of these sentinels species.

5. Acknowledgments

Authors wish to thank the following structures or people for otter and/or osprey conservation and study or samples providing (see text): Muséum d'Orléans (sampling coordination), MEEDDM, DREAL Centre, LPO, UFCS, CRBPO, ONCFS, ONEMA, ONF, RTE, EDF, R. Wahl, private land owners and companies, Ph. Guillet & M.-F. Larigauderie of the City of Orléans Museum, D. Vey & the technical team of VetAgro Sup Toxicology unit, "Loiret Nature Environnement" and other environmental associations.

This study was financially supported by: European Commission (FEDER), « Plan Loire Grandeur Nature 2007-2013 », Etablissement Public Loire, Agence de l'Eau Loire-Bretagne, French Ministry of Environment (MEEDDM, DREAL Centre, Plan National d'Actions pour le balbuzard pêcheur en France), VetAgro Sup, Ville d'Orléans, Office National des Forêts, Parc naturel régional des Volcans d'Auvergne, Parc interrégional du Marais poitevin, and CNRS.

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17

Is Pesticide Use Sustainable in Lowland Rice Intensification in West Africa?

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1. Introduction

Rice is a major staple food for about 3 billion people (Nguyen and Ferrero 2006). In West Africa, it is indeed no longer a luxury food and has become a major source of calories for the urban poor. The poorest urban households obtain 33% of their cereal-based calories from rice (NISER, 2005). Urbanization, changes in employment patterns, income levels, and rapid population growth have contributed to widening the gap between supply and demand (Figure 1). The gap between production and consumption is made up by imports, which are estimated at 2 million metric tones per annum.

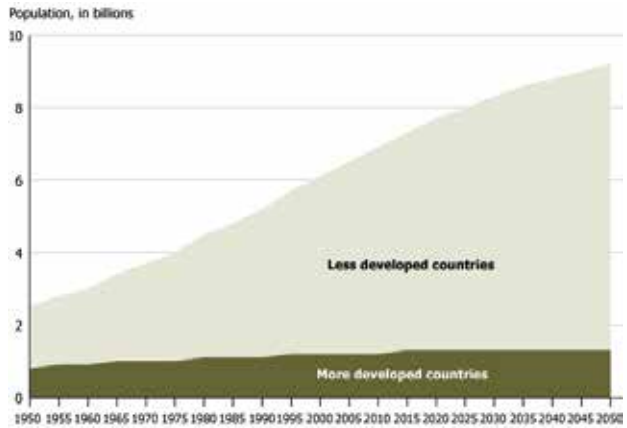

Fig. 1. World Population Growth 1950–2050 (Source: United Nations, 2009).

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Rice remains one major crop in which West Africa can easily become self-sufficient given the potentials that abound in the region. The potential land area for rice production in West Africa is between 4.6 million and 4.9 million ha. Out of this, only about 3.7 million ha—or 75 percent of the available land area—is presently cropped for rice. Cultivable land to rice is spread over five ecologies, namely: rainfed upland, rainfed lowland or shallow swamp, irrigated rice, deepwater or floating rice and tidal mangrove swamp. The commonly used ecosystems and share of rice area for the rice ecosystems are presented in Table 1.

Ecology	Share (%)		Yield (t/ha)	
	Area	Production	Current	Potential
Upland	40	37	1.0	1.5-4.5
Rainfed lowland	48	49	1.4	2.5-5.0
Irrigated lowland	6	14	2.8	5.0-7.0

Upland: area expansion and yield increase may be fulfilled
Rainfed lowland: most promising
Irrigated lowland: dam construction is too expensive at current rice price
Source: Sakurai, 2006

Table 1. Rice production ecology in West Africa

Amongst these, lowland rice has the highest priority, being the ecology that represents the largest share of rice area and rice production. Smallholder farmers with farm holdings of less than 1 ha cultivate most of the rice produced in West Africa. However, rice productivity and production at the farm level are constrained by several factors. These constraints include insufficient appropriate technologies, poor supply of inputs, ineffective farmer organizations and groups, poor quality of rice, poor marketing arrangements, inconsistent agricultural input and rice trade policies, and environmental constraints. These environmental constraints include poor drainage and iron toxicity in undeveloped lowland swamps, poor maintenance of developed lowland swamps, drought, deficiencies of N and P, poor soil management practices, seasonal over-flooding of rice fields, pests and diseases.

1.1 Hypothetical shift in production system

With the increasing awareness of the limited potential for intensification of rice production in the uplands, farmers are gradually moving into the lowlands, which are less fragile (permits residual moisture use), more fertile and ecologically robust. The lowland areas are underutilized in West Africa. The lowland areas are expected to meet the growing demand for rice in West Africa because they provide potential for expansion, diversification and intensification of rice production in the region. This change in farming practice has been accompanied by an increase in the use of agrochemicals (pesticides and fertilisers), high-yielding varieties and monoculture/continuous cropping, which further disrupt traditional farming and natural ecosystem functioning. Most West African countries are currently undergoing intensification in rice production to cope with the high population pressure. However, these may have adverse consequences on pest outbreaks, if it lacked the vision on the conservation of renewable inputs (biodiversity), and the whole issue of sustainability. This prediction is based on the hypothesis that with the improvement in the irrigation system, farmers will be able to grow high-yielding, photo-insensitive rice crops, and more crops per year on the same field. In such systems, the pest and beneficial cycles will be uninterrupted.

1.2 Insect pests of rice in West Africa

The major insect pests of lowland rice in West Africa include: the African rice gall midge (AfRGM), *Orseolia oryzivora* Harris and Gagné (Diptera: Cecidomyiidae); the rice stem borer complex: the stalk-eyed flies – *Diopsis* spp. (Diptera: Diopsidae); the African white borer – *Maliarpha separatella* Ragonot (Lepidoptera: Pyralidae); the yellow stem borers – *Scirpophaga* spp. (Lepidoptera: Pyralidae). Other important pests of rice include: vectors of rice yellow mottle virus (*Trichispa sericea* Guérin, *Chaetocnema pulla* Chapius, *Chnootriba similis* Thunberg and *Oxya hyla* Serville, etc.). All these pests are indigenous to West Africa except *Maliarpha separatella* that can also be found in Asia.

1.3 Yield losses caused by insect pests

Serious damage to the rice crop by the complex of insect pests result in significant yield losses which are typically in the range 10-30% yields and, in some regions or years, may exceed 90% (Nguu, 2008) (Table 2). Pests cause considerable and unacceptable crop losses in the field and in storage. The very high food losses in West Africa, attributable to pests, highlight their role in causing food shortages that lead to hunger.

Country	Pests	Estimated crop loss
Ghana	Stem borers	30%
Nigeria	Stem borers	25 – 30%
	African rice gall midge	10 – 35%
Burkina Faso	Stem borers	10 – 40%
	African rice gall midge	20 – 60%
Mali	African rice gall midge	20 – 35%
Cameroon	Stem borers	26 – 30%

Source: Youdeowei (1989, 2004)

Table 2. Examples of average losses attributable to pests of rice in selected West African countries

1.4 The race for pesticide sales in West Africa

The yield potential of rice cultivated in the intensified systems is continually challenged by chronic pest infestations and by pest outbreaks. This challenge is seen by the chemical industries mostly based in Asia and developing countries as an avenue to aggressively market their products in West Africa. The products are sold without proper training of smallholder farmers on how to safely apply it and without warning of the harmful effect on the environment. The sad aspect of the race for pesticide sales is that many banned pesticides in Asia and other developed countries of the world are being dumped in West Africa. The indiscriminate use of the pesticides has posed a lot of danger to the environment and ecosystem making human life to be under threat. The current article, therefore, not only meets a demand expressed from the regional entomologists but also makes an important contribution to raise alarm of the danger of pesticides in lowland rice which is currently being targeted for intensification.

2. Importation of pesticides into West Africa

As agricultural production system moves more and more from subsistence to market – oriented large scale farming, a concomitant increase in pesticide usage arise (Sosan *et al,*

2008). The climatic conditions of West Africa especially rainfed lowland ecology is conducive for build up of pest populations. Pesticide use in Africa accounts for less than 5% of global pesticide use and per hectare averages are low, estimated at around 1 kg/ha active ingredient applied (compared with 3- 7kg/ha in Latin America and Asia (PAN, 2010). However, low use volumes do not necessarily equate to low risk, particularly as some of the most toxic pesticides continue to be applied in Africa especially in West Africa, often under extremely dangerous conditions (PAN, 2010). Though, there are differences in the rate of agrochemicals application across the agroecological zones, pesticide use was high in dry savannah of West Africa (Ephraim *et al.,* 2010). Pesticide use in Africa accounts for only 2–4% of the global pesticide market of US$31 billion (Williamson *et al.,* 2008). Although Africa is currently neither a major consumer nor producer of chemicals in global terms, pesticides use in the African agricultural sector is likely to increase as a result of the growing commercialization as well as the growing focus of development agencies on improving yields of small farmers (Nelson *et al.,* 2006). Most African countries were net importers of pesticides. In Ghana, the number of pesticides dumped by the chemical industries was between 163 to 180 units as at 2002 (Suglo, 2002). In Kabba area of Kogi State, Nigeria, the number of pesticide users increased dramatically from 42% in 1971 to 78% in 1998 (Youm *et al.,* 1990). Importation of agrochemicals into sub-Sahara Africa increased in monetary values from $16.1 million in 1973 to $30 million in 1977 (Youm *et al.,* 1990). Most of the pesticides brought into West African countries have been banned. Pesticide that is banned for agricultural purposes in 52 countries due to its hazardous nature is being used in Ghanaian agriculture (Glover *et al.,* 2008). Most farmers in Africa increasingly depend on pesticides alone to control insect pest, and without satisfactory understanding of the associated hazards. Nigeria ranked first among West African countries in terms of quantities of pesticides use (Abete *et al.,* 2000). Thus, Nigeria alone accounted for nearly 93% of UK pesticide exports to West African countries. Pesticides are the main sources of pollution in

Fig. 2. Pesticide application on rice field

the Senegal River Valley of Senegal, Mauritania, and Mali principally for vegetable production and herbicides/fertilizers for irrigated rice cultivation. Overall, we do not want to experience pest and disease resurgence as a result of high use of chemical pesticides (Figure 2). The only way we can prevent it or reduce the negative effect is to educate irrigated rice farmers on the danger ahead of the indiscriminately use of pesticides. There is high overuse of chemical fertilizers and pesticides in cotton compared to rice in West Africa.

3. Cases of pesticide mis-use

Over the decades, chemical pesticide use has posed a threat to subsistence farming in West Africa because of the well known technical drawbacks such as high cost, lack of adequate protection for the user, absence of safety warnings, excessive and wasteful use leading to environmental pollution. A case in point is the Gezira irrigation scheme in Sudan, where continuous use of pesticides against the cotton jassid, *Empoaca lybica* has led to resistance in the whitefly (*Bemisia tabaci*), cotton bollworm (*Helicoverpa armigera*), and aphids (*Aphis gossypii*). This, in turn, has led to even higher rates of pesticide application and the consequent emergence of secondary pest outbreaks due to the selective removal of natural enemies from the crop system. For instance, citrus leafminer (*Liriomyza trifolii*) is native to Asia but has been a minor pest of citrus in Africa until recent years when it is now considered as the major threat to citrus (Abete *et al*., 2000). The picture was not different in Madagascar where *Spodoptera littoralis* became a serious pest due to over-use of chlorinated hydrocarbons including monophos-DDT against cotton pests. Pesticide overuse to control pests in other crops such as cotton, coffee, cacao, groundnuts has resulted in the development of resistance to dieldrin and DDT by two mosquito species, *Anopheles gambiae* Giles and *Anopheles rofipes* (Gough) in the West African countries of Ivory Coast, Nigeria, Ghana, Mali, Burkina Faso, Togo, and Senegal.

In South East Asia, Brown Planthopper (BPH), a secondary pest of rice, suddenly became a major pest due to insecticide misuse. Since 2005, outbreaks of rice BPH have occurred in East-Asian countries such as Vietnam, China and Japan.

3.1 Destruction of non-target organisms and natural enemies

Non-target organisms are organisms that the pesticides are not intended to kill. Natural enemies include insect predators, insect parasitoids, and insect pathogens. Over 98% of sprayed insecticides reach a destination other than their target species, including non- target species. Successful biological control using five exotic parasitoids against the potato tuber moth, *Phthorimaea operculella*, both native of South America was achieved in Zimbabwe and Zambia. Unfortunately, this system has broken down due to increase in pesticide use by farmers unaware of the value of biological control, and due to the need other pests. Overuse of pesticides in Ghana to control cocoa mirids resulted in the killing of numerous non-target beneficial organisms. As a consequence, the shield bug, *Batl1ycoelia thalassina* (HerrichSchaeffer), a secondary pest resurged and caused a yield loss of 18% of the cocoa crop in Ghana's Eastern and Brong-Ahafo Regions (Owusu, 1971, Alfred *et al.,* 2001,).

3.2 Human and animal health hazards

Chemicals pollute the water body thus making it unsafe for human use e.g. drinking, washing of farm produce, etc. Many of the pesticides used are persistent soil contaminants,

whose impact may endure for decades and adversely affect soil conservation (USEPA, 2007). Pesticide related poisoning deaths are often caused by using pesticide packages or containers after they are emptied of toxicants. It was reported by Youm *et al.* (1990) that forty six residents in Ilorin area of Nigeria were hospitalized as a result of "mistakenly drinking or eating pesticides". Also, in a study conducted by Hotton *et al.* (2010) in the northeastern part of Nigeria on effect of pesticide use, he found out various ailments associated with pesticide use and the use of pesticide container. These include: bronchilis chest pain, asthma, cough, running nose, vomiting, nausea, excessive sweating, diarrhea, burning on urination, abdominal pain, irritation of eye, temporarily and permanent lost of vision, weakness of arms, hands and legs, stiffeners of the waist, fatigue, etc. Empty pesticide containers are used to store food because of a lack of understanding on dangers of pesticides, poor pesticide labeling, and a low literacy rate. Pesticides that are applied to crops can volatilize and may be blown by winds into nearby areas, potentially posing a threat to wildlife (Sequoia & Kings, 2007). More importantly, the remains of these pesticides flow back to the streams and river. Some people at the other end will fetch it for drinking and for other domestic activities thus resulting to one ailment or the other depending on the concentration. Fish and other aquatic biota may be harmed by pesticide-contaminated water (Collin *et al.,* 2008).

4. Beyond pesticide application

Resistance in pests due to chemical application is one of the major factors disrupting traditional pest management practices in West Africa. In order to maximize rice production and agricultural intensification while minimizing reliance on expensive pesticides, a long-term pest management strategies including varietal resistance, biological control and improved cultural practices is needed. IPM seeks to integrate multidisciplinary approach (combination of options) with limited pesticide use to provide effective environmentally sound, socially acceptable and economically safe solution to pest problems. AfricaRice and partners have developed some chemical free products for smallholder farmers in West Africa. Specific examples are provided below:

4.1 Varietal resistance

Improving varietal resistance or tolerance to insect pests is one of the most promising options for managing insect pests in West Africa. AfricaRice and partners have identified several Oyrza sativa varieties with resistance/tolerance to the AfRGM. Cisadane (from Indonesia) has been selected as a variety tolerant to AfRGM and released in Nigeria as FARO 51 based on initial selection at NCRI and on-farm studies in Abakaliki by AfricaRice. BW 348-1 (from Sri Lanka) has good tolerance to AfRGM *and* iron toxicity under field conditions. It has been released in Burkina Faso and Mali (WARDA, 2003). Leizhung (from South Korea) is another tolerant variety to AfRGM released in Mali. Suitable lowland NERICAs being screened for insect resistance or tolerance include: NERICA L-25 and NERICA L-49 (Nwilene *et al.,* unpubl. data). AfricaRice identified one tropical *O. sativa* variety (TOS 14519 from The Gambia) with moderate resistance to AfRGM, which is currently used as a resistant check variety in screening. Several traditional *Oryza glaberrimas* (e.g. TOG 7106 – from Mali, TOG 7206 – from Côte d'Ivoire, TOG 7442 – from Nigeria and TOG 6346 – from Liberia) have been found to be highly resistant to the pest (Nwilene *et al.,* 2002).

4.2 Biological control

Biological control is a major component of sustainable agricultural systems that are designed and managed to reduce dependence on chemical and other energy-based inputs, minimize ecological risk resulting from farming practices, and enhance agricultural productivity in relation to resources available. To ensure that biological control will contribute to sustainable agriculture, AfricaRice identified the gregarious endoparasitoid *Platygaster diplosisae* (Hymenoptera: Platygastridae) and the solitary ectoparasitoid *Aprostocetus procerae* (Hymenoptera: Eulophidae) are the most important wasps attacking AfRGM. The *Paspalum* gall midge (PGM) *Orseolia bonzii* Harris (Diptera: Cecidomyiidae) which infests *Paspalum scrobiculatum* L. (Poaceae), a common weed in rice agroecosystems, is distinct from AfRGM, and is an alternative host for the two main parasitoids of AfRGM. The delay between the destruction of *Paspalum scrobiculatum* and the appearance of AfRGM populations on a rice crop means that the large majority of the parasitoids from *O. bonzii* die before AfRGM population is available – asynchrony between pest and associated natural enemies. AfricaRice has shown that habitat manipulation with *Paspalum scrobiculatum* management at the edge of rice fields had significantly increased the carry-over of parasitoids from *Paspalum* gall midge (*Orseolia bonzii*) to AfRGM. The combination of beneficial organisms, tolerant varieties and habitat management suppressed AfRGM, restored nature's balance, and resulted in increased rice yields (Nwilene et al., 2008a).

4.3 Chemical-free products

Chemical free products for insect pest control include the use of botanicals and biological control using pathogens. AfricaRice has demonstrated that neem seed powder and neem oil can provide effective control against termites in West Africa (Nwilene et al., 2008b). Termites constitute a major biotic constraint to upland rice production in West Africa. The control of termites has largely relied on broad spectrum and persistent organochlorine insecticides. Land use practices can affect the flow of water and persistent pesticides along toposequences from the fragile upland to the lowlands thereby causing harmful effect to humans. To meet the needs of upland rice farmers in West Africa, AfricaRice has shown that the biological control pathogen – the entomopathogenic fungus *Metarhizium anisopliae* is effective against termites on rice fields and can also be used as alternative to persistent chemical pesticides because of the serious health and environmental risks in terms of pollution, destruction/death of non-target/useful insects, and the reduction of biodiversity.

4.4 Adoption of IPM practices

The Food and Agriculture Organization of the United Nations in collaboration with technical assistance from AfricaRice introduced the concept of IPM training in farmer field school (FFS) to West Africa through a series of technical cooperation projects in irrigated rice schemes in Ghana, Cote d'Ivoire and Burkina Faso. Following the success of this programme, IPM farmer field school projects were extended to several other countries in West, Eastern and Southern Africa. The initial results obtained by farmers who applied IPM practices for irrigated rice production in Ghana showed that yields of rice were consistently higher in IPM fields than in fields where conventional farming practices were adopted. In the rice fields where farmers adopted IPM practices, pesticide use for pest control was reduced by over 90% and savings on pesticide use amounted to $100 per ha. Net returns from such fields were 32% higher than in farmer practice fields. Data from Mali show

conclusively that by adopting IPM practices farmers are able to increase the production of rice by 9 - 21%, increase revenue by 14% to 35% while at the same time significantly reducing pesticide use by up to 100% (Nacro, 2000; Youdeowei, 2001 and 2004).

5. Conclusion

The need to increase rice yields for present and future generation requires that solutions to these pest problems are found that are both sustainable and adoptable in the socio-economic environment of farming communities. Why are there abundant and diverse natural enemies in West Africa rice ecosystems? The answer is simple – low use of pesticides in rice fields. The high cost of pesticides means that few farmers have access to them at present. AfricaRice has demonstrated that managing, rather than destroying, a "friendly weed" (*Paspalum scrobiculatum*) at the edge of rice fields (good sources of parasitoids - *Platygaster diplosisae* & *Aprostocetus procerae* close to the rice crop) offers African farmers free, non-chemical control of the continent's worst rice insect pest - African rice gall midge (Nwilene *et al.,* 2008b). It is significant to note that the natural enemy populations of rice pests are high and the species diverse in West African rice ecosystems. This is evident in the high levels of parasitism of the AfRGM. This may be the reason why West Africa has not suffered the insect resurgent crisis, that occur in Asian rice ecosystems, where the natural enemies of the brown planthopper, *Nilaparvata lugens* (Stål) are being killed through the misuse and inefficient application of insecticides. This provides evidence that pesticide use is less in West African rice ecosystems and that the natural enemies are being conserved. All future IPM strategies development should be designed to preserve, and possibly enhance, the existing and abundant natural enemy populations in West African rice ecosystems. Whereas the challenge in Asia is to stop farmers from overuse of pesticides, in West Africa, it is to prevent future overuse of pesticides. IPM is the only preventive approach and the way out for pest management in lowland rice ecology. In the long-term, everyone benefits from a healthier environment. This generation must rise up to the task of saving the global environment from pollution by discouraging production and importation of synthetic pesticides into West Africa. Smallholder farmers who use pesticides are often unaware of the adverse effect of pesticide applications. In implementing integrated pest management options, existing farmers' knowledge should be carefully analyzed, refined and integrated into the basket of options for them to choose from. There is a need to revisit a number of national policies related to food production and protection, in order to encourage partnership and participation in the identification, analysis, advocacy, and follow-up of plant protection issues as well as public awareness of the effect of pesticides on food and the environment.

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18

Transgenic Pesticidal Crops and the Environment: The Case of Bt Maize and Natural Enemies

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1. Introduction

Stem borers are the most destructive field insect pests of maize (see Plate 1) in sub-Saharan Africa (SSA) (Insect Resistant Maize for Africa [IRMA], 2001; Songa et al., 2001). Important stem borer species include *Busseola fusca* Fuller (Lepidoptera: Noctuidae), *Chilo partellus* Swinhoe (Lepidoptera: Crambidae) and *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae) (Overholt et al., 1994).

Plate 1. *Sesamia calamistis* larvae feeding on a maize leaf

Stem borer control approaches that have been used (with varied degrees of success) fall into four broad categories: chemical (application of insecticides); cultural (use of a range of farm practices to delay or reduce insect attack); biological (use of natural enemies of stem borers); and host plant resistance (the plant offers its own resistance to insects). The use of Bt maize (genetically modified maize expressing a δ-endotoxin from *Bacillus thuringiensis* and therefore having an in-built ability to produce pesticidal toxins) has been found to be effective in the management of stem borers in other parts of the world (Sharma & Rodomiro, 2000). However, this strategy has not been widely employed in Africa despite recent efforts to develop Bt maize suitable for different agro-ecological zones in the region (Muhammad & Underwood, 2004). Also, there is still significant debate regarding the possible risks posed by this technology (Obonyo et al., 2010). Fears

that have been raised include; food safety and human health concerns, environmental concerns, possible impact on agricultural systems, and socio-economic issues. Regulatory decisions on whether or not to adopt genetically modified (GM) crops should therefore take all these concerns into consideration.

Because the Bt toxin is embodied in the plant itself, Bt crops are regulated as pesticides in some jurisdictions. For example, the US Environmental Protection Agency (EPA) has for a long time regulated Bt crops under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (Frisvold & Reeves, 2010). The Food and Agriculture Organization (FAO) defines a pesticide as "any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies". The term includes substances intended for use as plant growth regulators, defoliants, desiccants, agents for thinning fruit or preventing the premature fall of fruit, or any substances applied to crops either before or after harvest to protect crop produce from deterioration during storage and transport (Food and Agriculture Organization of the United Nations [FAO], 2002).

Used within the context of Integrated Pest Management (IPM), Bt crops offer a number of advantages. They are safe and easy to use, requiring only planting seeds of an adapted, resistant cultivar (Kennedy, 2008). In general, resistant cultivars have been compatible with other IPM tactics, including cultural, biological and chemical controls (Smith, 2005, as cited in Kennedy, 2008). However, it is well established that plant-borne factors that affect herbivores also interact with natural enemies and consequently with the biological control function they provide. Natural enemies such as predators and parasitoids fulfil an important ecological and economic function by helping to keep stem borer populations below the economic injury level and thus contributing to sustainable IPM systems (Romeis et al., 2008a). Most IPM systems aim to enhance biological control through conservation of existing natural enemies (Bale et. al., 2008 as cited in Romeis et al., 2008a). Thus it is important to minimize the non-target effects of other components of IPM such as pesticides or habitat manipulation (Romeis et al., 2008a).

Transgenic insecticidal plants can have impacts on natural enemies (Kennedy & Gould, 2007, as cited in Romeis et al., 2008a); this may stem from changes in either the plant structure, or primary/secondary metabolites. Adverse effects may occur, for example, if the natural enemy is exposed to, and is susceptible to the plant-borne insecticidal factor. These factors can cause population level effects which might lead to changes in the level of biological control that natural enemies provide (Kennedy & Gould, 2007, as cited in Romeis et al., 2008a). This chapter reviews published literature on impacts of Bt maize on stem borer natural enemies, with particular attention to stem borer parasitoids. This is aimed at consolidating information pertaining to the potential impacts of Bt maize on the development and behaviour of maize stem borers and their natural enemies, with special emphasis on stem borer parasitoids such as the larval parasitoids *Cotesia flavipes* Cameron (Hymenoptera: Braconidae), *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae) and *Xanthopimpla stemmmator* Thunberg (Hymenoptera: Ichneumonidae). However, this is not

an environmental risk assessment of Bt maize but an analysis of the possible impacts of Bt maize on one category of beneficial organisms in the ecosystem.

2. Bt maize

The Bt maize plant has a built-in system that consistently delivers the Bt toxins to the target pest throughout the growing season. Bt maize has been used to control a common maize stem borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae), in the northern temperate region (Matilde et al., 2006). Indeed, Bt maize has been commercially grown in the U.S.A since 1995 and the area under its cultivation is increasing (Minorsky, 2001; Sakiko, 2006). In Africa, Bt maize is commercially grown only in South Africa, though its cultivation is expected to spread to other countries of SSA (James, 2001).

Bacillus thuringiensis toxins expressed in current commercially available Bt maize hybrids are selective in their mode of action (Swadener, 1994). Therefore some authors (e.g., Schuler et al., 1999a) claim that the effects of Bt maize on non-target arthropods associated with maize should be minimal. However, it was Bt maize that was involved in significant controversy (the "monarch butterfly controversy"), following the publication, in *Nature,* of a preliminary study by Losey et al. (1999) (Minorsky 2001). Indeed, Losey et al. (1999) raised serious concerns about the ecological safety of Bt maize cultivation to non-target lepidopterans, in particular the larvae of the monarch butterfly, *Danaus plexipus* L. (Lepidoptera: Danainae). On the basis of laboratory assays, the authors concluded that monarch larvae reared on milkweed (*Asclepias syriaca*) leaves dusted with pollen from Bt maize ate less, grew more slowly, and suffered higher mortality than those reared on leaves dusted with non-transformed maize or on leaves without pollen. The conclusions of Losey et al. (1999) were challenged by other scientists on three grounds. First, the pollen doses used by Losey et al. (1999) were not quantitatively measured but were gauged by eye to match pollen dustings on milkweed leaves collected in the field. This raised concerns about subconscious biases on the part of the researchers. Secondly, concerns were raised as to the validity of extrapolating from the results of Losey at al. (1999), which considered only one type of pollen, to all types of Bt maize pollen. Lastly, the soundness of extrapolating from laboratory assays to the field was uncertain, although a subsequent field study by Jesse & Obrycki (2000) did seem to confirm the fears raised by the Losey et al. (1999) study (Minorsky, 2001). However, it should be emphasised that the continuous expression of the Bt toxins in the plant tissues throughout the growing period (Baumgarte & Tebbe, 2005) increases the chances and degree of exposure to non-target insects of ecological and economic importance. Hence, there are concerns about the possible adverse impacts of this novel pest control technology on the higher trophic level non-target arthropods (such as pollinators, pollen feeders and natural enemies of pests) through crop plant-based food chains.

2.1 Bacillus thuringiensis and genetic engineering technology

Bacillus thuringiensis is a soil-dwelling bacterium that produces large amounts of insecticidal δ-endotoxin when it sporulates into a resting stage. This bacterium, abundantly found in grain dust from silos and other grain storage facilities, was discovered in Japan in 1901 by Ishawata (Baum et al., 1999). Bt is related to two other important spore-forming bacilli, *B. cereus* and *B. anthracis* and is differentiated largely on the basis of containing several plasmid-encoded protoxin genes (Aronson & Shai, 2001). There are hundreds of Bt

subspecies and most produce, primarily during sporulation, one or more parasporal inclusions each comprised of either one or several related insecticidal protoxins, the so-called δ-endotoxins (Schnepf et al., 1998). These endotoxins are biologically inactive protein toxins that crystallize into characteristic shapes. In bacteria, the endotoxins are mixtures of several specific crystalline protein toxins (hence referred to as Cry proteins, Ostlie et al., 1997) that are divided into several numbered classes; these are in turn subdivided into subclasses (Andow & Hilbeck, 2004). The mode of action of *B. thuringiensis* toxins (each of which is active on a subset of insect larvae from at least three orders of insects - Coleoptera, Lepidoptera, and Diptera, Gould and Keeton, 1996) involves ingestion followed by crystal solubilisation and proteolytic activation of protoxin in the insect midgut. Activated toxin binds to receptors in the midgut epithelial membrane and inserts into the membrane, leading to cell lysis and death of the insect (Schnepf et al., 1998). Because of unique but overlapping specificity profiles, Bt subspecies are generally effective against a broad range of insects, usually within a particular order of insects (Aronson & Shai, 2001). Also, many produce, during growth, less well characterized insecticidal proteins, the so-called vegetative insecticidal proteins (Estruch et al., 1996) as well as other pathogenic factors (Agaisee et al., 1999).

Researchers have isolated the δ-endotoxin gene from different strains of Bt, and have expressed it in several crops in order to control lepidopteran and coleopteran pests (Groot & Dicke, 2002). Several of the isolated proteins have selective insecticidal properties against specific insect species (Andow & Hutchison, 1998). Therefore not all commercial Bt maize hybrids express the same insecticidal protein. Moreover, Bt maize plants may not express the protein uniformly throughout the plant, nor continuously throughout the crop season. Bt maize hybrids containing and expressing one of four proteins *Cry1Ab*, *Cry1Ac* or *Cry9C*, and *Cry1F* have been developed and made available since 1996. Cry genes from *B. thuringiensis* are randomly inserted into plant chromosomes at different insertion sites via microprojectile bombardment using a particle gun technique (Bohorova et al., 1999). A promoter, a DNA sequence that regulates where, when, and to what degree an associated Cry gene is expressed (Ostlie et al., 1997), is attached to a Cry gene before it is inserted into a maize chromosome. A successful insertion of the new genetic package containing the modified Bt gene into a plant is called a transformation event (Rice & Pilcher, 1998). Different transformation events (in maize) provide varying levels of resistance to insect pest targets (Williams et al., 1997).

2.2 Plant-Insect tritrophic systems and Bt crops

Natural enemies have an important role to play in the co-evolution of plants and insects (Romeis et al., 2008a). "The third trophic level must be considered part of a plant's battery of defences against herbivores" (Price et al., 1980 as cited in Romeis et al., 2008a). Plant protection by natural enemies is well documented and has been manipulated in the development of biological control strategies in many crops (Dicke & Sabelis, 1988; Whitman, 1994). Plants are well placed to influence the efficiency of parasitism and predation and they mediate numerous interactions between entomophagous arthropods and herbivores. Their structures and products often supply essential resources for parasitoids and predators. In addition, chemical and morphological plant attributes may affect the efficacy of biological control agents by influencing their abundance, survival, development time, fecundity and

rate of attack (De Moraes et al., 2000). Moreover, plants influence the quality of parasitoids' herbivorous hosts by determining the quality of the host's nutrient intake (Vinson & Barbosa, 1987). Several studies show that secondary compounds ingested by the host affect parasitoids, either negatively or positively (De Moraes et al., 2000). Toxins and low nutritional quality may weaken the herbivore's immune system thus affecting its capacity to defend itself against parasitoid eggs (Benrey & Denno, 1997; Van den Berg & Van Wyk, 2007; Vinson & Barbosa, 1987).

In order to exploit arthropod herbivores, natural enemies must be able to locate small, highly dispersed targets within a complex spatial and chemical environment (De Moraes et al., 2000). Besides, herbivores have evolved numerous adaptations to avoid being discovered and attacked (Vet & Dicke, 1992). Plants provide both olfactory and visual signals used as foraging cues by parasitic and predaceous arthropods (Ma et al., 1992; Powell & Wright, 1991; Turlings et al., 1995). Some parasitoids use volatiles emitted by undamaged plants to locate the habitat and possibly microhabitat of their host (Ma et al., 1992; Ngi-Song et al., 1996). Plant volatiles released in response to mechanical damage by herbivores are known to be attractive to various parasitoids (Mattiaci et al., 1994; Steinberg et al., 1993). Volatiles released in response to herbivore feeding are generally reliable indicators of herbivore presence and can potentially bring parasitoids in close proximity to their hosts (De Moraes et al., 2000). Indeed, plants are actively involved in the production and release of chemical cues that guide foraging parasitoids (Turlings et al., 1995). Therefore Bt maize may affect, negatively or otherwise, host finding through the volatile emissions.

Extensive research has been published on the impacts of Bt plants on natural enemies within the context of agro-ecosystems (O' Callaghan et al., 2005; Romeis et al., 2006). Long-term, large scale field studies have indicated no meaningful impacts of Bt maize on predator populations even when the predator has acquired the toxin by feeding on intoxicated prey (Hellmich et al., 2005, as cited in Shelton et al., 2008). In addition, studies in which Bt crops were compared to conventional crops treated with insecticides have demonstrated the latter to be far more harmful to predators (Shelton et al., 2008). The situation, however, appears to be more complex for parasitoids. While an insect predator is characterised by feeding on multiple and various hosts during its lifetime, a parasitoid usually completes its entire lifetime within a single host and derives all its nutritional requirements by feeding on the host tissues. This intimate relationship between a parasitoid and its host would put the parasitoid at greater risk to any hazard its host encounters (Shelton et al., 2008). Parasitoids inside dead lepidopteran larvae that are exposed to *B. thuringiensis* usually suffer the same fate as the larvae. Thus, death of herbivore larvae caused by *B. thuringiensis* toxins may be detrimental to populations of parasitoids. Indeed, studies have found that herbivore larvae that were exposed to *B. thuringiensis*, but were themselves resistant to its effects, supported the normal development of parasitoids (Chilcutt & Tabashnik, 1999; Schuler et al., 1999a). Because the strains of *B. thuringiensis* currently in use are largely specific to Lepidoptera, there may be no direct consequences of *B. thuringiensis* on predators and parasites of herbivores (Agrawal, 2000). However, *B. thuringiensis* may have indirect negative effects on the populations of natural enemies of herbivores through the consumption of sick, dead, or dying herbivores (Agrawal, 2000). Critical questions that need to be considered in assessing the effect of Bt on natural enemies include: Do predators and parasitoids of herbivores avoid Bt exposed prey? Could behavioural mechanisms in parasitoids potentially reduce the

indirect negative effect of Bt? Because the feeding of susceptible caterpillars on Bt plants is severely reduced, and plant damage attracts parasitoids, parasitoids may preferentially be attracted to either resistant larvae or susceptible larvae on Bt plants (Schuler et al., 1999b). Thus, a potential tri-trophic benefit of employing *B. thuringiensis* in agriculture is that parasitoids may act as agents for minimizing the evolution of resistance to *B. thuringiensis* in pests (Agrawal, 2000).

Bt toxins may have indirect effects on beneficial insects such as parasitoids either by killing the intoxicated host (Schuler et al., 1999a), or rendering the host nutritionally unsuitable (Down et al., 2000). In turn, parasitoids' host quality may be influenced by host plants, giving rise to tri-trophic interactions (Price et al., 1980). For example, Ashouri et al. (2001) reported reduced weight of adult *Aphidius nigripes* Ashmead (Hymenoptera: Braconidae) developing on *Macrosiphum euphorbiae* Thomas (Homoptera: Aphididae) that was feeding on Bt potato. Other studies (e.g. Atwood et al., 1997a,b; Liu et al., 2004) showed that when host larvae were fed on a diet containing Bt protein, larval duration, pupal weight, body weight of the newly emerged adult, parasitoid emergence rates and adult longevity were negatively affected. *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) developing inside *Pseudoplusia includens* Walker (Lepidoptera: Noctuidae) larvae that was feeding on Bt cotton suffered reduced longevity, and females had fewer ova (Baur and Boethel, 2003). *Cotesia flavipes* larval emergence was lower in Bt fed *C. partellus* larvae (23%), compared with non-Bt fed *C. partellus*, (83%) (Prütz & Dettner, 2004). Cocoon numbers and cocoon weight of parasitoids were decreased when *Helicoverpa armirgera* Hubner (Lepidoptera: Noctuidae) larvae fed on diet containing transgenic cotton leaf powder containing *Cry1A* plus *CpT* (Ren et al., 2004). Liu et al. (2005a), in studies on the effects of transgenic cotton on *Campoketis chloridae* Uchida (Hymenoptera: Ichneumonidae) observed that the body weight of larvae of the parasitoids were significantly reduced when parasitized hosts fed on transgenic cotton leaves compared to those fed on traditional cotton. Duration of egg and larvae stage were significantly prolonged while pupal and adult weight of *C. chloridae* was decreased when the host larvae fed on transgenic cotton leaves longer than 48 hours. Development of the larval parasitoid, *Microplitis mediator* Haliday (Hymenoptera: Braconidae) was negatively affected when the host, *H. armirgera*, larvae were reared on a diet containing *Cry1Ac* toxin (Liu et al., 2005b).

Nonetheless, a number of investigations show that Bt toxins are not pathogenic to parasitoids developing in infected hosts. For example, Orr & Landis (1997) observed that parasitism of European corn borer larvae by *Eriborus terebrans* Gravenhorst and *Macrocentrus grandii* Goidanich (Hymenoptera: Braconidae) was not significantly different in transgenic and non-transgenic plots. Schuler et al. (2004) observed that *Cotesia plutellae* Kurdjumov (Hymenoptera: Braconidae) eggs laid in Bt resistant *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae), fed on Bt oilseed rape leaves, developed to maturity and there was no effect of Bt plants on percentage parasitism, time to emergence from hosts, time to adult emergence, and percentage adult emergence from cocoons. Parasitoids reared on Bt susceptible hosts hatched, although premature host mortality did not allow the *C. plutellae* larvae to complete their development. This may support the thesis that the Bt toxin has no direct impacts on parasitoids, but rather that the impacts may be due to reduced host quality. Data so far indicate that parasitoids, in general, may be more susceptible to host quality and host-mediated impacts of GM crops compared with to predators (Lovei & Arpaia, 2005).

From the foregoing, it is apparent that Bt plants may affect natural enemies either directly or indirectly. For the insecticidal proteins of insect resistant GM plants to directly affect an individual natural enemy, the organism has to not only be exposed to the toxin but also be susceptible to it. Thus, an organism is not affected by the GM plant when either exposure or sensitivity (hazard) does not occur. However, for an effect to be of ecological relevance it must result in changes in population or community processes. Similarly, direct or indirect effects of the GM plant on individual natural enemies, natural enemy species or groups/guilds of natural enemies might not lead to a decreased biological control function (Romeis et al., 2008a). Moreover, natural enemies may be affected indirectly by the GM plant when they feed on sublethally impaired herbivores (sick prey). Such effects appear to be caused by declines in nutritional quality of the host/prey organism. These prey/host quality mediated effects appear to account for most (if not all) of the Bt plants' effects on natural enemies that have been reported from laboratory and glasshouse studies (Romeis et al., 2006). It is well established that parasitoids are especially vulnerable to changes in their hosts' quality, since they usually complete their development in a single host (Godfray, 1994). Therefore this review lays particular emphasis on the potential impacts of Bt maize on stem borer parasitoids. Bt maize are deployed to control Lepidoptera, which implies that lepidopteran parasitoid hosts would (as a direct consequence of being affected by the Bt toxin) invariably be less suitable for parasitoid development. Thus it is not surprising that parasitoid life-table parameters are significantly affected when the host suffers (Romeis et al., 2006). In extreme cases, parasitoids attack sublethally affected hosts that die before the parasitoid offspring completes development (Davidson et al., 2006; Schuler et al., 2004). Sections 2.2.1 to 2.2.5 provides a review of the potential impacts of Bt maize on stem borers and their natural enemies (specifically parasitoids).

2.2.1 Effect of Bt maize on stem borer oviposition preference

One of the major risks associated with the use of transgenic pesticidal crops is that pests can develop resistance which could reduce the efficacy of such crops as plant protection tools (Wolfenbarger & Phifer, 2000). Furthermore, if larvae developed resistance to the Bt toxin, there could be greater chances of natural enemies getting host-mediated exposure to the toxin (Obonyo et al., 2008a). When the US EPA reviewed the first registration for Bt plants, there was considerable concern in some sectors that resistance to the plants would rapidly occur and that not only would this be a concern to growers of Bt crops but also to organic farmers who relied on Bt as a foliar spray (Shelton et al, 2008). The high dose/refuge strategy (the use of high doses of one or more toxins, combined with a refuge of non-Bt plants) has been proposed as a likely means to delay the development of resistance by insects against transgenic plants (Bates et al., 2005). This strategy emphasises the presence of susceptible insect populations; these may slow down the evolution of resistance (Bentur et al., 2000; Shelton et al., 2000; Tang et al., 2001). The premise is that susceptible insects, if present in sufficient numbers, would mate with resistant insects and dilute resistance genes. However, several biological factors that influence the number of insects exposed to Bt toxin may substantially affect the success of the high dose/refuge strategy (Ives & Andow, 2002). One such factor is oviposition preference. Preference for Bt maize would require more refuge plants to counter an increased selection pressure. However, preference for refuge plants could have the opposite effect. From a resistance management perspective, an ideal

plant, in addition to killing larvae, should repel adult oviposition (Hellmich et al., 1999). This would reduce selection for resistance because fewer larvae would be exposed to plant toxins. Potential effects of Bt transgenic maize on stem borer natural enemies could therefore partly depend on the oviposition preferences of stem borers, either for Bt or non-Bt maize.
Various studies have been conducted on effects of Bt maize on stem borer oviposition behaviour. In field tests, the number of eggs laid by susceptible European corn borer females did not differ between Bt corn (containing *Cry1Ab*) and non-Bt corn (Orr & Landis, 1997). Pilcher & Rice (2001) observed that *O. nubilalis* females did not show any oviposition preference towards non-Bt or Bt maize (using Event 176 and Bt11). Van den Berg & van Wyk (2007) reported that *S. calamistis* adults did not differentiate between Bt and non-Bt maize plants in oviposition choice experiments. More recently, Obonyo et al. (2008a) observed that *C. partellus* and *S. calamistis* moths did not discriminate between Bt and non-Bt maize plants for egg laying. This non-discriminatory oviposition behaviour could be due to the fact that the ratios of caterpillar-induced odour emissions of Bt maize plants are identical to those of non-Bt plants (Turlings et al., 2005) since genetic modification does not alter the volatile profile of undamaged maize plants (Dean & De Moraes, 2006). These results have important implications for pest resistance management and monitoring. Because oviposition is not affected by the Bt toxin, and females are exposed equally to Bt maize and non-Bt maize refuges, it can be assumed that eggs will be distributed equally between Bt and non-Bt maize hence there will always be a pool of insects on susceptible crops, which is necessary for resistance management and hence ensuring that the development of resistance is delayed as much as possible. Furthermore, since the development of resistance against Bt toxins requires the survival and development of at least two exposed larvae into a male and a female (Kumar, 2004) and since Bt maize causes up to 100% mortality (Obonyo et al., 2008a) the possibility of resistance development would be further restricted.

2.2.2 Effect of Bt maize on stem borer development and mortality

An understanding of the effect of Bt toxins on development of herbivorous insects is important because host development time could have a direct effect on natural enemies by influencing the 'window of vulnerability', the period during which the host is exposed to natural enemies (Schoenmaker et al., 2001; Schuler et al., 1999a; Wallner et al,. 1983). Also, the combined effects of developmental delays may result in temporal asynchrony of moths emerging from Bt and non-Bt maize- resulting in susceptible individuals mating before resistant adults emerge (Horner et al., 2003). Since the success of the refuge strategy requires that any resistant individuals mate with susceptible ones, such asynchrony in emergence from Bt and non-Bt maize plants could compromise the strategy and hence weaken the potential of Bt maize as an option for stem borer control. Furthermore, effects of Bt plants on host development could impact on the biology of a natural enemy developing in such a host (Walker et al., 2007; Weseloh, 1984).
Obonyo et al. (2008b) observed that Bt maize had significant effects on stem borer development time. Feeding of stem borer larvae on Bt plant tissue at the 3rd and 4th instars significantly lengthened the duration of the respective instars (but not the subsequent ones) while overall larval development time was not affected probably because the larvae were exposed to Bt for a relatively short duration. Schoenmaker et al. (2001) suggested that ingestion (by lepidopteran larvae) of sublethal doses of Bt toxin prolonged development time by temporarily inhibiting feeding. Continuous exposure to Bt toxin

prolonged development of *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) while exposure to toxin for shortened durations had no significant effects on larval development time (Dutton et al., 2005a). Therefore it seems that larvae may recover from the effects of the Bt-toxin, following transient exposure. Other lepidopteran larvae that ingest sublethal doses of Bt also resume normal development after a few days (Moreau and Bauce, 2003; Siegfried et al., 2001). Dutton et al. (2005a) reported that there were no significant effects on overall larval development when 3rd instars of *S. littoralis* larvae were exposed to Bt sprayed plants because the effect of the toxin did not persist for long due to rapid degradation of the Bt spray (Haddad et al., 2005). In contrast, significant effects, attributed to long toxin persistence, were reported when larvae were reared for four days on Bt maize (Haddad et al., 2005). Huang et al. (2006) observed larval development inhibition of *O. nubilalis*, *D. grandiosella* and *Diatraea saccharalis* F. (Lepidoptera: Pyralidae) fed on a diet prepared from *Cry1Ab* protein (extracted from Bt corn leaves). Similarly, transgenic maize containing *Cry1Ab* delayed larval development of *H. zeae* (Horner et al., 2003; Stewart et al., 2001) and *D. plexippus* (Dively et al., 2004). Development time of the 5th instar of *C. partellus* larvae subjected to transient feeding on Bt maize at the same growth stage was not affected (Obonyo et al., 2008b), possibly because pupation follows shortly after the 5th larval stage in this species at which time the larvae are relatively inactive and do not feed much (Tettamanti et al., 2007); and their large sizes enable them to tolerate more toxin (Huang et al., 1999). Overall, larval development time in these larvae was significantly longer as a consequence of Bt exposure (Obonyo et al., 2008b). This indicates a disturbance to the "normal" development cycle, from which the larvae may eventually recover. The increase in larval development time therefore increases the window of vulnerability during which stem borer parasitoids can get host mediated exposure to the Bt toxin. This in itself may not be cause for concern but rather the consequences of such exposure. Possible consequences of host-mediated exposure to Bt toxins are discussed in subsequent sections of this chapter.

2.2.3 Effect of Bt maize on the ability of parasitoids to locate hosts

The success of biological control agents depends on their efficiency to search for, and locate target hosts (Nordlund et al., 1988). Parasitoid host finding behaviour is complex and influenced by many factors (Ngi-Song et al., 1996; van Leerdam et al., 1985). One important factor is volatiles emitted by the host plant. There are significant quantitative (Turlings et al., 2005) and qualitative (Dean and De Moraes, 2006) differences in volatile emissions between Bt and non-Bt plants. Both the quantity and composition of emitted volatiles influence host finding by *Cotesia* species (Steinberg et al., 1993). Host species odours are also used by parasitoids for host location and hence any change in host physiology may alter parasitoids' host location behaviour (Takasu & Lewis, 2003).

Bt maize may influence host species odours and thus parasitoid host finding behaviour. Obonyo (2009) showed that damaged but uninfested Bt and non-Bt maize were similarly attractive to females of the larval parasitoids *C. flavipes* and *C. sesamiae*, and that both were also more attractive than the control air flow from a plantless cage. Females of *C. flavipes* and *C. sesamiae* were equally attracted to stem borer infested maize plants (irrespective of Bt status). This suggests that females of *C. flavipes* and *C. sesamiae* do not distinguish among plant- and host-derived cues from Bt and non-Bt maize when searching for stem borer hosts. Therefore the presence of Bt toxin in maize plants apparently did not affect the host location

process of these parasitoids. Similar findings have been reported elsewhere (Ngi-Song and Overholt, 1997; Potting et al., 1997). Also, *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) and Microplitis rufiventris Kokujev (Hymenoptera: Braconidae), which are important larval parasitoids, were not able to distinguish between the odours of a Bt maize event and its near-isogenic line (Turlings et al., 2005). This indicates that growing Bt maize is not likely to affect host finding by stem borer larval parasitoids.

Furthermore, plant volatiles may act as cues for host location by pupal parasitoids (Obonyo 2009). Chemical analyses of collected odours between Bt and non-Bt maize revealed significant quantitative differences (Turlings et al., 2005); this could possibly affect host location by pupal parasitoids such as *X. stemmator*. Indeed, Obonyo (2009) found that *X. stemmator* parasitoids preferred host plant odours compared to odours from a blank control. However, volatiles from Bt plants were deterrent to *X. stemmator*. Oviposition preference of insects has been predicted to correlate with host suitability for offspring development (preference – performance hypothesis) (Jaenike, 1978). This hypothesis (known as the 'mother knows best' principle) (Johnson et al., 2006) was developed for herbivorous insects but is assumed to play an important role in parasitic Hymenoptera as well (Vinson and Iwantsch, 1980). According to this preference – performance hypothesis, *X. stemmator* avoids the Bt plants because potential hosts have a lower quality when feeding on Bt maize. In parasitoids, host organisms are the only source of nutrients for the immature stages (Sequeria & Mackauer, 1992), and thus parental fitness depends on the accurate assessment of host sites for their potential to sustain the development of their larvae (Meyling & Pell, 2006). Therefore adaptation to reliable cues, enabling the evaluation of the quality of potential hosts, is a selective advantage for ovipositing females. Chemical cues may not only attract but also deter parasitoids from entering host sites. Naive females of the solitary ectoparasitoid, *Lariophagus distinguendus* Forster (Hymenoptera: Pteromalidae) which parasitizes immature stages of several stored-product infesting beetle species avoid odours from mouldy grains which are unsuitable for the development of their larvae (Steiner et al., 2007).

Considering that Bt maize has an adverse effect on the host location behaviour of *X. stemmator*, it could possibly compromise the biocontrol potential of this parasitoid, hence impacting negatively on the use of Bt maize as part of IPM strategies.

2.2.4 Effect of Bt maize on parasitoid biology

Changes in host plant chemistry may negatively affect natural enemy fitness through reducing survivorship, clutch size, body size and/or fecundity (Ode, 2006). Such negative impacts may occur either directly (when the natural enemy encounters the toxin in its host or prey) or indirectly (when natural enemy fitness is reduced due to lower prey/host size or quality). A number of studies have found variable results on effects of Bt toxins on parasitoid life history parameters; these include no apparent negative effect (Obonyo, 2009; Schuler et al., 1999a), synergism between the transgenic plants and parasitoids (Tounou et al., 2007), lower parasitoid survival (Blumberg et al., 1997), or emergence rates (Atwood et al., 1997b; Liu et al., 2005a), increased parasitoid development times (Liu et al., 2005a; Liu et al., 2005b; Vojtech et al., 2005), reduced longevity (Baur & Boethel, 2003), reduced body mass (Ashouri et al., 2001; Liu et al., 2005a), and altered parasitoid sex ratios (Wallner et al., 1983).

Changes in host plant chemistry may also affect acceptance of the plants by their hosts, with consequences on associated natural enemies. Obonyo (2009), however, showed that there were

no significant differences in host acceptance ratio between Bt exposed and non-Bt reared larvae. Turlings et al. (2005) observed that braconid parasitoids did not distinguish between odours of Bt and non-Bt maize plants in olfactometer experiments. Although a number of studies have found no significant differences in oviposition choice between hosts fed on transgenic and non-transgenic diets (Bell et al., 1999, Schoenmaker et al., 2001; Schuler et al., 1999b), there are cases where parasitoids seem to distinguish by host quality (reviewed in Steidle and van Loon, 2003; Overholt et al., 1994; Sallam et al., 1999). More recently, Obonyo (2009) reported a higher host acceptance ratio of *C. flavipes* for *C. partellus* compared with *S. calamistis*. These contrasting results could be due to the different dietary material (plant material and microbial formulations) used in the various studies. However, it is more likely that the lack of significant effects of Bt was due to the transient feeding of the host on Bt maize (Obonyo, 2009). Negative effects of Bt toxins on parasitoids are often indirect, occurring via reduced host quality (Chen et al., 2008; Vojtech et al., 2005; Walker et al., 2007) but larvae exposed to the Bt toxin and subsequently transferred to a Bt-free diet may recover by replacing damaged mid-gut cells and excreting the toxin (Tounou et al., 2007).

As already mentioned, host quality directly impacts on parasitoid development. Ingestion of Bt toxins by stem borer larvae could therefore affect parasitoid developing within these larvae. Temerak (1980), Salama et al. (1991) and Atwood et al. (1997b) observed that incorporation of microbial Bt formulations in host food decreased the emergence of parasitoids larvae. Wanyama (2004) found that Bt contaminated diets significantly increased *C. partellus* cocoon development time. Bernal et al. (2002) found a longer development time of *Parallorghas pyralophagus* Marsh (Hymenoptera: Braconidae) on Bt fed hosts. In contrast, Obonyo (2009) and Prutz & Dettner (2004) observed no effects of host ingested Bt toxins on the mortality of *C. flavipes* inside cocoons. Also, Prutz & Dettner (2004) found no significant effects of Bt-contaminated diets on *C. flavipes* pre-cocoon development time.

Besides, the proportion of female parasitoids produced in each generation is an important factor in the success and survival of parasitoid populations (Godfray, 1994). A female biased sex ratio is an important characteristic of a biocontrol agent, especially parasitoids, because only females contribute to pest mortality upon release (Waage, 1982). In fact, the failures of numerous biological control projects have been attributed to male biased sex ratios (Stouthamer et al., 1992). A number of studies (e.g., Bernal et al., 2002; Obonyo, 2009, Prutz & Dettner, 2004; Walker et al., 2007) have reported no effect of Bt toxin on sex ratios of parasitoid progeny. Therefore adoption of Bt maize is not likely to impact on larval parasitoid sex ratios and/or parasitoid populations. It is clear though that Bt maize may have variable effects on the biology (and hence the effectiveness) of stem borer parasitoids.

2.2.5 Effect of Bt maize on fluctuating asymmetry of parasitoids and parasitoid size

Environmental or genetic stress can cause an increase in the fluctuating asymmetry (FA) of bilaterally morphological traits (Parsons, 1990) and hence may be used as a measure of the ability of individuals to cope with different kinds of environmental stresses (Jones, 1987; Leary and Allendorf, 1989; Parsons, 1990). FA refers to random deviations from symmetry of otherwise bilaterally symmetric traits; it occurs when an individual is unable to undergo identical development on both sides of a bilaterally symmetrical trait (Liu et al., 2005c). Environmental stress, including extreme temperatures (Mpho et al., 2002; Sciulli et al., 1979),

pesticides (Hoffmann & Parsons, 1990), and qualitative and/or quantitative food deficiency (Liu et al., 2005c; Parsons, 1990) may cause FA in morphological traits during development. For example, the FA value of the third segment of antenna in aphids was significantly higher on Bt cotton compared to control cotton (Liu et al., 2005c). Generally, there is a negative correlation between the degree of FA and the fitness of populations (Moller, 1997). For instance, the lifespan of *Malacosoma disstria* Hubner (Lepidoptera: Lasiocampidae) shortened as the degree of FA of the first segment of foreleg tarsi increased (Naugler and Leech, 1994). Wing asymmetry, in contrast, may influence flight ability and hence the ability of parasitoids to reach hosts (Bennet & Hoffmann, 1998).

There have been variable results on effects of exposure to Bt toxins on FA values. Obonyo (2009) observed that transient feeding of hosts on Bt maize had a number of effects on FA depending on the trait under consideration as well as the host and parasitoids species. Transient feeding of *C. partellus* and *S. calamistis* hosts on Bt maize adversely affected *C. sesamiae* and *C. flavipes* parasitoids, respectively; this was reflected in the higher FA values for antennal length and wing length in *C. sesamiae* and *C. flavipes*, respectively that were developing on hosts subjected to transient feeding on Bt maize. In contrast, *C. sesamiae* exposed to *S. calamistis* hosts that had been subjected to transient feeding on Bt maize showed lower FA values. The lower FA values could be due to the improved performance of *C. sesamiae* on Bt exposed *S. calamistis* thus indicating that there could be instances where Bt maize could actually enhance parasitoid performance. Similarly, Tounou et al. (2007) observed positive effects of Bt intoxicated *S. calamistis* larvae on *C. sesamiae*. Such positive effects (on exposure to Bt) could be attributed to the weakening of the immune system of the host, resulting in lower encapsulation rate of the parasitoids eggs by the host larva (Tounou et al., 2007). Encapsulation of *C. sesamiae* eggs has been reported in *S. calamistis* (Gitau et al., 2007; Hailemichael, 1998). It is possible that this encapsulation reaction could affect parasitoid development. The success of the encapsulation reaction depends on the vigour of the herbivore (Siva-Jothy & Thompson, 2002) which may be reduced by host plant induced stresses (Blumberg, 1997, Souissi & Le-Ru, 1998, Turlings & Benrey, 1998). The parasitoid *Cotesia kazak* Telenga (Hymenoptera: Braconidae) has more success on its host *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae), fed on less toxic Bt-amended diets (Walker et al., 2007) compared with *Tranosema rostrale rostrale* Brishke (Hymenoptera: Ichneumonidae) developing on Bt fed spruce budworm *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae) (Schoenmaker et al., 2001). Transient feeding of hosts on Bt maize apparently had no significant effects on FA of antennae and wing lengths in the other host parasitoid combinations (Obonyo, 2009). This was possibly because the levels of toxin ingested were not high enough to affect these traits in these host-parasitoid combinations or alternatively because these traits are not sensitive to the effects of the Bt toxins. Transient feeding of *S. calamistis* on Bt maize did not adversely affect *C. flavipes* development. However, *C. sesamiae* developing on *C. partellus* subjected to transient feeding on Bt maize had greater FA values compared with those developing on *C. partellus* exclusively reared on non-Bt maize. Similarly, Wanyama (2004) did not detect any significant effects of transient feeding of host on Bt toxins on parasitoid development time. Even though a significant difference was not detected in the percentage of dissected pupae that contained immature parasitoids, they were much fewer on pupae from larvae exposed to transient feeding on Bt maize. Wanyama (2004) found a significantly higher proportion of dead parasitoids in pupae of Bt fed hosts.

Female parasitoids are usually able to control the proportion of fertilized eggs, which they oviposit, depending on host size and quality (Charnov and Stevens, 1988). The strong male bias in Bt fed hosts could indicate inferior host quality. Obonyo (2009) and Liu et al., (2005d) showed that longevity of adult parasitoid wasps was unaffected by transient feeding on Bt maize, indicating that adoption of Bt maize may not have significant effects on parasitoid longevity and may therefore not adversely impact on parasitoid biocontrol potential.

Therefore it is clear that where transient feeding of hosts on Bt maize increases FA, it does so in a trait-specific way. The lack of consistency in FA responses across traits may reflect variation between trait types in their susceptibility to environmental stress (Woods et al, 1999). We conclude that the FA of some traits (and consequently parasitoid fitness) in the parasitoids may be affected by transient feeding of their hosts on Bt maize. Thus in assessing the risk posed to non-target organisms by transgenic plants it may be necessary to ascertain the relationship between fitness parameters and the FA values of the non-target organisms.

In addition to FA, parasitoid size may also have an impact on parasitoid fitness. Many studies have established positive associations between adult body size and standard laboratory and field fitness measures (e.g., Godfray, 1994; Bennet & Hoffmann, 1998). Increase in hind tibia length increased the fitness of *Trichogramma carverae* Oatman and Pinto (Hymenoptera: Trichogrammatidae) (Bennet and Hoffmann, 1998). Parasitoid size may be determined indirectly by measuring hind tibia length (Bennet & Hoffmann, 1998; Kazmer & Luck, 1995; West et al., 1996), antennal length and wing length. Studies (e.g., Obonyo, 2009) show that transient feeding of hosts on Bt maize has various effects on trait sizes depending on the trait, host and parasitoid species. For example, transient feeding of *C. partellus* on Bt maize adversely affected *C. flavipes* by reducing their antennal length. In contrast, antennal length was significantly increased in *C. sesamiae* developing on *S. calamistis* hosts subjected to transient feeding on Bt maize. The longer antenna lengths in *C. sesamiae* on *S. calamistis* subjected to transient feeding on Bt maize could be due to the improved performance of *C. sesamiae* on Bt exposed *S. calamistis*.

The effect of Bt toxins on wing length and hind tibia length of parasitoids have been reported in a number of studies. Obonyo (2009) showed that wing length of some parasitoid species were affected by feeding of hosts on Bt maize. He also found that hind tibia length were significantly reduced on *C. flavipes* developing on *S. calamistis* hosts subjected to transient feeding on Bt maize, and *C. sesamiae* developing on *C. partellus* subjected to transient feeding on Bt maize. In contrast, hind tibia lengths of *C. sesamiae* were not significantly affected following transient feeding of their *S. calamistis* hosts on Bt maize (Obonyo, 2009). Similarly, Obonyo (2009) did not observe significant effects on parasitoid hind tibia lengths following exposure of *X. stemmator* to hosts that had been subjected to transient feeding on Bt maize. This indicates that Bt toxins probably had no significant effect on parasitoid size. Therefore it is expected that *X. stemmator* emerging from hosts subjected to transient feeding on Bt maize would perform equally well in the field as those emerging from non-Bt fed hosts. Also, the lack of significant differences in FA values between parasitoids reared on Bt fed and non-Bt maize fed hosts may suggest that the Bt toxin did not provide a stressful environment for the developing parasitoids. Although exposure of *X. stemmator* to hosts subjected to transient feeding on Bt maize did not affect the biology of *X. stemmator*, it significantly reduced the proportion of female progeny (Obonyo, 2009). A

female biased sex ratio is important for biocontrol agents, especially parasitoids because only females contribute to pest mortality upon release (Waage, 1982).
It is clear from the foregoing that Bt maize may impact on parasitoid fitness as well as sex ratios and hence could possibly affect parasitoid biocontrol potential.

3. Conclusion

It is difficult to determine, from existing literature, whether the observed host mediated effects of Bt maize on parasitoids are direct or indirect. Parasitoid performance can be affected as a result of the Bt toxin reducing host's biomass (Farrar and Ridgeway, 1995; Deml et al., 1998), or changing the host hemolymph-pH, hemolymph ion concentration, and nutrient concentration (Tanada and Kaya, 1993), which in turn can affect the parasitoid larvae living in the hemolymph. Alternatively, parasitoid larvae may be affected by ingesting the Bt toxin present in the hemolymph. Whatever the case, the most important consideration should be whether Bt maize can cause significant harm to stem borer natural enemies and most significantly the overall environmental consequences of such impacts. In order to determine the overall impact of growing Bt maize on stem borer natural enemies, the issues raised in this chapter should be placed in the context of real-life scenarios, taking into account *inter alia*, local agricultural practices, agro-ecological conditions, trade policies etc.
This review has confined itself to potential impacts of Bt maize on stem borer natural enemies, specifically parasitoids and it is therefore not possible to make generalizations. Any judgment on the potential impact (s) of Bt maize, or any transgenic crop for that matter, should be made on a case-by-case basis using a rational, evidence-based scientific approach. In contrast to application of chemical insecticides with contact toxicity, insecticidal proteins expressed by GM plants have to be ingested to affect parasitoids. Consequently, when assessing the potential impacts of growing Bt maize on natural enemies it may be necessary to assess which organism (s) may be exposed under actual field conditions, and at what level. Indeed, the level at which an organism can be exposed to a plant expressed insecticidal protein may vary depending on the concentration of the toxin in the plant or environment, the plant tissue in which the protein is expressed, and the feeding behaviour of the non-target organism (Dutton et al., 2005b; Romeis et al, 2008a,b,c). Therefore exposure pathways can be predicted only if the relevant information for the GM plant, the environment and the natural enemy is available. Also, it may be necessary to make comparisons between the potential consequences of Bt maize on natural enemies and the use of conventional insecticides. Evidently, most studies have focussed mainly on making comparisons between Bt maize and non-Bt maize, without looking at the alternatives. It is only when a comparative approach is adopted that it may be possible to make prudent judgements regarding this novel method of insecticide delivery. Therefore in assessing the potential impact of Bt maize on the environment, it would be useful to pose the question: how would the use of Bt maize compare to the alternative (s)?" When assessment is not comparative decision making becomes non-objective. For example, it may be known that Bt maize reduces wing lengths of some stem borer parasitoids. However, with this information alone it may not be possible to reach useful conclusions. In contrast, if comparisons were made between the effects of Bt maize and the alternative (s) e.g., broad spectrum insecticides (effect on natural enemies), it would be easier to make more informed and objective conclusions (and hence decisions) regarding this novel technology.

4. Disclaimer

The views expressed in this article are those of the individual authors and do not necessarily reflect the views and policies of the International Centre for Genetic Engineering and Biotechnology or the University of Venda.

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19

Colony Elimination of Subterranean Termites by Bait Application Using Benzoylphenylurea Compounds, with Special Reference to Bistrifluron

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1. Introduction

1.1 Termite as pest insect

Termites are insects that belong to Termitidae and from systematic point of view cockroaches are most closely-related to them. Although termites had been formed the independent order, Isoptera, recently they became a part of Blattodea (Inward et al., 2007). While cockroach is one of the most important household and hygiene pest insects all over the world, termite is also occupying a high position as a household pest insect. However their position is bit different from their closest relatives and is a structural pest which attacks and gives serious damage to wooden structures such as residences [c.f., in developing countries they are pests to trees and agricultural products (Constantino, 2002)]. In Japan, several hundred million dollars are spent annually for the prevention and control of termites (Yoshimura, 2001; Tsunoda, 2003). One thing to be addressed here is that termites are very important decomposers of cellulose in the ecosystem. They become pest insects when their habitats overlap those of humans.

Fig. 1. Left: workers and soldiers of *Coptotermes formosanus* in their nest. Right: Damage on a residential structure caused by foraging termites of *C. formosanus*

Termites are eusocial insects similarly as Hymenopterous insects, like ants and wasps, which are taxonomically distantly-related groups. They usually build up queen-and-king-centered

colonies. Thousands to millions of worker termites are usually deployed in each colony. They are systematically mobilized to attack and feed wooden materials, therefore sometimes give critical damages to wooden structures. Among them, economically the most important group in temperate and tropical regions is Rhinotermitidae which includes genera *Coptotermes* and *Reticulitermes*. Termites belonging to Rhinotermitidae are 'subterranean termites' as they build their nests in the ground. Since they come on underground tunnel and often invade through underfloor space of structures, few people are aware of them until their houses are assaulted. *Coptotermes* and *Reticulitermes* termites widely occur in human habitat and are seriously harmful to structures. *Coptotermes* termites, like *Coptotermes formosanus* Shiraki, *Coptotermes gestroi* (Wasmann), *Coptotermes acinaciformis* (Froggatt) and so on, build huge colonies and extremely problematic species around the Pacific countries (Fig. 1).

1.2 Chemical control of termites and colony elimination by insecticidal baiting

In general, methods to control termites using insecticides are preventive: typically liquid formulations are applied to perimeter or underfloor of a premise or to wooden materials directly. However, their unique characteristics of eusocial nature had been drawn large interests of researchers to control their whole colonies by application of toxic baits. Actually such an approach has been examined for its feasibility for decades and various kinds of insecticide have been evaluated as active ingredients (table 1). Although some insecticides succeeded in reducing population of a colony for the meanwhile, they could seldom bring about elimination of it. In the mid of 1990's benzoylphenylurea (BPU) compounds such as hexaflumuron were proven to be effective as bait toxicants for eliminating rhinotermitid colonies (Su 1994; Su et al., 1997). Since then many studies have documented that BPUs can successfully eliminate subterranean termite colonies in the field (Tsunoda et al., 1998; Peters & Fitzgerald, 1999, 2003; Getty et al., 2000; Sajap et al., 2000; Su et al., 2000; Lee, 2002; Tsunoda et al., 2005; Husseneder et al., 2007; Ripa et al., 2007).

Chemical class or mode of action	Active ingredient	Source
Juvenile hormone analogue	Methoprene	e.g., Su et al., (1985)
	Fenoxycarb	e.g., Jones, (1989)
Metabolic inhibitor	A-9248	e.g., Su & Sheffrahn, (1996)
	Sulfluramid	e.g., Su & Sheffrahn, (1996)
	Hydramethylnon	e.g., Powson & Gold (1996)
Phenylpyrazol	Fipronil	e.g., Huang et al., (2006)
Benzoylphenylurea	Hexaflumuron	e.g., Su, (1994)
	Diflubenzuron	e.g., Rojas & Morales-Ramos (2003)
	Lufenouron	e.g., Haverty et al., (2010)
	Chlorfluazuron	e.g., Peters & Fitzgerald (2003)
	Noviflumuron	e.g., Cabrera & Thoms (2006)

Table 1. Active ingredients that have been evaluated as bait toxicant for termite control in the field

Up to now, only BPUs have been successful as bait toxicants in terms of elimination of subterranean termites' colonies. As BPUs are chitin synthesis inhibitor, it is presumed that their success as bait toxicant should be attributed to 1) their extreme slow action, 2) less dose dependency and 3) less feeding deterrency (Su & Scheffrahn 1993, 1996). However, there have been few reports that tried to examine such properties of BPUs.

1.3 Bistrifluron

Bistrifluron (Fig. 2) is one of BPUs (Kim et al., 2000), therefore it was expected to show good efficacy in terms of colony elimination of subterranean termites. Author conducted several laboratory studies to evaluate inseciticidal efficacy of the compound against subterranean termites in order to examine how baiting with BPUs bring about colony elimination of termites. *C. formosanus* and *Reticulitermes speratus* (Kolbe) which are the most important pest species in Japan were used for the studies.

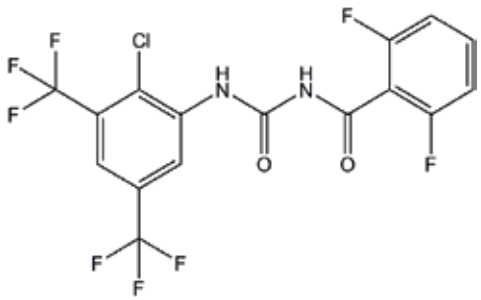

Fig. 2. Chemical structure of bistrifluron

2. Termiticidal activity of bistrifluron and its speed of action

In order to quantitatively evaluate feeding toxicity of bistrifluron against termite, no-choice and two-choice feeding tests with filter paper baits were conducted with *C. formosanus* workers in the laboratory (Kubota et al., 2006).

2.1 Evaluation methods

In the no-choice feeding test, filter paper disks treated with 0.005, 0.05, 0.5 and 5.0% (w/w) of bistrifluron were air-dried and weighed. Each disk was placed in a small plastic cup (ca. 14 ml) with small entry holes to allow termite access. Plastic cups were then put into separate plastic containers (200 ml) and each container retaining 100 of *C. formosanus* workers. The bottom of this larger container was covered with 2-3 mm of plaster and had several small holes made in the base. Assembled units were placed on a damp cotton pad in an incubation chamber so that termites could uptake water through the plaster. Five units were prepared for toxic baits and untreated controls. The units were maintained under appropriate condition for 12 weeks. Dead or moribund termites were counted at given interval to determine the change in mortality over time.

In the two-choice feeding tests, Filter paper disks were treated with 0.5% (w/w) of bistrifluron. The test container was a plastic Petri dish (140 mm in diameter) with approximately 5 mm-thick agar [4% (w/w)] on the bottom. Four wells (35 mm in diameter) were made through the agar and plugged by two treated and two untreated disks. One hundred termites were introduced before the dish was covered with a lid and sealed with parafilm. Units were kept under appropriate condition. Mortality and weight of disks consumed were checked at given intervals after termites were released. Five replications were made.

2.2 Speed of action

In the no-choice test, mortality increases significantly faster as bistrifluron concentration were higher (Fig. 3). When termites were exposed to 0.5% bistrifluron (possible concentration in a commercial product), there had been no significant increase in mortality

until the 4th week. Even when exposed to overdosing 5.0% bistrifluron, 10 times higher concentration, there was no significant increase in mortality at the 1st week. These results indicate that bistrifluron shows very slow action on termites. In comparison to sulfluramid which is often used for ant bait, *C. formosanus* workers exposed to 0.01% (w/w) sulfluramid would die off within a week (Grace et al., 2000). On the other hand, dose dependency of bistrifluron's speed of action suggests that faster or better effect will be obtained by larger dosing. The no-choice feeding test with various concentrations of bistrifluron baits was also conducted against Japanese *R. speratus* workers and similar dose dependency and very slow action at even high doses were also shown (Kubota et al., 2007).

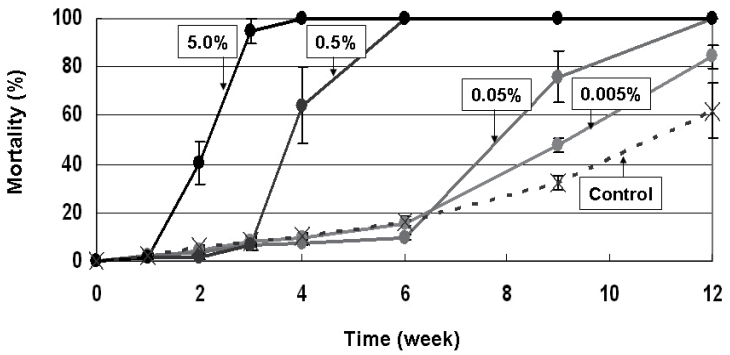

Fig. 3. Time-course change of mortality of *C. formosanus* workers exposed to 0.005, 0.05, 0.5 and 5.0% bistrifluron baits and blank bait in the no-choice feeding test (data from Kubota et al., 2006)

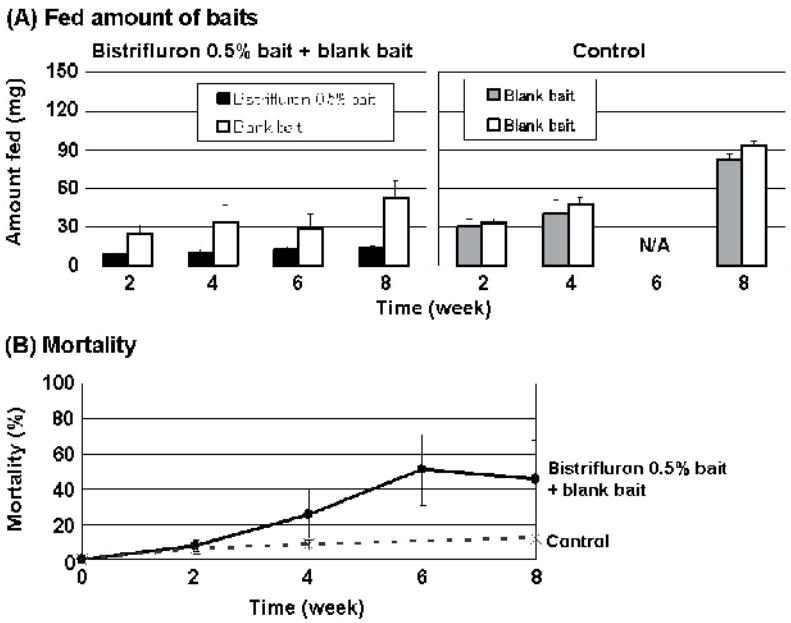

Fig. 4. The results of two-choice feeding test: A) fed mounts of bistrifluron 0.5% baits vs. blank baits, and also blank baits vs. blank baits in controls; B) time-coarse change of mortality of *C. formosanus* workers exposed to 0.5% bistrifluron baits and blank baits (data from Kubota et al., 2006)

In the two-choice test, fed amounts of baits treated with 0.5% bistrifluron seemed less than untreated baits and significant effect on termite survival was not shown during 8-week test period. These results also indicate that feeding preference of termites will greatly influence on performance of a bait product.

2.3 Allogrooming inhibition effect

Some subterranean termites build their nest in the ground, form tunnel networks and extend their territories. Workers and soldiers are patrolling every time and everywhere in their territories because they are always under threat of attacks from various kinds of enemies. Various microbes are living very next to termites' territory in the ground and trying to take every opportunity to invade it. Some of them are pathogenetic to termites. It is considered that termites have developed behaviours to protect their territories from attacks of many invaders. Allogrooming, the behaviour that a worker grooms other colony member's body, is one of the most important behaviour since microbes attached onto termite's body are effectively removed through the behaviour (Thorne & Traniello, 2003).
It is considered natural that incompetence of the above described behaviour should be involved in the mechanism of colony elimination by bait application. That is, once large part of workers are intoxicated with bait toxicant and fail in colony protecting behaviours like allogrooming their colony will collapse by intruding enemies shortly. To examine such a effect of bait toxicant, allogrooming inhibition effect of bistrifluron was evaluated by the no-choice feeding test similarly designed as the test described in the section 2.1 (Kubota et al., 2006).
In the no-choice feeding test *C. frmosanus* workers were exposed to filter paper baits treated with 0.5% (w/w) bistrifluron during the test period (5 weeks). At every week termites fed on the bait were stained with red dye, erythrosin (0.5 g/l), on their tergites. Five red-stained workers were left together on a moistened filter paper in a petri dish for 3 hours then the intensity of the red dye that remained on each termite was rated as follows: (1) red dye unchanged, (2) faded or partly disappeared, and (3) no dye remaining at all. Five replications were made for each evaluation time.
When termites were exposed to untreated filter paper no red dye were remaining on all the termites throughout the study period. On the other hand, when they were exposed to toxic bait there were two termites with red dye partly remaining even at 1st week and 24 termites with dye partly remaining or intact at the 2nd week. These results indicate that workers uptaking more than a critical dose of bistrifluron soon become unable to do their regular works to maintain colony health and that it will then result in acceleration of the collapse of a colony.

3. Colony elimination in the field cases

Colony elimination performance of bistrifluron has been examined in the field. In this section the results of the Japanese and Malaysian cases with *Coptotermes* are described. Aside from these studies, Colony elimination performance of bistrifluron was demonstrated against Australian *C. acinaciformis* (Evans 2010) and Malaysian *Globitermes sulphreus* (Neoh et al. 2011).

3.1 Field trial against *Coptotermes formosanus* in Japan

A bait system using bistrifluron as an active ingredient was applied to a *C. formosanus* colony which naturally occurred in Okayama city, Okayama prefecture in Japan (Aki, 2005). The bait system worked in the following procedure: bait stations containing wood blocks

were installed in the ground. Every station was investigated as to whether wood blocks were infested with termites at given timings. If any station was infested with termites, infested blocks were replaced with paper baits impregnated with 0.5% (w/w) bistrifluron. The location of their nest was identified in this case: their nest had been established within a tree trunk in the vicinity of the premise being attacked. Therefore a couple of bait stations were installed in the ground around the trunk.
The progress and results were as follows: bait stations containing wood blocks were installed on June 29, 2002. Toxic baits were applied on July 18, 2002 as some wood blocks were infested. After some inspections of bait stations there was no live termite and were a lot of dead soldiers on September 3, 2002 at any place where they had been seen before. Inspection within the nest with a microfiber scope revealed that there was also no living termite in their nest. Therefore it can be concluded that the colony had been eliminated by the 1.5-month bait application.

3.2 Field trial against *Coptotermes gestroi* in Malaysia

Efficacy of bistrifluron as a bait toxicant was evaluated against *C. gestroi* which is the most dominant species in Malaysia (Lee, 2007). The study was performed using premises in Penang Island, which had been suffered from their heavy infestation. Blank paper baits stored in plastic cases (the side to which an infested site was attached was open) were applied onto several infested sites of each premise. Since blank baits had been infested at all the premises, 30-50% of them were replaced with tablet bait made of powder cellulose incorporated with 0.5 or 1.0% (w/w) bistrifluron for each premise. All the bait stations were monitored on a weekly basis. Baits with less than 20% remaining matrix were replaced with new ones. The replaced bait matrix were dried and weighed to determine consumed amount. At the time point when there was no termite activity in all the stations applied to each premise it was concluded that the colony was eliminated.
In the four cases applied with 0.5% bistrifluron baits every colony has been eliminated 6-8 weeks after application of toxic baits. Consumed amounts of toxic baits were 211.4-645.2 g per colony, which were equivalent to 1.06-3.23 g of the active ingredient. In the other four cases applied with 1.0% bistrifluron baits every colony has been eliminated 4 or 5 weeks after application of toxic baits. Consumed mounts of toxic baits were 172.9-833.9 g, which were equivalent to 1.73–8.34 g of the active ingredient. Although how much amount of and how intensively baits are consumed would depend on some factors like colony population and activity, faster elimination was obtained with 1.0% bistrifluron baits than 0.5% baits in this study.

3.3 Discussion on the field studies

As in most cases location of each nest will be out of reach and unidentified, judgement of colony elimination by absence of termite in their nest is difficult and unrealistic. Whether colony elimination has been achieved should be determined by monitoring all the bait stations for a certain period after no termite activity was observed in all the stations by baiting, like the Malaysian case described above. If status of no termite activity continues for the given period (EPA guideline mentions that it should be more than 12 months) then it should be concluded that the colony applied with bait has been eliminated (US EPA, 2004). On the other hand, in the Japanese case colony elimination was confirmed by direct inspection of inside of the nest. Separately from this case, there were many Japanese cases that colony elimination seemed to

be achieved by application of bistrifluron bait based on the above described procedure (unpublished data). These data indicate that application of bistrifluron bait will successfully eliminate colonies of *Coptotermes* termites within 1–2 months. Although numbers of foraging termites varies from ten thousands to over a million, it will take weeks that single colony fall into the collapse after large part of foraging termites of single colony take more than a critical amount of bistrifluron and become unable to take part in maintaining colony's health. The laboratory study described above also showed that individual workers will become incompetent and die within a couple of weeks. Combining such laboratory results with the fact that colonies having hundreds of thousands to a million of foragers were eliminated in 1–2 months, it is suggested that large part of foragers took a critical amount of bait toxicant in very short time and it is an interesting fact for discussion of termite's feeding behaviour. A manner in which termites will take up bait toxicant will be discussed in the next section.

4. Feeding behaviour of termites and kinetics of bistrifluron in the termite body

To discuss how termites will take up a critical amount of bistrifluron through their feeding acitivities, some laboratory studies were conducted to determine its lethal dose and kinetics in their bodies (Kubota et al., 2008). Firstly analytical method had to be established to perform these tests.

4.1 Analytical method

Method to chemically analyze bistrifluron amount in termite body was examined using high performance liquid chromatography (HPLC). The outline of analytical method established for the study is as follows.

The termites were analyzed by HPLC immediately after they were collected. The termites were kept chilled in a vial on ice until they were crushed and homogenized in a mortar with a pestle after being rinsed with solvent. A preliminary chemical assay demonstrated that there was no peak of bistrifluron in HPLC analysis with termites that had been exposed to blank bait. When a given amount of bistrifluron was mixed well with homogenized termites in solvent, 100% bistrifluron was recovered by the above-described procedure. The amount of bistrifluron recovered from solvent that was used to clean termites that had been exposed to 0.5% bistrifluron bait for 1 or 2 weeks was much smaller than 5% of that recovered from whole bodies. The amount of bistrifluron that remained on the external surface of termites was considered to be negligibly small. Homogenized termites were washed into a flask with acetonitrile and then subjected to ultrasonication (42 kHz ± 6%) for more than 1 hour in an ultrasonic device to extract bistrifluron from termites. The acetonitrile suspension was filtered with a filter, PTFE of 0.45 μm. HPLC analysis was performed by a Shimadzu LC-10Avp (Shimadzu Corporation, Kyoto, Japan), with a column of SUMIPAX ODS A-217 (4.6 mm in internal diameter, length 150 mm, Sumika Chemical Analysis Service, Ltd., Tokyo, Japan). Flow rate, injection volume and wave length were 0.5 ml/min, 20 μl and 254 nm, respectively. Mobile phase was acetonitrile/water = 80/20. Analytical-grade 2, 4, 6–anilinetrichloride was used as an internal standard. HPLC-grade acetonitrile was used as a solvent.

4.2 Lethal dose of bistrifluron to workers of *Coptotermes formosanus*

How much amount of bait should be fed by individual foragers was investigated to examine how much amount of bistrifluron should be necessary to be taken to give a critical effect on them. The similar no-choice test with *C. formosanus* workers as described in the 2nd section was designed to examine how long termites should be exposed to 0.5% (w/w) bistrifluron

bait to feed enough amounts. Time-course changes of mortality for 3-, 7- and 14-day exposures were recorded for 12 weeks and compared to that in the untreated control. In each case of 3-, 7- or 14-day exposure toxic baits were replaced with blank baits just after exposure to toxic for given periods. Five replications were made for each exposure period.
While 3-day exposure did not cause significant increase in termite mortality in comparison to untreated control, 7- and 14-day exposures resulted in mortality increase at the 6th week and 4th week, respectively, and for both cases all the termites had been dead by the end of test period. It is indicated that 7-day exposure should be enough for most of workers to take up a lethal dose under the test condition. LT_{50} (50% lethal time) was ca. 6 weeks.

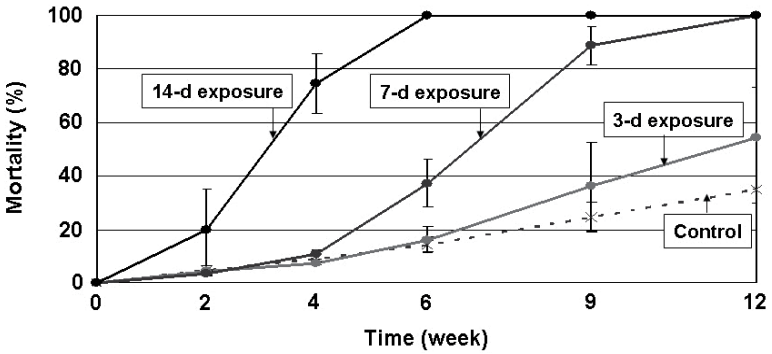

Fig. 5. Time-coarse change of mortality of *C. formosanus* workers exposed to 0.5% bistrifluron bait for 3, 7 or 14 days (data from Kubota et al., 2008)

Based on these results, *C. formosanus* workers exposed to 0.5% bistrifluron baits for 1 week and those intoxicated were analyzed at the 6th week after the test initiation in order to determine how much amount of bistrifluron would remain and how bistrifluron moves in a termite body. The amount of bistrifluron that was detected from moribund termites at the 6th week was 397.7 ng/termite, which indicates that the uptake and accumulation of ≥400 ng bistrifluron by an individual termite could possibly provide slow-acting insecticidal efficacy.

4.3 Distribution and kinetics of bistrifluron in the termite body

Distribution of bistrifluron in the termite body was examined. *C. formosanus* workers were exposed to 0.5% bistrifluron bait for 7 days in the above-described no-choice feeding test. The termites were analyzed right after 1-week exposure to toxic bait and also after subsequent 2-week exposure to blank bait. They were dissected into heads, legs, alimentary tracts, and remaining bodies under a stereoscopic microscope. Each body part from ten termites was analyzed collectively and four replications were made.
The amounts of bistrifluron recovered from heads, legs and other body parts of termites were 90.5, 4.5 and 559.1 ng/termite, respectively, just after *C. formosanus* workers were exposed to toxic bait for 1 week (Fig. 6). These amounts were 95.8, 6.4 and 385.7 ng/termite, respectively, after termites were then exposed to blank bait for 2 weeks (Fig .6). There was no significant difference in the amount of bistrifluron between the post-exposure periods for any of the body parts. The amounts of bistrifluron recovered from alimentary tracts were 60.8 and 48.8 ng/termite, respectively, whose levels were approximately equivalent to 10% of that from a whole termite body. These results indicate that bistrifluron molecules should move quickly from inside the alimentary tract toward each body part and then they would exist in each body part stably.

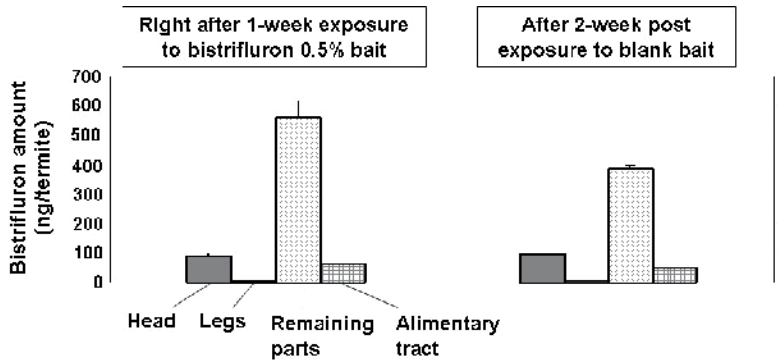

Fig. 6. Amounts of Bistrifluron recovered from each body part of *C. formosanus* workers exposed to 0.5% bistrifluron bait for 1 week (data from Kubota et al., 2008)

Finally how much amount of bistrifluron would be transferred and/or lost from an individual termite which fed toxic bait to their nestmates by trophallaxis. As termites transfer materials which they foraged to their nestmates from their mouths, bait toxicant may be also transferred from foragers to other nestmates. It is also possible that a large proportion of bistrifluron is not present in a termite body in a stable form, but just circulates in a termite body and among individual termites by trophallaxis. Similarly as the previous tests *C. fromosanus* workers were exposed to 0.5% (w/w) bistrifluron bait for 1 week. Right after 1-week exposure to toxic bait 10 exposed termites (donors) were left together with 10 intact workers (recipients) and blank bait. Recipients were replaced with new intact workers every week. Donors and recipients were analyzed every week to monitor transfer/lost of bistrifluron between termites. Ten termites were collectively analyzed and four replications were made for both donors and recipients at each time point.

The amount of bistrifluron detected in the donors was 546.0 ng/termite just after the exposure for 1 week, and this significantly decreased to 250–300 ng/termite after contact with recipients for 1-3 weeks (Fig. 7). However, the loss of bistrifluron from donors was not significant during the 1-3 weeks of contact with recipient. The amount of bistrifluron recovered from the recipients was 32.6 ng/termite after 1 week of contact with donors, and the amounts were very small after an additional period of contact for 1 and 2 weeks (2.2 and 1.6 ng/termite, respectively).

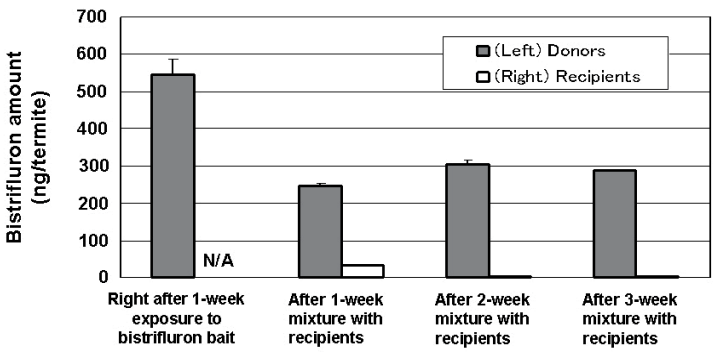

Fig. 7. Changes in the amount of bistrifluron regularly recovered from *C. formosanus* worker donors and recipients after the 1-week exposure to 0.5% bistrifluron bait (data from Kubota et al., 2008)

When termites were first exposed to 0.5% bistrifluron bait for 1 week and then mixed with the same number of unexposed nestmates, 6% of the bistrifluron taken by donors was transferred to recipients, and some bistrifluron was lost from the donors during the 1-week period of mixing. However, bistrifluron appeared to remain unchanged inside the termite body because there was no significant difference in the bistrifluron amount in the donors at the 2nd, 3rd and 4th weeks and very little bistrifluron was detected in recipients at the 2nd and 3rd weeks.
These results support the notion that once a large amount of bistrifluron is taken by a *C. formosanus* worker, it stably exists in a termite body for several weeks. Unfortunately, there is no evidence regarding how much bistrifluron can be transferred among nestmates in the field to compare the results further. However, it is possible that some proportion of bistrifluron is transferred to nestmates by frequent trophallaxis within a short period of time. For example, the largest proportion of hexaflumuron taken by *Reticulitermes hesperus* Banks in California was transferred from donors to recipients after exposure to hexaflumuron bait for a day (Haagsma & Rust, 2005). In addition, the materials transferred from donors to recipients seem to be retransferred to other nestmates by cascade events (Sùarez & Thorne 2000).

5. Process of colony elimination by the uptake of bait toxicant through termites' foraging activity

The results reported in section 4 were all from laboratory evaluation and possibly based on termites' feeding behaviours under controlled conditions. They should be examined by comparing them with outcomes obtained in a simulated study under the field condition. Threfore, bistrifluron baits were applied to a colony of *C. formosanus* and foraging termites were collected and analyzed for bistrifluron amount at given timing, which would give an insight in terms of how bistrifluron would be taken up by foragers and distributed among colony members (Kubota et al., 2009).

5.1 Experimental setup

A colony of *C. formosanus* obtained from the field was used for the study. The nest of the colony originally infested residence in Wakayama Prefecture, Japan, and collected in December 2006, and then placed in a Styrofoam box (52 cm wide x 35 cm long x 44 cm high) to form an artificial nest.
Experimental baits were prepared by folding three sheets of paper towels (ca. 20 g). Bistrifluron-treated baits were prepared by pretreatment of core paper towels with an acetone solution of bistrifluron to give 0.1 g of bistrifluron per bait [ca. 0.5% (w/w)].
The laboratory arena was set up with a plastic container (77 cm wide x 122 cm long x 21 cm deep) containing four concrete blocks (39 cm wide x 19 cm long x 10 cm high) aligned so that adjacent blocks were in contact. The Styrofoam box, the artificial nest, was placed over two of the concrete blocks. Water was added to the plastic container to supply termites with water and to create a moat around the blocks to prevent termite from escaping. A wood block of *Pinus thunbergii* Parl. was placed on the concrete block in the opposite side from the nest. Alignment of the materials is roughly illustrated in Fig. 8. The laboratory arena, with nest and wood block, was set up in December 2006. By the end of January 2007, the termites had constructed a mud tunnel between the nest and the block. On 10 September 2007, one blank bait each was placed on the mud tunnel at three sites (site 1, 2, and 3), and each bait was covered with an aluminum cup (ca. 200 ml). Because foraging termites started consuming blank baits immediately after they were installed, the bait at site 1 was replaced with a bistrifluron bait on 25 September 2007. Allocation of bait (toxic or blank) at each baiting site is shown in Fig. 9.

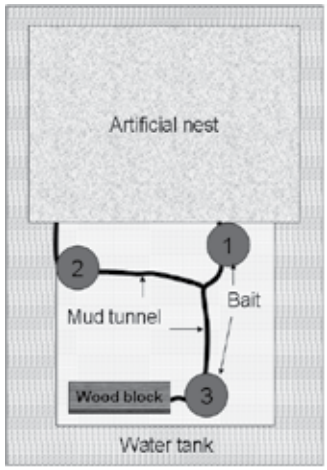

Fig. 8. Illustration of the experimental arena and alignment of the artificial nest, wood block, tunnels and baits (copied from Kubota et al., 2009) This is a copy from Sociobiology 2009.

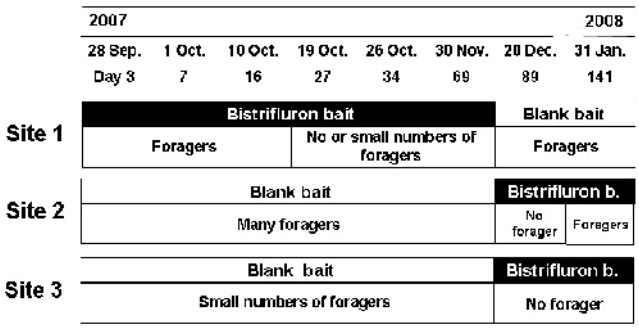

Fig. 9. Allocation of bistrifluron/blank bait and status of termite activity at given dates at each baiting site (site 1, 2 and 3)

5.2 Collection of foraging workers and analysis

Termites were separately collected from each site (~5 termites/bait) on 28 September 2007 (day 3 after installation of a bistrifluron bait), and individual termites were subjected to chemical analysis of bistrifluron in the same way with HPLC as section 4.1. Termites were also collected in the same manner on five other dates: 1 October (day 7), 10 October (day 16), 19 October (day 27), 26 October (day 34), and 30 November (day 69) before baits were renewed. Because termite foraging always decreased shortly after toxic baits were added (termites appeared to stop feeding on baits for about 1 month after placement of a toxic bait), all three baits were renewed on 30 November 2007 (a blank bait was placed at site 1 and a toxic bait was placed at sites 2 and 3, see Fig. 9). Additional termites were collected for bistrifluron analysis on 20 December 2007 (day 89) and 31 January 2008 (day 141).

5.3 Confirmation of colony elimination

The baits were more heavily consumed by termites at site 2 than at sites 1 and 3, regardless of treatment with bistrifluron. Foraging activity appeared to decline with time after toxic baits were added as evidenced by the decreased number of foragers at all baiting sites (both

treated and untreated). Because no forager was present at site 1 (treated bait added on 25 September) on sampling dates from 26 October to 30 November, all baits were replaced with new ones on 20 November as previously noted: toxic baits were added at sites 2 and 3 and a blank bait was added at site 1. Foraging termites were seen at site 1 on 30 December, and foraging termites were observed at site 2. One possible explanation for the decreased foraging population and disappearance of foragers from site 1 is feeding deterrence by bistrifluron, as previously suggested in the two-choice test described in section 2.2. Although it is unclear why foragers came back to the toxic bait at site 2, the shuffle of treated bait sites might lead to reinfestation of the treated bait by foragers at site 2 where termites previously fed on untreated bait. Dead workers and soldiers were found at site 1 and site 2 several days after the last collection (31 January 2008), and no live termite was present at these sites. Author concluded that the colony was eliminated because no live termites were found over half a year after application of toxic baits. Although the current study demonstrated that *C. formosanus* colonies could be eliminated by application of bistrifluron, the results clearly supported the importance of placement of baits.

5.4 Manner of the uptake of bait toxicant by foraging termites

Bistrifluron amounts recovered from each termites collected from each site are shown in Fig. 10. The recovered amounts of bistrifluron ranged from 101 to 1026 ng/termite when five foragers were collected from site 1 (with toxic bait) on 28 September (day 3) and 1 October (day 7). On the other hand, when blank baits were present at sites 2 and 3, only two of 17 foragers contained bistrifluron, and the quantities detected were small (45 and 57 ng/termite). Between 10 October and 30 November 2007 (toxic bait at site 1 and blank baits at sites 2 and 3), bistrifluron was detected from seven foragers collected from site 1, and the amounts ranged from 31 – 477 ng/termite. During this same period, analyses of 28 foraging workers from sites 2 and 3 indicated that 17 termites contained bistrifluron (22 – 196 ng/termite) and 11 termites did not. These results appear to demonstrate that up to a few hundred milligrams of bistrifluron was taken up by an individual worker through foraging activities.

Because much smaller amounts of bistrifluron were recovered from workers collected at sites 2 and 3 than site 1, it is uncertain whether those workers were donors or recipients of bistrifluron among colony members. If they were recipients, they received some bistrifluron from donors that originally took bistrifluron at site 1. This was quite possible because only a small portion of materials taken up by donors would be transferred to recipients (Sùarez & Thorne 2000; Haagsma & Rust 2005; Buczkowski et al., 2007). If they were donors, they consumed bistrifluron at site 1 and were caught at sites 2 or 3 after losing some portion of the bistrifluron through trophallaxis and metabolism. Unfortunately, distinguishing between donors and recipients will be difficult until foraging behaviour of termites is better understood: after feeding at one site, whether do foragers feed at other sites on their way back to the nest? And how frequently do they go the rounds of their feeding sites?

Analyses of eight termites collected on 31 January 2008 (several days before colony elimination) revealed that these termites consumed sufficient amounts of bistrifluron (483 – 1380 ng/termite) to cause death by 31 January 2008. Because of the lack of data describing the temporal change in the quantity of bistrifluron taken up by termites during this period, we were unable to delineate how bistrifluron spread within the colony, although the results clearly showed that almost all foragers acquired a lethal dose of bistrifluron. Slow mortality will allow the foragers to move too far to identify source of toxin and is therefore essential if the bait is to eliminate colonies of subterranean termites like *C. formosanus*. Workers of *C. formosanus* exposed to 0.5% (w/w) noviflumuron bait could move as far as 50 m before they were killed by the insecticidal effect of noviflumuron (Su, 2005).

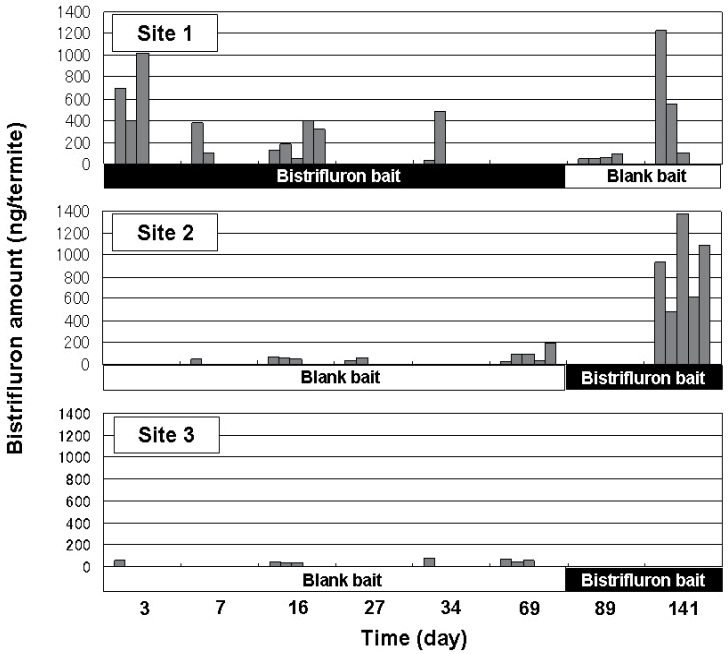

Fig. 10. Bistrifluron amounts recovered from foraging *C. formosanus* at each site from 28 September 2007 to 31 January 2008

It is quite reasonable that the colony was eliminated soon after colony mates acquired sufficient amounts of bistrifluron. They could not maintain the health of the nest by allogrooming or by fighting against invading microbes, even if almost of them were still live as discussed in section 3. Many dead soldiers were found at the bait sites, indicating that they came there for feeding or for escaping from a microorganism-contaminated nest. The present study showed that colony elimination by bistrifluron involves acquisition of lethal quantities of bistrifluron by large portion of foragers. Further extensive studies on artificial or natural colonies are needed to more completely understand the mechanisms and processes of colony elimination by baits.

6. Conclusions

It can be concluded as below by a series of experiments:

- Bistrifluron acts on termites extremely slowly, however, its efficacy is significantly dose-dependent.
- Bistrifluron inhibits colony-maintaining activities of termites like allogrooming behavior before the colony is eliminated.
- A lethal dose of bistrifluron against *C. formosanus* is ≥400 ng per termite and some portion of bistrifluron once taken up by foraging termites would remain in termite body for weeks, while the rest of bistrifluron is discharged.
- Sufficient amount of bistrifluron can be taken up by foraging termites owing to its slow action and subsequently bistrifluron would be transferred their colony mates.
- Feeding deterrency of bistrifluron as a reflection of its dose-dependence is not always an unfavorable characteristics since the improvement of feeding preference by termites could lead to the faster colony elimination.

7. Acknowledgment

The author thanks to Prof. Kunio Tsunoda of Kyoto University and Prof. Chow-Yang Lee of Universiti Sains Malaysia for reviewing the manuscript draft. The author also thank to Dongbu Hannong Co., Ltd. (Seoul, South Korea) to provide the opportunity to evaluate efficacy of bistrifluron against termites.

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Camouflage of Seeds, a Control Method of the Bird Mortality in Grain Crops

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1. Introduction

This chapter discusses the development of a mitigation measure against impacts caused by the ingestion of poisoned seeds by insecticides like carbofuran and carbosulfan in wheat, corn and rice fields.

In accordance with Brazilian Federal Decree nº 4.074 of January 2002, agrotoxins should be added to seeds mixed with dye to avoid the risk of being ingested by humans. The toxic dye rhodamine B, whose reddish-purple colour has been used for this purpose, it's apparent and attractive to granivorous wild birds such as Columbidae and Icterinae.

Fig. 1. Eared-dove (*Zenaida auriculata*) fatally poisoned by eating weath treated with carbofuran and rhodamine B. Harvested crops are attractive to the granivorous birds and may increase the mortality rates during the next sowing.

Seeds treated with carbofuran and rhodamine B, which are not completely covered during mechanical seeding, are picked up by granivorous birds foraging for food. This leads to direct mortality (Figures 1, 2, 7, 11) by deliberate ingestion or secondary poisoning if predators ingest these poisoned preys. This problem is not confined to Brazil but is also an issue in several countries where seeds covered with agrotoxins are used.

Most measures to mitigate the damage caused by birds attacking the production and / or mitigation of the mortality of birds by the use of pesticides, has been to repel the birds of the site, avoiding the consumption of seeds by acoustic, tactile, visual and/or gustative repellents. However, the use of repellents spend resources of high operational and financial costs, has not produced satisfactory results and often violates humanitarian principles causing injury and suffering to the birds.

Despite these findings and public criticism, several recent studies evolve in this direction (Tobin, 2002), seeking chemical repellents and/or color repellents to reduce the damage to production and reduce the risk of pesticide poisoning wildlife (Avery, 2002).

Fig. 2. Rufous–collared sparrow (*Zonotrichia capensis*) killed by eating poisoned wheat seeds treated with carbofuran and rhodamine B, which can be seen beside the bird.

Poorly developed sense of taste is a plausible hypothesis to explain the limited results of some authors, using chemical repellents in the absence of alternative food sources for birds: Avery et al. (1998) with anthraquinone, Moran (2001) metil antranilato, Almeida et al. (2010a) with carbosulfan, Almeida & Almeida (in press) employing carbosulfan and methiocarb. Taking the example of these cases, when grain stained with 'aversive' colors were used, there were also ingestion rates (Avery et al., 1999, Hartley et al., 1999, 2000) which can result in high mortality rates in situations where the density of birds is high. The combination of chemical repellents for aversive colors has generated more effective results as a method of controlling agricultural damage (Avery & Mason, 1997; Nelms & Avery, 1997).

Fig. 3. Camouflaged seeds in the absence of irregularities in the surface soil can be more conspicuous to the birds. Note that the color and roughness of the camouflage, conferred by the dye powder, help hide the seeds in the soil.

However, seeds with aposematic and/or aversive colors become very conspicuous, which may represent a high risk of mortality. This can be inferred, considering that, despite the lack of consumption of blue color seeds in some experiments (Hartley et al., 1999, 2000, Almeida et al. 2010b) the use of carbofuran in the granular formulation of the same color has been banned in Virginia, USA and Canada, due to bird mortality caused by direct ingestion of the product (Almeida et al., 2010b).

Moreover, there has been no communication observed between poisoned birds and healthy ones, that could stop the consumption, even when expressive mortality rates occur and in the presence of obvious agonistic behaviors caused by the ingestion of seeds treated with carbofuran, methiocarb carbosulfan or in combination with conspicuous colors such as beige and red (Almeida et al., 2010a; Almeida & Almeida, in press). In these experiments, it was noteworthy that gustative repellents (carbosulfan and methiocarb) were superseded by the fact that carbofuran has the unpleasant distinction of being so hazardous to wildlife, that it simply cannot be effectively regulated or managed accordingly without mortality risk occur (Richards & Mineau, in press). However, the camouflage of seeds (Figures 3 and 4) has been presented as a method capable of significant reducing the mortality of birds in comparison with the traditional way of planting seeds treated with carbofuran and rhodamine B, and compared to other possible mitigation alternatives, such as gustative repellents and/or conspicuous colors, which could be aposematic, leading to neophobia and aversion by birds (Almeida et al., 2010a, b; Almeida & Almeida, in press).

A cryptic species is one whose color and/or morphology resemble relevant aspects of the habitat where it lives. The ecological literature is rich in documentation of cryptic species that can survive in the presence of predators. The ability of many species of reptiles, amphibians and fish to change their pigmentation according to the substrate where they are found, it is indicative of the remarkable evolutionary success of cryptic behavior, which is common in nature.

The Bionics, defined as: ´Living creatures science-based ', which one of the most oldest and best know applications is the camouflage, whose inspiration would come from the ability of

cryptic animals such as zebra and chameleons. Camouflage comes from the French word 'camoufler' which means to disguise or conceal through a disguise and its evolutionary significance has been gaining wide scientific support as a mechanism that reduces the risk of detection (Merilaita et al., 1998, Zug et al. 2001; Merilaita, 2003, Schmidt et al., 2004, Frankel et al. 2004; Merilaita & Lind, 2005, Cuthill et al., 2005).

The principle of camouflage inspired and guided the development of this research. Seeds of wheat, corn and rice, when treated with systemic pesticides, would receive dyed camouflage, making them similar to the soil where they were planted. This cover would preferably be processed on the same machine that performs pesticide treatment, moreover, it could not impair the mechanical planting or germination. The camouflage should have low operational and financial cost, as well as been non-toxic. Thus, seeds that were not buried by the planting machine, remaining dangerously exposed to granivorous birds, would count with a camouflage whose effectiveness would be favored by organic matter not decomposed, by the irregularities of the soil, and by the contrasts between light and shadows on the soil surface, even though the birds actively seeked them on the ground.

Birds attack the agricultural fields because they are abundant sources of food readily accessible, requiring low energy expenditure. If food becomes difficult to find, to ingest, and to digest, birds will spend more time and energy to forage. Having them difficulty to maintain a certain rate-making energy, the theory of optimal foraging predicts that animals will look for another food source (Avery, 2002, Begon et al., 2006).

The aim of this paper is to present the method of concealment of seeds and how it was designed, with respect to the material tests and seed germination. Results obtained from these assessments show that camouflages can increase the germination rate of seeds and that 'home-made' forms of camouflage can be as efficient, if not better than camouflage produced by industrial processes. Also, camouflage do not need high operating or financial costs, it can be applied to seeds using the usual machinery of seed pesticides treatment on commercial scale plantations.

Fig. 4. Camouflaged seeds can be visible, representing risk of poisoning for birds, if it were not completely buried and were seeded outside the plowed soil of the planting, or in absence of non decomposed organic matter on the soil surface.

However, before, results of research on the effectiveness of the forms of mitigation that has been tested, among which different types of camouflage, whose forms of development and germination tests are being discussed here, will be presented.
The mitigation method will be contextualized regarding the impacts caused by the usual ways of planting wheat and corn with seeds poisoned by carbamates pesticides, such as carbofuran and carbosulfan. Then we will present a discussion on results obtained with different types of ingredients, used in preparing camouflage, regarding the effectiveness of camouflage, cover durability and environmental factors that influence the trial in agricultural fields.
Finally, there will be a discussion about determinant factors of the camouflage effectiveness in some species in experimental conditions, explaining the results obtained by this research.

2. Methods of controlling bird mortality

An study consolidating three experiments was conducted in Paraná and São Paulo States to evaluate if camouflaged seeds could minimize the mortality of wild birds, caused by the ingestion of commercial seeds treated with pesticides and rhodamine B (Almeida et al., 2010a). When put all together, the three experiments were summing up to 15,896 kg of seeds, sowed in 111.46 hectares. Alternative strategies for reducing mortality, like the use of carbofuran without rhodamine B and the use of carbosulfan. Carbofuran without rhodamine B gives a 'beige' hue to seeds and could make them less attractive to birds, representing economic savings at planting. Carbosulfan, a carbamate with insecticidal and nematicidal properties and with a lethal dose circa 20 times weaker than carbofuran, could act as a chemical repellent if the birds were able to link the poisoning symptoms to the food, halting the consumption before dying.
Experiments in rice and wheat plantations were performed in regions where mortality of birds, coming from the ingestion of seeds treated with pesticides, was reported by farmers and they were performed in the planting seasons, when numerous flocks of birds were present.

Fig. 5. Installation of an experiment of planting in commercial scale using seeds poisoned with carbofuran.

The powdered brown-colored dye used in to camouflage the seed was obtained through mixing red, black, blue, brown and yellow powdered dyes, reaching a hue similar to the soil samples previously gathered. The liquid dye used in the other camouflaging was obtained by mixing red, blue, yellow, black and brown pigments in water, and then adding the slurry to the carbofuran syrup in proportions which allowed a proper camouflage to match the region's soil color. The process of camouflaging seeds was performed in the same machine used in the treatment of seeds with carbofuran or carbosulfan.

For treating the seeds, carbofuran in a concentrated suspension of 350 g/l was used. The dosage of carbofuran used for wheat or rice seeds was 2 l for 100 kg. Carbofuran's toxicity (human LD_{50} = 8-12 mg/kg) was so high that small (~ 40 g) and medium-sized birds (~120 g) ingesting seeds from the experiment often died *in situ*. Sowing was conventional in dry land, made by a tractor-mounted gravity seed drill implemented with a seed drill over plowed soil in the traditional planting system (Figure 5).

The tested treatments were disposed in parcels no further than 700 m away from each other. The amount of seeds in parcels and among treatments was balanced. The average plot size was 8.54 hectares (n = 6; standard deviation = 7.8), and there were 144.2 kg of seeds per hectare. Two searches for dead birds were made per day in every parcel. The searches parties were composed of two, three or sometimes four people walking slowly (~ 3 km/h) or in a pick-up truck (~ 5 km/h) and were gathered within and around the parcels (Figure 6).

Fig. 6. Birds feeding on seeds poisoned with carbofuran in a experiment of planting.

Bird carcasses were collected and identified. The food present in the digestive tract of bird carcasses was collected separately as it follows: i) crop and esophagus; ii) proventriculus; and iii) gizzard (Figure 7). The seeds from each treatment were counted. The numerical variations from dead birds and consumed seeds between treatments were checked with analysis on variance, considering days of experimental exposure as repeated samples. Birds which were able to ingest seeds from more than one treatment were excluded from the analysis.

The Experiment 1 was conducted using wheat seeds, and the treatments were: i) red seeds treated with carbofuran and rhodamine B; ii) brown seeds camouflaged with powder dye, treated with carbofuran.

The total experimental area consisted of 10 ha, divided into 1 ha parcels. Five hectares were assigned to each treatment. The amount of seeds in parcels and among treatments was balanced. A total of 1,350 kg of seeds were used.
Experiment 2. The treatments applied to the rice seeds were: i) red seeds treated with carbofuran and rhodamine B; ii) brown seeds, camouflaged with powdered dye and treated with carbofuran; iii) red seeds treated with carbosulfan and rhodamine B.
For treating the seeds, powder carbosulfan, in a concentration of 350 g / kg, was used; the dosage was 3 kg powder every one hundred of seeds. Carbosulfan toxicity for humans was LD_{50} = 212 mg / kg. Treatments were made to seven parcels of 0.26 ha, adding up to 5.46 ha. 100 kg of seeds were used per hectare. The amount of seeds in parcels and among treatments was balanced.
Experiment 3. The following treatments, applied to the wheat seeds, were tested: i) red seeds treated with carbofuran and rhodamine B; ii) brown seeds, camouflaged with liquid dye, treated with carbofuran; iii) beige seeds, treated with carbofuran without rhodamine B; iv) brown seeds, camouflaged with powder dye, treated with carbofuran.
The total assay area was 96 ha, divided into four equal parcels of 24 ha, each one receiving 3,500 kg of seeds related to the treatments (145 kg / ha).
The mortality mounted to 296 birds, distributed into 11 species. Eared doves were the most numerous victims, with 263 deaths. The consumption of camouflaged seeds was lower than that of commercial seeds treated with rhodamine. The mortality caused by seeds with rhodamine was higher than the one caused by camouflaged seeds. When powder dye camouflaging was used, the mortality range decrease in 75.5 to 100%, and the range of consumption decrease in 57.4 to 99%, relative to the other treatments.

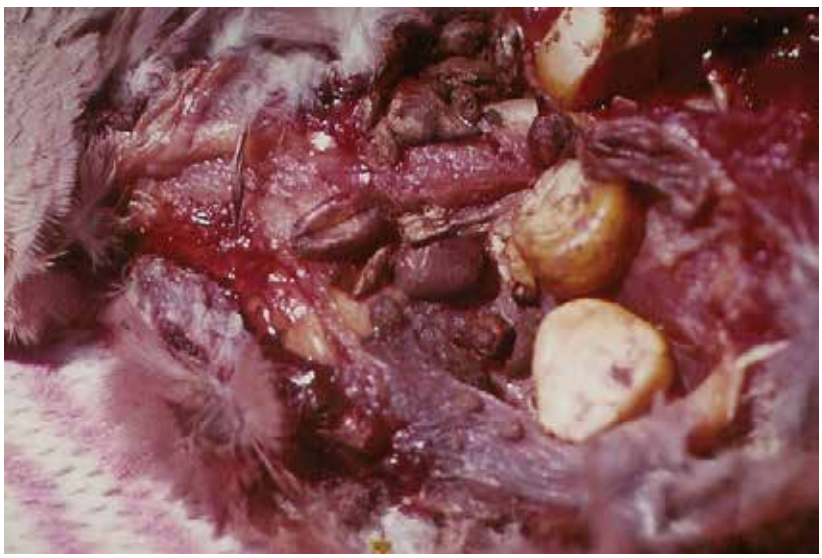

Fig. 7. The dissection of the Ruddy ground dove (*Columbina talpacoti*), showing one wheat camouflaged seed, which killed the bird. This bird was one of the 73 victims who died with only one seed in the crop, poisoned early in digestion.

The brown color and the powder dye, rich in iron oxide, decrease the spectral reflectance (Hartley et al., 2000; Demattê et al., 2003; Espig et al., 2005; Almeida, 2006) of seeds in the wave lengths visible by the Passeriforms and Columbidae (Hart, 2001), making them similar

to the soil. In addition, camouflaged coating, made with the powder, presents an opaque surface and, for this reason, has less shine than seeds treated with rhodamine, being less conspicuous (Schmidt et al., 2004; Cuthill et al., 2005). Besides, it is possible that there wasn't search image for brown seeds, and in accordance with the optimal foraging theorem, greater energy would be required to consume and locate such seeds (Avery, 2002; Begon et al. 2006). However, the ingestion of small grits to help crush foods is a common aspect in the behavior of several bird Families (Sick, 1997). So, it is possible that brown-dyed seeds are less removed due to a set of factors linked to the camouflaging phenomenon like background match, avoiding well defined contours and color contrasts (Merilaita 2003, Frankel et al., 2004, Schmidt et al., 2004) and not only due to the aversion to the brown color, as tested by Hartley et al. (1999, 2000). They tried blue and brown with relative success at avoiding the consumption of poisoned grains by birds, suggesting such colors would cause aversion.

The notable ingestion of red seeds suggests that this color, attributed by rhodamine B, is not aversive, but attractive (Schmidt et al., 2004; Cuthill et al., 2005), which is in contradiction to the findings of Avery & Mason (1997) and Nelms & Avery (1997). This color's spectral behavior, in the wavelengths visible by the birds, is similar to yellow and orange (Schmidt et al., 2004), which are contrasting and conspicuous and similar to beige. This may explain the high consumption of seeds treated with carbofuran without rhodamine B. Thus, whether the reddish hue from rhodamine B, or the beige color, coming from the syrup of carbofuran without rhodamine B, there is a higher probability of birds detecting and ingesting less camouflaged seeds.

The replacement of carbofuran with carbosulfan also appeared to reduce mortality, but carbosulfan mortality is thought to have been underestimated because of the apparent movement of affected birds after exposure. Besides, the possible carbosulfan action as a gustative repellent was not checked and, in addition, birds intoxicated by carbosulfan – vulnerable to predator attacks – were able to reach shelters, like forest reserves. Effects of pesticides over the agricultural matrix may propagate through forest fragments, affecting negatively its quality and aggravating the problem of habitat fragmentation, one of the main causes of the biological diversity erosion. These aspects are contrary to the use of carbosulfan and other products, which are up to 20 times less toxic than carbofuran, for the treatment of seeds or as gustative repellents.

The authors had concluded, if seeds treated with pesticides must receive a different color to prevent accidents with humans, the camouflaging method may be used for mitigating the mortality of birds in plantations, substituting rhodamine B (Almeida et al. 2010a).

Another study focusing on mitigation methods was realized consolidating two experiments, witch compares the environmental impacts associated with the use of carbofuran, carbosulfan and methiocarb-treated seeds, with the latter two carbamate compounds being considered as alternatives to carbofuran, and all coloured with Rhodamine B (Almeida & Almeida, in press). Methiocarb (Mesurol) also is a carbamate insecticide, causes vomiting and paralysis when ingested by birds, acting as a secondary chemical repellent (Calvi et al., 1976; Dolbeer, 1994).

The rice and wheat experimentation occurred in similar conditions of bird abundance, in the same geographical region, and using equals sowing method and carbofuran/carbosulfan treatments of the anterior study. Likewise, the data collect method and the analysis were the same. The experiments were done with 664 kg of seeds, sowed in 5.74 hectares. A comparison between the number of deaths consistent with ingesting seeds treated with carbamates was made with the treatments: in the first rice trial: i) carbofuran and

Rhodamine B, ii) carbosulfan and Rhodamine B; and in the second wheat trial: i) carbofuran and Rhodamine B; ii) carbosulfan and Rhodamine B; iii) carbofuran and Rhodamine B plus methiocarb. Methiocarb was added to the seeds, after they received treatment with carbofuran and Rhodamine B. The dose of methiocarb powder was equal to 1 kg for each 100 kg seeds.

The resulting mortality of this experiment mounted to three species, distributed into 314 birds, which 308 were Eared Doves deaths. In the first trial plots, where seeds treated with carbofuran were sown, 109 birds were found dead, and in those treated with carbosulfan, 40 were recovered. These results differed significantly, suggesting that a greater mortality was caused by carbofuran, and that the use of carbosulfan may lessen the impact of grain farming on wild birds. In turn, data clearly showed that carbosulfan use will still result in notable mortality. Further, some birds were noted as having been visibly poisoned by carbosulfan, yet managed to leave the test plot and (though disorientated) reach refuge outside the test area (ca 550 metres away). It was not possible to know if these poisoned birds recovered or died after escaping the experimental plot. Given these points, just like in the anterior experiment, it's necessary consider: i) the number of deaths recorded was not accurate, since poisoned birds may have left the test plot; ii) carbosulfan didn't act as a secondary repellent which interrupts consumption; iii) birds poisoned with carbosulfan had a greater tendency to reach the adjacent forest, where they may be preyed upon by forest species, therefore be hidden from farmers, researchers and hence, public opinion. These points raise questions regarding the principle that carbosulfan may not be used as a substitute for carbofuran. The findings reported here suggest that carbosulfan is probably not an effective alternative to carbofuran.

Fig. 8. The distribution of seeds in a plot of the removal experiment.

In the second assay, the possible repellent effects of methiocarb and carbosulfan were not statically proven. In the treatment with methiocarb, 62 birds died, while similar results were obtained using carbofuran, where 82 birds died. Although fewer birds died when carbosulfan was used, as in the previous trial and experiment, many birds visibly affected by carbosulfan escaped the test plot, making an accurate estimation of mortality difficult.

Although previous experiments have shown the relative efficacy of methiocarb as a secondary repellent to birds (Nelms & Avery 1997; Avery *et al.* 2001; Avery 2002), its use in association with carbofuran did not generate results showing it to be a mitigating factor in bird mortality, probably because carbofuran is an extremely toxic pesticide, needing only one treated seed to kill a small or medium sized bird (Almeida et al., 2010a). Thus the toxicity of carbofuran would overrun any of the potentially beneficial effects of methiocarb.

Finally, a third field study, performed in two trials (Almeida et al., 2010b) aimed at evaluating if camouflaged seeds would be less consumed by wild birds in comparison to commercial seeds coated with red-colored rhodamine B and blue seeds (Avery et al., 1999; Hartley et al., 1999; 2000). Seed removal experiments were performed in an area reserved for annual planting of 2.78 ha in southeast of Brazil, where, because of the frequent planting of corn and other cultures, a great deal of granivorous birds could be found at the site. Experimental parcels (1.5 x 1.5 m) were randomly distributed in the area circa 50 m from each other. Seeds from each treatment were disposed in columns over the ground approximately 11 cm apart (Figures 8, 9 and 10). Daily samplings were taken for six days after setup, checking the number of remaining seed in these periods.

Camouflages tested corresponded to the treatments created with the ingredients: liquid dye and powder dye, both colored brown and/or terracota. The brown-colored powder dye was shaded and thus adjusted by comparison to soil samples from the area. The camouflaged coating elicited by powder dye gave the seeds a dark brown, rugged and opaque surface.

The brown-colored liquid dye was a novel product which attributed to the seeds a smooth, opaque coating, with a shade slightly lighter than the soil in the experiment location. Acrylic glue was added to the dye to enhance adhesion and resistance to camouflaging. Dye and glue were applied to seed as an aqueous solution. Soil of the trials' site was tested as a substitute to artificial dyes and/or as an accessory camouflaging element, aiming for a higher coincidence in shades of camouflaging colors in relation to the soil.

Camouflages were applied onto seeds with either natural or purplish-red colors previously treated with rhodamine B dye, the product ordinarily and legally used in Brazil to distinguish pesticides-treated seeds.

To evaluate the effect of the search image (Begon et al., 2006) of birds for non-dyed natural grains in comparison to the other treatments, ordinary corn seeds were also used to mimic the situation wild birds find in harvest wastes. Finally, to evaluate the possible aposematic or neophobic effect of blue color, seeds were treated with mid-blue powder dye.

None of the tested treatments had pesticides. Variance analyses of the daily seed permanence in the trials were performed.

Trial 1. To evaluate camouflaged corn seed remaining, natural and rhodamine B-colored, twelve squares with seven rows of seeds each were distributed. Five types of brown camouflage treatments were tested, natural undyed corn and corn with rhodamine B only. The soil under five parcels was weeded and leveled to elicit studying the influence of irregularities and litter on seed removal. The yellow corn grain (natural) and rhodamine B-treated corn columns could be more attractive to the birds, possibly exposing columns close to groups one and two to higher pressure than those more distant. For that reason, the order

of columns was modified in each parcel so that all treatments could bear the same influence of closeness to the control groups.

Trial 2. The differences in the permanence of wheat, corn and rice seeds with the following treatments were evaluated: i) natural, undyed seeds; ii) rhodamine B-treated seeds; ii) brown powder dye camouflaged seeds; iv) industrialized camouflage with liquid brown dye; v) seeds camouflaged with mid-blue powder dye (Figure 13). Permanence differences among treatment (colors) and botanical species were also evaluated in using twelve experimental parcels (four parcels per grain species). All the parcels were weeded and leveled for making the counting of smaller seeds (wheat and rice) more accurate. Because columns with either natural colors, blue or rhodamine B could be more attractive to birds, the order of the columns was changed in each parcel, exposing the camouflage treatments to influences identical to the closeness to groups with attractive colors.

Fig. 9. Corn camouflaged in a plot of the removal experiment

The following findings were obtained: i) the camouflaged seeds were less removed than seeds with rhodamine B and natural colors, noticing that the removing of powder dye camouflaged seeds, standed between 84.5% to 98% bellow of those with rhodamine B; ii) the camouflaging was more effective in the presence of irregularities and litter; iii) there was no removal of blue-colored seeds.

Regarding the seed camouflaging, the authors concluded that the method presents low operational and financial costs, does not cause suffering to birds and may be used to diminish bird´s mortality by carbofuran in wheat, corn and rice plantations. In concern of the blue seeds, the absolute absence consumption reinforces the theory that there is aversion to this color, according to Avery et al. (1999), Hartley et al. (1999, 2000), with the possible occurrence of aposematism (Joron, 2003) and neophobia (Thomas et al., 2004). Nevertheless, as the usage of blue-colored granular carbofuran had been prohibited because of the mortality of birds caused by the direct ingestion, this color probably should not represent a reliable mitigation method.

Fig. 10. Domestic pigeons (*Columba livia*) feeding near to the plots of the removal experiment

2.1 The impact of poisoning by seeds treated with pesticides in birds

All the studies realized to compare bird mortality with the possible mitigation methods, like camouflaged, beige color, carbosulfan and methiocarb-treated seeds (Almeida, 2006; Almeida et al., 2010a; Almeida & Almeida, in press) allowed the evaluation the environmental impact of traditional modes of wheat, corn and rice cultivation used in Brazil, on dry and ploughed soil, with an emphasis on avian mortality caused by seeds dyed using Rhodamine B and treated with carbofuran and carbosulfan (Almeida & Almeida , in press).

This work partially described above, accounts results from seven field trials, employing 7,339 kg of seeds sowed in 57.24 hectares (Almeida & Almeida, in press). The rice and wheat experimentation occurred in similar conditions of Almeida et al., (2010a).

In these experiments, an evaluation of the effect of carbofuran on wild birds was undertaken in these experiments. The mean, standard deviation and confidence interval (p = 0.05%) of mortality was calculated, and the confidence intervals were computed using the Student's *t* distribution (Almeida & Almeida, in press).

Picazuro pigeons (*Patagioenas picazuro*) and small species such as the eared dove (*Zenaida auriculata*), ruddy ground dove (*Columbina talpacoti*) and Chopi blackbird (*Gnorimopsar chopi*) were the predominant species poisoned by seeds treated with carbofuran and Rhodamine B. All these granivorous bird species are common in rural areas of the south-eastern Brazil.

Granivorous birds represented 97% of the species poisoned, and the eared dove accounted for 89% of the total number of individuals found dead. The mortality mounted to 465 birds of 13 species, including partial carcasses and piles of feathers found (feather spots data, Figure 12), which represent birds that were partially consumed or totally removed by avian and mammals predators.

The minimum number of birds poisoned was between 25 and 183 where eared dove flocks were present. The estimated number of birds expected to be poisoned was 77.5 ± 55.7 (n=6; standard deviation=69.6, p=0.05). Therefore, we would expect an average of approximately 9 deaths per hectare (between 2.5 - 15.6 birds per hectare).

Fig. 11. Eared dove (*Zenaida auriculata*) killed by ingestion of wheat seeds treated with carbofuran. Eared dove were the most numerous casualties in the plantation experiments.

Aware of the fact that some birds poisoned by carbosulfan escaped into the surrounding habitat before dying, such estimates need to consider that the mortality caused by carbosulfan was probably conservative and not accurate. Moreover, counting did not provide a full tally of secondary poisoning mortality rates, since predators left the area or those carcasses were removed by scavengers before being counted (as described by Mineau 2005).
The estimative of the number of birds potentially poisoned on Brazilian farms are very worrying. Agrotoxins like carbofuran are used on a very large scale in Brazil, and improper use occurs throughout the agricultural landscape. Governmental control is very absent, and millions of hectares are devoted to rice, wheat, and corn production. Species such as the eared dove are abundant in some regions, and they may be both, victims and vectors, effectively passing the agrotoxin legacy up the food chain to predators (some of whom may be rare) as the poisoned doves are predated or scavenged.

Fig. 12. Activity of predators removing poisoned bird, as evidenced by the pile of feathers.

The high mortality rates noted in Brazil are equivalent to existing findings (Eisler, 1985; Agriculture Canada, 1993; Mineau et al., 1999; Mineau, 2005) regarding the potential impact carbofuran exposure can have on wild birds.
Given these findings, research must be intensified to evaluate the magnitude of the impact that pesticides are having on Brazilian fauna, since these application methods and formulations have been banned in developed countries, but they are still used in Brazil, and probably in other Latin America countries, on large commercial scales, with government permission. The Brazilian Federal Government should be pressured, since the continued use of carbofuran and Rhodamine B is in conflict with Brazilian Law (Number 7802, July 11, 1989) because they may cause environmental damage.

Fig. 13. Seeds treatments tested in the second trial of the removal experiment: camouflaging with industrialized liquid dye (above, left), camouflaging with powder dye (above, right), aposematic mid-blue seed (center), yellow natural corn (below, left), rhodamine B treated seeds (below, right).

3. How to do camouflaged seeds?

3.1 In search of camouflage

The aim of the first experiment in coloring, which took place in March 1981, was to get a hue close to the soil color, this way, camouflaging the wheat seeds, making them imperceptible to birds. An adequate coverage should effectively camouflage the seeds without affecting germination, having low cost and being non-toxic. On the first experiment with color, we used several different shades of the commercial product called Powder Chess ® (hereinafter: powder dye), commonly used in paintings. This product was applied directly to the seed without use of adhesives.

The chemical composition of this dye was based mainly on iron oxides (FeO) in red and yellow colors, and silicatate colloidal sulfur of the FeO in blue color, these being the three colors forming the mixtures (camouflage).
The proportions in grams of the pigments, according to the colors of soil, ranged as follows: i) 15 red, 4 yellow: 5 blue, ii) 15 red: 14 yellow: 5 blue, iii) 15 red: 4 yellow: 15 blue. The analysis of the camouflage covers was done visually, comparing them with soil samples from Londrina (PR).
Results showed that, as proportions of each pigment were varied, different tones could be obtained. This fact contributes positively to the camouflage of seeds, enabling it to create coatings with colors that can approach the tone of croplands, from light sandy soils to dark-reddish clayey ones. For example, to color 110 kg of wheat seeds according to a sample of dark red latosol from Londrina-PR, it took 2.4 kg of mixed colors. The individual quantities of dye to 100 kg of seed were: 1.5 kilograms of red pigment, 0.40 kg of yellow pigment, and 0.50 kg of blue pigment.

3.2 Camouflage improvements

Searching for the development of camouflage techniques of seeds treated with syrup of carbofuran, which would provide color and texture similar to the ground, in order to prevent wild birds to recognize them, experiments were carried out in two phases to the selection of appropriate material. Later, the ingredients used for camouflage were selected, according to criteria relevant to seeds germination and their treatment on the machines that apply the Carbofuran syrup. This syrup, composed of Carbofuran, Rhodamine B had a commercial commonly used concentrated suspension of 350 g/L (hereinafter: carbofuran syrup).
From all the materials tested, those which did not showed favorable characteristics to the staining process were:

i. Plaster: after the drying process, it had become extremely brittle, loosening up the seed with ease. Besides, while still damp, caused clumping of the seeds during the treatment.
ii. Calcium carbonate: caused clumping of the seeds during the treatment.
iii. Ink (synthetic pigment concentrate, Coral ®): caused clumping of the seeds during the treatment and the drying process took a long time.
iv. Dolomitic limestone with magnesium: when dry, promoted a heterogeneous brittle coverage.

The items which initially showed favorable characteristics to the staining process were:

i. Powder dye, used with water and/or carbofuran syrup, presented coverage, opacity and appropriate fluidity, as well as quick drying. Did not cause seeds clumping.
ii. Liquid dye (Chess ®), used with water and/or carbofuran syrup, presented adequate coverage and smooth, as well as fast drying, however caused higher brightness. Did not cause seeds clumping.

3.2.1 Material selection for seeds camouflage: phase 1

This phase started in January 2002 aimed at testing different materials to be possibly used in the process of concealment of the seeds. Features like the coloring capacity of the pigments, its viscosity, seeds agglutination during the treatments, homogeneity and cover resistance were observed, and so the materials deemed suitable were selected for phase 2.

The material was tested in phase 1: white plaster, calcium carbonate, powder dye, liquid dye, ink (synthetic pigment concentrate), white acrylic glue, dolomitic limestone with magnesium (Hidrax ®) and carbofuran syrup.
In each test (i.e. for every mixture type), a portion of corn (100 g) was poured into a plastic cup. In a separate bowl, the material to be tested was mixed up, adding soon after this mixture to the corn. The closed vessel was shaken to simulate the machine where seeds normally receive carbofuran syrup.
Experiments were carried out by varying the proportions of material used, resulting in 75 samples of treated seeds. Samples that showed notable coloring failures were excluded. From 75 experiments, 20 samples of shades of different colors were obtained, going from dark brown to lighter shades, varying also the covering texture of the seed by the mixtures.
As for colourings, the best results were obtained with the following proportions: 2 to 4 kg of dye powder to 100 kg of seeds, using 2 l of carbofuran syrup and adding 2 to 3 l of water.
Particularly for the red latosol, the best results in coloring tests were shades tones achieved by mixing the following proportions of powder dye (for 100 kg of seeds, using 2.0 liters of carbofuran syrup): yellow powder (2.6 kg), red powder (0.7 kg), brown powder (0.4 kg), totaling 3.7 kilograms of dyes.
These proportions resulted in a medium brown shade. In order to obtain similar shades of dark or medium brown, the ratios mentioned above must be changed. For a darker ground, it is necessary to use more brown and less yellow, for a lighter soil, one should use more yellow and less brown.
The use of soil as the main element to camouflage corn seed showed good results for colour and texture. The proportions that showed the best results in the laboratory were: 6 l of water, 0.5 l of acrylic glue, 2 l of carbofuran syrup, 50 kg of sieved soil. The better amount of water to 1 to 2 kg of powdered dye was 1.5 to 2 l (using 2 l of syrup) to 100 kg of seeds. The amount of acrylic glue that brought satisfactory results was 0.2 l per 100 kg of treated seeds with 2 l of syrup.

Fig. 14. Prototypes of camouflaged seeds, during the experiment in color and texture. These treatments were selected for the germination experiment

The mixture of dry soil sieved with acrylic glue and water covering the seeds presented an adequate concealment from the ground as the main element of camouflage. The powder dye mixed with carbofuran syrup and water; and the mix of soil, water, and glue; were the treatments that showed the best colouring results, and the proportions were:

i. soil as a secondary element (untreated seeds): 1 kg of brown powder dye, 2 l of carbofuran syrup, 4 l of water, 1 l of glue, 30 kg of sieved soil;
ii. soil as a secondary element (previously carbofuran syrup-treated seeds): 0.3 kg of brown powder dye, 0.7 kg of red powder, 2.6 kg of yellow powder dye, 0.2 l of acrilic glue, 3 l of water, 20 kg of sieved soil.

3.2.2 Material selection for seeds camouflaging: phase 2

This phase, conducted between February and March 2002, aimed to:

i. Determine the ideal minimum proportions of pigments in order to obtain a homogeneous covering of the seeds and complete disappearance of the reddish color caused by the Rhodamine B, present dye in the carbofuran syrup.
ii. Determine the minimum proportions of pigments in order to achieve coloring shades next to the various shades of the soil.
iii. Determine the minimal amount of water to be added to the mix.
iv. Determine the minimal amount of glue, necessary to the best pigment adhesion to the seeds, in the case of seeds already treated with carbofuran syrup.
v. Test the possibility of using soil as the main element or secondary one in the camouflage of seeds.

Corn and pigments (powder dye) were weighed on an analytical balance. Corn seeds were divided into portions of 100 g. Powder dye in brown, yellow and red colors, were divided into different portions from 0.1 g to 2.0 g.

In each test (i.e. for every mixture type), a portion of corn (100 g) was poured into a plastic cup. In a separate bowl, the material to be tested was mixed up, adding soon after this mixture to the corn. The closed vessel was shaken to simulate the machine where seeds normally receive carbofuran syrup.

When the sifted soil was used, first the seeds received the coverage mentioned above, after, while they were still wet, they received a layer of dry soil. Then, it was added water and glue, and more servings of dry ground, until it reached the proper coverage. Next, the seeds were placed in plastic containers for dry and then received visual analysis.

Treatments (or prototypes) of camouflaging obtained with the ingredients that gave the best results (Figure 14) were evaluated for germination and can be found in the Table 1.

3.3 Camouflaged seeds in germination tests

3.3.1 Seeds camouflaging with powder dye

This experiment, conducted in January 1982, aimed to assess whether the pigments used in the process of concealment of seed germination are inhibitors of wheat germination, with or without the presence of carbofuran and the fungicide that is applied in the comercialized seeds.

Eight treatments were established:

i. Red powder dye.
ii. Blue powder dye.
iii. Yellow powder dye.

iv. Red + yellow powder dye + fungicide + carbofuran.
v. Red + yellow + blue powder dye + fungicide + carbofuran.
vi. Fungicide.
vii. Fungicide and carbofuran.
viii. Control.

For each treatment was prepared two replicates with 49 seeds in each container, spaced about 1.5 cm apart. The filter paper was adequately dampened and the samples were covered with plastic film to retain moisture, and then placed in an incubator at a temperature of 25 ° C. The samples were verified on the fifth day after sowing, when they took an inventory of germinated seeds.

As suggested in Table 1, the powder dye did not cause negative effects on the wheat germination. The experiment suggests the contrary, since it was observed a dark-greenish coloring in the seedlings of 1 to 5 treatments, and also larger seedlings from treatments 4 and 5, probably due to the nutritional action of the iron element contained in the powder dye.

Treatments	%
Blue	100
Red + yellow + fungicide + carbofuran	100
Red + yellow + blue + fungicide + carbofuran	100
Fungicide	100
Control	100
Red	98
Yellow	98
Fungicide + carbofuran	98

Table 1. Effects of powder dye in the weath germination

3.3.2 Seeds camouflage with liquid dye

To know the influence of liquid colorants in the germination of rice seeds, the second experiment was done in February 1982, where the pigments used in the process of seeds concealment, with or without the presence of carbofuran were tested. Twelve treatments were established:

i. Ocher liquid dye.
ii. Orange liquid dye.
iii. Blue liquid dye.
iv. Yellow liquid dye.
v. Black liquid dye
vi. Red liquid dye.
vii. Red, yellow and black liquid dye.
viii. Red, yellow, black liquid dye, and Carbofuran.
ix. Blue, orange, yellow and ocher liquid dye.
x. Blue, orange, yellow, ocher liquid dye, and Carbofuran.
xi. Carbofuran.
xii. Control.

For each treatment four replicates were prepared with 49 seeds in each container, spaced about 1.5 cm apart. The filter paper was adequately dampened and the samples were covered with plastic film to keep it damp. Seeds were placed in an incubator at a temperature of 25 ° C. The samples were verified in the second and fifth days after sowing, when they took an inventory of the seeds and subsequent analysis of nonparametric variance.

The results of this trial are shown in Table 2 and Figure 15, showing that according to the Kruskal-Wallis test there was heterogeneity (H = 470, p = 0.000, df = 11) on the rice germination under different formulations and formulations combinations of the liquid dye. The germination varied from 92% to 100% on fith day in greenhouse. The grains that less germinated belonged to orange, blue, yellow, and blue + orange + yellow + ocher treatments. This finding prosibly stems from the different composition of organic and inorganic pigments of each dye, which negatively affected the rice germination in the case of treatments 2, 3, 4 and 7.

Treatments	Treat	*X*	%
Black	5	49	100
Red	6	49	100
Blue + orange + red + yellow + ocher + carbofuran	8	49	100
Control	12	49	100
Carbofuran	11	48.1	98
Ocher	1	47.04	96
Red + yellow + black	9	47	96
Red + yellow + black + carbofuran	10	47	96
Orange	2	46.06	94
Blue	3	45.1	92
Yellow	4	45.1	92
Blue + orange + red + yellow + ocher + carbofuran	7	45.08	92

Legend: Treat – treatment number; % - Percentage of seed germination; *X* – Germination average

Table 2. Effects of liquid dye in the rice germination

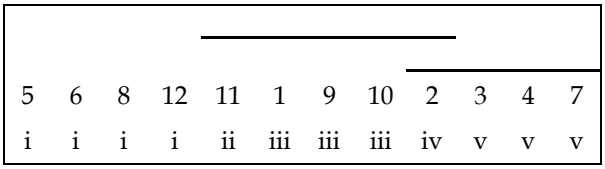

Fig. 15. Variation of the germination average of rice treatments. Arabic figures represent treatments according to Table 2. Roman figures represent decreasing ranking of the seedlings number. Treatments means united to bars are equal to 5% of significance level.

Black, red and ocher dyes should be preferred to others in the seeds camouflage, since they don't adversely affect germination. Existing the need for making colors compositions to match the camouflage to the soil, the dyes cited above must be used, as they undo the action of dyes that inhibit germination. This fact could be observed in seed germination of treatment 8 (blue + orange + red + yellow + ocher + carbofuran).

3.3.3 Improved camouflages

These new tests started in April 2002, they aimed evaluate whether the ingredients used in the process of improving the camouflage (section 3.1.2.2, Figure 14) would be inhibitors of corn seeds germination. For each treatment, four replicates were prepared with 14 seeds in each container, spaced about 1.5 cm apart. The treatments were based in the Table 3, where can be find the ingredients and proportions for each treatments.

Treatments	Powder dye	Powder dye	Powder dye	Liquid dye	Syrup	Glue	Soil	Water
Corn without carbofuran syrup	**Brown (g)**	**Red (g)**	**Yellow (g)**	**Brown (ml)**	**(ml)**	**(ml)**	**(g)**	**(ml)**
Powder dye, liquid dye and water	0.5	-	-	0.5	-	-	-	2
Powder dye, glue and water	0.5	0.2	-	-	-	0.2	-	2
Powder dye, syrup and water	1	-	-	-	2	-	-	2
Powder dye, syrup, soil and water	1	-	-	-	2	-	20	2
Corn with carbofuran syrup								
Powder dye, liquid dye and water	0.3	0.1	-	0.2	-	-	-	2
Powder dye, glue and water	0.4	-	0.4	-	.	0.2	-	2
Powder dye, glue, soil and water	0.3	0.7	2.6	-	.	0.2	10	3
Corn with carbofuran syrup and fungicida								
Powder dye, liquid dye and water	0.4	-	0.8	-	.	0.2	.	2
Powder dye, liquid dye and water	0.5	-	-	0.5	.	-	.	2

Table 3. The best treatments obtained in the material selection for seeds camouflagin, phase 2.

Seeds were placed in an incubator at a temperature of 25°C. The samples were scanned in the second and fifth days after sowing, when an inventory of the seeds and subsequent analysis of nonparametric variance were performed.

In two days in the germination chamber there was heterogeneity ($H = 31.29$, $p = 0.001$ $df = 11$) in the behavior of the tested seeds. Treatments 4, 3, 7 and 8 had the greatest germination average in relation to other, and treatment 8 occupies an intermediate position between these two groups (Table 4, Figure 16).

According to Table 4 and Figure 16, the association (or not) of the liquid and the powder to carbofuran syrup, besides the glue, didn't impair seed germination. By contrast, germination was better with these associations in relation to treatment 1 (carbofuran syrup) and 6 (control). Probably the nutrient action of the powder pigment, rich in iron, favors this result.

Tratamentos	Treat	*X*	%
Carbofuran syrup + p. dye + glue + water	4	13.5	96
Carbofuran syrup + p. dye + liq. dye + water	3	11.75	83
P. dye + liq. dye + water	7	11	78
P. dye + glue + water	8	10	71
Carbofuran syrup + p. dye + water + soil	12	8.75	62
Carbofuran syrup + p. dye + glue + water + soil	9	6.5	46
Carbofuran syrup + fungicide	2	5.25	37
Carbofuran syrup	1	4.5	32
Carbofuran syrup + fungicide + p. dye + glue + water	5	3.5	25
Carbofuran syrup + p. dye + water	11	3.5	25
Control	6	3	21
Carbofuran syrup + fungicide + p. dye + liq. dye + water	10	3	21

Legend: Treat – treatment number; % - percentage of germination; *X* – média de germinações; *X* – Germination average; P. dye – Powder dye; Liq. dye – Liquid dye

Table 4. Effects of camouflagin in the corn seeds germination, after two days

4	3	7	8	12	9	2	1	5	11	6	10
i	ii	iii	iv	v	vi	vii	viii	ix	ix	x	x

Fig. 16. Variation of the germination average of corn treatments in two days. Arabic figures represent treatments according to Table 11. Roman figures represent decreasing ranking of the seedlings number. Treatments means united to bars are equal to 5% of significance level.

Tratamentos	Trat	*X*	%
Control	6	14	100
P. dye + liq. dye + water	7	14	100
P. dye + glue + water	8	14	100
Carbofuran syrup + p. dye + water + soil	12	14	100
Carbofuran syrup + p. dye + water	11	13,75	98
Carbofuran syrup + p. dye + glue + water	4	13,5	96
Carbofuran syrup + p. dye + liq. dye + water	3	12,75	91
Carbofuran syrup + fungicide + p. dye + liq. dye + water	10	12,5	89
Carbofuran syrup + p. dye + glue + water + soil	9	12,25	87
Carbofuran syrup + fungicide	2	11	78
Carbofuran syrup	1	10,5	75
Carbofuran syrup + fungicide + p. dye + glue + water	5	9,5	67

Legend: Treat – treatment number; % - percentage of germination; X – média de germinações; X – Germination average; P. dye – Powder dye; Liq. dye – Liquid dye

Table 5. Effects of the camouflagin in the corn seeds germination, after five days

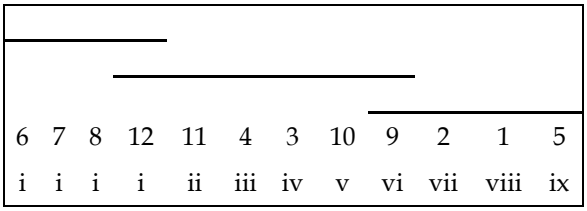

Fig. 17. Variation of the germination averages of corn treatments in five days. Arabic figures represent treatments according to Table 5. Roman figures represent decreasing ranking of the seedlings number. Treatment averages united to bars are equal to 5% of significance level.

Components that may have inhibited germination in two days were: i) soil associated with carbofuran syrup (treatment 12); i) soil and carbofuran syrup (treatment 9), i) carbofuran syrup and fungicide (treatments 2, 5, 10), iv) dye powder and carbofuran syrup (treatment 11).

With over three days in a germination chamber, the analysis model of variance suggests that the heterogeneity increased ($H = 36.74$, $p = 0.0001$, $df = 11$), with the segregation of three distinct groups (Figure 17). The best averages germination were obtained in the absence of the fungicide and the carbofuran syrup, which inhibited the emergence of the seed embryo, respectively in the first and second scale, in the fifth day at the germination chamber. The dye powder, liquid dye and glue did not inhibit the corn germination.

4. Camouflaging on the seed treatment machines

In commercial scales plantations there are large quantities of seeds treated with pesticides such as carbofuran and carbosulfan. Machines made specifically for this purpose are usually used. Aiming to test the compatibility of these machines to camouflage seed, tests were carried out during seven experiments of planting described in Almeida et al. (2010a) and Almeida & Almeida (in press). In these tests, dyes were added to the carbofuran syrup with or without Rhodamine B, since in certain circumstances, the syrup of carbofuran is found only on sale together with Rhodamine B. More recently, an eighth test was conducted with the objective to test dyes proportions in a new treatment machine, made by another enterprise.

The results of all tests were subjected to visual inspection, observing subsequent drying to coloring, assemblage, texture and adhesion of seeds.

In all seven initial tests, the process of seeds concealment of with powdered or liquid dye was carried out with ease, there were no problems operating the machines by FMC, bad seed coating or agglutination due to the presence of camouflage dyes, except for carbosulfan liquid syrup manufactured by FMC (Marshal 25 ST Brown). This syrup presented showed an irregular coating at a rate of 3 kg of the syrup to 100 kg of seeds, being necessary to increase the amount of dye syrup to 5 kg, to have more homogeneity in seeds coverage. The use of camouflage made with powdered dyes was more frequent and was obtained using 4 kg of powder to 100 kg of seeds.

The latest tests were performed in June 2002 using the Fersol company machine (Figure 18) that worked best with the proportion of 1.5 liters to 2 liters of a solution of carbofuran syrup with Rhodamine B, and 1.9 kg of powdered dye to 20 kg of seeds.

Fig. 18. Seed treatment machine, used to test the application of camouflage in corn seeds. On the floor, in plastic sacks, results of three tests of camouflage by varying the amount of carbofuran syrup, dye powder and water.

Evidently, the color is an important aspect for the camouflage effectiveness (Hartley et al., 1999, 2000), however the presence of Rhodamine B in the application of the camouflaged coating on a commercial scale impair the obtention of the desired brown shades.
During field removal experiment, camouflaged coats previously with similar colors, shades differ in terms of nuances during the time spent in the field. Nonetheless, there were no differences on removal rates among camouflaged seeds. Apparently it may not be necessary having major similarities of shades between camouflaged seeds and the soil to prevent higher consumption rates (Almeida et al., 2010b). Such observation is supported by Merilaita & Lind (2005), who concluded that the exact similarity of color among the prey and the background is neither enough, nor necessary to minimize the probability of detection by the predator.
In order to have a greater similarity between the camouflage colors and soil, with Rhodamine B in the mixture, it may be necessary to perform two mechanical treatments. First one, to apply carbofuran syrup and when it dries, it proceeds with the camouflaged covers powder or liquids dyes. Once using Carbofuran without Rhodamine B, one can be apply the camouflaged cover concurrently, there being no need for two treatments. Aside from this drawback, the concealment process of seeds is easily obtained using the machines to seed treatment with carbofuran syrup, there being no need for mechanical adjustments.

5. Discussing on the field performance of the prototypes

Improved camouflage prototypes, whose ingredients were evaluated in Section 3.1.2.2, were tested for germination (section 3.2.3), regarding the seeds removal by birds in agricultural field (described in section 2, Almeida et al. 2010b) and then using this same experiment, the performance of these prototypes will be discussed, regarding the response of the

camouflaged covers to weather during five days of exposure, usually when the seeds germinate until the third day after sowing.
The lowest numbers of removals were found for seeds which camouflage was based on powder dye, water, liquid dye, and acrylic glue (Table 5, treatments 3, 4, 5). From the third day on, a significant removal of seeds from treatment 7 (camouflaged with soil, glue and water) happened, as a result of rupture of the coating layer, which exposed the yellow color to the birds. This result, like the seeds removal from treatment 6 (dye powder, soil, glue and water), suggests that soil use as an camouflage ingredient would be inadequate, probably due to less coverage durability to weather with each passing day. In fact, at the end of the trial, the coatings containing soil were broken, leaving the seeds in evidence.
In despite of non significative at 5%, the highest removal of seeds camouflaged with industrial liquid dye in the treatment 5, shows the importance of superficial grooving in the coverage, attributed to the powder dye in the least removed camouflaged seeds in Trials 1 and 2 (Almeida et al., 2010b). These results suggest that the efficiency of the camouflaging is not only characterized by the brown dye, or because of the similarity of shades between the soil and the seeds, but also by a group of factors attributed to the seed coverage and the background, like opacity and superficial grooving in the seed, and the presence of irregularities and organic matters on the soil surface. In this case, opacity and superficial grooving were provided by the powder dye in the 'home-made' camouflaging (treatments 3 and 4), which, at least to the human view, notably were less conspicuous in the agricultural soil.
In addition to lower rates of seed removal as well as bird mortality, germination rates obtained with the powder dye camouflage (Tables 4 and 5) suggest that in terms of nutrition to the seedlings, the use of these covers can interest the farmer. This part will be appropriate for farmers who wish to camouflage their seeds on their own, without depending on agrochemical companies. Results from tests with materials and methods that have allowed the improvement on the camouflaging methods, including experiments with dye and texture, effects of camouflaged coatings on seed germination and procedures taken during seed treatment with camouflages, dyes and pesticides can be helpful in this issue.
In some trials using powder dye camouflaging without adhesives, as acrylic glue, insectivorous birds were unexpected victims. A cloud of powder released by the seed drill next to the ground was observed during sowing, and it may have poisoned arthropods, which were then consumed by insectivorous birds (Almeida et al., 2010a; Almeida & Almeida, in press). However, in the process of improving camouflage, the use of acrylic glue prevented the detachment of the dye powder from the seeds. Therefore, adhesives should be required ingredients when there is an association of pesticide to powder camouflage.
The experimentation revealed that the better ingredients to create camouflage covers for seed were: i) powder dye, acrylic glue and water; ii) powder dye and water; iii) powder dye, soil and water; iv) powder dye, liquid dye and water. They show that 'home-made' camouflages with low financial and operational costs present good results in the field, sometimes better than industrial ones.

6. Conclusions: and so, what matter at now and to the future?

As research results, in 2003 were granted licenses by the Ministry of Health and the Brazilian Institute of Environment and Natural Resources (IBAMA) for the commercial use of the camouflaged carbofuran syrup without Rhodamine B.

Currently, camouflaged seeds in the industrial process have more shine and do not have as much surface roughness compared to those of 'hand-made' camouflage. Thus, the effectiveness of the industry seed depends more on the similarity of their colors in relation to the soil. However, probably due to issues associated with large commercial scale production and financial costs, it is common for industrial seed showing clear tone differences from the ground in certain areas, which can compromise the effectiveness of camouflage.

If camouflage seeds fail to effectiveness desired in not attracting birds and are sold at higher prices than seeds stained with Rhodamine B, the tendency is that these seeds are sold only in regions where extremely dense granivorous birds populations threaten to undermine the crops, as soon as they are sowed.

Thus, an appropriate color to the soil and the camouflaged seeds commercialization would be restricted to the occurrence of bird pests outbreaks, as it's noticed in the western of São Paulo state, whose Eared doves population (*Zenaida auriculata*) have been causing huge losses in agriculture (Bucher & Ranvaud, 2006; Ranvaud & Bucher, 2006;).

Many bird species that usually die poisoned by seeds treated with Rhodamine B and carbofuran damage the plantations (Almeida & Almeida, in press). In regions where there were outbreaks of Eared doves, sometimes farmers deliberately poisoned them offering wheat treated with Rhodamine B and carbofuran (Almeida & Almeida, in press). So, indeed, many farmers do not care about the accidental plague bird deaths in their crops, and they are probably unaware of the secondary poisoning risks that take predators and scavengers. As the amount of remaining forest in certain agricultural landscapes is so scarce, it may be plausible that the impact of secondary poisoning is jeopardizing the survival of scarce predators populations that depend on forests, with risks of local extinctions of wildlife populations such as mammals: Canidae, Felidae, Mustelidae, Procyonidae, and birds: Accipitridae, Falconidae, Strigidae and Cathartidae.

In order to minimize the risks outlined above, this text strongly emphasizes that, when it comes to planting seeds, the Brazilian government should ban the use of dyes such as Rhodamine B, which are attractive to birds and should at least, make obligatory the use of dyes in similar shades with to the soil in each region.

If according to the Brazilian Federal Decree 4.074 (January 4, 2002) agrochemical defensives must be added to the seeds in association with dyes, to reduce risk of human poisoning, the camouflaging should be employed, substituting Rhodamine B, since the use of carbofuran and Rhodamine B is confliting with Brazilian Law nº 7.802 (July 11, 1989), which prohibits the registration of pesticides and components that may cause environmental damage.

The Brazilian government should invest more to reduce conflicts between agricultural production and wildlife. Research is needed on methods to mitigate the impacts from various pesticides, as well as their use. Impacts assessments of secondary wildlife poisoning should be performed. There is lack of control on the agrochemicals use, as well as in divulgation of the poisonings in the wildlife. There is lack in the efforts in basic education in concern the conservation and in more sustainable ways of producing food.

The method of camouflaging seeds does not solve the problem of various damage types caused by highly toxic pesticides in the Brazilian agricultural landscape, it is suitable for a particular case, transcript in this text, it equals to a conservationist flag at an iceberg tip, whose enormous base represents huge poisoning problems, which are not visible by most Brazilians, themselves consumers and potential primary victims.

As the carbofuran, several other pesticide formulations and their application forms have been banned in most developed countries like the U.S. and Canada, however in less developed countries, such pesticides are still produced, used, imported and exported (Richards, in press). The governments of the 'in development countries' should be concerned to monitor the evolution of this knowledge, adopting similar restrictions and mitigation measures.

7. Acknowledgements

We also thanks to Florindo Orsi and Antonio C. Zen for technical information; to Antonio Natal Gonçalves for information about colours and paints; to Paulo Y. Kageyama for the germination chambers; to Fersol Indústria e Comércio and to FMC Agricultural Products by the financing research and logistic support.
Alexandre thanks to the Arlinda Coelho and Edisiene de S. Correia, for allowing your dedication to this chapter, during the work time in the SENAI. He also thanks the Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) for the post-doctoral fellowship (2319/2009), when part of the text was wrote.

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Part 3

Pesticides Mobility, Transport and Fate

21

Geochemical Indicators of Organo-Chloro Pesticides in Lake Sediments

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1. Introduction

The study area, Lake Liangzi is regarded as one of the least polluted lakes in Central China. This is because the local people have made great efforts to protect the lake from the use of all forms of chemicals for crop and fish farming activities. Besides this measure, other uses such as recreation, water supply and electricity production are also not permitted. In fact, it is noteworthy to mention that the lake supplies one of the most popular and delicious types of fish in Central China. It is called "Wuchang fish" and is very popular in most leading restaurants in Central China and quite expensive for its delicacy.
However, since modern technology has revealed that organic compounds such as Organo chloro-pesticides (OCPs) could be deposited into lakes and other water bodies from remote areas, far from the point of application, it will be worthwhile to undertake investigation to ascertain whether the lake is free from these contaminants. The path of investigation used dated sediment core since according to Venkatesan, et al, (1998), the history of chlorinated hydrocarbon contamination can be followed using contaminants from dated sediment cores, since sediments integrate and retain records of influx of recalcitrant organic contaminants. It should be mentioned that this volume of work is part of the author's PhD research and also one of the pioneering research works on pesticides in the lake.
OCPs are a group of common pollutants belonging to the class of pollutants called Persistent Organic Pollutants (POPs). One of the most important families of Persistent Organic Pollutants (POPs) that has a significant impact upon the environment are pesticides.
According to Sierra Club of Canada, (1998), pesticides are the only man-made toxic chemicals deliberately released over large areas. They are poisonous almost by definition. Pesticides poison the food chain, contaminate water supplies and are implicated in the declining populations of many biotic species.
In this study, a single drilled undisturbed sedimentary core extracted from Lake Liangzi in Central China was analyzed for target compounds using highly sophisticated instruments. Even though other organic compounds (such as Polycyclic Hydrocarbons; PAHs, Aliphatic Hydrocarbons; Ahs and heavy metals) were detected, they are not discussed in this article.. The focus of discussion are the organocgloro-pesticides, including DDT, DDD and DDE and the geochemical relationship between them.

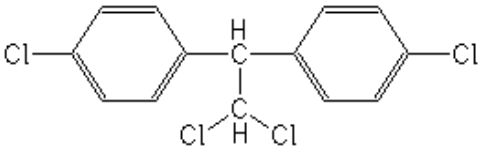
1.1 Brief background to DDT, DDD and DDE

According to Environmental Protection Agency (EPA) of USA, (1989), the term DDT refers to the collection of all forms of DDT, DDE, and DDD.

The primary forms or isomers of DDT, DDE, and DDD are (namely *p,p'*-DDT, *p,p'*-DDE, and *p,p'*-DDD) (US EPA,1989). US EPA, (1989), also indicate that DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane) is a pesticide that was once widely used to control insects on agricultural crops and insects that carry diseases like malaria but is now used in only a few countries to control malaria. Technical-grade DDT is a mixture of three forms, *p,p'*-DDT (85%), *o,p'*-DDT (15%), and *o,o'*-DDT (trace amounts). All of these are white, crystalline, tasteless, and almost odorless solids. Technical grade DDT may also contain DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) and DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethane) as contaminants. US EPA (1989), further indicate that both DDE and DDD are breakdown metabolites of DDT and that DDT does not occur naturally in the environment. They may occur in the atmosphere as a vapor or be attached to solids in air. Vapor phase DDT, DDE, and DDD may break down in the atmosphere due to reactions caused by the sun. DDT, DDE, and DDD last in the soil for a very long time, potentially for hundreds of years. Most DDT breaks down slowly into DDE and DDD, generally by the action of microorganisms. These chemicals may also evaporate into the air and be deposited in other places (EPA, 1989). These organic compounds, according to Agency for Toxic Substances and Disease Registry (ATSDR 2002), are easily broken down in air with a half-life of two days. Also according to ATSDR (2002), DDT and especially DDE build up in plants and in fatty tissues of fish, birds, and other animals.

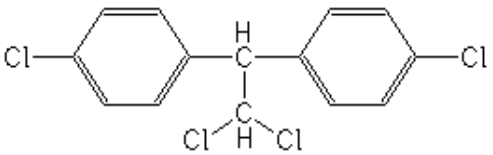
DDT

Chemical formula: ($C_{14}H_9Cl_5$).
Structure:



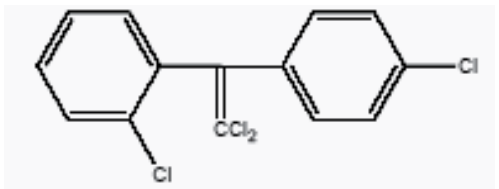
DDD

Chemical formula, ($C_{14}H_{10}Cl_4$).
Structure:



DDE

Chemical formula ($C_{14}H_8Cl_4$)
Structure:



1.2 Study area

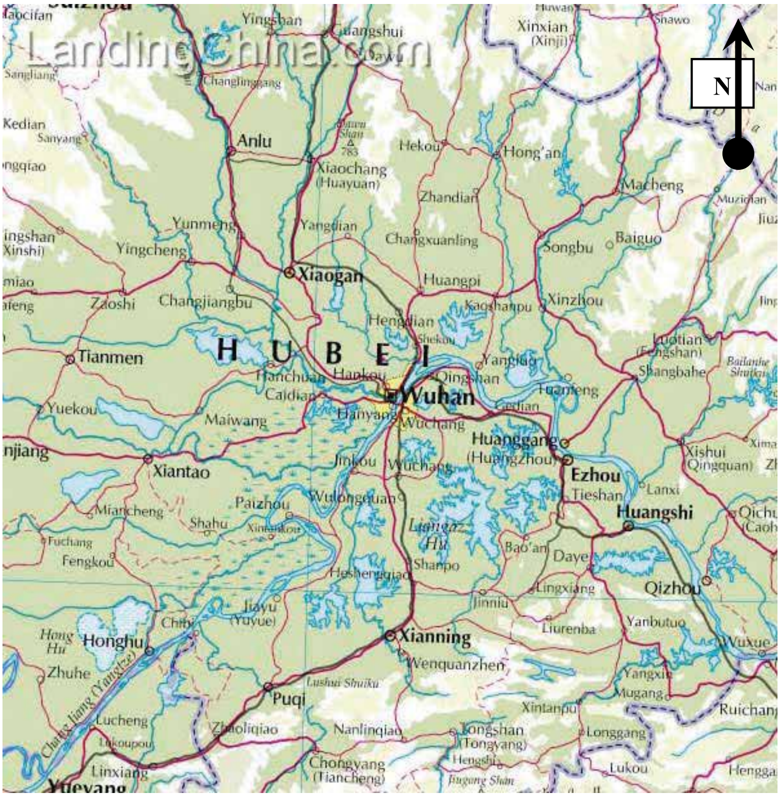


Fig. 1. Map of Wuhan and other major cities in Hubei Province (including the study area)
Source: LandingChina.com; "The Professional Travelling guide to China", 2004

1.2.1 The limnological data of the Lake Liangzi

The limnology according to Donyinah et al. (2007), is based on the information supplied by J. Xiangtan (1995). The lake which lies within the southeast area of the heavily industrialized city of Wuhan is the 13th largest freshwater lake in China and the second largest shallow lake of Hubei Province. It is a low-lying lake, which is located not far from the southern bank of the Yangtze River. In the 1950s, the actual surface area of the lake was 458.5km.^{2} (177.0 mi^{2}), but in the 1980s and 1990s, the area of the lake shrank to 227.15km^{2} (87.70 mi^{2}) because of reclamation of the lake for agricultural purposes. Also in the 1950s the lake was 2.25-2.8 m (7.38-9.18 ft) deep and the deepest area was 1.5 m (4.9 ft) during winter, when the water was lowest. In 1997 and 1998, the lake measured between 1.2 and 4.2 m (3.9 and 13.7 ft) and the average depth was 2.8 m (9.2 ft). The circumference of the lake totals more than 470 km (292 mi) and the shoreline is irregular. The vegetation around the lake is grass with a few trees. Except in the east, there are many small uplands and hills around the lake; the hills are, however, some distance away. Few croplands near the small hamlets around the extended edges of the lake. Rainfall is abundant, with the average rainfall range over several years being 1263.4mm/yr (49.7 in./yr). The average air temperature area the lake is about 17ºC. The highest monthly air temperature is 28.6ºC and the lowest is 3.3ºC. The wind circulation pattern consists of cold winds from the north blowing in the winter and spring and northeast and east winds blowing in the summer and fall, respectively. The temperature

of the water in the lake is higher than the surrounding air. Whereas the average temperature of the surrounding air over several years is 17.5ºC, the highest monthly average temperature of the lake is about 30ºC and lowest is 4.7ºC. Annually, there are 243 days when the temperature of the lake water is higher than 10ºC. No significant difference exists between the temperatures of each layer of water in the lake; the difference between the upper layer and the bottom layer is not more than 1ºC. The lake water is very clear, but the shallow water near the edges becomes turbid when there are stormy waves. The transparency changes between 0.3 and 1.4 m (1 and 4.5 ft) and the average is 0.8 m (2.6ft). Generally, the transparency in spring and winter is high, and the lake's water appears light green. The water is alkaline, and the pH is commonly about 8.1, with no distinct differences between the upper layer and the bottom layer. The main ions in the water are bicarbonate class. The iron content of the lake water is fairly high, and the average is 7 mg.

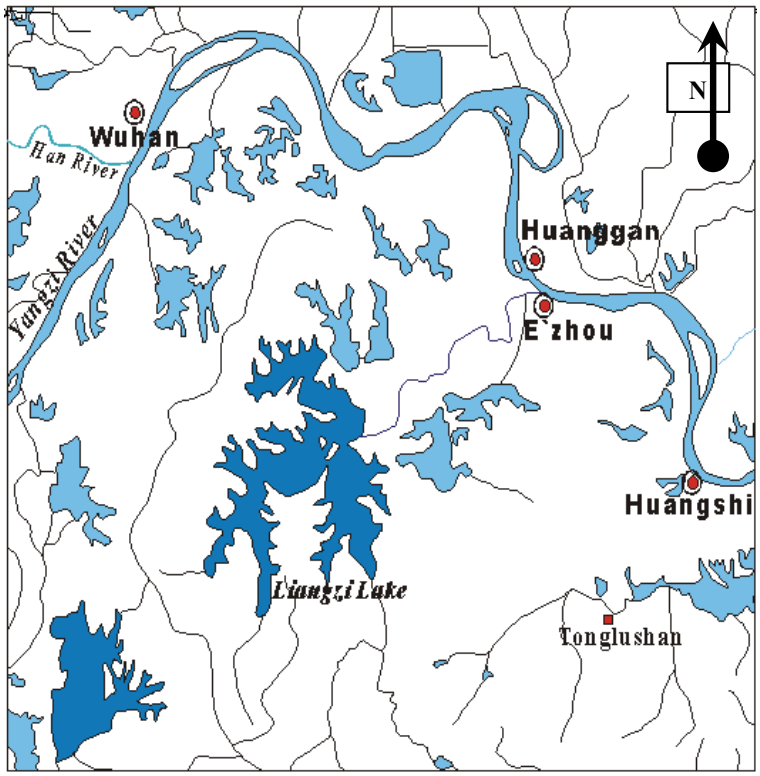


Fig. 2. A Map Showing the Location of Lake Liangzi

2. Sampling and sampling techhniques

The location chosen for sampling was at the middle and the deepest part of the Lake, the sediment core samples will thus assumed to be a true representative of the Lake's sediments and therefore, reliable for all analyses.

Sampling was carried out from a small local motorized fishing boat, using a soft manual hand-driven core-drill (KC Kajak Sediment Core Sampler). Core samples were taking from a depth of 70~80cm in the lake's bed and each core sample was immediately sliced (from top to bottom) into fractions of (1cm thick), using a well polished and treated metallic soft-core

sediment cutter. The sliced samples were immediately put into fresh plastic sample bags and serially well labeled, using coloured felt pens. Maximum precaution was taken to avoid any contamination, such as skin exposure, especially the bare hands (since the touching with the bare hands could contaminate the samples from the possible lipids of the hands by touching with the bare hands) and thus sampling was carried out by wearing fresh plastic disposable gloves. In order to preserve the freshness of the samples, they were immediately stored in a freezer at -20°C until the time for the laboratory analysis. The handling and the transportation of the samples from the lake to the laboratory were carefully and cautiously handled to avoid any possible contamination.

2.1 Laboratory methods

For the purpose of instrumental analyses, the target compounds were extracted from the solid samples and transferred into liquid form. This initial extraction process is called the pretreatment of samples. The pre-treatment was performed at the Environmental Laboratory of the School of Environmental Studies, China University of Geosciences, Wuhan. Standard laboratory procedures were followed throughout the process.

The reagents used for the pretreatment were hexanes, dichloromethane, surrogate standards, silica gel, aluminum oxide and distilled water (for washing and rinsing of the apparatuses before usage).

A total of seventy-seven (77) samples were analyzed. One hundred milliliters of the initial solvent, dichloromethane, was poured into the sample for the extraction process. The surrogate standard was introduced into one selected sample in each set of batch samples. A set of six batches of samples were kept on hot water bath (Model HHS-6; temperature range 37^{o}C-100^{o}C) and Soxhlet extractor at fixed temperature of 50^{o}C and left for 48 hrs. The temperature fluctuation during the period was within an accuracy of ±1^{o}C. The extractor was then filtered into another round-bottomed flask and concentrated by rotation evaporation using a rotary evaporator (ZFQ-85A; speed range 0-200 rpm) and Auto Science vacuum and pressure (model AP-01; pressure range = 0- 30 mm Hg [0-4 kPa]). The pressure used for the evaporation was fixed at 20 mm Hg (2.6 kPa) for all the samples. The concentrate was carefully transferred into special 5 mL sample bottles using special pipettes and immediately stored in a freezer. After the transfer of each concentrate, the round-bottomed flask was thoroughly rinsed with dichloromethane. The process was repeated for all the 77 samples. The extracts were then left to evaporate naturally at room (ambient) temperature and then mixed with hexanes until all the dichloromethane totally evaporated.

These extracts were then column fractionated into the target compound (OCPs) using silica and aluminium oxide. The particle size of the aluminium oxide and the silica gel was 100-200 mesh. Care was taken to label each extracted solution. The column extraction was conducted through columns of aluminium oxide, and silica gel was placed in 100 mL burettes. The aluminium oxide formed the top column (5 mL), whereas the silica gel was the bottom column (15 mL). Prior to passing the samples through the columns; 30 mL of dichloromethane was initially passed through the columns to wash any possible contaminants in the column. Another 30 mL of the dichloromethane was passed through the columns after each sample was introduced into the column for the extraction of target organic compounds. These fractionated solutions were again concentrated by rotation evaporation using the Rotary evaporator (ZFQ-85A), and bottled in 8 milliliter cell bottles. They were then evaporated again, using a Nitrogen manifold setup with a gentle stream of nitrogen gas (99.9% purity) to about 5mL. The final extracted samples of analysis were analyzed by gas chromatography (GC) and GC-mass spectrometry (GC-MS).

2.2 Instrumental analyses

2.2.1 Gas chromatography and gas chromatography /mass spectrometry

2.2.1.1 Instrument

GC: HP 6890 GC/FID (flame ionization detector).
GC-MS: HP5890II GC equipped with HP5972 MSD.

2.2.1.2 GC parameters

Column: HP-5 fused silica capillary column (30m×0.25mm×0.25um i.d.), constant flow1.5;
Inlet: splitless, temperature 290°C
Oven: initial 60°C keep 1 min, 6°C /m to 290°C keeps 20 min;
Detector: flame ionization detector (FID), temperature 300°C.

2.2.2 Qualitative analysis and quantitative analysis

2.2.2.1 The qualitative and quantitative analysis of PAH

The GC-MS was calibrated with standard compounds of known concentrations at eight different concentrations: 0.2 ppm[1], 0.4 ppm, 0.8 ppm, 1 ppm, 2 ppm, 4 ppm, 8 ppm, and 10 ppm. The calibration curves of 16 PAHs based on the average response factors were used for concentration determination. Target compounds were qualified using the internal standard technique. Their concentrations were reported as nanogram/gram (μg/g) based on dry sediment weight.

2.2.2.2 The qualitative and quantitative analysis of aliphatic compositions

The lack of standard compounds seems to bring difficulty to the qualitative and quantitative analysis of aliphatic composition, but the equally-spaced retention time makes it possible to determine the carbon number of aliphatic compositions properly once one of a series of samples was subject to GC-MS analysis. Because the response factors of aliphatic compositions with different carbon number are almost the same, the quantitative analysis was accomplished in accordance to the response factor of internal standard $C_{20}d_{42}$.

2.2.3 QC/QA

A strict Quality Control/Quality Assurance (QC/QA) measurement was followed throughout the process of analysis.
The recovery of the analytic procedure was determined by measuring the amounts of surrogate compounds added prior to sample extraction. The recoveries range from 44.2% to 118.3% (except Nap-d8 volatile even at room temperature), within the restrictive range of US EPA 610 method Therefore, except Nap whose values can only serve as reference, other data satisfy the demand for quantitative analysis. All data have not been revised and all concentration values are according to the instrumental recoveries. All the PAH concentrations were reported on a dry weight sediment basis.
The relative standard derivation for duplicate samples ranged from 0 to 15%.
A method blank experiment was carried out for every 12 samples.
The solvent blanks were checked and none of the investigated substances were found in the blanks.

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[1] ppm (parts per million)

2.3 Dating of sediments

The dating of the samples was carried out at the State Key Lab of Organic Geochemistry (SKLOG), Guangzhou Institute of Geochemistry, and Chinese Academy of Sciences.

Lead isotope 210 (^{210}Pb) was used for dating the samples and the results obtained showed that at a depth of 18 cm from the surface of the lake's bed, the age of the samples was a little over 100 years, (precisely 101 years). A graph of depth age was plotted and indication showed that the age of the lake sediment at any depth could be found by simple extrapolation from the graph.

A regression graph was also plotted to indicate the accuracy of the technique of analysis. Both graphs are presented below.

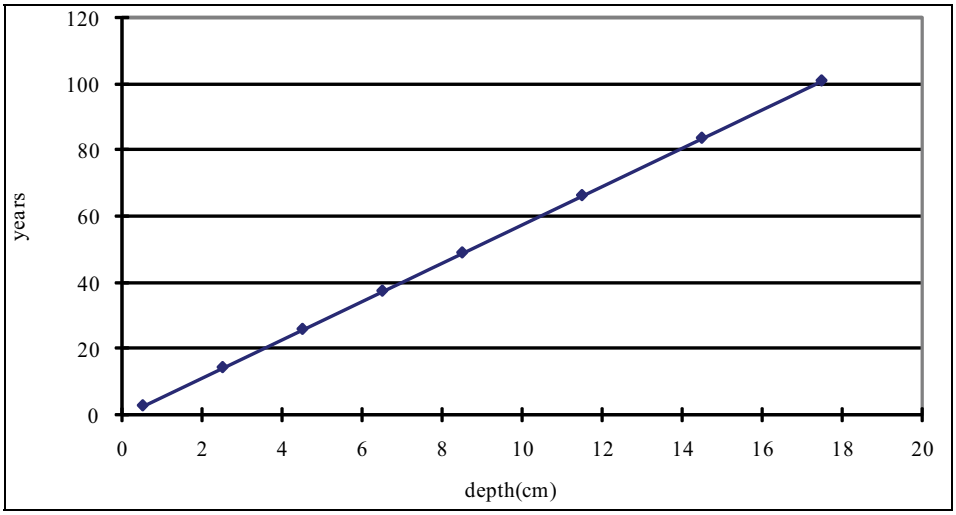

Fig. 3a. Graph of Dating (years) verses depth (cm)

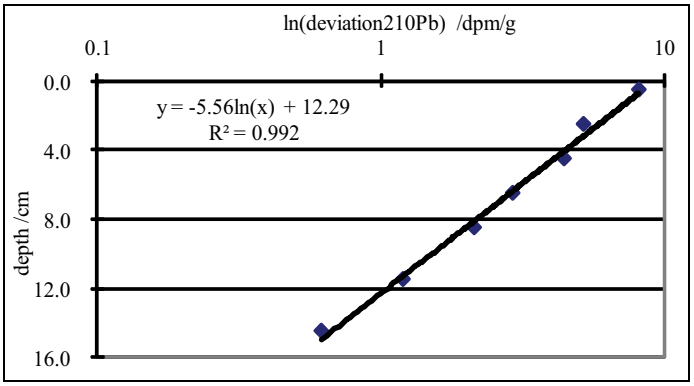

Fig. 3b. Regression Diagram of accuracy of Results of Dating by (210Pb)

3. Results and discussions

The results derived from the instrumental analysis were processed using Excel and Original (Version b) and presented in a tabular form (tables 1 to 4 refer). Graphical illustrations are represented in figures 4 to 9.

Depth (cm)	TCMX	a-HCH	b-HCH	d-HCH	r-HCH	heptachlor	Aldrin
-3	0.30	0.28	1.42	1.36	0.80	0.09	0.07
-4	0.19	0.11	0.49	1.25	0.11	0.00	0.00
-5	0.12	0.10	0.80	0.45	0.35	0.00	0.00
-6	0.72	0.09	0.33	0.50	0.00	0.00	0.00
-7	0.05	0.08	1.99	0.23	0.10	0.00	0.00
-8	0.06	0.05	0.77	0.20	0.07	0.00	0.16
-9	0.05	0.04	0.29	0.42	0.10	0.00	0.01
-10	0.05	0.02	0.13	0.15	0.09	0.00	0.00
-11	0.05	0.01	0.11	0.12	0.07	0.00	0.00
-12	0.11	0.04	0.07	0.31	0.11	0.04	0.06
-13	0.00	0.01	0.08	0.09	0.08	0.00	0.04
-14	0.28	0.04	0.11	0.27	0.07	0.00	0.00
-15	0.12	0.04	0.10	0.13	0.03	0.00	0.03
-17	0.04	0.01	0.06	0.25	0.15	0.00	0.14
-18	0.08	0.00	0.07	0.18	0.06	0.00	0.03
-19	0.84	0.06	0.11	0.11	0.07	0.05	0.04
-20	0.54	0.07	0.07	0.10	0.08	0.00	0.00
-21	0.33	0.06	0.26	0.22	0.03	0.01	0.08
-22	0.40	0.08	0.10	0.56	0.06	0.01	0.06
-23	0.13	0.05	0.06	0.13	0.05	0.00	0.01
-24	0.28	0.08	0.05	0.26	0.11	0.03	0.00
-25	0.02	0.00	0.00	0.42	0.01	0.02	0.00
-26	0.03	0.01	0.06	0.13	0.07	0.04	0.02
-27	0.08	0.02	0.14	0.31	0.15	0.00	0.06
-28	0.07	0.03	0.06	0.18	0.08	0.00	0.00
-29	0.00	0.01	0.04	0.11	0.08	0.00	0.01
-30	0.05	0.03	0.07	0.54	0.13	0.03	0.00
-31	0.04	0.03	0.13	0.19	0.08	0.03	0.00
-32	0.02	0.02	0.16	0.20	0.18	0.04	0.00
-33	0.06	0.00	0.03	0.07	0.04	0.00	0.01
-34	0.04	0.06	0.08	0.11	0.02	0.00	0.00
-35	0.03	0.06	0.19	0.26	0.04	0.03	0.02
-36	0.04	0.04	0.07	0.10	0.09	0.00	0.00
-37	0.03	0.03	0.16	0.08	0.00	0.09	0.04
-39	0.03	0.02	0.03	0.09	0.05	0.00	0.00
-40	0.02	0.00	0.03	0.23	0.06	0.00	0.01
-41	0.05	0.03	0.09	0.17	0.07	0.00	0.00
-42	0.03	0.00	0.00	0.17	0.02	0.00	0.00
-43	0.03	0.01	0.06	0.06	0.05	0.01	0.00
-44	0.05	0.03	0.06	0.17	0.07	0.00	0.00
-45	0.04	0.03	0.06	0.32	0.07	0.01	0.00
-46	0.06	0.03	0.07	0.23	0.07	0.00	0.00
-47	0.01	0.00	0.00	0.11	0.02	0.00	0.00
-48	0.04	0.03	0.12	0.14	0.05	0.01	0.00
-49	0.03	0.01	0.05	0.14	0.03	0.00	0.00
-50	0.03	0.02	0.06	0.19	0.05	0.01	0.00
-51	0.03	0.02	0.05	0.18	0.05	0.01	0.00
-52	0.04	0.03	0.15	0.12	0.05	0.01	0.00
-53	0.06	0.01	0.05	0.24	0.10	0.02	0.00
-54	0.48	0.03	0.05	0.15	0.03	0.01	0.02
-55	0.00	0.03	0.03	0.14	0.01	0.01	0.01
-56	0.65	0.05	0.11	0.21	0.05	0.03	0.03
-57	0.00	0.04	0.03	0.06	0.00	0.00	0.01
-58	0.02	0.01	0.04	0.08	0.04	0.00	0.00
-59	0.00	0.03	0.00	0.10	0.05	0.01	0.02
-60	0.02	0.02	0.04	0.27	0.02	0.01	0.00

Table 1. Results of Organochloro-Pesticides (OCPs) (concentration in ng/g)

Depth (cm)	heptachlor epoxide	a-chlordane	endosulfan 1+r-chlordane	p,p'-DDE	Diedrin	o,p'-DDE	Endrin
-3	0.19	0.28	0.38	7.56	0.27	2.25	0.44
-4	0.00	0.18	0.12	6.95	0.00	1.38	0.00
-5	0.00	0.09	0.15	7.55	0.00	1.29	0.00
-6	0.00	0.06	0.10	8.30	0.00	1.27	0.00
-7	0.00	0.08	0.07	9.36	0.00	1.42	0.00
-8	0.00	0.01	0.00	3.93	0.00	0.49	0.00
-9	0.00	0.02	0.00	2.01	0.00	0.27	0.00
-10	0.00	0.00	0.04	0.96	0.00	0.15	0.00
-11	0.00	0.00	0.03	0.79	0.00	0.12	0.00
-12	0.00	0.05	0.02	0.47	0.00	0.20	0.00
-13	0.00	0.07	0.05	0.27	0.00	0.21	0.00
-14	0.00	0.05	0.04	0.19	0.00	0.00	0.00
-15	0.00	0.00	0.03	0.31	0.00	0.14	0.00
-17	0.02	0.05	0.02	0.13	0.02	0.00	0.00
-18	0.00	0.10	0.05	0.15	0.00	0.00	0.00
-19	0.00	0.03	0.06	0.13	0.00	0.14	0.15
-20	0.00	0.00	0.00	0.12	0.00	0.00	0.00
-21	0.00	0.08	0.22	0.11	0.02	0.17	0.25
-22	0.00	0.07	0.02	0.13	0.00	0.06	0.09
-23	0.00	0.01	0.01	0.12	0.00	0.00	0.11
-24	0.00	0.00	0.02	0.07	0.00	0.14	0.81
-25	0.00	0.02	0.02	0.56	0.00	0.10	0.00
-26	0.00	0.01	0.02	0.06	0.00	0.13	0.00
-27	0.24	0.05	0.03	0.06	0.00	0.91	0.04
-28	0.00	0.05	0.04	0.10	0.00	0.00	0.00
-29	0.00	0.02	0.02	0.14	0.00	0.06	0.00
-30	0.00	0.19	0.09	0.09	0.00	0.13	0.00
-31	0.00	0.01	0.06	0.07	0.00	0.34	0.00
-32	0.00	0.12	0.21	0.10	0.00	1.93	0.00
-33	0.00	0.02	0.02	0.08	0.00	0.04	0.00
-34	0.00	0.02	0.02	0.07	0.00	0.00	0.00
-35	0.03	0.07	0.38	0.05	0.01	0.14	0.09
-36	0.00	0.03	0.02	0.05	0.00	0.06	0.00
-37	0.00	0.04	0.10	0.03	0.00	0.08	0.07
-39	0.00	0.01	0.01	0.03	0.00	0.00	0.00
-40	0.00	0.03	0.04	0.04	0.00	0.07	0.00
-41	0.00	0.01	0.07	0.03	0.00	0.22	0.00
-42	0.00	0.02	0.07	0.03	0.00	0.13	0.00
-43	0.00	0.00	0.02	0.03	0.00	0.13	0.00
-44	0.00	0.01	0.05	0.03	0.00	0.15	0.00
-45	0.00	0.09	0.06	0.02	0.00	0.09	0.00
-46	0.00	0.00	0.04	0.03	0.00	0.11	0.00
-47	0.00	0.01	0.01	0.02	0.00	0.02	0.00
-48	0.00	0.01	0.02	0.02	0.00	0.00	0.00
-49	0.00	0.01	0.03	0.03	0.00	0.09	0.00
-50	0.00	0.01	0.02	0.03	0.00	0.05	0.00
-51	0.00	0.01	0.02	0.03	0.00	0.08	0.00
-52	0.00	0.02	0.02	0.03	0.00	0.13	0.00
-53	0.00	0.05	0.03	0.03	0.00	0.07	0.00
-54	0.00	0.01	0.02	0.04	0.00	0.06	0.00

Table 2. Results of OCPs (concentration in ng/g) (Continue)

(cm)	Endulsufan 2	p,p'-DDD	o,p'-DDT	Endrin aldehyde	endosulfan sulfate	p,p'-DDT	Endrin ketone
-3	0.33	1.30	0.66	0.33	0.35	0.95	0.32
-4	0.12	0.73	0.32	0.05	0.07	0.44	0.20
-5	0.05	0.76	0.29	0.00	0.00	0.41	0.00
-6	0.00	0.84	0.13	0.00	0.00	0.24	0.35
-7	0.00	0.85	0.12	0.00	0.00	0.16	0.00
-8	0.01	0.24	0.15	0.00	0.00	0.23	0.02
-9	0.00	0.17	0.11	0.00	0.00	0.07	0.00
-10	0.06	0.11	0.51	0.00	0.00	0.00	0.00
-11	0.05	0.09	0.42	0.00	0.00	0.00	0.00
-12	0.05	0.06	0.41	0.00	0.00	0.04	0.03
-13	0.08	0.02	0.72	0.00	0.00	0.05	0.00
-14	0.01	0.02	0.06	0.00	0.00	0.03	0.01
-15	0.05	0.10	0.19	0.06	0.00	0.46	0.01
-17	0.08	0.01	0.81	0.00	0.00	0.08	0.03
-18	0.01	0.02	0.15	0.00	0.00	0.04	0.00
-19	0.08	0.05	0.31	0.00	0.11	0.37	0.02
-20	0.06	0.04	0.14	0.00	0.00	0.06	0.05
-21	0.10	0.07	0.34	0.06	0.04	0.22	0.12
-22	0.04	0.02	0.31	0.00	0.05	0.09	0.03
-23	0.01	0.02	0.07	0.00	0.00	0.03	0.00
-24	0.09	0.05	0.28	0.00	0.00	0.05	0.01
-25	0.01	0.05	0.12	0.00	0.00	0.14	0.11
-26	0.00	0.01	0.17	0.00	0.00	0.06	0.09
-27	0.16	0.04	0.20	0.10	0.12	0.32	0.20
-28	0.02	0.02	0.12	0.00	0.00	0.05	0.11
-29	0.02	0.01	0.23	0.00	0.00	0.03	0.11
-30	0.03	0.05	0.32	0.08	0.06	0.14	0.17
-31	0.05	0.02	0.10	0.00	0.00	0.09	0.11
-32	0.24	0.05	0.14	0.07	0.16	0.21	0.13
-33	0.01	0.01	0.10	0.00	0.00	0.06	0.08
-34	0.02	0.03	0.22	0.00	0.00	0.00	0.05
-35	0.08	0.06	0.17	0.08	0.11	0.33	0.07
-36	0.02	0.02	0.09	0.00	0.02	0.02	0.06
-37	0.10	0.05	0.16	0.04	0.15	0.16	0.12
-39	0.01	0.01	0.06	0.00	0.01	0.04	0.06
-40	0.02	0.02	0.12	0.00	0.00	0.06	0.13
-41	0.08	0.02	0.11	0.00	0.03	0.10	0.07
-42	0.05	0.02	0.10	0.00	0.00	0.04	0.08
-43	0.03	0.02	0.11	0.00	0.02	0.05	0.09
-44	0.05	0.02	0.13	0.00	0.00	0.09	0.10
-45	0.00	0.00	0.17	0.00	0.00	0.08	0.08
-46	0.05	0.02	0.12	0.00	0.00	0.08	0.07
-47	0.00	0.01	0.05	0.00	0.00	0.03	0.04
-48	0.00	0.00	0.20	0.00	0.00	0.09	0.08
-49	0.02	0.02	0.08	0.00	0.00	0.03	0.04
-50	0.01	0.01	0.13	0.00	0.00	0.05	0.11
-51	0.00	0.01	0.11	0.00	0.00	0.06	0.08

Table 3. Results of OCPs (concentration in ng/g) (Continue)

Depth (cm))	methyoxychlor	PCB209	Totals without ISTD
-3	0.14	1.22	21.30
-4	0.48	0.42	13.61
-5	0.62	0.00	13.03
-6	0.52	1.81	15.27
-7	0.00	0.00	14.51
-8	0.04	0.00	6.44
-9	0.18	0.00	3.75
-10	0.09	0.00	2.35
-11	0.08	0.00	1.95
-12	0.04	0.61	2.71
-13	0.09	0.00	1.86
-14	0.03	0.00	1.22
-15	0.02	0.00	1.81
-17	0.10	0.00	2.00
-18	0.08	0.52	1.55
-19	0.05	0.00	2.76
-20	0.02	0.00	1.33
-21	0.64	0.00	3.43
-22	0.09	0.06	2.32
-23	0.13	0.00	0.93
-24	0.21	1.23	3.76
-25	0.07	0.00	1.67
-26	0.06	0.00	0.97
-27	0.11	0.00	3.33
-28	0.11	0.00	1.05
-29	0.05	0.00	0.92
-30	0.94	0.57	3.72
-31	0.11	0.00	1.45
-32	0.52	0.00	4.53
-33	0.18	0.00	0.81
-34	0.03	0.00	0.77
-35	0.22	0.00	2.52
-36	0.01	0.10	0.84
-37	0.01	0.00	1.55
-39	0.12	0.00	0.59
-40	0.08	0.04	1.01
-41	0.00	0.00	1.15
-42	0.00	0.24	1.01
-43	0.16	0.00	0.88
-44	0.03	0.00	1.03
-45	0.04	0.00	1.15
-46	0.00	0.00	0.99
-47	0.02	0.00	0.36
-48	0.02	0.00	0.84
-49	0.02	0.00	0.63
-50	0.06	0.05	0.89
-51	0.07	0.00	0.81
-52	0.02	0.00	0.99
-53	0.11	0.77	1.84
-54	0.12	0.00	1.22

Table 4. Results of OCPs (concentration in ng/g) (Continue)

A critical appraisal of the data indicated that concentration values were higher at the lake bed surface and near surface for all detected organic compounds. The shape of the graphs of concentration with depth as well as age-concentration values conformed to this general trend.

From activities around the lake, there was no indication of direct sources of supply of pesticide entry into the lake. This is due to a concerted effort by the local people who avoid the use and application of pesticides in and around the lake due to the importance of the local fishing industry. According to ATSDR (2002), one of the modes of transmission of these pesticides is through the atmosphere and therefore, it could be inferred that the only possible source of deposition into the lake is through atmospheric transfer.

The mode of transfer for pesticides into water bodies such as rivers, streams, lakes and estuaries can be either by direct point source or non-direct point source. The situation in Lake Liangzi involves non-point source, as there is no evidence of a direct point source within the lake's environs. The catchment area does not have any rivers or streams inflow into the lake (fig 2) and since the use of any pesticides for farming activities around the lake is prohibited, run-offs could not be a source of deposition.

According to Sierra Club, (1998), properties attributed to pesticides show that upon their release into the atmosphere they can travel far from the point of application. In view of this, it could be inferred that the possible source of these pesticides could only be from remote areas and through atmospheric transfer. A significant indication presented by the sediment core analysis and the presence of the organochloro-pesticides was that their concentration levels conform to the time of usage for chemical application in agriculture in China. According to Chiras (2000), the traditional Chinese pest control methods were the practice for farming until very recent times, when modern chemical application became popular. This practice was when the people dug trenches and directly confronted the pests by catching them. Furthermore, the correlation of the concentrations to the sediment dating is not a surprise as China only adopted modern chemical pesticides after 1949. Chen et al. (1996), mentioned the use of DDT in the 1960's, a peak period of use occurring during the 1970's and the chemical banned in the early 80's due to its toxic effect. It should therefore, be noted that the indication of DDT in the sediment core is from the residue of previous usage. Again, this should not be a surprise as these pesticides belong to POPs and can persist for years post deposition.

3.1 Geochemical degradation of DDT

The geochemical degradation of DDT was observed from the variable concentrations of its derivative compounds, DDD and DDE. According to ATSDR (2002), DDD and DDE enter the atmosphere as contaminants of toxic breakdown products of DDT. A critical examination of these three organochloro-pesticides reflects the organic processional relationships that exist between them. These organic compounds, according to ASTDR, are easily broken down in the atmosphere with a half-life of two days. DDT in soils breaks down slowly to DDD and DDE through micro-organism activity at a half-life of between two and fifteen days. This partially explains the presence of these compounds at variable concentrations at different levels of the sediment core, which did not conform to the trend indicated by the other pesticides (Compare figures 4 to 9).

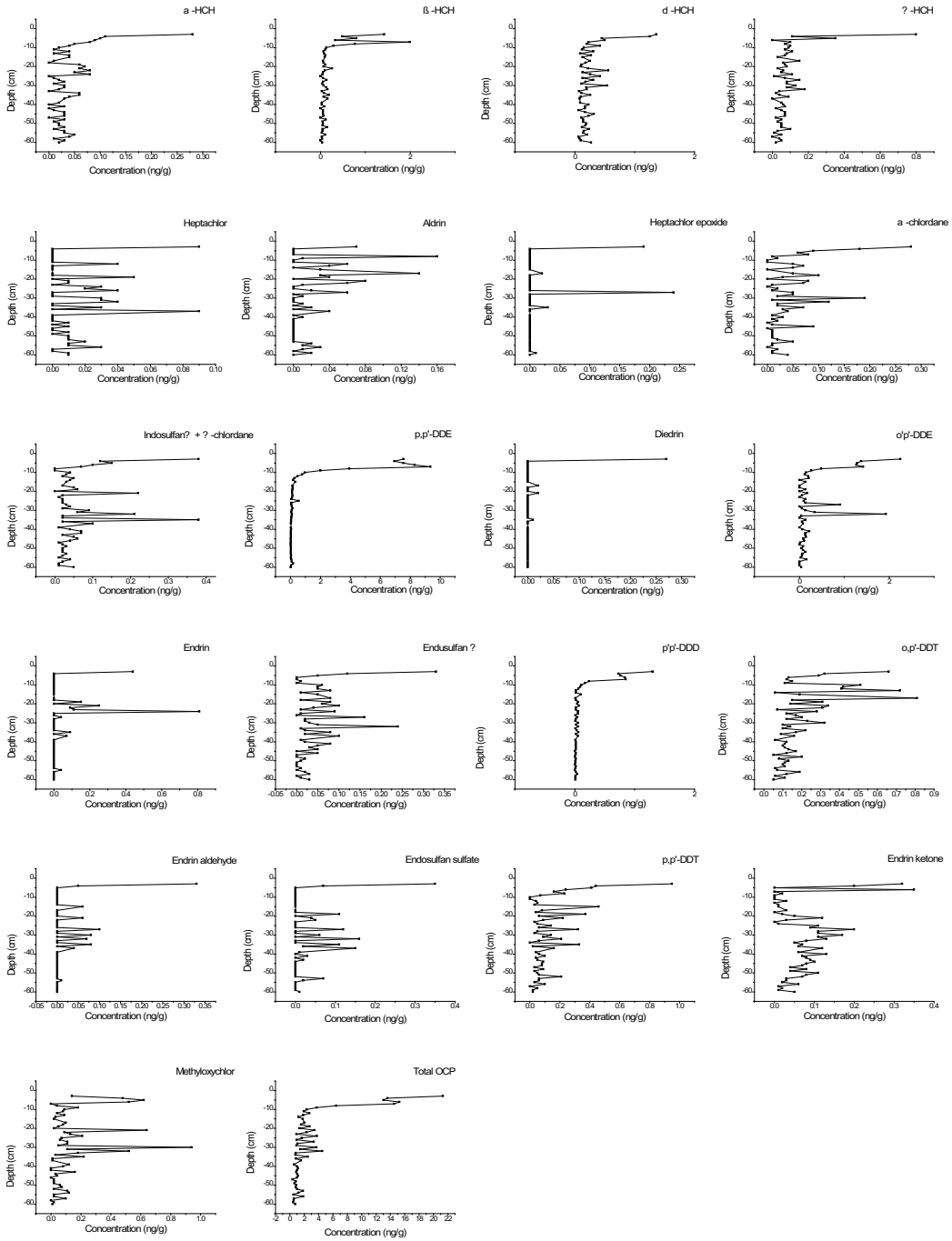

Fig. 4. Graphs of OCPs

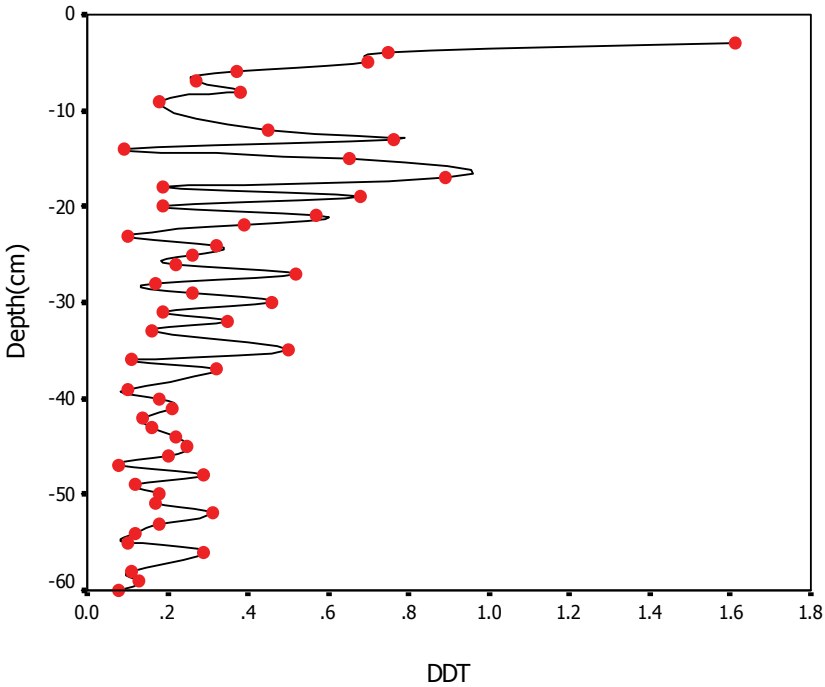

Fig. 5. Graph of Concentration (ng/g) of DDT verses Depth (cm)

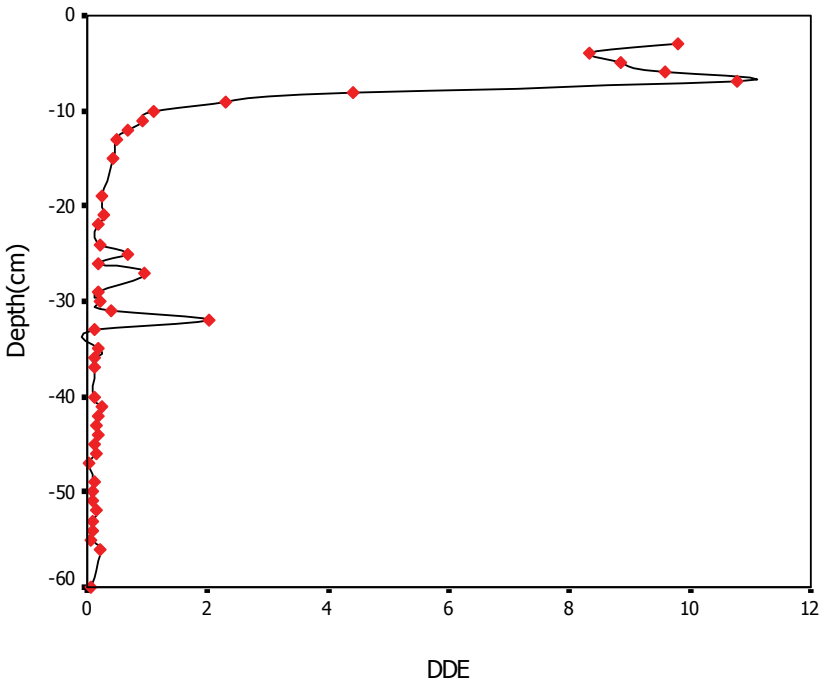

Fig. 6. Graph of Concentration (ng/g) of DDE verses Depth (cm)

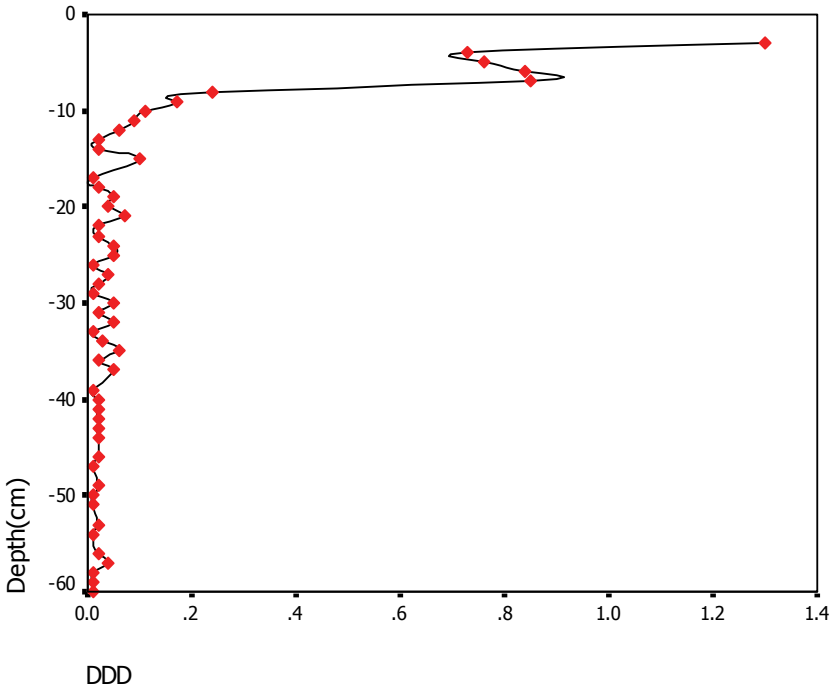

Fig. 7. Graph of Concentration (ng/g) of DDD verses Depth (cm)

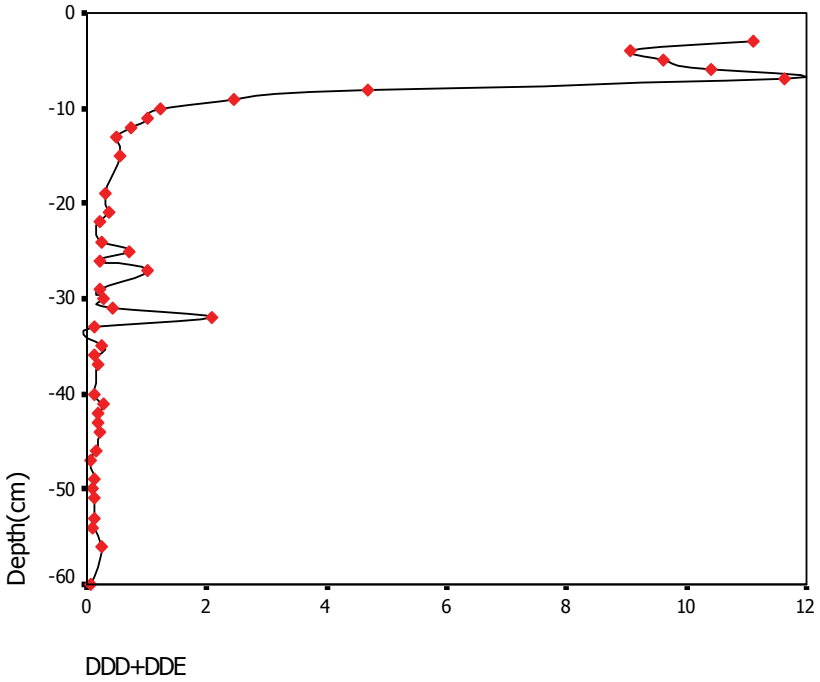

Fig. 8. Graph of Concentration (ng/g) of ∑ DDD and DDE

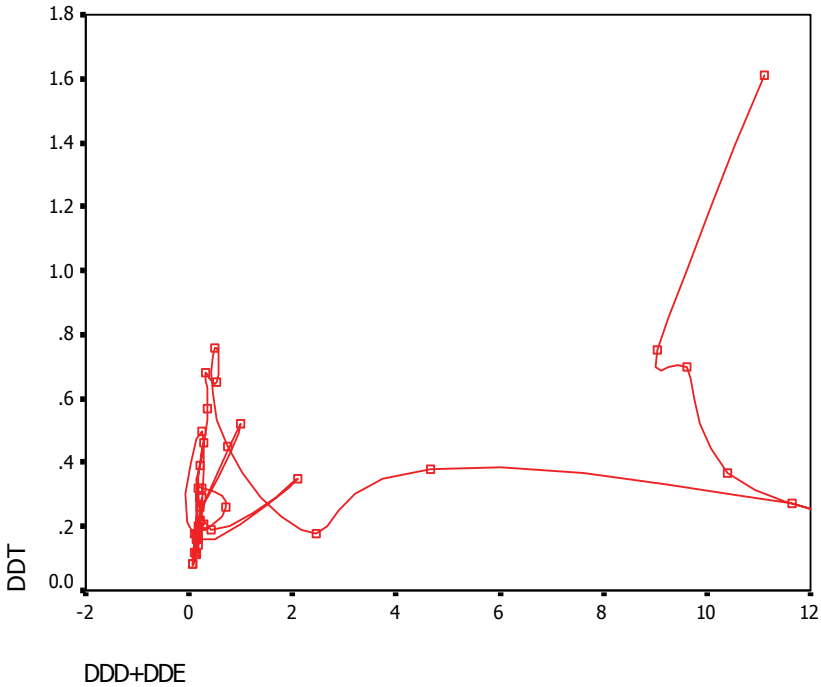

Fig. 9. Graph of ratio of DDT to ∑ DDD and DDD

The most remarkable indication is the relatively higher concentrations of DDE over DDD. This is because DDE accumulates in plant and animal tissues. The extremely high concentration value for DDE at different levels is thus indicative of this phenomenon. It has been assumed that after the transformation from DDT or DDD the resultant DDE compound bio-accumulated in the fatty tissue of aquatic species (including micro-organisms) then decomposed and re-deposited in the lake sediments. The "apparent anomalous" indication of DDE and its predominance over other compounds detected is thus explained through this phenomenon of bioturbation. Also, according to Wedemeyer (1967), and Baxtor (1990), DDT undergoes slow degradation in comparison to DDD and DDE by chemical and biological processes in the natural environment. The degradation rate and degradation products are controlled by the parameters of environment conditions such as pH, redox condition and microbial activity. The ratio of various degradation products may, therefore, reflect some of the localized environmental conditions attributing to the degradation process.

An analysis of the general trend of the other detected organochloro-pesticides in the sediment core indicate that the variable concentrations may be attributed to leaching or post depositional geochemical processes within the sediment. In the analysis of sediment core, consideration will have to be given to the physical processes continually at work within sediments. This according to Sanders et al. (1992), could lead to a gradual alteration and possible disturbance of accumulating stratigraphy. Such mechanisms according to Sanders et al, eventually result in partial loss of temporal resolution within the core. Another issue to be considered according to Sanders et al. (1992), is the fate of a compound following deposition to a water surface, and the potential losses incurred during its passage through the water column and after incorporation into the sediment profile. They further indicate that biotic and abiotic degradation may serve to deplete certain susceptible compounds, and enhance levels of more recalcitrant components. It should be noted that a lake is an open

water body that is exposed to natural conditions such as the geochemical cycle, solar energy, atmospheric and aquatic effects. From the combination of these processes, and in view of the fact that these organochloro- pesticides are all of recent deposition into the lake, the equilibrium between deposition and maturity is yet to be reached. It should also be noted that the concentration levels of the pesticides detected are as a result of the recalcitrant nature of all the pesticides detected. It is suggested that the variability and distribution of the concentrations from high to low for most of the compounds detected throughout the sediment column could be attributed to these processes. Also sediment trap studies by Sanders (1993), suggest that fractions of organic pollutants entering the water column becomes incorporated into the bottom sediment and that large proportions of remainder is returned to the atmosphere, following outgassing across the water/air surface. Sanders indicate that it is important to acknowledge that historical sediment records do not quantifiably reflect inputs to a water body, but rather provide an over all qualitative time-trend assessment of the remaining resistant component.

4. Conclusion

Twenty one (21) different organo-chloro pesticides were detected from the single drilled sedimentary core from the bed of Lake Liangzi. All the 21 organochloro-pesticides detected indicated high values at the surface and decreased down in the sediment column. They all fall within the second generation of pesticides and the class of the organocholoro-pesticides or organo-chlorines, commonly called OCPs. The organochloro-pesticides detected in the sediment core analysis included the most dangerous types; Dichloro Diphenyl Trichloro Ethane (DDT), Dichloro Diphenyl Dichloro Ethane (DDD) and Dichloro Diphenyl Dichloro Ethylene (DDE), which are among the ecological high risk class of organochloro-pesticides. The Organochoro detected were: Hexachlorocyclohexane (HCH, a,b,c), Heptachlor,Aldrin, Heptachlor Epoxide, Chlordane, Endosulfane 1 + r-Chlordane, Dieldrin, Endrin, Endolsufane II, Endrin Aldehyde, Endosulfane Sulfate, Endrin Ketone, Methyoxychlor, p,p' DDE, o,p'DDE, p,p'DDD, o,p'DDT, and p,p'DDT.

The general trend observed from the analysis indicated variable concentrations of the compounds throughout the column. Concentrations were relatively higher at the surface and near-surface of the column which is in conformity with dates during which pesticide use was prevalent in China. The geochemical degradation of DDT to DDD and DDE was also observed. The relatively higher concentration of DDE is due to the process of bioaccumulation. Most of the pesticides detected are from the residue of previous chemical composition, since DDT and other pesticides have been banned in China. The sources of deposition into the lake was atmospheric transfer, and their point source may be remote as there is no evidence of direct contamination for these organic compounds. The general trend observed indicated that the levels of concentration correlated with recent depositions for these organochloro- pesticides.

It could therefore, be inferred that Lake Liangzi has not been spared the menace of pollution, despite the attempts by the people to avoid the use and applications of any chemicals for farming activities.

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Pesticides and Their Movement Surface Water and Ground Water

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1. Introduction

Pesticides are poisons designed to kill pests such as rodents, insects, weeds and fungi. Pesticides are, by their nature, toxic chemicals; since many pesticides may potentially leave residues on foods available for human consumption, there is much concern regarding the potential health risks of pesticides in the human diet. Pesticides used in agriculture to control pests, such as insects, weeds, and plant diseases, have been subject to considerable legislative, regulatory, and consumer scrutiny over the past few decades. Pesticides, with their high degree of toxicity, constitute a very important group of target compounds in environmental samples. Those presentnin waters may have an agricultural, domestic or industrial origin, the most harmful effect being their inclusion in the so-called "nutrition-chain" (Vinas et al., 2002). Many common pesticides contain potent neurotoxic chemicals that attack and disable portions of the nervous system and brain. The use of pesticides in commercial agriculture has led to an increase in farm productivity (Guler et al., 2010). Pesticides also present environmental concerns including water and soil contamination, air pollution, destruction of natural vegetation, reductions in natural pest populations, effects upon non-target organisms including fish, wildlife, and livestock, creation of secondary pest problems, and the evolution of pesticide resistance (Winter, 2004). Many pesticides were used on a global scale from the 1950s to the mid-80s, most of which are stable and persistent in the environment (Barra et al., 2001).

The use of pesticides in agriculture is necessary to combat a variety of pests that could destroy crops and to improve the quality of the food produced. The advantages and disadvantages of pesticide pollution controlling technique are determined by many factors, which require a comprehensive evaluation method adopted in the evaluation of pesticide pollution controlling techniques. Exposure to high levels of pesticides can cause a range of acute, flu- and malaria-like symptoms including headaches, weakness, nausea, respiratory distress, convulsions, coma, and death, accounting for an estimated 20,000 fatalities per year (Jiang and Wan, 2009; Guler et al., 2010).

In a recent USEPA summary report defined vulnerability applied to risk assessment as a four component system: (1) susceptibility or sensitivity of the human or ecological receptors; (2) differential exposures of the receptors; (3) differential preparedness of the receptor to withstand the insult from exposure; (4) differential ability to recover from these effects. All of these components are pertinent to systems undergoing development from the fetus

through childhood. For example, differences in the chemical biotransformation capacity of the human fetus and developing child can be both protective and potentially detrimental to normal development Regarding this point, there is little direct information regarding the specific metabolism of xenobiotics, much less pesticides, in children or the fetus. Overriding differences in biotransformation in the fetus is the probable role of maternal metabolism of xenobiotics affecting the level of fetal toxicant exposure. Polymorphisms of maternal phase 1 and phase 2 enzymes may play a key role in these exposure events (Garry, 2004).
Deterioration of surface and ground water quality represent the most significant adverse environmental impact associated with agricultural production. Degradation of surface and ground water quality has been identified as the primary concern with respect to the impact of agriculture on the environment. The degradation may occur as a result of the leaching of agricultural chemicals soil or biological organisms to surface waters. In this study, it is evaluated the surface and ground water contamination by pesticides.

2. Pesticide properties

The physical and chemical properties that make pesticides effective for pest control also create a potential for surface and ground-water contamination. The fate of a pesticide applied to soil depends largely on two of its properties: persistence and adsorption (adsorption is inversely related to solubility). Persistence is the "lasting power" of a pesticide. Most pesticides in the soil break down or "degrade" over time as a result of several chemical and microbiological reactions.
Generally, chemical reactions result in only partial deactivation of pesticides whereas soil microorganisms can completely break down many pesticides to carbon dioxide, water and other inorganic constituents. Some pesticides produce intermediate substances called metabolites as they degrade. The biological activity of these substances may or may not have environmental significance. Microbes decrease rapidly below the root zone so pesticides leached below this depth are less likely to be microbially degraded. However, some pesticides will continue to degrade by chemical reactions after they have left the root zone.
Degradation time is measured in half-life. Half-life refers to the amount of time it takes for a pesticide in soil to reach half the activity level it had at the time of application (i. e., for a pesticide with a half-life of 30 days, 50 percent of the pesticide will have degraded after 30 day). Pesticides having short half-lives often do not persist in the soil long enough to leach into groundwater. Chemicals with long half-lives are highly persistent and have a greater change of leaching into groundwater. To describe potential persistence, scientists classify pesticides as follows:

1. Non-persistent chemicals — Half-life less than 30 days
2. Moderately persistent chemicals — Half-life of 30 to 100 days
3. Persistent chemicals — Half-life greater than 100 days (Mahler et al., 1997).

Pesticides are divided into many classes. The pesticide classes are shown in Table 1(Squibb, 2002). The adsorption process binds pesticides to soil particles, like iron fillings or paper clips stick to a magnet. Adsorption occurs because of the attraction between chemicals and soil particles. Pesticide molecules are positively charged. For example, are attracted to and can bind to negatively charged clay particles. Strongly adsorbed pesticides are less subject to through soil than weakly adsorbed pesticides. On the other hand, strongly adsorbed pesticides are more subject to loss via surface runoff. Factors controlling pesticides

Group	Subgroups	Examples
Organochlorines		DDT Endrin Aldrin Dieldin Endosulfan α-, β-, γ-, Hexachlorocyclohexane
Anticholinesterases	Organophophates (Ops)	Malathion Fenitrothion Dichlorvos
	Carbamates	Diazinon Carbaryl Aldicarb
Pyrethrins and synthetic Pyrethroids		Pyrethrum Permethrin Cypermethrin Flumethrin
Natural compounds, other than pyrethrins		Abamectin Ivermectin Rotatone Nicotine
Substances which interfere with system specific to insect	Juvenile hormone analogues Chitin synthesis inhibitors Ecdysone agonists Formamidine $GABA_A$ blocker	Cyromazine Diflubenziron Tebufenozite Amitraz
Miscellanous synthetic insecticides		Fibronil

Table 1. The Main classes of Pesticides

adsorption include pesticide charge; soil pH, temperature and water content; the presence of previously adsorbed chemicals that have a stronger bond to soil particles; and the amount and type of organic matter present. In general, pesticide adsorption relates inversely to pesticide solubility in water. Highly soluble pesticides are weakly adsorbed and pose a greater threat of groundwater contamination.

Four chemical properties that affect pesticide movement are solubility, adsorption, volatility and degradation.

Solubility: The tendency of a pesticide to dissolve in water affects its leaching potential. As water seeps downward through soil, it carries with it water- soluble chemicals. This process is called leaching. Water solubility greater than 30 mg/L has been identified as the flag for a

potential leached. Highly soluble pesticides have a tendency to be carried in surface runoff and to be leached from the soil to groundwater. Poorly soluble pesticides applied to soil but not incorporated have a high potential for loss through runoff or erosion.

Adsorption: Adsorption refers to the attraction between a chemical and soil particles. Many pesticides do not leach because they are adsorbed, or tightly held by soil particles. Pesticides which are weakly adsorbed will leach in varying degrees depending on their solubility. Adsorption depends not only on the chemical properties of the pesticide but also on the soil type and amount of soil organic matter present. Even strongly adsorbed pesticides can be carried with eroded soil particles in surface runoff. The potential for a pesticide to be adsorbed is called the adsorption partition coefficient (K_d). The lower the partition coefficient is the greater the pesticide leaching potential.

Volatility: The tendency of a pesticide to become a gas, similar to the evaporation of water will affect its loss to the atmosphere by volatilization. If a pesticide is highly volatile (has a high vapour pressure) and is not very water soluble, it is likely to be lost to the atmosphere and less will be available for leaching to groundwater. Highly volatile compounds may be come groundwater contaminants, however if they are highly soluble in water. For most pesticides, loss through volatilization is insignificant compared with leaching or surface losses. Volatile pesticides may cause water contamination or other problems from aerial drift. Environmental conditions such as temperature, humidity and wind speed affect volatilization losses. Special surfactants or carriers can be used to reduce volatilization losses.

Degradation: A pesticides rate of degradation (persistence) in soil also affects leaching potential. Pesticides are degraded or broken down into other chemical forms by sunlight (photodecomposition) by microorganisms in the soil and by a variety of chemical and physical reactions. The longer the compound lasts before it is broken down that is the longer it persist the longer it is subject to the forces of leaching and runoff (Hairston, 1995).

3. Pesticide movement in surface water and ground water

For an agricultural system to be sustainable, adverse environmental effects of agricultural production must be minimized while competitiveness and profitability are maintained or enhanced. Degradation of surface and ground water quality has been identified as the primary concern with respect to the impact of agriculture on the environment. The degradation may occur as a result of the leaching of agricultural chemicals soil or biological organisms to surface waters. Contamination of surface water is less serious than is the case for groundwater. Properly applied pesticides may reach surface water and groundwater in three basic ways: runoff, run-in, and leaching. Runoff is the physical transport of pollutants over the soil surface by rainwater that does not soak into the soil. Pesticides move from fields while dissolved or suspended in runoff water or adsorbed (chemically attached) to eroded sediment. Run-in is the physical transport of pollutants directly to groundwater. For example, this can occur in areas of limestone (Karst-carbonate) aquifers, which contain sinkholes and porous or fractured bedrock. Rain or irrigation water can carry pesticides through sinkholes or fractured bedrock directly into groundwater. Leaching is the movement of pollutants through the soil by rain or irrigation water as the water moves downward through the soil. Soil organic matter content, clay content and permeability all affect the potential for pesticides to leach in soils. In general, soils with moderate to high organic matter and clay content and moderate or slow permeability are less likely to leach

pesticides into groundwater. In fine textured soils, macropores, which are principally root channels and wormholes, may contribute to the leaching of pesticides.

The advantages and disadvantages of pesticide pollution controlling technique are determined by many factors, which require a comprehensive evaluation method adopted in the evaluation of pesticide pollution controlling techniques. But in the average comparison experiment of pesticide pollution controlling techniques, an intuitive analysis and simple nature description of the ecologic, economic factors under the technique effects are made and the analysis results are independent to each other, a systematic and comprehensive evaluation of advantages and disadvantages of candidate techniques compared is difficult to be made. The change and development of these factors themselves is a grey change process. The Grey System Theory put forward by Deng Julong in 1980s is a new method of solving problems of few data, poor information and uncertainty, which takes the systems of "small sample", "poor information" and "uncertainty" with part information known and part unknown as the subject, mainly by finding valuable information through creation and development from the "part" information known, so as to achieve correct description and effective monitoring of rules of the system operation and evolution. At present, the Grey System Theory is widely applied in many scientific fields, but no literatures of pesticide pollution controlling evaluation can be found. During the pesticide pollution controlling evaluation, the information provided by limit system investigation and spatial–temporal detection data is not complete and certain and the vegetable field pesticide controlling system is a grey system. Based on this point, this paper has made a comprehensive comparison of the pesticide pollution controlling techniques in the vegetable production by adopting a relational analysis method of the Grey System Theory. The chemical pesticide provides a necessary guarantee for the output increase, but the pesticide abuse has led to daily worsen of the ecosystem of agricultural fields (Jiang et al., 2009).

3.1 Pesticides in groundwater

Pesticides in groundwater are an extremely serious problem. The turnover rate for groundwater may be as a few months, but more commonly years and decades are needed to replace the water in an oxygen-free environment are much less effective in breaking down pesticide chemicals. Extremely slow dilution and breakdown means that the contaminant will be present for a long time. The most critical hazard of contaminated groundwater is the potential for toxic effects in man and domestic animals that drink the water. Contamination of an underground aquifer cannot be easily corrected. Doing so requires drilling purge wells and pumping the water to the surface. Pumping may have to be continued for a long time to remove all the contaminated water. The process is extremely expensive. Preventing groundwater contamination is the best solution to what could be a hazardous situation. Numerous instances of groundwater contaminated with pesticides have been identified. In some cases, small communities have had to use bottled water until other sources of drinking water were developed. At this time, the full extent of groundwater contamination is not known. Pesticides have been found in groundwater in numerous instances, however, and it seems apparent that more instances will be discovered as more and more underground aquifers are sampled and tested for the presence of pesticides. The time it takes for pesticides to travel to groundwater decreases as the depth to groundwater decreases. Generally, the depth to groundwater is least in spring and greatest in late summer. If spring rains come shortly after pesticide application and water table is close to the surface, a greater potential for groundwater contamination exists.

3.2 Pesticides in surface water

The presence of pesticides in surface water, even in very small amounts, compromises the life cycle of aquatic organisms, such as algae and fish (tumors, interference with hormonal systems, respiration, growth, reproduction, etc.). Pesticides are harmful to the environment and a threat to the health of those who use these substances, notably those working in the agriculture (headaches, fertility loss, carcinogenic effect, etc.). But most importantly, the prolonged consumption of drinking water, fruits, and vegetables containing pesticides, even at very low doses, presents long-term risks to health. The question of pesticides brings up particular concerns in the area of drinking water production and wastewater treatment because these are among the principal pollutants that impact water resources.Surface water also can be contaminated directly by pesticide spray drift the travel and deposition of fine pesticide spray droplets away from their intended target when the spray is applied too close to water. Drift incidents can result in greater surface water contamination than either runoff or leaching. Obvious, acute effects such as fish kills can occur. Most surface waters (except deep lakes) have a rapid turnover rate, which means that fresh water dilutes the concentration of the contaminant quickly. In addition, most surface waters contain free oxygen, which enhances the rate at which pesticides are broken down by microorganisms. Contamination of surface waters should not be treated casually. An extremely toxic pesticide can cause the death of fish and other aquatic organisms even at low concentrations. Rivers and streams are receptors of toxic wastes generated on land. Pesticides impair beneficial uses of these waters and their biological resources. Pesticides are a group of organic compounds which have been found in aquatic systems worldwide (Rovedatti et al., 2001).

4. Pesticide transport in air, water and soil

In structured soils, macropore flow often causes rapid nonuniform leaching via preferential flow paths, where a fraction of the contaminant percolates into ground water before it can degrade or be adsorbed by the soil. As a result of agricultural practices, pesticides have been detected in many aquifers and surface waters. With regard to pesticides, moderately sorbed compounds with relatively short half-lives are particularly affected. Travel times for pesticides preferentially leached below the root zone are comparable to those for conservative solutes, with losses of typically less than 1% of the applied dose, but reaching up to 5% of the applied mass. These apparently small numbers can be put into perspective by considering the EU drinking water standard, which states that concentrations of a single pesticide may not exceed 0.1 μg l^{-1}. For a dose of 0.2 kg ha^{-1} and an annual recharge of 200 mm, this implies a maximum allowed leaching loss of only 0.1% of the applied amount. Hence, macropore flow should be considered in risk assessment of ground water contamination with pesticides. Pesticide leaching through the vadose zone to ground water is a complex process controlled by a range of soil and environmental conditions.

Accordingly, pesticide fate models account for a variety of processes including soil water flow, solute transport, heat transport, pesticide sorption, transformation and degradation, volatilization, crop uptake, and surface runoff. A particular modeling challenge is to predict pesticide transport at very low leaching levels important for pesticide registration. On the other hand, it has been argued that for very low concentrations, approaching the level of quantification, the criteria for accuracy need not be as rigorous, particularly when the analysis takes into account the uncertainty of data and model outcome.

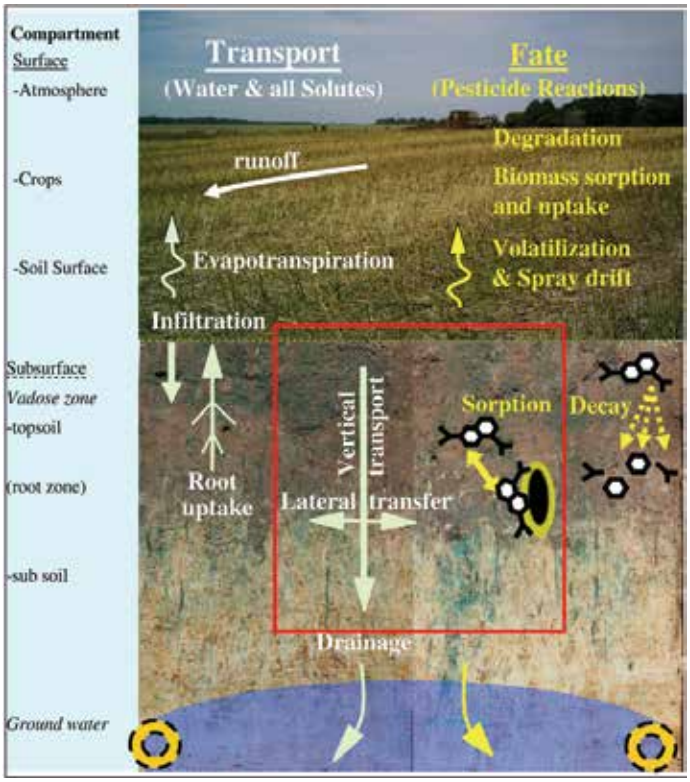

Fig. 1. Principal processes governing pesticide transport and fate in agricultural structured soil systems. The central frame is explained in Fig. 2.

The principal processes governing pesticide transport and fate in agricultural structured soil systems are illustrated in Fig. 1. Soil matrix and macropore characteristics invoking different transport patterns are highlighted in Fig. 2. Descriptions of models for simulating transport of pesticides (and other chemicals) can be found in several reviews and model comparison studies (Colume et al., 2001; Köhne et al., 2009).

Once applied to cropland, a pesticide may be taken up by plants, adsorbed to plant surfaces, broken down by sunlight (photodegradation), or ingested by animals, insect, worms or microorganisms in the soil. It may be downward in the soil and either adhere to soil particles or dissolve in soil water. The pesticide may be vaporize and enter the atmosphere (volatization) or breakdown via microbial and chemical pathways into less toxic compounds. Pesticides may be leached out of the root zone by rain or irrigation water or wash off the surface of the land. Pesticides applied to the soil and immediately incorporated are protected from photodegradation, volatization and dew, which can cause hydrolysis (decomposition by reaction with water). Properly applied pesticides can reach surface and under-ground waters in two ways: in runoff and by leaching. Runoff is the physical transport of pollutants (chemical or soil) over the ground surface by rainwater, snowmelt or irrigation water that does not penetrate the soil. In the leaching process, pollutants are carried through the soil by rain or irrigation water as it moves downward.

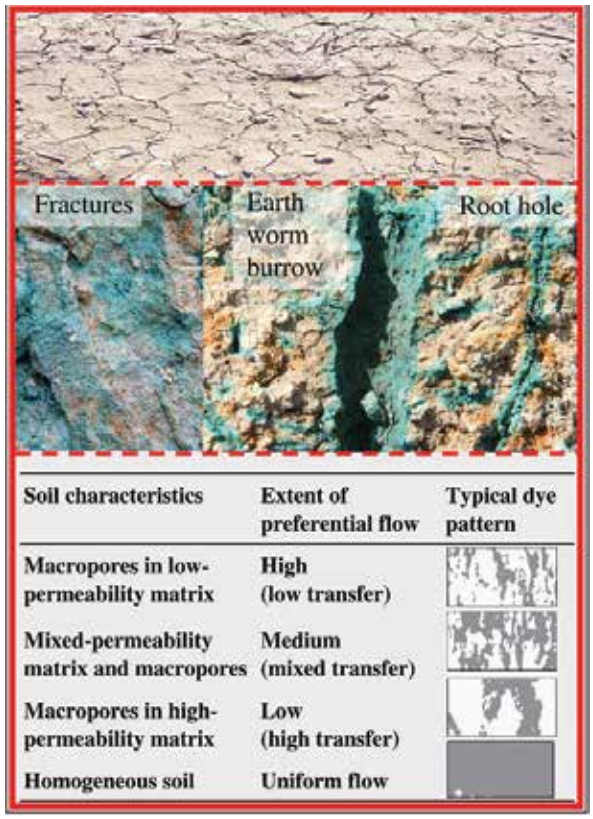

Fig. 2. Fractures and microtopography are triggers for preferential infiltration (top), Diverse structure/matrix interfaces stained by dye tracer visualize different preferential transport paths; these interfaces may affect lateral diffusion, sorption and degradation (middle). Soil matrix and macropore characteristics and resulting transport patterns; actual patterns also depend on the characteristics of rainfall and of overlaying soil horizons

4.1 Factors affecting pesticide movement

Pesticides are primarily moved from agricultural fields to surface waters in surface run-off. The amount lost from fields and transported to surface waters depends on several factors, including soil characteristics, topography, weather, agricultural practices, and chemical and environmental properties of individual pesticides (Colume et al., 2001). Pesticides that are susceptible to leaching do not move through all soils and into ground water at the same rate. Leaching and runoff are nonpoint pollution processes that depend on five sets of factors, some of which are controllable and some not.

1. **Application factors:** These include the application site (crop or weed plants or soil surface or subsurface), the formulation (e. g., granules or suspended powder or liquid), and the application amount and frequency). The management practices that affect movement of pesticides are application methods, application rates and timing, and handling practices. The way in which a pesticide is applied determines leaching potential. Injection or incorporation into the soil, as in the case of nematocides, makes the pesticide most readily available for leaching. Most of the pesticides which have been

detected in groundwater are those which are incorporated into the soil rather than sprayed onto growing crops. Pesticides sprayed onto crops, however are more susceptible to volatilization and surface runoff losses. Application rates and timing of a pesticides application also are critical in determining whether it will leach to groundwater. The larger the amount used and the closer the time of application to a heavy rainfall or irrigation, the more likely that some pesticide will leach to groundwater. Particular care should be taken when practicining chemigation because of the risks of back-siphoning and leaching. Properly storing and mixing pesticides and properly disposing of the containers are other factors that can contribute significantly to the contamination of surface water or groundwater. Quick and proper cleanup of spills is also important.

2. **Pesticide persistence and mobility:** Some pesticide-soil combinations result in such strong binding of the pesticide to soil particles that the pesticide is moved only if the soil is moved, i. e., if erosion occurs. Many pesticides now in use are degraded so quickly on soil and crop surfaces that rainfall must occur within a few days after application for significant transport to occur. Pesticides must be relatively persistent and mobile to leach to ground water because the travel time for water to percolate to deep aquifers can range from months to years. However, once a pesticide has leached into subsurface soils, the biological activity and binding capacity there are often less than in soils near the surface. Thus, the pesticide becomes more persistent and mobile. Persistent and mobile pesticides also are more a threat for runoff. However, that part of pesticide residues which is most available for runoff –the part at the topmost surface of soils is the part most rapidly dissipated by evaporation and photodegradation. Moreover, runoff transport can be complete in hours, and erosion can transport immobile pesticides attached to soil. Thus, pesticide runoff is less dependent on the pesticide properties than pesticide leaching, and much more dependent on how soon runoff occurs after application.
3. **Soil and field topography:** Soils differ greatly in their capacity to absorb water. The slope and drainage pattern of a field or a watershed greatly affect its potential to generate runoff water. Fast-draining soils such as sands and sandy loams have the greatest leaching potential; slow-draining clays and silty clays have the greatest runoff potential. Watershed size has an important effect on runoff pesticide concentration patterns; small streams adjacent to treated fields can have very high peak concentrations of hundred of ppb, but concentrations decrease quickly to low values. In large rivers, peak concentrations are much lower but concentrations may be elevated longer. The properties of soils that affect pesticide movement are texture, permeability and organic matter content. Soil texture is determined by relative proportions of sand, silt and clay. Texture affects movement of water through soil (infiltration) and therefore, movement of dissolved chemicals such as pesticides. The sandier the greater the change of a pesticide reaching groundwater. Coarse- textured sands and gravels have high infiltration capacities and water tends to percolate through the soil rather than to runoff over the soil surface or be adsorbed to soil particles. Therefore, coarse-textured soils generally have high potential for leaching of pesticides to groundwater but low potential for surface loss to streams and lakes. On the other hand, fine-textured soils such as clays and clay loams generally have low infiltration capacities and water tends to runoff rather than to percolate. Soils with more clay and organic matter also have more surface area for adsorption of pesticides and higher populations of

microorganisms to breakdown pesticides. Therefore, fine-textured soils have low potential for leaching of pesticides to groundwater and high potential for pesticide surface loss. Highly permeable soils are susceptible to leaching. Soil permeability is a measure of how fast water can move downward through a particular soil and can typically be inferred from soil texture. Since water moves quickly through highly permeable soils, these soils may lose dissolved chemicals with the percolating water. In highly permeable soil, the timing and the method of pesticide application need to be carefully designed to minimize leaching losses. Soils high in organic matter have a low leaching potential. Soil organic matter influences how much water a soil can hold and how well it will be able to adsorb pesticides and prevent their movement. In addition, high organic matter may reduce potential for surface loss by increasing the soils ability to hold both water and dissolved pesticides in thr root zone where they will be available to plants. High organic matter also supports much of the microbial activity that decomposes pesticides.

4. **Weather and climate:** Climate affects the type of grown, the intensity of pest problems, and the persistence of pesticides used. The intensity of rainfall and its timing with respect to pesticide application determines how much pesticide transport occurs. While these factors are not controllable, probabilities of pesticide runoff and leaching can be estimated, and avoiding pesticide application when rain is imminent is often possible. Areas with high rates of rainfall or irrigation may have large amounts of water percolating through the soil and therefore, are highly susceptible to leaching of pesticides especially if the soils are highly permeable. Intensity, duration and frequency of occurrence of rainfall also affect storm water runoff and losses of surface- applied pesticides.
5. **Farm management:** Pesticides manufacturers are making an effort to provide farmers with the information needed for pollution prevention. The farmer has considerable control over the pollution probabilities: knowledge of erosion control and of best application techniques, and an eye on the weather, is the first lines of defence against pollution (Wauchope et al., 1994; Vinas et al., 2002).

4.2 Methods of prevention

Farm pesticides are regulated by state and federal laws. It can be held liable for any damage to people, animals, fish, or wildlife resulting from your pesticide use and handling practices. Protect and the environment by using pesticides on labelled crops at label rates. Safely store and transport pesticides and all potential pollutants to reduce the chance of an accident or spill. This can be accomplished by following two basic steps.

1. Select the proper chemical for the pest to be controlled. Identify the pest by pictures and descriptions in publications available from agricultural agencies, public libraries or local garden centres. Select only a pesticide that is recommended both for the pest and the plant or location affected.
2. After deciding on the pesticide formulation and appropriate application method, thoroughly read, understand and follow label directions.

Pesticide users should be aware of several specific situations when analyzing their pesticide use practices in the context of potential groundwater contamination. All need to be carefully considered. Correcting one bad practice will not help when another bad practice may represent a bigger problem.

Storage: Checking storage facilities should be first step in the chain of events involved with pesticide use. Containers are frequently opened in the storage area and the possibility of a

spill cannot be ignored. Spilling a concentrated formulation is a more serious matter than applying diluted material on a field. Storage facilities should have a concrete floor so that spilled concentrate can be cleaned up and disposed of properly thereby avoiding soil and water contamination.

Mixing and loading: Mixing and loading sites are areas where a lot of pesticide can be inadvertently spilled on the ground. Repeated spills increase the concentration of the pesticide in the soil and increase the possibility of materials leaching through the soil to groundwater. Growers who apply a lot of pesticide should construct a pit lined with clay or preferably concrete and filled with rock and soil. Mixing and loading can be carried out over the pit so that any spill is contained and the active ingredient is broken down without the possibility of leaching to groundwater. The pit must be large enough to accommodate the maximum pesticide use anticipated for an operation. Many commercial pesticide applicators now have such a pit, which is often covered with a concrete slab sloped toward a drain in the centre that provides access to the pit.

Application: Multiple applications to the same area have been responsible for groundwater contamination in several locations in the growers who depend on multiple pesticide applications for a crop should be analyze their pest management practices carefully with a view toward reducing the number of applications, the total amount applied, using a different pesticide less likely to leach through the soil profile, or using nonchemical methods to manage the pests. The problem is particularly acute when applications are made to sandy soil in areas with a high water table. Growers in such a situation will want to enlist help of pest management specialists in order to design a program that minimizes the possibility of groundwater contamination without sacrificing effective pest control.

Rinsing tanks: Rinsing spray tanks can be a source of possible contamination. The best solution is to drain rinse water into a pit, as described in the section on mixing and loading. If no pit is available, users should not dump or spray the rinse water in the same place repeatedly. Always remember that the more pesticide applied to the same area, the greater the possibility of the active ingredient leaching to groundwater. It is also important to calculate accurately the amount of spray solution needed to avoid the need for disposing of excess spray solution.

Rinsing and disposing of containers: Rinsing and disposing of containers is the last in the sequence of operations involved in the use of pesticides. A container is never completely empty and the concentrated formulation remaining represents a troublesome source of future contamination. Containers should be rinsed three times with the rinsate being added to the spray solution and then punctured so they can't be used for another purpose and disposed of in a sanitary landfill. Some landfill operators will not accept the containers unless they are crushed so they will take up less space in the landfill. Paper or cardboard containers should be emptied as completely as possible then punctured and disposed of in landfill. The pesticides are never completely combusted and represent a potentially hazardous source of exposure for the person doing the burning (Noyes et al., 1991; Wauchope et al., 1994).

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Should We Be Concerned with Long-Term Health Problems Associated with Pesticides in Namibian Groundwater?

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1. Introduction

In the modern world there are several sources of pesticides and other phenyl type organic compounds. Why do we use pesticides? Simply that the world's agricultural practices have commercialized to feed an overgrowing world population. Agriculture has also become a very lucrative business, where various players try to find niches to market their products. At the same time, societies have continued to become more aware about their natural environment and how to sustain it. As far back as 500 A.D., the city of Athens passed a law requiring all refuse to be disposed in a designated landfill outside the city walls (Zakrzewski, 1997); this was followed by many such laws in Europe. However direct linking of disease and health to environmental occupation was done by Dr. Percival Pott, in 1775 (quoted in Zakrzewski, 1997), who correctly linked the "chimney workers" with the unusual high rate of scrotal cancer, which he associated with exposure to soot in their work environment (Zakrzewski, 1997).

In a city like Windhoek, pesticides may arise from the control of grasses on road pavements (Mapani, 2005); or from small scale urban agricultural gardens behind homes. However the major source of pesticides is by far from the farming industry. It is estimated that in the United States alone, close to 98% of the sprayed insecticides and 95% of the herbicides finally reach an unintended sink (destination) (Miller, 2004). The effect of this is obvious when it comes to infiltration of water into the ground that has been used in the irrigation of crops. Pesticides are one type of compounds that undergo "biomagnification" in the food chain (US EPA, 2003). This aspect is especially dangerous as it ensures that the threat from the pesticide is not decreased with time, but rather increased. The United States Environmental Protection Agency (US EPA, 2003) has classified pesticides into four categories; namely; (i) organophospates; (ii) N-methyl carbomates; (iii) triazines and (iv) chloroacetanilides. Some specific pesticide compounds have common mechanisms of toxicity, and require cumulative risk assessment over a relative long period, in order to define the potential risks. However the danger to aquatic life as described below in section 2 is well known, but the danger to human health is ill defined in comparison; with only a few

pesticides that have been well studied such as DDT (dichloro-diphenyl-trichloroethane), which was used widely across the world in the 1960's and 1970's.

In Karst areas such the Tsumeb-Grootfontein-Otavi (TGO) area of Namibia (Figure 1), that is underlain by limestone and dolomite aquifers and has in excess of 4000 square km where commercial agriculture is practiced, the danger from pesticide poisoning can not be over emphasised. The area receives on average about 760 mm of rain per annum. The area also serves as a major source of groundwater for the capital city Windhoek, which lies some 500 km to the south. Such relationships (groundwater source versus commercial agriculture) are generally present but the effect of pesticide use is not generally given the attention it deserves. No systematic studies have been conducted to especially ascertain attenuation rates of pesticides used on agricultural land.

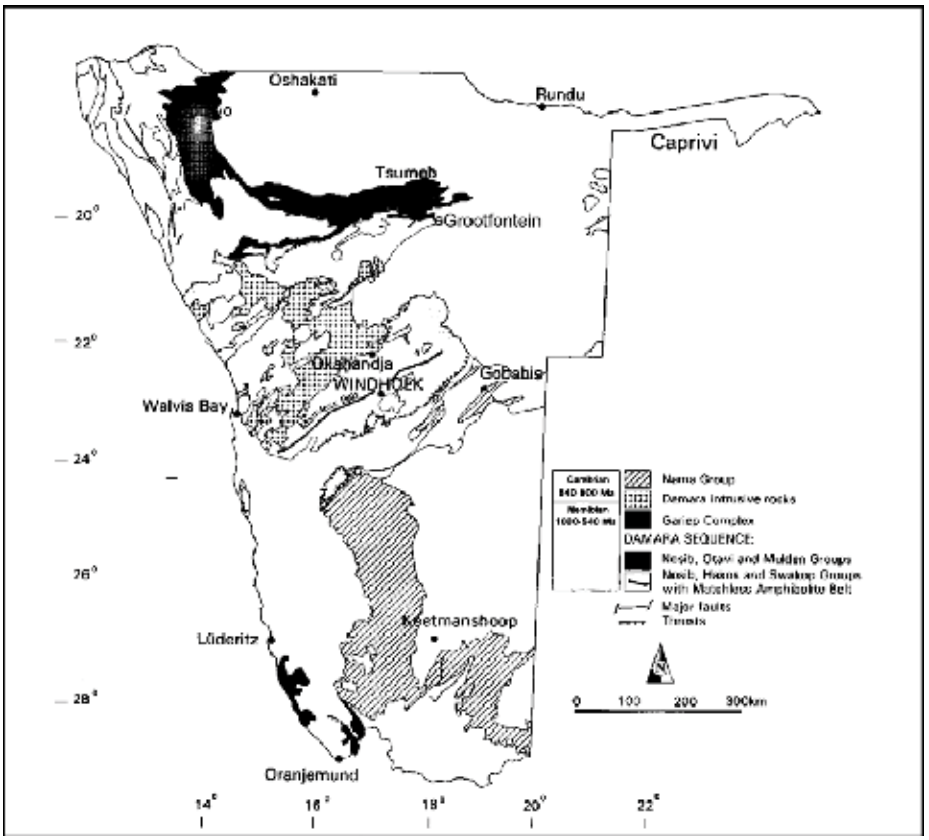

Fig. 1. Simplified Geological Map of Namibia, showing the crystalline carbonates of the Otavimountainland (Grootfontein-Tsumeb) area shown in black.(Geological Survey Namibia, 2000).

It must be borne in mind that treatment of drinking water only eliminates biological threats, and does not usually address pesticides. Generally water analyses do not target phenyl compounds routinely; such that it is not possible to always know whether a particular area is affected or not. Pesticides may be point sources, i.e., only emanating from one particular location, with one general pesticide; or they could be termed as multiple sources or mixtures

of point sources. When mixtures of point sources or multiple point sources occur in one particular area, then pesticides may present completely different mechanisms of toxicity. It has also now been known that pesticides can persist in rivers and in groundwater (Chilton, 2000) for long periods of time. This coupled with the fact that these xenobiotics are bio-cumulative in the ecosystems and in humans (USEPA, 2003), the concern is then raised as to how long their effects take or whether or not the effects will appear as diseases in humans after a critical period of time.

In this paper, the conditions are examined, that should raise concern on the possibility of severe effects appearing among the general population that uses groundwater obtained from areas that have been affected by pesticide exposure. These areas are mainly the Grootfontein-Tsumeb-Otavi (GTO) area in the northern part of the country, including the Tsumeb townlands; the Windhoek area and Okahandja towns, that are sustained from Kombat and Berg Aukas aquifers in the GTO area and the Stampriet area, in the south between Windhoek and Keetmanshoop where the Stampriet aquifer is both used as a water source for domestic use and for farming purposes (Figure 1); the Mariental area, which receives its water from the Hardap dam, whose catchment is a traditionally farming area, situated some 200 km north of Keetmanshoop (Figure 1).

2. Effect of pesticides on groundwater, aquatic life, humans and other biota

Pesticides degrade, and their products of degradation (degredates) can find their way into groundwater and surface water (Chilton et al., 2000; Foster et al., 1991). However as the some authors have pointed out, e.g., Barret (1996), Houtman et al (2004); the process of degradation takes very long that the effects of these xenobiotic compounds generally do affect aquatic life in surface waters. The danger to aquatic life is better defined compared to human health (USEPA, 2003). To human health pesticides have long term effects that need to be known and avoided where possible.As indicated above at least 98% of sprayed insecticides and 95% of herbicides reach an unintended destination, including surface and groundwater (Miller et al., 2004). This implies that pesticides are ending up in areas where they must not be present, and its here that the trouble begins. Once pesticides have entered the hydrological cycle, their elimination is difficult, and in most places impossible, especially so with groundwater. However when pesticides percolate together with surface water, towards the groundwater table, certain quantities may be trapped by clay minerals (Mapani & Schreiber, 2008). The effectiveness of this trapping process (attenuation) depends on the soil types developed in a particular area. In areas where montmorillonite clays are abundant, this process is much better, although it may not completely eliminate the danger. In arid areas such as Namibia, soils are not well developed and the trapping ability of soils is virtually non-existent.

2.1 Effects of pesticides on groundwater

In the area around Windhoek, Mapani (2005) reported the use of pesticides and herbicides in the removal of weeds in paved areas of the city. This kind of usage is continuous, and poses a major threat to groundwater, as it is continuous and the threat is present in all seasons. Windhoek is further disadvantaged by having a thin soil cover, less than 30 cm in most places before bedrock is reached (Figure 2). Thus contamination via percolation of rainwater into the aquifer is made much easier and faster. In an arid country like Namibia,

where some agricultural activities such as livestock watering, vegetable and horticultural farming are mainly supported by irrigation, there occurs a danger to recycle the pesticides via groundwater pollution, through groundwater abstraction and usage. As a result, percolating irrigation water on farms that have been treated by herbicides and insecticides allow these chemicals to reach down to the groundwater water table. The percolation times (displacement times) are varied, and slower in very arid areas, or where thick soil acts as a filter. In alluvial aquifers of ephemeral rivers as is the case for Namibia, these displacement times are rapid and get flushed at least once a year when these streams are in flood. In zones away from ephemeral rivers and streams, this process of displacement time can take months to years. For example, in Windhoek, the water table occurs at 70-76 m below surface; and the displacement time can tale up to ten (10) years due to the nature of the fractured aquifer (Mapani & Schreiber, 2008). In other aquifers with semi-arid conditions, chemicals used today may take 50 years to reach the groundwater table. The displacement times for the pesticides and other contaminants in groundwater are different for different aquifers, and also depend on other factors such as soil composition, soil thickness and rainfall. In areas of high rainfall with thick loamy and clayey soils, the displacement times are longer; however if the soils are sandy, and high rainfall is characteristic to the area, the displacement time is accelerated. A general rule is that with increasing permeability, and absence of clay minerals, displacement times for pollutants are shorter; and become longer with increasing volume of clay minerals and deeper levels to the groundwater table.

Once the pesticides have found their way to the water table, cleaning them is difficult. The pesticides will then be ingested by both livestock and humans through drinking water. In Namibia, livestock farming forms the largest source of export oriented agricultural activity (Table 1). In arid areas, which is mainly in the freehold zones of the country, groundwater is the only source of water for livestock. This is especially the case in the southern part of the country, where cropping is not practiced.

Fig. 2. Windhoek soil profile measuring 20 cm from the Northern Industrial area. The bottom of the pen shows the bedrock-soil profile contact. Note the immaturity of the soil horizon.

Tenure	Cattle	Sheep	Goats	Pigs	Poultry	Ostriches
Freehold	824 207	1 727 210	479 930	9 035		51 464
Communal	1 368 152	359 224	1 230 260	5 671		929
TOTAL	2 192 359	2 086 434	1 710 190	14 706	403 937	52 393

Table 1. Livestock statistics in Namibia as of 1998 census (source: National planning commission)

Once pesticides have found their way to the groundwater, uptake of this water by livestock will ensure the beginning of bioaccumulation in the animal tissues. The accumulated pesticides will then accumulate in the food chain and remain there for a considerable time (Chilton et al., 2000). Pesticides and insecticides are some of the inorganic compounds with a property of bio-magnification. This property implies that if one particular pesticide or herbicide is used over a long time, it may eventually reach the maximum contaminant limit (MCL) in the organism. This danger is very great to areas where groundwater is used for domestic use; for livestock watering in areas where livestock is also slaughtered for food.

2.2 Effect of pesticides on surface water

Pesticide routes into humans are through water, vegetables, fruits, dust and aerosols. The pesticides that are sprayed as aerosols can drift long distances on windy days and end up in surface water sinks, whether they be dams or running river systems. The northern part of Namibia is characterized by subsistence farming (communal land tenure), where river water and shallow wells are the main source of water. For such populations, which depend on cultivation of vegetables, crops, and fruits for their livelihoods are as much exposed to the danger of pesticides as are the commercial farmers to the south. Application of pesticides and herbicides, especially for crops such as cotton, have become a norm to try and maximize crop yields for rural populations. Commercial agriculture that has recently commenced on the banks of the Okavango and Zambezi rivers is also a possible new source of pesticides. Along the Orange river in the south, commercial agriculture in fruit farming (grapes) has traditionally been practiced for a long time. Farm workers experience the greatest exposure to pesticides through direct contact with these chemicals. This is further proved by studies that show that nearly every human being has a small percentage of pesticides in their fat samples (DPR, 2008).

Pesticide contaminated surface water has been known to kill aquatic life, such as fish, frogs and zooplankton (Science Daily, 2006). Zooplankton is one of the major sources of food for fish in surface water bodies. These pesticides also kill insects on which some fish feeds. The herbicide 'atrazine' has been shown to turn some male frogs into hermaphrodites, thus decreasing their ability to reproduce (Science, Daily, 2006). Thus these pesticides become xenobiotic in aquatic life forms, and when MCL for each organism is reached, due to bio-accumulation and bio-magnification, they die off. This is probably the most dangerous aspect about pesticides; their bio-magnification and bio-accumulation properties.

Pesticides can also persist in river systems for a long time, up to time spans of years. This aspect of persistence ensures that aquatic biota can be continuously contaminated during that time span (Kolpin et al.,1996). This is a common feature of pesticides, their resilience in natural systems. Kolpin et al (1996) found significant amounts of pesticide residue and their metabolites in near surface aquifers of the mid-western United States. The British situation on pesticides in water has been well documented, showing that a definite threat exists; for

instance, Lawrence and Foster (1987) showed how agricultural pesticides end up in surface and groundwater aquifers. It is clear that nearly every country where some research has been done, reflects the potential of pesticides to affect the surface water. Li and Zhang (1999) studied the situation in China, and found diffuse pollution from agricultural pesticides in surface and ground waters. The situation can not be expected to be any different in Namibia.
Pesticides that remain in residual amounts in soils have other negative effects on ecosystems. Firstly they degrade nitrogen fixation in soils (Fox et al., 2007; Potera, 2007). This is caused especially by some organochlorine pesticides where they degrade natural nitrogen fixing bacteria (Fox et al., 2007; Potera, 2007). This leads to soil degradation and poor crop yields; then farmers react by adding more fertilisers to the soils. The pesticides that hinder this important natural process are dichloro-diphenyltrichloroethane (DDT); methylparathion and pentachlorophenol. These hinder legume rhizobium chemical signaling, and larger quantities of fertilisers are then required.
It has been shown by several authors that endocrine disrupting compounds (EDCs) that are xenobiotic can cause reproductive impairment in fish even when they have been in the aqueous ecosystem for some time (e.g., Jobling et al., 1996; Gray and Metcalfe, 1997; Gronnen et al., 1999 Metcalfe et al., 2000). The EDCs washed is surface water systems have a debilitating effect on wildlife.
All the fishes that partake of pesticides that end in surface waters will inevitably end up in humans through fish consumption. This will continue to happen until such time that intolerable levels are reached and humans get ill from pesticide effects. As they continually accumulate in surface waters, so do they as well in the food chain. These have long lived effects on fishing rural societies that couple with agriculture.

2.3 Pesticides and insecticides in air and in household pest control

Many households spray their homes for pest control, in one form or another. Concern exists over long term exposure of pesticides and insecticides in infants. Some authors have attributed the early onset of leukemia on pesticides (Lowengart et al., 1987). Fenske et al. (1990) have shown that pesticides in air have serious consequences when sprayed in rooms to eradicate pests. Concentrations that affect humans and children in particular can linger in the air for more than 24 hours post application period (Fenske, et al., 1990; Ritter et al., 2007; Woody, 1984). Woody (1984) has shown that children were intoxicated by pesticides with organophosphate active ingredients after houses were sprayed with dichlorvos in Arkansas in the USA. In Texas, USA, thirty seven (37) children were hospitalized after their homes were sprayed with organophosphate and carbamate containing pesticides (Zwiener& Ginsburg, 1988). This implies that these xenobiotics can accumulate in humans also from this route and bio-accumulate in our systems, and when a critical level is reached they become a health hazard. These results of how pesticides and insecticides affect humans are direct evidence that these xenobiotics can cause serious harm to human health.

3. Results and discussion

3.1 Results

Work done around the Windhoek area has shown that the main threats to the groundwater are pesticides, crude oil products such as diesel, petro and motor oil. The areas around Windhoek show very thin soil cover, with an average maximum depth of 30 cm. This soil is mainly made of kaolinites, smectites and illites (Table 2). This data is also summarized in figure 3. This clay distribution map, gives an idea of the most vulnerable zones to pesticide infiltration into the groundwater. Some 97% of the country have soils with a clay content of

less than 5%, with two deserts lying to the east (Kalahari) and to the west (Namib), devoid of clay soil material. This implies a low holding water capacity, and the soils are generally deficient in nutrients such as Mn, Fe, and Zn. This data suggests that natural attenuation factors suggested by Lerner et al. (2000) and Mapani and Schreiber (2008) are greatly reduced in sandy soils.

Area & geology	**Soil Type**	**Capacity to retard effluents and pollutants**
Windhoek area	Mainly kaolinite, illite and smectites	Little capacity
SE of the country- area is dominated by Kalahari sands, a few granite and basaltic rocks	Covered by Kalahari sands, dominated by micas and illite, chlorite, mixed layer silicate and minor kaolinite	Very little capacity
North central part of the country – dominated by carbonates and schists	Some illite, smectites, palygorskite, kaolinite and minormontmorillonite	Medium to good capacity
Northern regions (wet areas)	Palygorskite and smectite	Medium capacity
Southern areas	Illites and chlorites, some smectites occur	Limited capacity

Table 2. Summarized Main soil types in Namibia

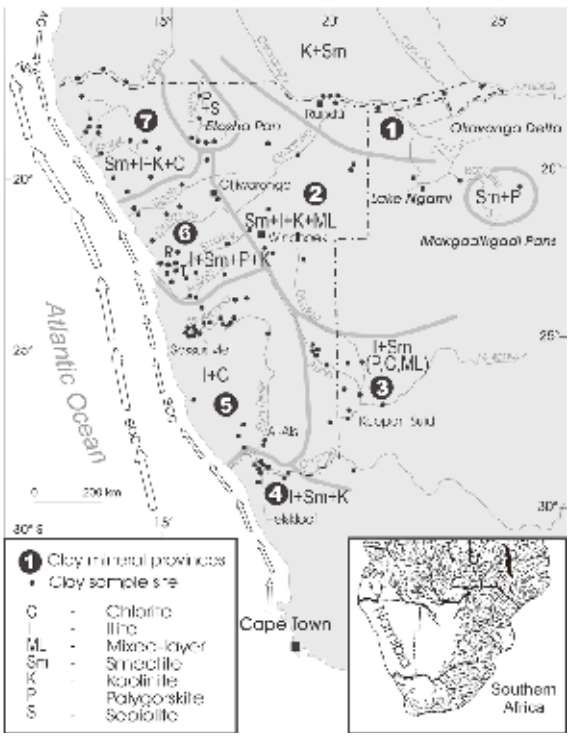

Fig. 3. Clay mineral distribution across Namibia (after Heine and Volkel, 2010). BOC = Benguela Ocean Current.

The northern part of the country, in the Oshana regions of Namibia, does receive infrequent floods in the summer. When this occurs, the generally sandy soils, do get an admixture of loamy sediments brought from the Angolan side of the border. However the shallow wells get flooded together with the fields where rain-fed agriculture is practiced. Fortunately only few farmers use pesticides on a regular basis, most subsistence farmers only use fertilisers. An example shown in figure 4, where a flood completely inundates the village, any pesticide/herbicide that were applied, get dispersed in the ecosystem, affecting large swaths of subsistence farmlands.

In Windhoek pesticides are applied for weed removal in paved areas such as airfields car parks, road pavements, and railway lines and sports facilities where grass is kempt for recreation purposes. These sources of pesticides pose serious problems, given the fact that the Windhoek aquifer is currently used as a major storage facility of fresh water.

Fig. 4. Flooding in the Oshakati area in 2009, some 800 km north of Windhoek.www.sulekha.com

3.2 Discussion

Plimmer et al. (1998) in a comparative study, showed that Africa, followed by Asia and South America experienced the largest crop losses due to insects and pathogens. This implies that the use of pesticides in these areas is a requirement to improve productivity. However the fate of pesticides in soils and water is least studied in these places compared to Europe and North America. Of the common pesticides and herbicides, Carbofuran, Alachlor, Ametryn, Atrazine and Isoproturon have been studied to see how they degrade in agricultural uses (e.g., Agrawal, 1999; Chilton et al., 1995; Harrison et al., 1998; Johnson et al., 1998; Johnson et. Al., 2000), although more work is still required similar to that of Houtman et al (2004) that traces endocrine disrupting compounds (EDCs) in sediments of river deltas. Most of the studies have been on laboratory batch experiments, a few on actual field samples of the groundwater and surface waters (Chilton et al., 1995; Johnson et al.,

1998 & 2000). Carbofuran breaks down to a 3-OH-7-phenol group when quantities of 12 kg per hectare are applied, with groundwater analyses picking up concentrations of 10–60 lg/l (Chilton et al., 2000). Alachlor is detected at the water table level, without much breakdown, together with Ametryn, when both are applied at a rate of 4 kg/hectare. Atrazine on the other hand may not be detected in wells, but will be detected in abstracted drinking water in the range 0.2–3 lg/l, implying that it has a less resident time at the water surface (Chilton et al., 2000). This behaviour of pesticides and common herbicides shows that these complex compounds pose a threat to all groundwater resources due to their long residence times, and their possibility to pass from one group of aquatic wildlife to another through the food chain. In the United Kingdom, Atrazine was banned for non-agricultural use (Chilton et al., 2000) because of its persistence in groundwater, and its effects on human health.

In the case of Namibia, alluvial aquifers in ephemeral river channels form a significant source of groundwater. These flow in general once or twice a year. This effect of once off flows and flooding, flush any substances such as pesticides down stream. This then leads to the lateral distribution of herbicides in the groundwater system once the rivers have stopped flowing, and water has percolated down into the aquifers. Some of the ephemeral rivers do not flow all the way into the ocean, the flow may stop mid stream due to insufficient water. This aspect is especially common, and the re-concentrating of pesticides in these ephemeral rivers occurs each year.

In Europe several studies have shown that pesticides and associated EDCs have effects on wildlife (Vos et al., 2000). In the Netherlands, quantities of EDCs analysed from sediments of the Rhone delta were generally low, but their effect on biota is significant (Houtman et al., 2004). In Japan, the work of Gray and Metcalfe (1997) and Gronnen et al. (1999) showed that there was an impairment of reproductive organs in fish as a result of xenobiotic compounds found in the fish. These xenobiotic compounds then can find their way into humans via oral ingestion. As fish forms one the largest source of protein for the human population, it is expected that bioaccumulation of these xenobiotic compounds will begin to show some health effects and in cases cause some poisoning (Weinbroum, 2005). Ollson et al (1998) discussed the effects of endocrine disruption substances in soils from around Sweden. In their discussion, Ollson et al (1998) conclude that these xenobiotic substances as pesticides impair the growth of animals and impair the reproductive systems. These effects lead to mutagens in species (Houtman et al., 2004). Brouwer et al. (1998) have shown that human health is also at risk from these organo-halogen compounds such as pesticides and herbicides. Feldman and Maibach (1974) carried out direct experiments on man, exposing the skin to the pesticides Carbaryl and Diquat. Subsequent urine tests showed that the human body easily absorbed Carbaryl and a little of Diquat. This study has direct implications on farm workers. The work of Chu et al.(1999) shows that there are circumstances when the rice can take up these xenobiotics, although the concentrations are low in the seeds but higher in the leaves. Fox et al. (2007) showed that pesticides directly reduced the efficiency of nitrogen fixing rhizobia and host plants.

4. Conclusions

Pesticides in groundwater are as a result of percolating irrigation water that has been treated with pesticieds into the groundwater. In areas where soil cover is thick with clays such as montmorillonite that have the capacity to trap organometallic and organic molecules, the attenuation of pesticides can be appreciable. Soil is not an effective filter, and some

groundwater aquifers will be contaminated where prolonged use of pesticides is practiced.In the case where ephemeral rivers flow throughirrigated land, they serve as a mechanism to re-distribute pesticides in the groundwater aquifers. Where flooding is frequent, the same mechanism of redistribution of pesticides occurs. The long-term effects pesticides in groundwater are much more long lived, and are difficult to remove from the aquifers. Hence their effects will be cumulative on the communities using the aquifers.The effects of pesticides on human health has now been shown to have several health effects with long term effects that need to be minimized or avoided.

5. Acknowledgements

The authors would like to thank the Earth Sciences department at the University of Gothenburg, Sweden for access to the electronic library. The authors thanks the anonymous reviewers who reviewed the paper.

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24

Transport of Carbon Tetrachloride in a Karst Aquifer in a Northern City, China

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1. Introduction

Carbon tetrachloride (CCl_4) has been used as a grain fumigant, pesticide, solvent for oils and fats, metal degreaser, fire extinguisher and flame retardant, and in the production of paint, ink, plastics, semi-conductors and petrol additives (Agency for Toxic Substances and Disease Registry (ATSDR), 1994). Its properties are shown in Table 1. CCl_4 is classified by the International Agency for Research on Cancer (IARC) and the US Environmental Protection Agency as a Group B2 carcinogen and also listed on the CERCLA Priority List of Hazardous Substances maintained by the Agency for Toxic Substances and Disease Registry (ATSDR, 2008). CCl_4 is a common contaminant in soil and groundwater. CCl_4 is found in approximately 20% of the US Superfund National Priority List sites (Ferguson & Pietari, 2000). But, there are limited published case studies of CCl_4 contamination in karst aquifer. Karst aquifers are distinguished by an abundance of large subsurface openings and are therefore especially vulnerable to chlorinated-solvent contamination (CCl_4, TCE, PCE). The release of chlorinated solvents into karst aquifers presents a difficult challenge to environmental scientists, managers, and regulators. The importance of karst aquifers to

Molecular weight	153.8 g/mol
Density(25 °C)	1.594 g/mL
Vapor pressure at 20 °C	12.2 kPa
Boiling point at 101.3kPa	76.72 °C
Melting point at 101.3kPa	22.92 °C
Critical pressure	4.6 Mpa
Critical temperature	283.2 °C
Solubility in water at 25 °C	785 mg/L
Henry's law constant at 24.8 °C	2.3×10^{-2} atm·m^3/mol
Heat of evaporation	194.7 kJ/kg
$LogK_{ow}$	2.64
$LogK_{oc}$	2.04

Table 1. Physical properties of carbon tetrachloride (Fouw, 1999)

water supply and their vulnerability to contamination by chlorinated solvents are reasons to seek improved understanding of how chlorinated solvents behave in karst aquifers (Wolfe et al., 1997). This chapter discusses CCl_4 transport and fate in a karst aquifer in a northern city of China based on *years of continuous monitoring* of CCl_4 concentrations.

2. Site characterization

2.1 Site location and pollution source

Karst aquifer investigated is located in the Northern China Plain. Karst aquifers provided averagely 25.8×10^4 m^3/d to urban public water supplies from 1981 to 2008 (Liu, 2010). As illustrated in the Fig.1, the karst water is in a relatively confined groundwater system unit and its east and west boundaries are coal seam water-resisting layers and the south and north are groundwater watershed. The karst groundwater system is composed several *relatively independent aquifers. The CCl_4 pollution occurs in the* southern Qiligou water-bearing basin.

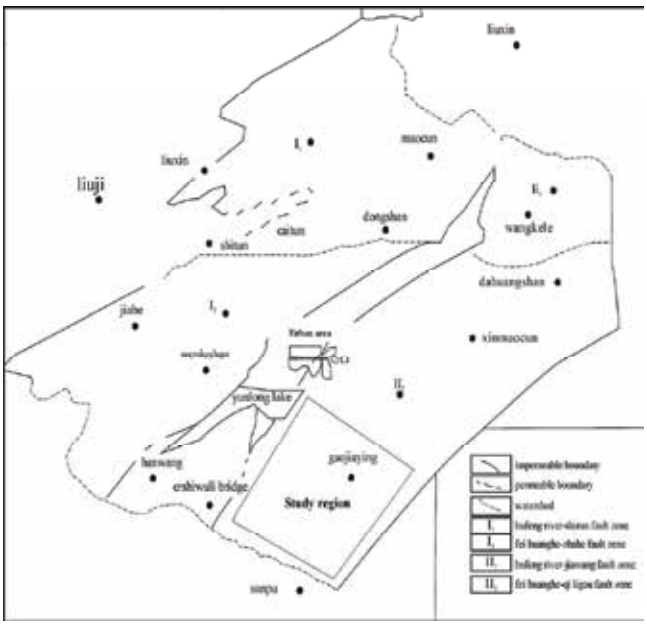

Fig. 1. *Hydro-geological zonation of the karst groundwater system in the city*

According to monitoring data obtained in November, 2000, it has been contaminated with CCl_4 in Qiligou water-bearing basin. The pollution source is a pesticide plant which produced a pesticide that used CCl_4 as a solvent and it has used more than 42 tons of CCl_4 in the past ten years. This plant is located at hill slope in southwestern recharge area of the karst aquifer (Fig. 2). However, emergency measures were taken in 2001, including closing the pesticide plant and intensive pumping from heavily polluted wells. Untill May 2001, carbon tetrachloride was found in 53 wells (contaminated area is about 17.3 km^2). The highest CCl_4 concentration in karst water was over 3900 μg/L in a water supply well approximately 465m away from the pesticide plant. The concentration in Chinese standards for drinking water quality is lower than 2μg/L (GB5749-2006) (China's Ministry of Heatlth, 2006). Since then, the contaminated wells have not been used for dinking water. While, some lightly contaminated wells have been pumping for *agricultural and industrial production*.

Fig. 2. View of the pesticide plant and its wastewater drainage

2.2 Geological and hydro-geological settings

As shown in the Fig.3, the contaminated site is a NE synclinal basin with area of approximately 200 km^2. Its southeastern and northwestern boundaries are two NE mountain chains composed of Cambrian and Ordovician limestone with elevations from 100 m to 248 m above the sea level. Quaternary deposits in the central lowlying area of the basin are composed of alluvium, proluvium, sand, sandyclay and subclay. The thickness of Quaternary is from 5 to 30 m, and the elevation varies from 30m to 40m above the sea level.

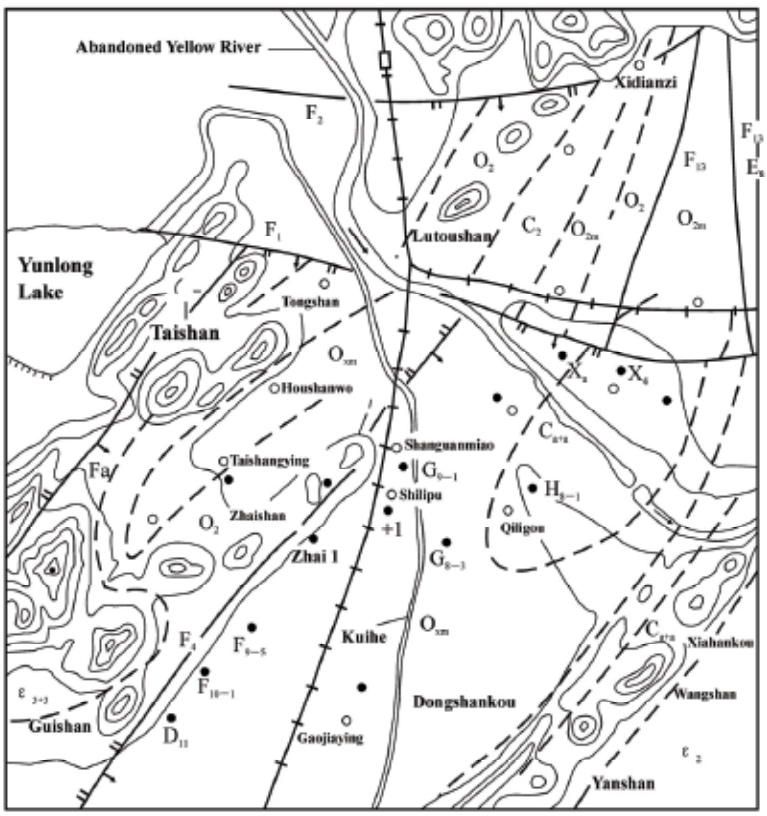

Fig. 3. Bedrock geologic map of Qiligou water-bearing basin

karren and *grooves* in exposed karst *area*

Corrosion on the surface of limestone

Fig. 4. Surface karst formation in the site

Limestone cropped out along hills contains abundant karst landforms such as caves, blind valleys and sinkholes, which provide pathways for the rapid transport of contaminants into the aquifer. Fig.4 presents the surface karst landform in the studied region.
Fig.5 demonstrates the *degree* of *development* of *karst* in the *subsurface*. Karst caves and fissures are the major structure of water storage. Especially, the honeycomb-like dissolved solution pores are quite well-developed.

Fig. 5. Corrosion of rock core samples

According to borehole data, there are four well developed underground karst or paleokarst zones and they are regarded as the horizontal runoff layers in the karst aquifer (Fig. 6). Of them, the third karst zone with a depth from 90 m to 150 m is the most important runoff layer of karst grounwater. Karst groundwater is recharged mainly from precipitation (about 835mm per year). Rain water seeps into karst aquifer from sinkholes, fissures in outcrop areas or in covered karst area. It first infiltrates into Quaternary phreatic aquifer in lowlying basin area, then infiltrates into underlying karst aquifer from recharge skylight. In middle sub-area, there is a layer of igneous rock and the karst aquifer can be divided into upper water-bearing zone and lower zone because of igneous rock watertight.

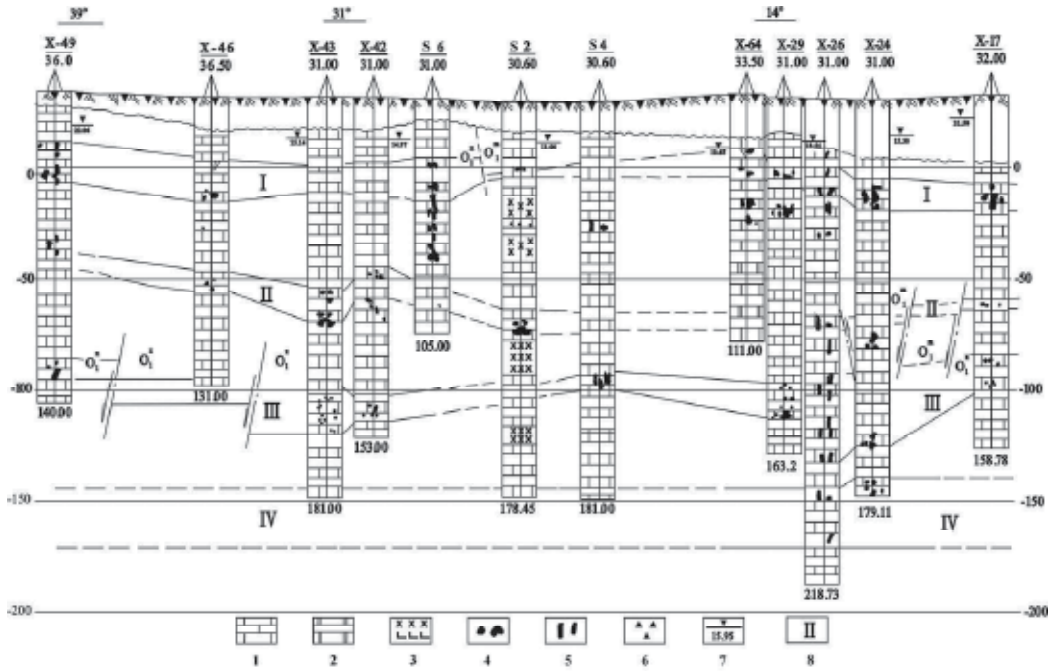

Fig. 6. Vertical zonation of karst development along the groundwater flow path in the site (1. limestone; 2. dolomite; 3. igneous rock; 4. cave; 5. karst fissure;6. fault fracture zone; 7 water table; 8 karst zone)

The variation in groundwater level from 2000 to 2008 is presented in Fig.7. Although the range of groundwater fluctuation in different wells is different, the trend is similar. It suggests there is a good hydraulic connection within the aquifer system, which makes the aquifer more vulnerable to contamination. Many years of groundwater level observations indicate that there is very little change in the karst groundwater flow field.

In conclusion, karst water-bearing medium in the site is distinguished as extreme anisotropic and heterogeneous. Hence it can be classified as the combination of fissure network and runoff zones type, which has unified hydraulic field. Tracer results indicates that the karst conduits are well developed along the syncline basin axis and the velocity of groundwater is fast in the runoff zone, which can attain 3027.8m/h when the water source in the mining conditions (Pei, 2007). Therefore the convection is predominant in mass transport of the pollutants in the aquifer. Hence the pollutant in the subsurface can move faster and further with the groundwater flow.

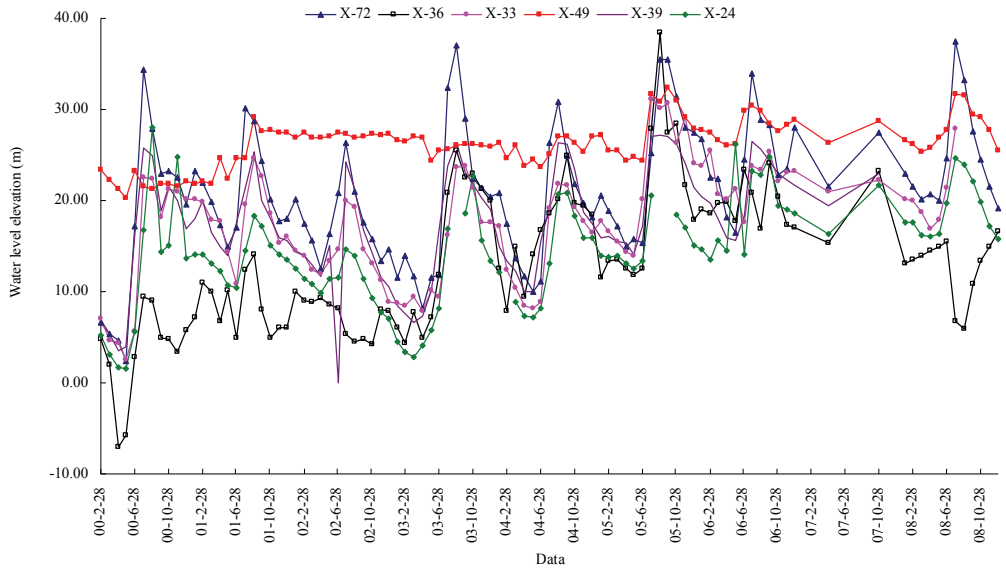

Fig. 7. Variation in piezometric levels in the research region

3. Pollution pathway analysis

3.1 Leakage test

The pesticide plant is located in the hill slope of bedrock. When CCl_4 was first found in November 2000, the drainage ditch running off wastewater was not built. The effluent with high concentrations of CCl_4 could directly leak into the karst aquifer (Fig.8). Under the intervention of provincial environment protection department, the plant built ditch in partial section (Fig.9). Leakage research was conducted to investigate the CCl_4 pollution pathways. Spot S1 to S6 were arranged along drainage ditch for leak off tests (Fig.9). During the flood period of 2001, two tests were performed and the results indicated that the discharged water leakage rate reached approximately 22% and leakage mainly happened in the bare limestone section, namely, effluent with high concentration CCl_4 can flow directly into karst aquifer (Table 2).

Fig. 8. Bare limestone along the wastewater ditch of the pesticide plant

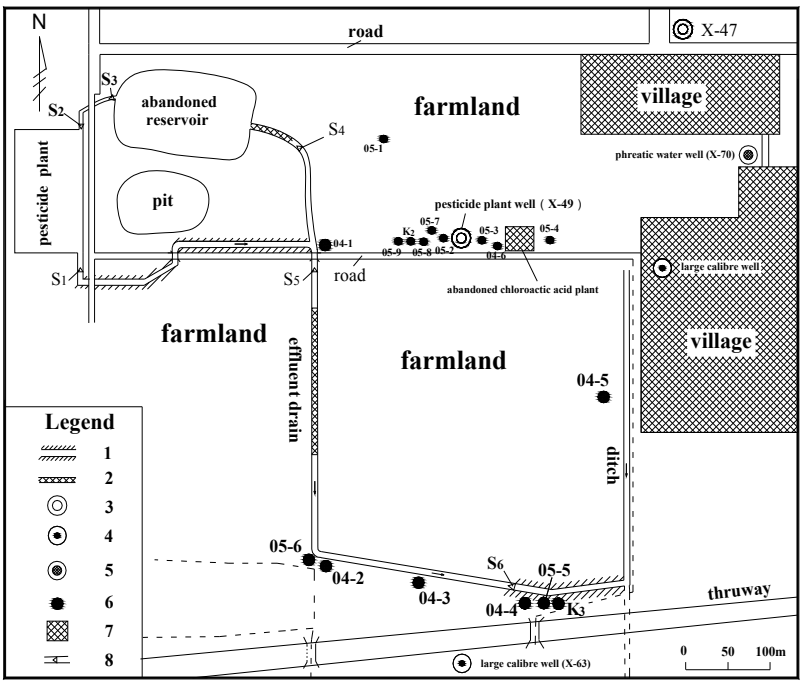

Fig. 9. Boreholes and leakage observation spots location in pollution source sub-area (1, lined ditch; 2, bared limestone; 3, karst groundwater well; 4, large calibre well; 5, phreatic water well; 6, borehole; 7, village; 8, leakage observation spot.)

Observation spot	Distance (m)	Discharge (m^3/h)		Leakage rate (%)	
		2001.7.22	2001.8.31	2001.7.22	2001.8.31
S1	1148.0	45.82	45.34	22.10	21.34
S6		35.66	35.66		

Table 2. Results of the leak off tests

3.2 Soil pollution investigation

In order to investigate the feature of effluent leakage into karst aquifer, 17 soil sampling boreholes numbered K2 and K3(2001), 04-1 to 04-6(2004) and 05-1 to 05-9(2005) were drilled with auto-driller (Model: DPP100-3B) and a total 206 soil samples were collected(for locations see Fig. 9). The quaternary deposits are 2.4-10.2m thick. CCl_4 and chloroform were detected in the soil (soil samples of K2 and K3 were analyzed only for CCl_4). CCl_4 was found in the drilling soil along the drainage ditch and nearby the west part of well X-49, and the highest concentration reached 47.1μg/kg (Table 3). The CCl_4 content of soil in the boreholes nearby well X-49, (e.g. 04-6, 05-2, 05-7 and 05-8) are much higher and their highest content is 34.0μg/kg, 42.2μg/kg, 33.5μg/kg and 47.1μg/kg respectively. In general, CCl_4 was found in the soil at depths than 3 meter, and the content increased with the increase of the soil depth. CCl_4 was not detected or was relatively low in the topsoil. Chloroform, the daughter product of CCl_4, was also detected, of which was in the range of 2.6 to 26.5μg/kg. For borehole 04-2, 04-3 and 04-3, the chloroform distribution was larger than that for CCl_4.

Samples with the highest content chloroform were colleted from clay-limestone interlayer (depth at 6.5 to 6.7m), intensive weathered igneous rock layer (depth at 9.0 to 9.2m) and fissured clay layer (depth 7.8-8.0m) respectively.

Borehole number	Borehole depth (m)	CCl_4			Chloroform		
		Detected depth (m)	Content (μg/kg)	Depth of maximum content (m)	Detected depth (m)	Content (μg/kg)	Depth of maximum content (m)
K2	9.1	0.8-8.7	1.3-2.9	4.6-4.8	NT	NT	NT
K3	6.0	0.8-5.3	1.1-7.7	6.5-7.0	NT	NT	NT
04-2	7.0	1.5-1.7, 6.5-7.0	0.9-2.8	6.5-6.7	5.0-6.7	9.5-19.8	6.5-6.7
04-3	10.5	4.0-6.7	0.7-1.1	5.5-5.7	2.5-10.2	5.3-11.7	9.0-9.2
04-4	9.2	5.0-8.7	0.7-1.1	8.5-8.7	7.8-8.0	15.8	7.8-8.0
04-6	5.0	0.5-4.7	0.7-34.0	4.0-4.2	ND	ND	ND
05-1	8.1	ND	ND	ND	0.5-8.1	5.3-9.2	5.0-5.2
05-2	4.7	2.5-4.7	3.3-42.2	4.0-4.2	0.2-4.7	2.7-26.5	4.0-4.2
05-3	5.7	ND	ND	ND	1.2-5.7	2.7-7.3	1.0-1.4
05-4	6.7	5.5-6.2	1.0-1.8	5.5-5.7	1.0-5.7	2.6-8.0	3.5-3.7
05-6	6.2	ND	ND	ND	0.2-6.2	6.9-11.3	2.5-2.7
05-7	5.7	2.7-4.9	7.6-33.5	4.2-4.4	ND	ND	ND
05-8	5.4	3.2-5.4	1.8-47.1	5.2-5.4	ND	ND	ND

ND- Not detected, NT-Not test.

Table 3. CCl_4 and chloroform contents in the soils

3.3 Pollution pathways

There are three pollution pathways of karst groundwater. Specifically: Wastewater directly entering the karst aquifer in the pesticide plant area (Fig.10); Flowing into the aquifer through bare section of limestone in drainage ditch; and Leaking under ditch by soil (Fig11).

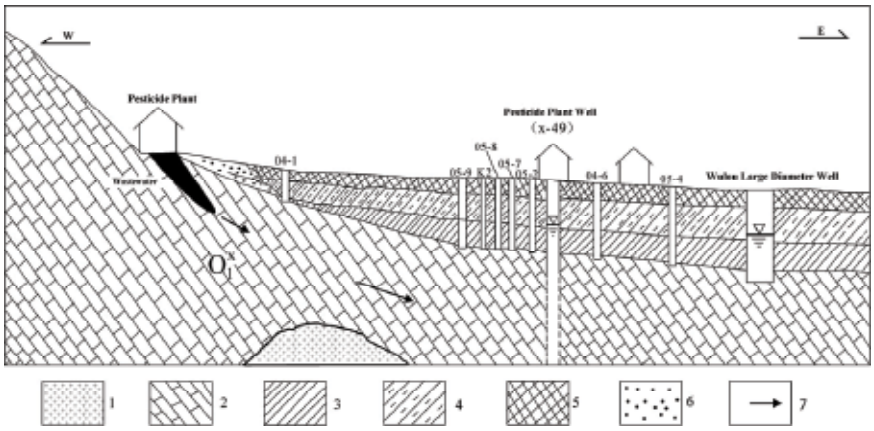

Fig. 10. Generalized pollution pathway of direct seepage into the karst aquifer within the pesticide plant (1. Intrusive igneous rock; 2. limestone; 3. clay; 4. silty clay; 5. cultivated soil; 6. sand; 7. Movement direction of CCl_4)

Fig. 11. Wastewater with CCl_4 seeping *through* the soil *into the karst aquifer*

4. Spatial distribution of CCl_4 in the karst aquifer

4.1 Plane distribution of the CCl_4 plume

The size and shape of the CCl_4 plume in the aquifer was confirmed by multiple samples from multiple water supply wells. In porous media aquifer or unconsolidated aquifer, the plume concentration decreases with the distance from the pollution source. But the plume distribution in the studied site was quite different. Karst conduits develop along preferential pathways between areas of groundwater recharge and discharge. CCl_4 in groundwater was recharged from the southern pollution source and transported into northern supply wells forming a long belt-like plume. Based on CCl_4 concentration data, the contaminated area can be divided into three sub-areas: southern pollution source sub-area, northern sub-area of artificial discharge center and transition sub-area or middle sub-area. The CCl_4 plume in the karst aquifer was "dumbbell" shaped, with high contamination located in the southern and northern sub-area and relatively light concentrations in the middle transitional sub-area, as shown in Fig. 12.

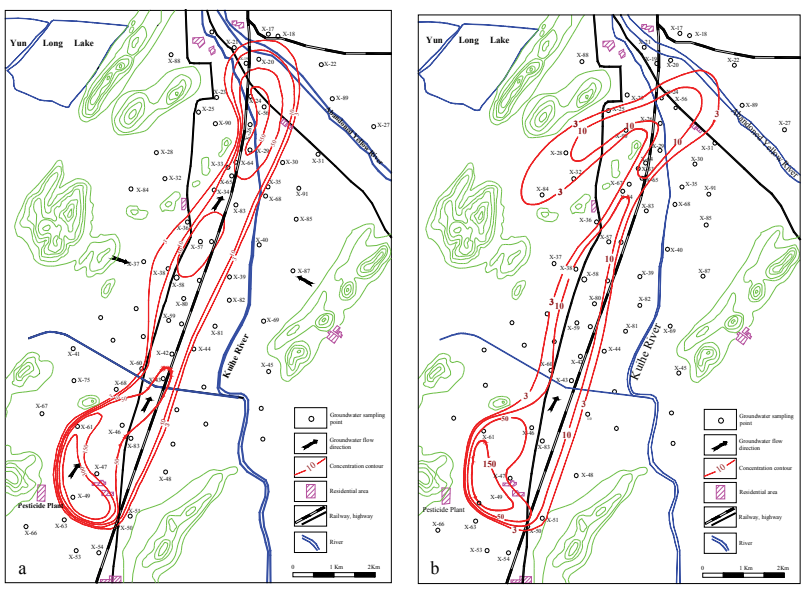

Fig. 12. CCl_4 plume distribution in the karst aquifer (a. 2001-4-18; b. 2008-4-30)

Because of the obstruction of higher-level water in the southern and western parts of the pollution source, polluted water could transport to northern sub-area along well-developed karst conduits. Transition sub-area has formed an obvious depression cone by artificial withdrawal and the water level was about 5.00 m lower than of the southern sub-area. Karst fissures and caves are most well developed in both horizontal and vertical direction in the northern sub-area. Water development experience in past fifty years has revealed this zone is the most water-yielding section and also the most intensive pumping area in the water-bearing basin. Consequently, northern sub-area is the centre of the depression cone and the CCl_4 is accumulated in this area. In the middle sub-area, there is a layer of diabase igneous rock aquifuge at a depth from 100 m to 150 m, which separates the aquifer into two individual layers without hydraulic connection. Because the depth of most wells in the middle sub-area is less than 150 m, CCl_4 concentration of the wells in this sub-area is relative lower.

4.2 Vertical distribution of CCl_4 in the karst aquifer

The high density and low viscosity of CCl_4 cause it to migrate downward until they encounter openings too small to enter. The influence of well depth on the CCl_4 concentration in wells was studied. CCl_4 concentration in Qiligou wells and Sanguanmiao wells increased with the increase of the well depth (Fig. 13). The transport of CCl_4 in the groundwater is controlled primarily with gravity under similar hydro-geological conditions. Therefore, with deeper the wells, there is higher CCl_4 concentration of groundwater is.

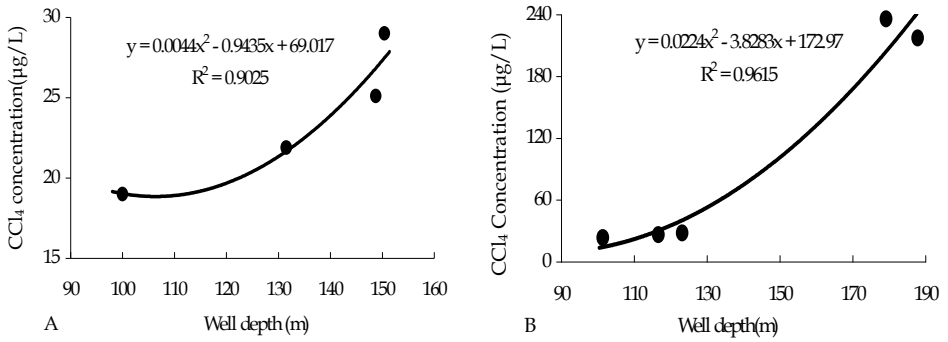

Fig. 13. Relationship between CCl_4 concentration and the well depth (A: Sanguanmiao wells; B: Qiligou wells)

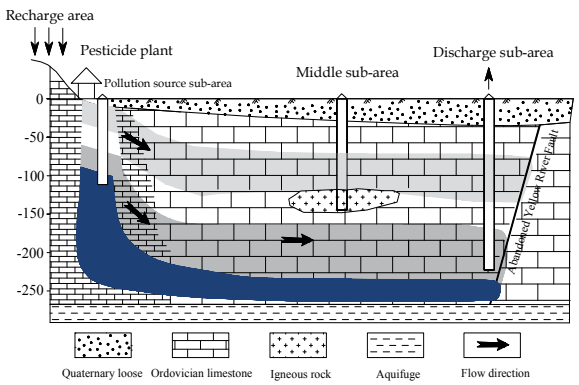

Fig. 14. Conceptual model for CCl_4 transport in the karst aquifer (Han et al., 2004)

According to geologic, hydro-geologic setting and monitoring data of CCl_4 concentration in the past years, transport of CCl_4 in the complex karst aquifer can be generalized as shown in Fig. 14.

5. Temporal change in CCl_4 plume in the karst aquifer

5.1 Temporal change in CCl_4 concentration in the aquifer

The changes in CCl_4 concentration in typical wells are presented in Table 4 and Fig.15. However, there is a general downward trend in concentration, CCl_4 concentration in most of the wells increased in 2010. This may be due to the decrease in groundwater exploitation.

Well		2001		2004		2005		2006		2007		2008		2009		2010	
X-49	25% quantile	n=16	1279.4	n=20	369.6	n=52	90.1	n=29	500.0	n=27	201.3	n=19	146.6	n=12	70.1	n=49	222.3
	50% quantile		1722.6		1662.6		104.6		815.0		264.5		218.3		119.6		272.5
	90% quantile		2584.3		2911.9		681.4		1313.6		620.5		587.9		193.8		627.6
X-61	25% quantile	n=12	181.5	n=20	248.0	n=54	61.8	n=32	17.7	n=27	9.1	n=25	13.0	n=12	20.3	n=55	65.1
	50% quantile		907.7		325.2		96.0		52.2		19.0		21.6		30.4		108.5
	90% quantile		2241.5		609.0		212.7		168.1		163.7		104.0		122.4		152.0
X-47	25% quantile	n=14	59.6	n=20	8.9	n=52	21.6	n=28	18.5	n=27	25.5	n=23	25.0	n=11	8.8	n=49	11.8
	50% quantile		69.0		29.3		68.5		53.4		74.5		48.5		18.4		15.3
	90% quantile		134.8		162.8		216.6		136.6		107.2		116.9		27.7		39.0
X-83	25% quantile					n=53	6.3	n=32	17.8	n=25	14.5	25	16.2	n=12	10.4	n=53	15.5
	50% quantile						25.1		27.3		45.4		24.6		11.3		18.9
	90% quantile						204.0		115.8		78.8		75.7		21.4		29.2
X-59	25% quantile			n=21	14.6	n=56	13.2	n=30	8.8	n=27	10.9	n=25	7.5	n=12	4.9	n=53	20.8
	50% quantile				24.2		23.1		14.0		14.2		11.3		6.5		23.6
	90% quantile				44.6		58.8		39.2		39.5		16.9		19.2		27.7
X-64	25% quantile			n=21	30.0	n=40	22.4	n=29	16.0	n=25	14.4	n=25	11.9	n=12	8.2	n=24	29.8
	50% quantile				40.2		31.0		23.6		16.2		14.4		8.7		33.1
	90% quantile				115.0		67.5		81.2		31.1		28.2		17.4		37.2
X-56	25% quantile			n=20	92.0	n=56	24.5	n=32	13.6	n=27	11.9	n=25	10.9	n=12	8.7	n=48	33.6
	50% quantile				115.8		43.2		23.8		18.1		13.5		9.9		37.8
	90% quantile				196.4		89.1		67.8		27.8		30.0		16.9		45.2

"n" is the number of the samples

Table 4. Summarizes of changes in the average CCl_4 concentrations with time (μg/L)

The Fig. 15 shows that: (1) the CCl_4 concentration in karst auifer has obvious seasonal variation. In general, CCl_4 concentration of groundwater during the drought period from February to June is relative lower and during the rainy period from August to October is much higher. (2) CCl_4 changes rapidly with time, which is notably different from the

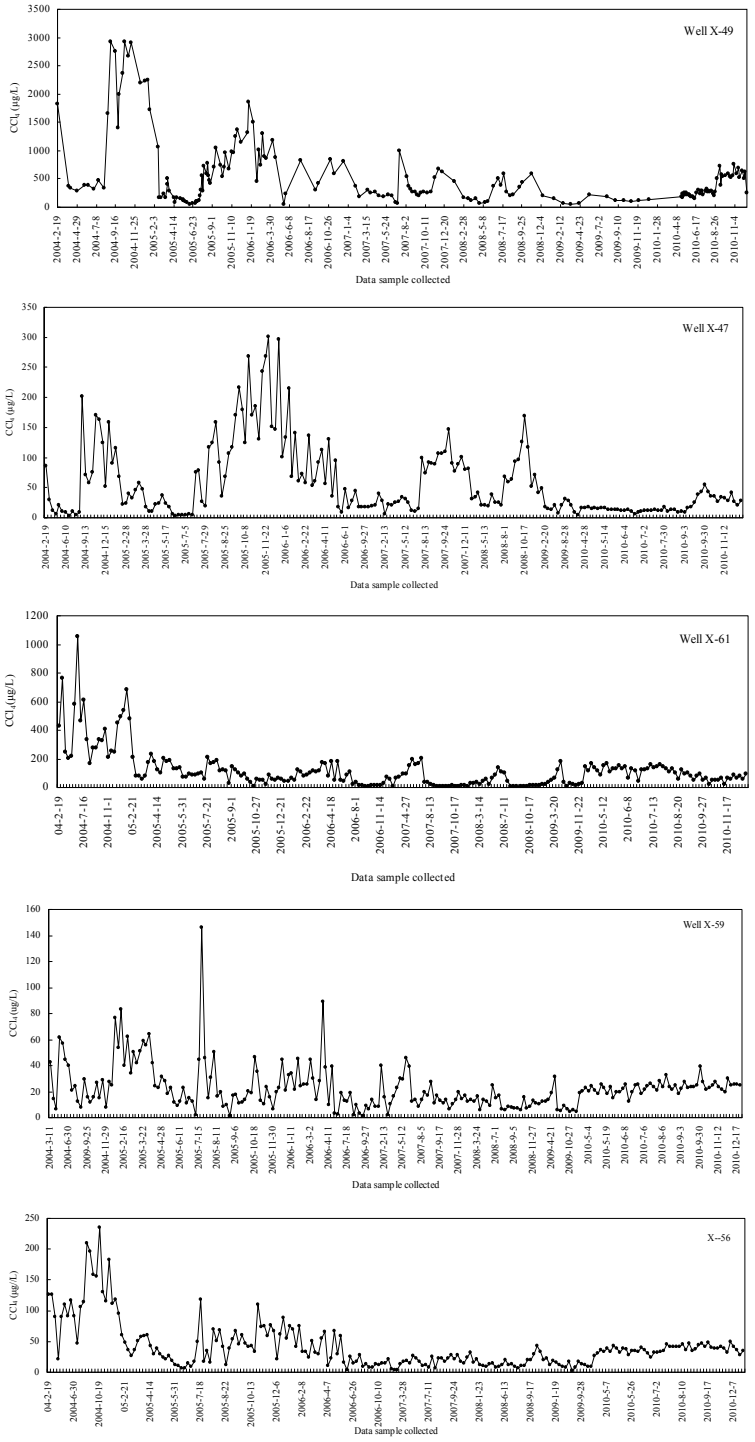

Fig. 15. The variation of CCl_4 over time in typical supply wells

common porous media aquifer. This may be due to the facts that: (1) The groundwater velocity in the aquifer is much higher than that in the porous media, which can reach 131.9m/h-3027.0 m/h. As a result, the advective flow dominates the movement of pollutant; (2) Local groundwater flow regime changed frequently. It was one of the important water supply source with over 80 wells owned by different departments for different purposes. Pump stopping and starting at different wells caused change in local flow field and subsequently cause change in CCl_4 concentration; and (3) CCl_4 transport channel is complex.

5.2 Mann-Kendall trend tests

The non-parametric Mann-Kendall test was used to detect monotonic (increasing or decreasing) trends in time-series of CCl_4 concentrations for the eight typical wells during the period 2004-2010. The Mann-Kendall test is widely used in environmental science for the detection of trends in time-series data. A 5% significance level was used to indicate statistically significant trends in the current study. The Mann-Kendall trend statistics (Z) indicates significant decreasing ($Z < -1.96$, $p < 0.05$) and increasing ($Z > 1.96$, $p < 0.05$) trends. Table 5 presents that there are a highly significant decreasing trend in CCl_4 in the karst aquifer and the decreasing trend in pollution source sub-area and the north sub-area area are more significant.

Well	Time	Z	Trend
X-49	2004.02-2010.09	-4.35532	Decreasing, Significant
X-62	2004.02-2010.09	-5.72119	Decreasing, Significant
X-47	2004.02-2010.09	-4.74661	Decreasing, Significant
X-83	2005.02-2010.09	-2.35838	Decreasing, Significant
X-43	2004.02-2008.12	-3.38838	Decreasing, Significant
X-59	2004.02-2010.09	-2.80019	Decreasing, Significant
X-74	2004.02-2010.08	-4.67132	Decreasing, Significant
X-56	2004.02-2010.09	-5.11015	Decreasing, Significant

Table 5. The Mann-Kendall trend statistic (Z, $p<0.05$) in CCl_4 concentration in typical wells

5.3 Temporal change in CCl_4 plume distribution

The Fig. 16 gives the change in CCl_4 plume over time. In almost ten years, there was very little change in the distribution of CCl_4 in the range of 3-10μg/L or 10-50 μg/L. There was a major reduction in the volume of groundwater containing concentrations between 50 and 300μg/L. The plume extended westward and eastward in northern sub-area to in the flood period every year. It should be noticed that the plume expanded eastward.

Based on observed trends in the development of a plume, plumes can be grouped as four categories: expanding, stable, shrinking and exhausted (Rice at al., 1995). In this study, the length of pollution plume of CCl_4 in the water-bearing aquifer was found to be stable while concentration is shrinking, which indicates that the plume decreased faster in concentration than in length.

Dynamic groundwater flow field is one of the most important factors controlling the CCl_4 plume (Han et al., 2006). CCl_4 plume in the aquifer is similar with the groundwater flow field, which is shown in Fig. 17. CCl_4 diffusion was confined by higher water level around the plume (Zhu et al., 2008). The scope of seriously polluted wells was similar with the center of cone of depression. CCl_4 concentration in the center of the local cone of depression was higher than that in the wells outside of the center.

Fig. 16.1 Change in CCl_4 plume with time in the karst aquifer (a, 2001-8-30; b, 2004-8-30; c, 2005-8-30; d, 2009-8-30)

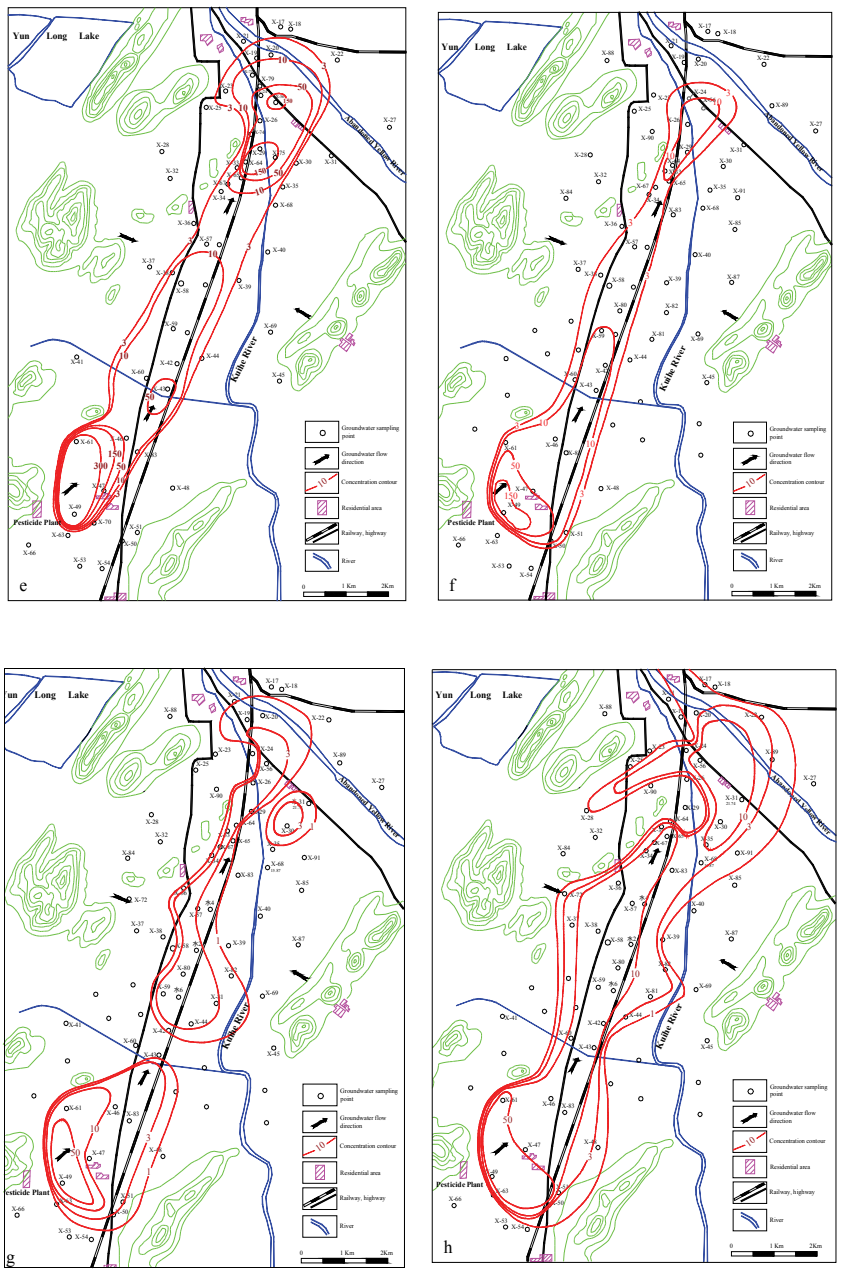

Fig. 16.2 Change in CCl_4 plume with time in the karst aquifer (e, 2004-12-30; f, 2008-12-30; g, 2009-12-30; h, 2010-12-30)

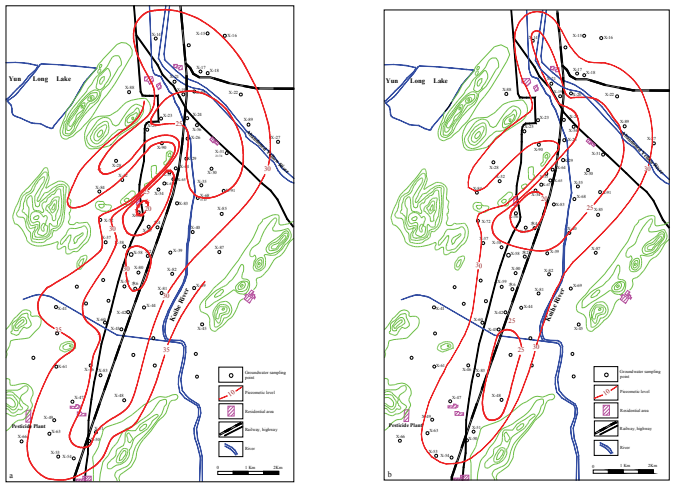

Fig. 17. Contours of the karst aquifer piezometric level (a, 2009-8-30; b, 2009-12-30)

5.4 Factor analysis of CCl_4 attenuation in the Karst aquifer

Concentration of CCl_4 decreased in the karst aquifer because of the influence of CCl_4 fate (volatilization, dilution, adsorption, chemical reaction, biological degradation) and it's difficult to describe CCl_4 attenuation in karst aquifer because of shortage of parameters. The main factors are as follows:

1. **Free-phase CCl_4 existence in observation well**

Most organics exist as the NAPLs which are the long-term sources of dissolved-phase organics in the aquifer. The EPA found the NAPL was the main-factor that affects the rate of pumping and it's important to determine its existence in the reservation well. Highly-concentration dissolved-organics are barely measured due to the low solubility of NAPL and dilution of reservation well. EPA presents an indirect way to measure the NAPL (1% principle): NAPL will exist if the concentration of chemical materials that related with NAPL was exceeded pure-phase or 1% of valid solubility. The pure-phase CCl_4 solubility is 785 mg/L at 25°C. The CCl_4 has been measured in groundwater at about 3909.9 μg/L, which is approximately 0.5 percent of its solubility, suggesting that there is no evidence to determine the CCl_4 NAPL existence.

2. **Passive extraction**

Passive extraction is the main factor decreasing the concentration of CCl_4 due to the fact that research area was the water supply source in the city with extraction of 2000×10^4 m^3/a. The groundwater exploitation has decreased dramatically since 2001, however, there are several irrigation wells and industrial wells situated within the contaminated area still in use. It is estimated that the discharge of CCl_4 for 2001, 2004, 2005 and 2008 were 5.42, 1.27, 0.26 and 0.14 tons respectively according to the groundwater exploitation volume and the average CCl4 concentration (Pei, 2009).

3. **Dilution**

Convection is one of the most important processes leading to dissolved-phase of contaminants transport in saturation area and concentration decrease. The trace experiment illustrates that convection is the dominant rather than dispersion during CCl_4 transport because of the high flow rate of karst water. Funnel-shape water levels were generated during pumping and the concentration of CCl_4 in reservation wells changed due to the water flow from the aquifer around the well.

4. **Volatilization**

Contaminants distribution in liquid-gas phase is governed by Henry's law. The trend between liquid phase and gas phase is determined by the Henry's constant. Volatile organics in groundwater could volatilize into atmosphere via soil. The volatilization should be considered if Henry's constant > $1x10^{-5}$ atm·m^3/mol and molecular weight < 200 g/mol. Thus the volatilization is dominant in the CCl_4 decrease according to Henry's constant of CCl_4 ($2.76x10^{-2}$ atm·m^3/mol) and molecular weight (153.82). It is estimated that CCl_4 volatilization for 2004, 2005 and 2008 were 5.38, 9.06 and 4.44 kg respectively (Pei, 2009).

5. **Adsorption**

Organic matters and clays are the most important factors which contribute to adsorption in aquifer and organic matters are dominant. The adsorption equilibrium of CCl_4 is associated with concentration of organics and K_{OC}. Silva (Silva et al., 2000) indicated that pore filling was main factor in solute distribution. The aquifer in Qiligou has the characteristics of high runoff, intense flash and less pore fillings, due to the high burial depth, long-term exploration and large production, which is shown in Fig.5. Therefore, adsorption has less influence on decrease of CCl_4.

6. **Biological degradation**

The CCl_4 was transformed into chloroform by biological degradation in the soil. It was manifested by the fact that CCl_4 and chloroform both existed in the soil samples around the pesticide plant and only CCl_4 was founded in the pore water (Zhu et al., 2006). Chloroform was not detected in the wastewater discharged from the pesticide and the karst groundwater. This suggests that the CCl_4 bio-degradation for its attenuation in the karst aquifer can be ignored.

6. Conclusions

In this chapter, the spatial distribution and temporal evolution of CCl_4 in the karst aquifer of a northern city in China were studied through groundwater and soil sampling and testing, groundwater level observation, analysis of water-bearing media, hydrodynamic conditions and artificial exploitation.

1. The water-bearing media is characterized as the multi-system of karstific apertures, fissures and caves. By the control of lithological and geological structure, the karst is extremely heterogeneous.
2. The CCl_4 plume in the karst aquifer was "dumbbell" shaped, with high contamination located in the southern and northern sub-area and relatively light concentrations in the middle transitional sub-area.
3. The concentration of CCl_4 in the aquifer is changed rapidly with time, which is different from the common porous medium aquifer because of the high groundwater velocity in the aquifer and migration channel, complex local flow field and good hydraulic connection. CCl_4 concentration was generally decreasing over time.
4. The length of pollution plume of CCl_4 in the water-bearing aquifer is stable while concentration is shrinking. The attenuation of CCl_4 in the water-bearing aquifer is controlled by passive pumping, volatilization, convection dilution and biodegradation.

7. Acknowledgment

We would like to thank the National Natural Science Foundation of China and Jiangsu Provincial Water Resources Bureau for financial support (40373044, 2007006 and 2009006).

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Study of the Presence of Pesticides in Treated Urban Wastewaters

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1. Introduction

The increase of the industrial activity and the consequent economic and social development have produced, at least until some years ago, a growth of big cities which leads and complicates the supply of one of the most important elements to the life, "water". Nowadays, faced with the growing water demand and the hydric resources shortage, the reuse of treated water is considered as a possible solution in order to increase existing resources. However, all effluents can`t be reused since environmental minimum flows have to be guaranteed downstream of the dumping (Ormad, 2011).

The *water reuse* is the application, before its return to the public water resources and the terrestrial maritime one to a new exclusive use of waters that, having been used by who derived them, they have been subjected to a process or processes of treatment established in the corresponding authorization of dumping and to the necessary in order to get the quality required in function of uses to which it goes to be destined. As well, reuse waters are treated wastewaters that have been subjected to an additional or complementary process which is capable to adapt its quality to the destined use. All this is carried out in *reclamation plants*, group of installations where treated wastewaters are subjected to additional treatments which can be needed in order to adapt the water quality to the destined use (Royal Decree 1620/2007).

Waters which go to be reclaimed, it is, treated wastewaters, are waters that must obey the specifications of Directive 91/271/EEC (Directive 91/271/EEC). However, in these waters can exist some substances which can be pollutants. In general, the composition of these waters mainly depends on two factors: the industrial contribution to the urban dumping and the type of water treatment in the wastewater treatment plant (WWTP). In spite of this, in general, these waters are characterized by:

- The presence of a great variety of pathogenic germs
- The presence of organic matter, with a Biologycal Oxygen Demand 5-day (BOD_5) up to 25 mg L^{-1} and a Chemical Oxygen Demand (COD) up to 125 mg L^{-1} (according to the current legislation). In this generic group, there are two types of organic matter:
    - *Non Hazardous Organic Matter*: This organic matter is mayoritary composed by compunds which haven't degraded in the WWTP. This is because they are refractary compounds or the achieved yields aren't of 100%; they are organic substances non toxic like carboxilic acids, esters, proteins, carbon hydrates, aminoacids, polihydroxilated alcohols, etc.

- *Hazardous Organic Matter:* persistent substances which haven't been removed in the WWTP. If these substances are present with low concentrations can derive in an environmental and health problem. They are pharmaceutical products, clean and care personal products, pesticides and plasticizers. Some of these compounds have characteristics toxic, cancinogenic, mutagenic, teratogenic, bioaccumulative or endocrin disruptors, and they are part of a big group of organic compounds, called Emerging Pollutants, which are received great attention in the last years (Hernando et al., 2006). All are hazardous compounds such as Directives 80/68/EEC, 2000/60/EC and 2006/11/EC establish (Directives 80/68/EEC, 2000/60/EC, 2006/11/EC).

- The presence of suspended solids, with a concentration up to 60 mg L^{-1}.
- High turbidiy
- The presence of inorganic pollutants, such as chlorides, nytrogen and phosphorus (with different concentrations depending on the plant of which come from, with or without nutrients removal) and, in some cases, heavy metals which haven't been removed in the WWTP and which concentration mainly depends on the industrial part that the urban dumping has.

Effluents of WWTPs can be reused to urban, agricultural, industrial, recreational and environmental uses. It is expected an urban use as irrigation of private gardens, emptying of healthy apparatus, irrigation of urban green places such as parks, sport fields and similar, cleaning of streets, systems against fires and industrial cleaning of vehicles. As agricultural use, it is expected the irrigation of cultivation which is consumed in fresh form or has an industrial treatment to its consume, irrigation of pasture to animal consumption, aquaculture, irrigation of woody cultivation, cultivation of ornamental flowers, nurseries, greenhouses and irrigation of industrial and no alimentary cultivation. It is considered the reuse as industrial use as cleaning and process water, in cooling towers and evaporative condensers. It is considered recreational use the irrigation of golf courses, ponds, water masses and ornamental circulated flows in which the public access to water is blocked. Finally, to environmental use it is expected the use of water to fill aquifers by percolation or direct injection, irrigation of woods and other green places which aren't accessible to people, silviculture, maintenance of wetlands, minimum flows and similar.

To carry out all of these uses, indicated in the Spanish current legislation about reclaimed water (Royal Decree 1620/2007) it is necessary to carry out a treatment which gets the minimum criteria of quality demanded in relation with several parameters. In general, this treatment has to remove the suspension matter, the turbidity, the hazardous substances and to disinfect the water, as a minimum (Metcalf and Eddy, 2002).

On the other hand, depend on the use of reclaimed water, apart from considering the quality criteria indicated in the Royal Decree 1620/2007, other criteria included in specific fields have to be considered; for example, Directive 2006/118/EC has to be considered, directive related to the protection of groundwaters against the pollution and deterioration when the reclaimed water is destined to environmental use (Directive 2006/118/EC). This norm includes Environmental Quality Standards (EQSs) related to nitrates, salinity, metals, trichloroethylene, tetrachloroetylene and other hazardous substances such as pesticides. Also the Directive 80/68/EEC establishes that it is necessary to impede the dumping of hazardous substance of the List I and to limit the dumping of the hazardous substances of the List II, lists in which a lot of pesticides are founded, in order to guarantee an effective protection of groundwaters. In the same way, the Spanish Royal Decree 60/2011 (Royal Decree 60/2011) by which quality objectives to some pollutants are fixed and the Reglament of Public Waters is modified, establishes EQSs to Preferential Hazardous Substances of the List II. Likewise, in the

application of the Directive 2000/60/EC the Decision 2455/2001 establishes the relation of Priority Hazardous Substances of the European Union which dumping to surface and groundwaters has to be limited. In this relation are included a lot of pesticides too.
The aim of this work is the characterization of urban treated wastewaters from different treatments with the purpose of knowing the presence of pesticides in these waters and if these waters can be reused or need an additional treatment for this. All studied WWTPs are located in the Ebro river Basin (Spain). Moreover, a bibliographic revision related to the pesticides mainly detected in this type of waters has been carried out.
This work has been carried out in collaboration with "Navarra Infraestucturas Locales S.A." (NILSA) by the Research Project "Regeneración de aguas depuradas mediante procesos de oxidación avanzada (CTM2008-01876/TECNO)".

2. Description of the selected WWTPs

The five selected WWTPs in this study are located in the "Comunidad Foral de Navarra" (Spain). Next a detailed description of the characteristics and process carried out in each WWTP are described.

2.1 WWTP-A

The description of this WWTP is as follow:
- Water origin: urban and industrial coming from companies of food and cans handling
- Equivalent inhabitants: 15869
- Designed flow: 2860 m^3 day^{-1}
- Treated flow: 2129 m^3 day^{-1}
- Designed organic load: 3000 kg BOD_5 day^{-1}
- Treated organic load: 2820 kg BOD_5 day^{-1}

Stages of the treatment in this WWTP are shown in the figure 1. Water arrives by sewers to the WWTP. After homogenizing water in a pond, the treatment consists of the separation of big and medium solids by sieves which act as desander/degreaser too. After water passes to the primary decanter during a retention time of 3-6 hours. Decanted muds are retired to treat them. The effluent of the decanter goes to biological reactor consisting of a percolator filter with plastic layer. After the secondary decantation, water goes to a lagoon system consisting of 4 lagoons put in series. Lagoons are 2.5 meters deep. Rests of organic matter are removed and the removal of pathogen germs happens too due to the effect of the solar radiation. The residence time of water in lagoons is about 25 days.

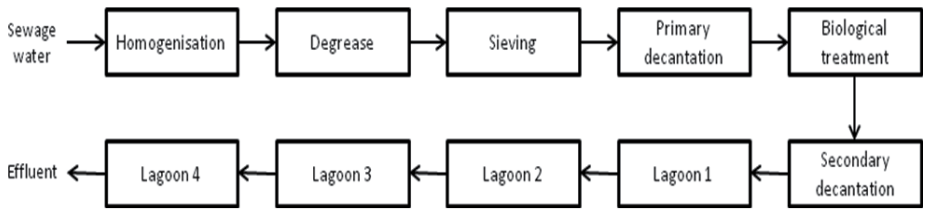

Fig. 1. Diagram of stages in the WWTP-A

2.2 WWTP-B

The description of this WWTP is as follow:
- Water origin: urban and industrial coming from chemical, textile and mechanical industries

- Equivalent inhabitants: 46237
- Designed flow: 20416 m^3 day^{-1}
- Treated flow: 16734 m^3 day^{-1}
- Designed organic load: 6876 kg BOD_5 day^{-1}
- Treated organic load: 5522 kg BOD_5 day^{-1}

Stages of the treatment in this WWTP are shown in the figure 2. Water arrives by a sewer system to the WWTP and it is subjected to a pretreatment consisting of the separations of thick solids by deflector sheets or sieves. After the degrease and desanding are carried out. This consisting of the extraction of mineral particles and the removal of greases, oils and floating material. Next obtained water goes to a primary decanter which purpose is decrease the suspension matter of the water. After this removal of matter, the biological treatment is carried out. The biological treatment is produced by bacterium layers. Finally, the biological mud is separated from water in the secondary decantation.

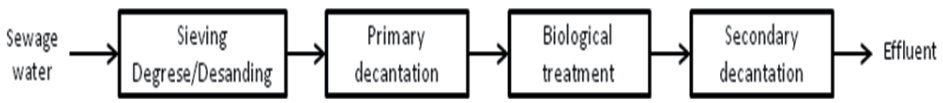

Fig. 2. Diagram of stages in the WWTP-B

2.3 WWTP-C

The description of this WWTP is as follow:

- Water origin: urban and industrial coming from carpentry industries, mechanical garages, vegetable preserved food industries and wineries
- Equivalent inhabitants: 3453
- Designed flow: 308 m^3 day^{-1}
- Treated flow: 361 m^3 day^{-1}
- Designed organic load: 110 kg BOD_5 day^{-1}
- Treated organic load: 149 kg BOD_5 day^{-1}

Stages of the treatment in this WWTP are shown in the figure 3. The sewage water arrives to the plant pass through a sieve where big solids are removed. After, water goes to the primary decanter in order to remove the suspended matter from water. Next water goes to the biological reactor which has a bacterium layer composed by stone and plastic where the organic load of water is reduced considerably. Aftherthat, the reduction of suspended matter is increased in a secondary decanter. After these processes, there is a tertiary treatment consisting of 4 lagoons. The residence time of water in lagoons is 5-6 days. The function of these lagoons is degrade the organic matter or the water and complete the disinfection by the effect of the solar radiation.

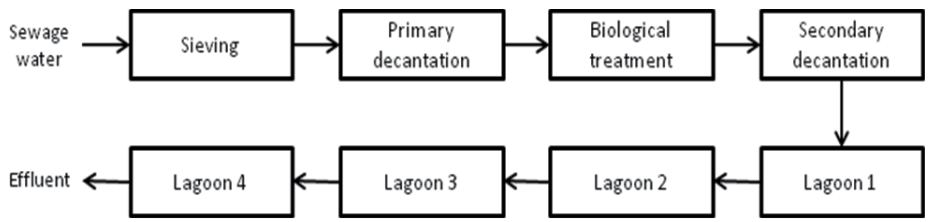

Fig. 3. Diagram of stages in the WWTP-C

2.4 WWTP-D

The description of this WWTP is as follow:

- Water origin: urban and agricultural
- Equivalent inhabitants: 4376
- Designed flow: 1800 m^3 day^{-1}
- Treated flow: 983 m^3 day^{-1}
- Designed organic load: 336 kg BOD_5 day^{-1}
- Treated organic load: 195 kg BOD_5 day^{-1}

Stages of the treatment in this WWTP are shown in the figure 4. Water which arrives to the WWTP is pumped to the sieve. The sieve operates as desander/degreaser too. After water goes to an anoxic biological reactor with agitation by which nitrogen and phosphorous is partially removed. Next, water goes to a decanter.

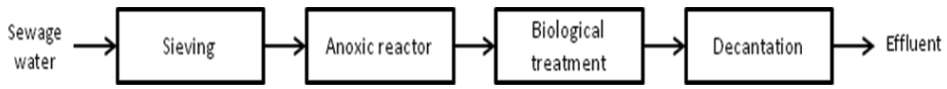

Fig. 4. Diagram of stages in the WWTP-D

2.5 WWTP-E

The description of this WWTP is as follow:

- Water origin: urban and industrial coming from winery industries
- Equivalent inhabitants: 27154
- Designed flow: 4633 m^3 day^{-1}
- Treated flow: 3400 m^3 day^{-1}
- Designed organic load: 1357 kg BOD_5 day^{-1}
- Treated organic load: 958 kg BOD_5 day^{-1}

Stages of the treatment in this WWTP are shown in the figure 5. Once sewage water arrives to the plant, it is subjected to a pretreatment. This pretreatment consists of a sieve which removes big solids and a pump that raises the water up to the degreaser/desander. Moreover, this stage has an auxiliary reactor which is used when peaks flow happens. Finally, the primary decanter removes a great part of solids. The secondary treatment consists of two biological reactors and one secondary decanter. In the first, water goes to a big lagoon in which the nitrogen is removed. In the bottom of this lagoon there are two stirrers which purpose is to avoid the decantation of solids. The residence time of water is a bit higher than in the aerobic reactor. The second part of the degradation is carried out in an aerobic reactor with two stages in series. In its interior there is a plastic layer in which the biomass is fixed.

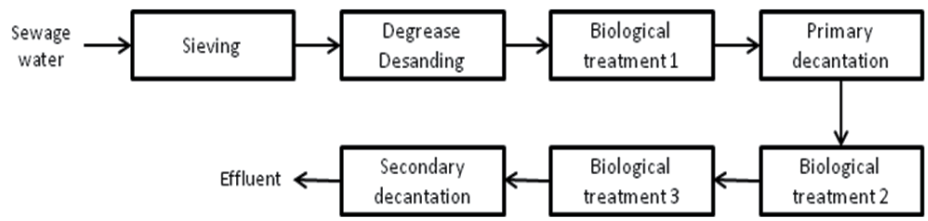

Fig. 5. Diagram of stages in the WWTP-E

3. Materials and methods

3.1 Samples

The sampling of WWTPs effluents, waters under study in this work, was carried out in May 2009 in the outlet of each WWTP. Samples were conserved refrigerated until their analysis.

3.2 Characterization of samples and methodology

The characterization of samples was carried out by the analysis of physical-chemical parameters and the methodology shown in the table 1.

Parameter	Analytic methodology	Reference
pH	Standard Method 4500-H^+ B	Eaton et al., 2005
Conductivity	Norm UNE-EN ISO 7888:1985	ISO 7888:1985
Turbidity	Norm ISO 7027:1999	ISO 7027:1999
Suspended solids concentration	Standard Method 2540 D	Eaton et al., 2005
Chemical Oxygen Demand	EPA Method 410.4	EPA Method 410.4
Biological Oxygen Demand 5-days	Standard Method 5210 B	Eaton et al., 2005

Table 1. Characterization of effluents of WWTPs

3.3 Analysis of pesticides

EPA method 525.5 (EPA Methods 525.2) is used in order to determine the presence of pesticides in samples. This method is based on gas chromatography and mass spectrometry with a previous solid-liquid extraction.

An Autotrace Workstation (Zymark) automatic extractor was used for the extraction. The chromatographic conditions and equipment used are shown in table 2 and the results of the methodology validation in table 3. The results were obtained using the Xcalibur POLARIS 1.2 version program (ThermoQuest).

Gas chromatographer TRACE GC 2000 (TermoFinnigan)	
Column	DB5-MS (J&W, 30 m, 0.25 mm, 0.25 µm)
Program of temperatures	90 ^oC (1 min) – 20 ^oC min^{-1} – 180 ^oC (1 min) – 2 ^oC min^{-1} – 240 ^oC (1 min) – 20 ^oC min^{-1} – 310 ^oC (10 min)
Injector temperature	250 ^oC
Injection volume	1 µL, splitless 0.8 min
Carrier gas	He (N55), 1mL min^{-1}
Mass spectrometer POLARIS (ThermoFinnigan)	
Ionization energy	70 eV
Acquisition mode	Full scan
Mass interval	50-450 amu
Screen speed	1 scan s^{-1}
Acquisition time	32.5 min

Table 2. Conditions of pesticides analysis

Pesticide	Quantification limit (µg L^{-1})		Calibration interval (µg L^{-1})	Validity interval (µg L^{-1})	Recovery interval (%)	
	Instrumental step	Full method			Instrumental step	Full method
Isoproturon	20	0.030	20-500	0.030-300	75-130	63-110
Diuron	20	0.030	20-500	0.030-300	82-128	70-123
3.4-Dichloroaniline	20	0.030	20-500	0.030-300	88-130	47-106
4-Isopropylaniline	20	0.030	20-500	0.030-300	80-130	60-125
Desethylatrazine	20	0.030	20-500	0.030-300	76-130	80-129
Trifluralin	20	0.015	20-500	0.030-300	70-130	70-127
Dimethoate	20	0.030	50-500	0.030-300	66-124	54-137
Simazine	50	0.030	20-500	0.030-600	75-135	64-127
Prometon	20	0.030	20-500	0.030-300	76-124	0-125
Atrazine	200	0.100	200-5000	0.100-300	78-130	75-127
Propazine	20	0.015	20-500	0.015-300	86-130	73-127
Terbuthylazine	20	0.015	20-500	0.015-300	79-130	83-128
Parathion methyl	50	0.030	50-500	0.030-300	78-139	72-130
Parathion ethyl	20	0.030	20-500	0.030-300	74-122	64-128
Alachlor	20	0.015	20-500	0.015-300	75-125	70-124
Ametryn	20	0.030	20-500	0.030-300	78-130	0-116
Prometryn	20	0.030	20-500	0.030-300	80-120	17-116
Terbutryn	20	0.030	20-500	0.030-300	80-120	13-114
Chlorpyrifos	20	0.015	20-500	0.015-300	75-120	73-116
Chlorfenvinfos	20	0.015	20-500	0.015-300	76-130	70-126
HCHs	20	0.015	20-500	0.015-300	84-124	70-120
Hexachlorobenzene	20	0.030	20-500	0.030-300	70-130	74-136
Heptachlor	20	0.015	20-500	0.015-300	75-130	58-113
Heptachlor epoxide A	20	0.015	20-500	0.015-300	85-125	62-112
Heptachlor epoxide B	20	0.015	20-500	0.015-300	84-130	58-113
Aldrin	20	0.015	20-500	0.015-300	85-125	64-126
4.4′-Dichlorobenzophenone	20	0.015	20-500	0.015-300	75-120	68-126
Isodrin	20	0.015	20-500	0.015-300	85-125	66-120
α-Endosulphan	20	0.015	20-500	0.015-300	70-125	70-93
pp′-DDE	20	0.015	20-500	0.015-300	89-122	64-107
Dieldrin	20	0.015	20-500	0.015-300	70-125	62-120
Endrin	20	0.015	20-500	0.015-300	80-125	74-122
pp′-DDD + op′-DDT	40	0.030	40-1000	0.030-600	79-125	66-139
Endosulphan-sulphate	20	0.015	20-500	0.015-300	83-125	73-126
pp′-DDT	20	0.030	20-500	0.030-300	76-130	50-120
Dicofol	50	0.030	50-500	0.030-300	80-148	63-136
Methoxychlor	20	0.015	20-500	0.015-300	77-126	75-130
Metholachlor	20	0.015	20-500	0.015–300	76-115	73-128
Molinate	20	0.015	20-500	0.015–300	91-130	75-113
Tetradifon	20	0.015	20-500	0.015-300	85-130	70-116

Table 3. Results of the methodology validation of pesticides analysis.

4. Results

4.1 Characterization of effluents of selected WWTPs

As it has been mentioned previously, the characterization of WWTPs effluents is carried out by several physical-chemical parameters, among them, turbidity and suspended solids concentration, which are parameters whose maximum admissible values are indicated in the Spanish legislation related to water reclamation (Royal Decree 1620/2007). Characterization results of WWTPs effluents are shown in the table 4.

Parameter	WWTP-A	WWTP-B	WWTP-C	WWTP-D	WWTP-E
pH (temperature in ^{o}C)	7.6 (14.7)	7.9(15.8)	7.4(11.1)	7.2(18.6)	7.3(13.5)
Conductivity (μS cm^{-1})	3220	1867	1086	2950	832
Turbidity (NTU)	34.0	12.3	1.7	34.7	5.4
Suspended solids concentration (mg L^{-1})	56	8	4	32	20
Chemical Oxygen Demand (mg L^{-1})	84	60	77	62	50
Oxygen Biological Demand 5-days (mg L^{-1})	16.4	17.5	15.3	15.5	12.5

Table 4. Results of the characterization of studied effluents

As well as this characterization, a bibliographic study was carried out. This study was done in order to confirm that the results of the selected WWTPs obtained are between typical values regarding characteristics of WWTPs effluents. In accordance with this study, habitual values of analyzed parameters in WWTPs effluents are shown in the table 5 (Imai et al., 2002; Mendoça et al., 2007; Hassanli et al., 2008; Kalavrouziotis et al., 2009; Santos et al., 2009).
In accordance with the obtained results it can be observed that the characterization values of the selected effluents are within usual intervals to effluents of WWTPs.

Parameter	Interval
pH	7 – 8
Conductivity (μS cm^{-1})	70 – 3200
Turbidity (NTU)	3 – 52
Suspended solids concentration (mg L^{-1})	4 – 92
Chemical Oxygen Demand (mg L^{-1})	19 – 98
Oxygen Biological Demand 5-days (mg L^{-1})	5 – 31

Table 5. Usual values of physical-chemical parameters in WWTPs effluents according to other references

4.2 Presence of pesticides in effluents of WWTPs

In general, in effluents of selected WWTPs exists the presence of several pesticides. Among all analyzed pesticides, 10 different pesticides have been detected: chlorfenvinfos, chlorpyrifos, dimethoate, isoproturon, prometon, metholachlor, 3,4-dichloroaniline, terbutryn, terbuthylazine and simazine. Chemical structures of detected pesticides in studied effluents are shown in the figure 6.

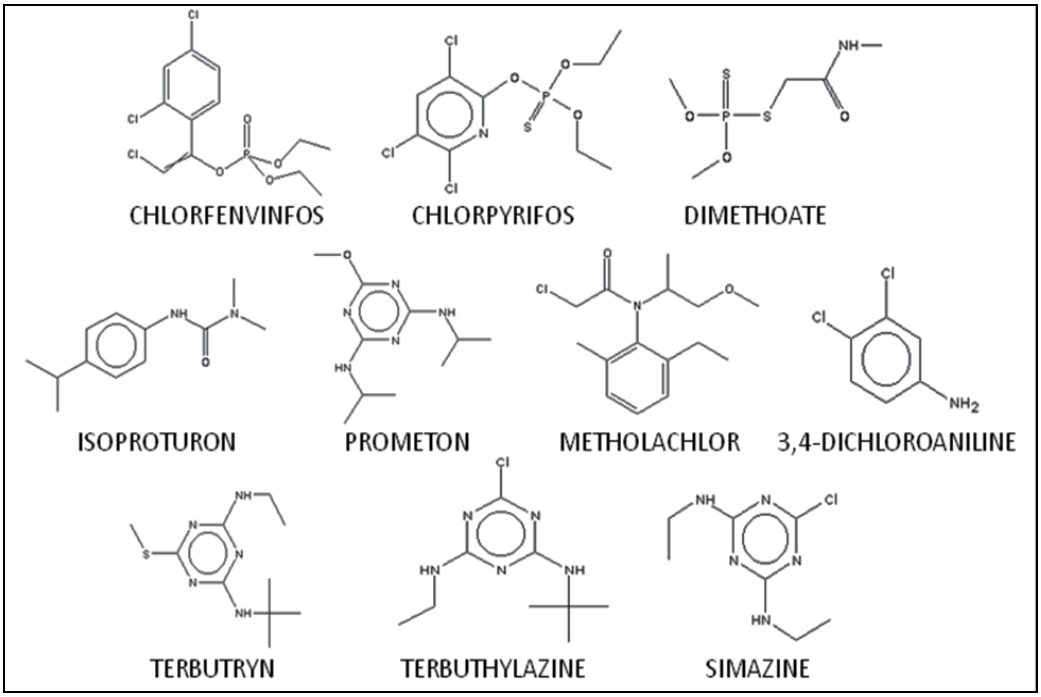

Fig. 6. Chemical structures of detected pesticides in effluents of studied WWTPs

Detected pesticides belong to different groups.

In the first place, organic-phosphorous pesticides such as chlorfenvinfos, chlopyrifos and dimethoate are detected. These pesticides are mainly used as insecticides. Chlorpyrifos and dimethoate are very toxic and chlorfenvinfos is extremely toxic. The main effects in the health caused by these pesticides are irritation of eyes and skin and if the exposition to these pesticides is long-term they can affect to nervous, respiratory and immunological systems.

Pesticides derived of urea such as isoproturon and diuron are detected too. These pesticides are mainly used as herbicides and they are moderately toxic compounds. They can cause irritation of skin, eyes and respiratory system, in addition to blood alterations in the case of extend expositions.

Some of detected pesticides belong to triazines group; they are terbutryn, terbuthylazine and simazine. These pesticides are mainly used as herbicides and they are moderately toxic substances. With respect to effects on health, the can cause irritation in eyes and skin and moreover, affections in liver, kidney, nervous and circulatory systems in the case of long-term contact.

Finally, the rest of detected pesticides are metholachlor and 3,4-dichloroaniline. Metholachlor belongs to anilide group, it is a moderately toxic substance and used as herbicide. It can cause irritation in eyes and in the case of long-term exposition it can provoke liver and blood conditions. 3.4-dichloroaniline is used as herbicide and it is a moderately toxic pesticide. It can cause dermatitis and damages in liver and kidney after long-term expositions.

Some of the pesticides detected in the studied effluents, such as chlorpyrifos, chlorfenvinfos, isoproturon and simazine were detected in other effluents of WWTPs such as it is shown in

other research works. As well as these pesticides, in these studies other pesticides were detected: α-endosulphan, hexachlorociclohexanes (α, β, χ, δ), atrazine, diuron, hexachlorobenzene, endrin, dieldrin, heptachlor, heptachlor epoxide B, pp'-DDD and pp'-DDE (Kastsoyiannis and Samara, 2004; Muñoz et al., 2008). Chemical structures of theses pesticides are shown in the figure 7.

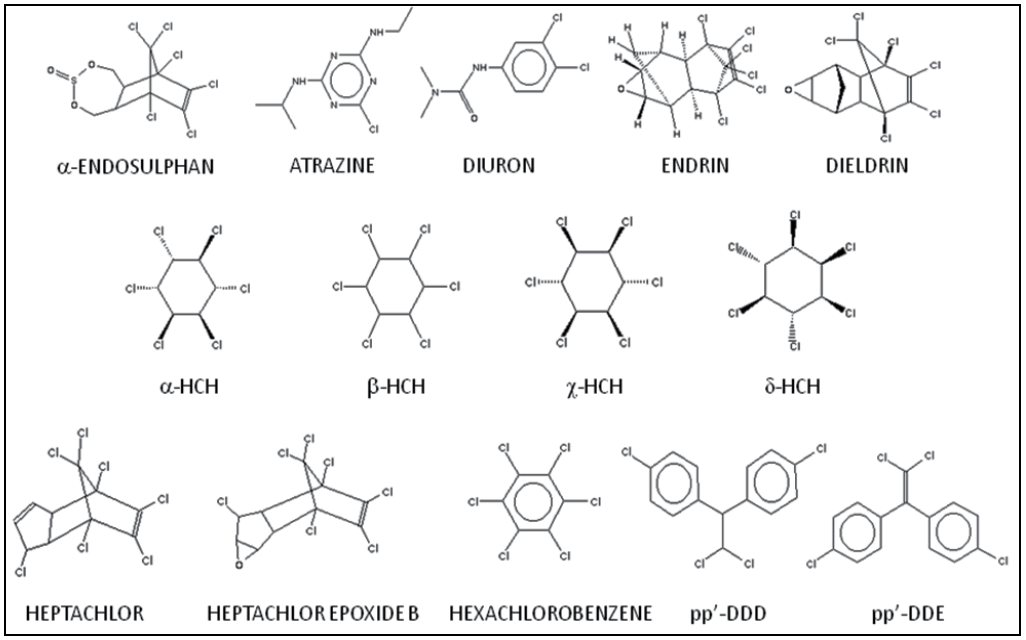

Fig. 7. Chemical structures of pesticides detected in other effluents of WWTPs (Kastsoyiannis and Samara, 2004; Muñoz et al., 2008)

Next, detected pesticides and their concentrations in studied effluents of each WWTP are detailed.

4.2.1 WWTP-A

Among all analyzed pesticides in the effluent of the WWTP-A, 3 pesticides were detected: prometon, terbuthylazine and isoproturon. Detected concentrations of these pesticides were:

- Prometon: 0.05 μg L^{-1}
- Terbuthylazine: 1.31 μg L^{-1}
- Isoproturon: 1.07 μg L^{-1}

4.2.2 WWTP-B

Among all analyzed pesticides in the effluent of the WWTP-B, only chlorpyrifos was detected. The concentration of chlorpyrifos in this effluent was 0.074 μg L^{-1}.

4.2.3 WWTP-C

Among all analyzed pesticides in the effluent of the WWTP-C, 4 pesticides were detected: chlorfenvinfos, dimethoate, terbuthylazine and simazine. Detected concentrations of these pesticides were:

- Chlorfenvinfos: 0.024 µg L^{-1}
- Dimethoate: 0.40 µg L^{-1}
- Terbuthylazine: 1.31 µg L^{-1}
- Simazine: 0.101 µg L^{-1}

4.2.4 WWTP-D

Among all pesticides analyzed in the effluent of the WWTP-D, 4 pesticides were detected: 3,4-dichloroaniline, terbutryn, terbuthylazine and metholachlor. Detected concentrations of these pesticides were:

- 3,4-Dichloroaniline: 0.045 µg L^{-1}
- Terbutryn: 0.077 µg L^{-1}
- Terbuthylazine: 0.89 µg L^{-1}
- Metholachlor: 0.035 µg L^{-1}

4.2.5 WWTP-E

Among all pesticides analyzed in the effluent of the WWTP-E, only dimethoate was detected. The concentration of this pesticide in this effluent was 0.272 µg L^{-1}. According to these results, the concentrations of detected pesticides in the 5 studied effluents are from 0.02 µg L^{-1} to more than 1 µg L^{-1}. This interval of concentrations is similar to ones detected in other WWTPs by other researches (Kastsoyiannis and Samara 2004; Muñoz et al., 2008). Moreover, after this study a direct relation between detected pesticides in the effluents and treatments carried out in each WWTP hasn't been observed.

4.3 Application in reclamation

According to the Spanish Royal Decree 1620/2007, water must guarantee quality criteria in order to it can be reused. These criteria change in function of the use of water. Maximum admissible values of enteric nematodes, Escherichia coli, turbidity and suspended solids as well as other criteria related to other pollutants.

In accordance with the presence of hazardous substance, among them, pesticides, EQSs established in the Directive 2008/105/EC (Directive 2008/105/EC) and in the Spanish Royal Decree 60/2011 (Royal Decree 60/2011) must be respected.

This study, about the application of effluents of WWTPs in reclamation, has been carried out not taking into account microbiological parameters. Next, the possible application of studied waters in reclamation is showed. This application has been divided in two parts: on one hand, taking into account values of turbidity and suspended solids in the law; and on other hand, the presence of pesticides.

4.3.1 Application in reclamation: turbidity and suspended solids

As it has been mentioned previously, in the annex I of Royal Decree 1620/2007 maximum admissible values of suspended solids and turbidity are indicated in order to reuse water and depending on its use. These values are shown in the table 6. In accordance with these values and the values obtained to the effluents of studied WWTPs, some conclusions can be extracted:

- WWTP-A:

  According to the obtained results of suspended solids concentration and turbidity, this water cannot be applied in any use without an additional treatment. It would be needed a treatment capable to decrease, as a minimum, suspended solids in water in order to apply it in some agricultural, industrial, recreational and environmental uses. They are:

USE OF WATER		MAXIMUM ADMISSIBLE VALUE	
		SUSPENDED SOLIDS (mg L^{-1})	TURBIDITY (NTU)
URBAN USES	Quality 1.1	10	2
	Quality 1.2	20	10
AGRICULTURAL USES	Quality 2.1	20	10
	Quality 2.2	35	-
	Quality 2.3	35	-
INDUSTRIAL USES	Quality 3.1 (a and b)	35	15
	Quality 3.1 (c)	35	-
	Quality 3.2	5	1
RECREATIONAL USES	Quality 4.1	20	10
	Quality 4.2	35	-
ENVIRONMENTAL USES	Quality 5.1	35	-
	Quality 5.2	10	2
	Quality 5.3	35	-
	Quality 5.4	Minimum required quality will be studied in each case	

Table 6. Maximum admissible values of suspended solids and turbidity to reclaimed waters (Royal Decree 1620/2007)

- Irrigation of products to human consumption with a system of water application which doesn't avoid the direct contact between reclaimed water and comestible parts and their consumption isn't in fresh form but with an industrial treatment later.
- Irrigation of pastures to consumption of animals producing milk or meat.
- Located irrigation of woody cultivation which impedes the contact between the reclaimed water and fruits consumed in the human diet.
- Irrigation of cultivation of ornamental flowers, nurseries, greenhouses without direct contact between reclaimed water and productions.
- Irrigation of non alimentary industrial cultivation, nurseries, ensilaged forages, cereals and oleaginous seeds.
- Process and cleaning water in the alimentary industry.
- Ponds, water masses and ornamental circulated flows in which the access of people is impeded.
- Recharge of aquifers by located percolation by the ground.
- Irrigation of forests, green places and of other type which isn't accessible to people.
- Silviculture.

As well as to apply a treatment capable to reduce the suspended solids concentration in water, it would be needed to reduce the turbidity too in order to apply this water in urban uses and the rest of uses contemplated and no mentioned previously.

- WWTP-B:

According to the obtained results of suspended solids concentration and turbidity, this water can be reused without applying any additional treatment to some uses and it

requires a posterior treatment if is destined to other uses. This water can be reused as is to some agricultural, industrial, recreational and environmental uses:

- Irrigation of products to human consumption with a system of water application which doesn't avoid the direct contact between reclaimed water and comestible parts and their consumption isn't in fresh form but with an industrial treatment later.
- Irrigation of pastures to consumption of animals producing milk or meat.
- Located irrigation of woody cultivation which impedes the contact between the reclaimed water and fruits consumed in the human diet.
- Irrigation of cultivation of ornamental flowers, nurseries, greenhouses without direct contact between reclaimed water and productions.
- Irrigation of non alimentary industrial cultivation, nurseries, ensilaged forages, cereals and oleaginous seeds.
- Process and cleaning water in the alimentary and no alimentary industry.
- Other industrial uses.
- Ponds, water masses and ornamental circulated flows in which the access of people is impeded.
- Recharge of aquifers by located percolation by the ground.
- Irrigation of forests, green places and of other type which isn't accessible to people.
- Silviculture.

In the case of urban uses it is necessary decrease the turbidity of water, just like to use in irrigation of golf courses and recharge of aquifers by direct injection. Its use in cooling towers and evaporative condensers is the only case in which, as well as reduce the turbidity, it is necessary decrease suspended solids in water.

- WWTP-C:

In view of characteristics of this water with regard to suspended solids and turbidity, this water could be used without applying it any additional treatment to all uses practically. Only it would be necessary an additional treatment which was capable to reduce suspended solids and turbidity of water in the case of one industrial use, in cooling towers and evaporative condensers.

- WWTP-D:

In this case, this water could be used in some sectors without applying any treatment and it would require an additional treatment in order to use in other uses. This water could be applied as is to:

- Irrigation of products to human consumption with a system of water application which doesn't avoid the direct contact between reclaimed water and comestible parts and their consumption isn't in fresh form but with an industrial treatment later.
- Irrigation of pastures to consumption of animals producing milk or meat.
- Aqueaculture.
- Located irrigation of woody cultivation which impedes the contact between the reclaimed water and fruits consumed in the human diet.
- Irrigation of cultivation of ornamental flowers, nurseries, greenhouses without direct contact between reclaimed water and productions.
- Irrigation of non alimentary industrial cultivation, nurseries, ensilaged forages, cereals and oleaginous seeds.
- Process and cleaning water in the alimentary industry.

- Ponds, water masses and ornamental circulated flows in which the access of people is impeded.
- Recharge of aquifers by located percolation by the ground.
- Irrigation of forests, green places and of other type which isn't accessible to people.
- Silviculture.

In order to reuse the water to the rest of uses could be necessary to apply a treatment capable to reduce the turbidity and suspended solids up to limits established in current legislation, except to its industrial use as process and cleaning water in the alimentary and no alimentary industrial for which only a reduction of the turbidity of water would be necessary.

- WWTP-E:

With respect to the effluent of the last selected WWTP, this could be used without an additonal treatment to the most of contemplated uses: urban, agricultural, industrial, recreational and environmental. Only a subsequent treatment, capable to reduce the turbidity and suspended solids, would be necessary in order to apply this water in the next uses:

- Irrigation of private gardens.
- Emptying of health apparatus.
- Cooling towers and evaporative condensers.
- Recharge of aquifers by direct injection.

4.3.2 Application in reclamation: pesticides

As it has been mentioned before, as well as to obey the maximum admissible values of turbidity and suspended solids, water can be reused if it observes EQSs indicated in the Directive 2008/105/EC and in the Spanish Royal Decree 60/2011 related to hazardous substances.

Specifically regarding to pesticides, maximum admissible concentrations of some of pesticides detected in effluents of studied WWTPs are established in legislation. These values are shown as annual average (AA) and as maximum admissible concentrations (MAC). Values considered in this study are MACs in order to conclude if analyzed waters are suitable or not for reuse with respect to the presence of pesticides.

EQSs to some of detected pesticides in effluents of WWTPs are shown in the table 7.

PESTICIDE	EQS-AA ($\mu g\ L^{-1}$)	EQS-MAC ($\mu g\ L^{-1}$)
Chlorfenvinfos	0.1	0.3
Chlorpyrifos	0.03	0.1
Isoproturon	0.3	1.0
Simazine	1.0	4.0
Metholachlor	1.0	No applicable
Terbuthylazine	1.0	1.0

Table 7. EQSs to some pesticides detected in WWTPs effluents (Directive 2008/105/EC, Royal Decree 60/2011)

In accordance with these values and the concentration of pesticides detected in WWTPs effluents, some conclusions can be extracted:

- WWTP-A:
  Due to the presence of pesticides this water couldn't be reused without applying an additional treatment.
  Detected concentrations of terbuthylazine and isoproturon exceed the maximum admissible concentrations indicated in the current legislation, therefore it is necessary to apply a treatment capable to degrade partially these pesticides in order to reuse this water. In this water, prometon is detected too but to this pesticide, an EQS isn't indicated.
- WWTP-B:
  The effluent of this WWTP could be reused without any additional treatment taking into account the presence of pesticides.
  In this water, only chlorpyrifos is detected but its concentration doesn't exceed the maximum admissible one indicated in the legislation.
- WWTP-C:
  In the case of this WWTP, the water couldn't be reused as is therefore an additional treatment would be necessary for this.
  The addtional treatment had to reduce the concentration of terbuthylazine of the water because detected concentration of this pesticide exceedes the corresponding EQS. In this water other pesticides are detected, chlorfenvinfos, simazine and dimethoathe. However, detected concentration of chlorfenfinfos and simazine don't exceed the EQSs and for dimethote, any EQS is established.
- WWTP-D:
  Regarding to the presence of pesticides, this water could be reused without treating it previously.
  In this effluent, some pesticides are detected by in any case the EQSs are exceeded or there aren't EQSs indicated in the legislation to these pesticides. 3,4-dichloroaniline, terbutryn and metholaclor are detected but these pesticides aren't comtemplated in the EQSs of the legistación. Terbuthylazina is detected too, but the detected concentration doesn't exceed the maximum admissible concentration legislated.
- WWTP-E:
  This effluent could be reused as is with respect to the presence of pesticides.
  Only dimethoathe is detected in this WWTP effluent but this pesticide isn't considered in the EQSs in the current legislation.

5. Conclusions

After carrying out this study, some conclusions can be extracted.
With respect to the presence of pesticides in effluents of WWTPs:

- Usual process carried out in WWTPs doesn't get the removal of hazardous substances from water, such as pesticides.
- After carrying out the study in five WWTPs located in the Ebro river basin (Spain), detected pesticides in WWTPs effluents are: chlorfenvinfos, chlorpyrifos, dimethoate, isoproturon, prometon, metholachlor, 3,4-dichloroaniline, terbutryn, terbuthylazine and simazine.
- As well as these pesticides, other researchers have detected more pesticides in WWTPs effluents, which is shown in other research works. These pesticides are: α-endosulphan, hexachlorociclohexanes, atrazine, diuron, hexachlorobezene, endrin, dieldrin, heptachlor, heptachlor epoxide B, pp'-DDD and pp'-DDE.

Regarding to the characteristic of the studied WWTPs effluents:

- Detected concentrations of pesticides in studied WWTPs are between 0.02 and 1.31 μg L-1.
- With respect to physical-chemical parameters considered in the legislation related to water reuse, turbidity and suspended solids, obtained results to studied effluents are the next: turbidity between 2 and 35 NTU and suspended solids concentration between 4 and 56 mg L^{-1}.

With regard to the reuse of WWTPs effluents:

- In general, regarding to results about turbidity and suspended solids, effluents of studied WWTPs could be used without applying any additional treatment to some agricultural, industrial, recreational and environmental uses. However, in order to use the water to the rest of uses of these groups and to urban uses, an additional treatment capable to reduce turbidity and suspended solids from water would be necessary.
- In accordance with the presence of pesticides and taking into account the EQSs indicated in the current legislation, three of the five selected effluents could be reused without any additional treatment. However, in order to reuse the other two effluents, a subsequent treatment capable to degrade partially some pesticides such as terbuthylazine and isoproturon would be necessary, since detected concentrations of these pesticides exceed the established EQSs.

6. Acknowledgements

This work has been carried out in collaboration with “Navarra de Infraestructuras Locales S.A. (NILSA)”, in the research project "Regeneración de aguas depuradas mediante procesos de oxidación avanzada (CTM2008-01876/TECNO)" financed by “Secretaría de Estado de Universidad e Investigación del Ministerio de Ciencia e Innovación”, “Diputación General de Aragón” and “Fundación Caixa Catalunya”.

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Interactions Between Ionic Pesticides and Model Systems for Soil Fractions

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1. Introduction

The extensive use of herbicides in agriculture and their potentially toxic effects have promoted studies investigating the physical, chemical and biological processes that determine the mobility, bioavailability and degradation of these compounds in soils (Blasioli et al., 2011). Knowledge of these processes will enable prediction of the transport and fate of herbicides in soils and aquatic systems, and thus enable measures to be taken to limit their environmental impact.

Retention is considered the main cause of the deactivation of herbicides in soils, and is important from the point of view of inhibiting the toxic properties of herbicides and of restricting their transport into aquatic systems (Jones & Bryan, 1980). Although not unique, adsorption reactions (i.e. accumulation of chemical species at the solid-solution interface) are the main cause of the retention of organic contaminants in soils, and their extent will depend on the physicochemical properties of both the adsorbent (soil) and the adsorbate (herbicide).

The chemical characteristics of organic compounds are largely responsible for their behaviour in soil, and the differences in adsorption of different herbicides in the same soil are attributed to their distinct chemical properties. Although herbicides are very diverse, two groups can be distinguished in order to interpret their interactions with soil components: those involving chemical forces and those involving physical forces. The first group comprises ionic or ionizable hydrophilic compounds, while the second group comprises non polar hydrophobic compounds.

Bipyridinium cations, such as paraquat (1,1'–dimethyl–4,4'–bipyridinium ion), are the best known members of the ionizable herbicides as they have been extensively used in agriculture and are consequently widely distributed in soils and waters. Paraquat (PQ) is applied as a dichloride or dibromide salt, which when dissolved in water releases the organic cation PQ^{2+}, which can be adsorbed on the soil surface, either by replacing inorganic cations or by an ionic interaction mechanism with negatively charged sites on the soil surface, in which the electrostatic effect will be determinant (Narine & Guy, 1982). PQ adsorbs on humic substances and the degree of adsorption increases as the pH increases, as

a result of the progressive ionization of the acid groups in the organic matter (Iglesias et al., 2009; Brigante et al., 2010). PQ shows affinity for iron oxides and clay minerals (Clausen & Fabricius, 2001; Seki & Yurdakoç, 2005; Pateiro-Moure et al., 2010). Studies of PQ adsorption to clays in the pH range 5.0-7.0 reveal surface concentrations of between 0.18 and 4 $\mu mol \cdot m^{-2}$, depending on the type of clay. The higher adsorption of PQ on clays is explained by the ability of the planar PQ molecules to enter the lattice layers (Bromilow, 2003) and the extent of the PQ adsorption on soils is determined by the amount and type of clay minerals present in the soils (Roberts et al., 2002).

Phenoxyalkanoic acids (such as MCPA) and their derivatives constitute a large group of herbicides that are used extensively in agriculture. This type of organic pesticide has a carboxyl group that is mainly responsible for the interactions with soil components (Tunega et al., 2004). At pH > 4 the acid group (pK_a = 3.10) will be ionized giving rise to significant adsorption of the anionic form of the pesticide on mineral oxides. It is known that the adsorption of pesticides of this type on goethite is strongly dependent on pH and ionic strength, and has been suggested to be due to the favourable interaction between the sorbing anions and the positively charged surface of the iron oxides (Celis et al., 1999; Spadoto & Hornsby, 2003). It is also known that the MCPA molecule has a phenolic lipophilic structure that makes this herbicide a model substance for the study of adsorption-desorption mechanisms in soils, which may involve both polar and hydrophobic interactions.

The soil surface, which comprises organic and inorganic components, is the primary site for chemical accumulation of organic herbicides, and its reactivity depends on the number and type of accessible functional groups. In addition to their specific reactivity, the reactivity of any functional group also depends on the proximity of other functional groups, so that the ionization of an acid group is enhanced by a nearby withdrawing substituent. Another factor to be taken into account is the accessibility of the functional groups to the adsorbates, as the association between different soil minerals and natural organic matter may make some functional groups inaccessible.

Oxides and hydroxides of iron and aluminium are particularly important inorganic soil components as although they are not major components, they are responsible for many chemical processes that take place in the soil because of their high specific surface and reactivity. For example, iron oxides contribute to the variable charge of soil and are known to play an important role in the adsorption of anions and cations (Cornell & Schwermann, 1996).

The organic matter on the soil surface constitutes the main source of the variable charge of surface horizons. Humic substances are the main source of pH-dependent changes in the soil organic matter (SOM), and the functional groups that generate the charge are mainly carboxylic groups. The ionized fraction of the acid groups increases as the pH increases and the negatively charged groups formed will interact electrostatically with the cations present in the environment.

Adsorption reactions in soils have historically been described by empirical adsorption isotherm equations. An adsorption isotherm is a plot of the concentration adsorbed on to a solid surface against the concentration in aqueous solution, for different total concentrations of a chemical species. These equations are popular because they are simple to use and the adjustable parameters are easily estimated, although they do not provide information about the chemical binding mechanisms (Sposito, 1982). Although adsorption isotherm equations are excellent for describing adsorption experiments, it must be taken into account that they are numerical relationships used to fit data, so that the equation parameters obtained are valid only for the chemical conditions under which the experiments are conducted.

Various chemical complexation models have been developed to describe adsorption data at the solid-solution interface. The major advantage of the complexation models over empirical approaches is that the former have the potential to be predictive and are applicable to more than one experimental condition. However, this potential has yet to be fulfilled in a general way. The most significant benefit provided by the models is the consideration of charge on both the adsorbate and the adsorbent surface, and the insight provided into the stoichiometry and reactivity of adsorbed species. These models have been widely applied to the reference materials selected as models of soil components, but have scarcely been used for mixtures of these components or for soils.

2. Model systems for the soil fractions

2.1 Organic matter

Humic substances constitute the largest fraction of natural organic matter, and therefore may be used as reference materials to elucidate the mechanism of interaction between ionic herbicides and SOM (Hesketh et al., 1996). The classical view (Stevenson, 1994) states that humic substances are polydisperse mixtures, and that their chemical behaviour is generally determined by two types of functional groups: carboxylic and phenolic groups. Most studies involving cation binding to humic sustances have been carried out in solution (Milne et al., 2003), and the information obtained for dissolved humic substances has been used to predict the binding properties of humic substances in soils (Tipping, 2002). However, some authors question whether the parameters calculated from laboratory experiments carried out with dissolved natural organic matter can be used to provide an accurate description of the properties of the solid (soil) organic matter (Merdy et al., 2006). In order to simulate more realistic conditions, peat soils with an organic matter content higher than 90%, can be used as reference materials to study the binding properties of humic substances in the solid phase.

2.2 Goethite

Iron mineral oxides and (hydr)oxides are common mineral compounds, particularly in soils. Goethite (α-FeOOH) is the most widely used reference material for (oxy)hydroxides as it is the most common and stable crystalline iron oxide under the conditions existing in soil. This mineral has a relatively high surface area, is very reactive (Cornell and Schwertmann, 1996) and therefore plays an important role in the migration of cations and anions in natural environments. Its properties have been studied in great detail in recent years, and there is therefore abundant information about its structure, chemical properties and adsorption, which facilitate the study of its interactions with ionic herbicides (Cornell & Schwertmann, 1996; Antelo et al., 2005; Brigante et al., 2010). Ionic pesticides may interact with reactive sites on the mineral surface via electrostatic interactions, cation exchange reactions and surface binding reactions, so that a high degree of adsorption might take place on mineral surfaces.

2.3 Humic acid-coated goethite

In soils and aquatic systems, mineral oxides are often found associated with natural organic matter and their mutual interaction will change the chemical and adsorptive properties of both the organic and the mineral fraction. Because soil colloids are multicomponent associations, to achieve a more realistic view of the behaviour of cationic and anionic pesticides in natural systems, the adsorption reactions that take place in these combined systems must be analysed. Therefore, once the adsorption of ionic herbicides on

organic and mineral fractions have been studied individually, the adsorption of herbicides on organic-mineral assemblages must be analysed. The simplest assemblage is a binary system in which the goethite particles are partially coated with humic acid, which will modify both the structural characteristics of the surface, as well as the electrostatic potential profile in the immediate surroundings (Saito et al., 2004). One approach for modeling adsorption on a complex assemblage is by component additivity (CA), which attempts to predict adsorption on the assemblage using the model parameters obtained for individual reference materials (Davis et al., 1998). One method used in the application of the component additivity approach (Weng et al., 2001, 2002) consists of using different adsorption models for different adsorbents, which may involve combining the charge distribution multisite complexation model (CD-MUSIC) (Hiemstra & van Riemsdijk, 1996) for goethite, and the non-ideal competitive adsorption (NICA) Donnan model (Benedetti et al., 1996) for humic acid.

2.4 Description of ion binding to soil organic matter

The negative charge on the humic molecules gives rise to a negative electrostatic potential in the immediate vicinity of the molecules and therefore to a salt dependence in the proton binding. Because of this, modelling of ion binding to humic substances requires a model that accounts for the heterogeneity of functional groups in terms of their affinity for protons, and the non-specific electrostatic interactions. The Donnan approach (Kinniburgh et al., 1999) has been used extensively and successfully in the last decade to describe ionic interactions with humic acids (Tipping, 2002; Companys et al., 2007; Vidali et al., 2009), and more recently with organic soils (Vasiliadis et al., 2007; Villaverde et al., 2009). It assumes that the surface charge, Q, is neutralized by counter-ions within a gel phase known as the Donnan volume, $V_{D.}$ This requires the use of the basic equation of electrical charge balance:

$$Q / V_D + \sum Z_j (C_{Dj} - C_j) = 0 \quad (1)$$

where C_{Dj} is the concentration of component j, with charge Z_j (including sign), in the Donnan volume, and C_j is its concentration in the bulk solution. Both concentrations are related by the Boltzmann factor, χ,

$$C_{Dj} = C_j \exp(-Z_j F \Psi_D / RT) = C_j \chi^{Zj} \quad (2)$$

where Ψ_D is the Donnan potential, F is the Faraday constant, R is the gas constant, and T is the temperature. The procedure used to solve the previous equations has been described in detail elsewhere (Benedetti et al., 1995). The only unknown is the Donnan volume, V_D, which varies with the ionic strength. Benedetti et al. (1996) showed that the apparent Donnan volume decreased with increasing ionic strength, in accordance with the following empirical relationship:

$$\log V_D = b(1 - \log I) - 1 \quad (3)$$

where I is the ionic strength, and b is constant.
The model enables calculation of the pH in the Donnan phase (pH_D) by use of equation 4:

$$pH_D = pH - \log(\chi) \quad (4)$$

The effect of ionic strength due to electrostatic interactions vanishes when the Q-pH curves obtained at the various inert electrolyte concentrations are plotted as a function of pH_D, to produce a unique master curve that can also be analysed to obtain the distribution of intrinsic proton binding affinity constants.
The NICA equation (Kinniburgh et al., 1999) for a bimodal affinity distribution describes the specific binding of protons to humic substances:

$$Q = Q_1 \frac{\left(K_1^{int}[H_D^+]\right)^{m_1}}{1+\left(K_1^{int}[H_D^+]\right)^{m_1}} + Q_2 \frac{\left(K_2^{int}[H_D^+]\right)^{m_2}}{1+\left(K_2^{int}[H_D^+]\right)^{m_2}} \quad (5)$$

where subscripts 1 and 2, respectively, indicate the carboxylic groups and the phenolic groups, K_i^{int} represents the intrinsic proton binding constants, and Q_i is the total number of available proton binding sites of each type. The exponent m is the width of the affinity distribution. Fitting of experimental data to the NICA-Donnan model can be conducted by means of different speciation and fitting programs (Kinniburgh, 1993; Keizer & van Riemsdijk, 1998; Gustafsson, 2010).
As in the case of the protons, modelling of the interaction between organic cations and SOM must take into account electrostatic and specific binding contributions. Interpretation of the effects of pH and ionic strength on cation binding is carried out with the previously described Donnan model. For this purpose, some electrostatic parameters derived from acid-base studies are required: (i) the magnitude of the negative charge of the organic matter sample at the values of pH and ionic strength corresponding to the experimental binding isotherms, and (ii) the optimized parameter *b,* which determines the Donnan volume for a given ionic strength. From these parameters, the electrostatic potential due to the negative charge on the natural organic matter can be calculated, and thus the concentration of cation within the Donnan phase, C_D, can be determined from equation 2. Once the electrostatic effect is accounted for, the Langmuir-Freundlich equation (6) enables us to obtain binding parameters under different experimental conditions:

$$M = M_{max} \frac{\left(K^{int}C_D\right)^m}{1+\left(K^{int}C_D\right)^m} \quad (6)$$

here M is the concentration of bound cation, M_{max} is the maximum concentration of the cation that can be bound, K^{int} is the intrinsic binding constant and m is the width of the affinity distribution. The advantage of the correction for the electrostatic effect arises from the fact that a unique set of binding parameters (K^{int}, M_{max} and m) enables prediction of the cation binding to SOM over a range of pH and ionic strengths. Note that equation 6 is restricted to a unique distribution of binding sites. This simplification was included since most of the cation binding experiments are conducted in experimental conditions (pH < 8) under which only carboxylic groups of the organic matter are ionized. Therefore, it is assumed that only these types of groups are involved in electrostatic interactions between SOM and cationic species.

2.5 CD-MUSIC model for description of adsorption processes on goethite

Surface complexation models (SCMs) are applied in order to understand and predict the reactivity at the solid-solution interface of mineral surfaces. SCMs describe the adsorption of cations, anions and small organic molecules on mineral surfaces by formation reactions for surface species on well defined coordination sites, in combination with an electrostatic model accounting for the influence of the electrostatic mean field potential of the charged surface. These models are usually divided into two main parts: one describing the solid surface, the type and reactivity of surface sites, the adsorbed species, the surface charge, etc, and the other describing the charge distribution and potential decay in the electrical double layer.

Among many SCMs that have been applied so far, the CD-MUSIC model has become one of the most popular models for describing the charging and adsorptive behaviour of goethite and other well crystallized mineral oxides. A complete description of the model has been published by Hiemstra & van Riemsdijk (1996, 2006), but a brief description is also given here, in which the surface equilibrium reactions considered are presented together with some remarks that help understand the main features of the model. This model describes the mineral surface populated with different surface groups, which can undergo protonation and can bind ions or molecules from the solution. In accordance with Hiemstra & van Riemsdijk (1996) within a pH interval 1-11, the charging behaviour of the goethite depends on the protonation/deprotonation reactions of singly, $\equiv FeOH^{-0.5}$, and triply coordinated, $\equiv Fe_3O^{-0.5}$, surface groups. On the other hand, doubly coordinated surface groups, $\equiv Fe_2OH^0$, are not reactive against protons and remain uncharged in this pH range. Protonation of the goethite surface can therefore be described by the following reactions:

$$\equiv FeOH^{-0.5} + H^+ \leftrightarrow \equiv FeOH_2^{+0.5} \qquad K_{H,1} \qquad (7)$$

$$\equiv Fe_3O^{-0.5} + H^+ \leftrightarrow \equiv Fe_3OH^{+0.5} \qquad K_{H,3} \qquad (8)$$

According to Venema et al. (1998) the proton affinity, log K_H, of the singly and triply coordinated surface groups present in the goethite are close to 8.0 and 11.7, respectively, suggesting that singly coordinated groups are more acidic. Nevertheless, for practical reasons, both log $K_{H,1}$ and log $K_{H,3}$ are made equal to the point of zero charge (PZC) of the goethite surface (Hiemstra & van Riemsdijk, 1996; Antelo et al., 2005). At sufficiently high pH, the goethite surface is mainly populated with $\equiv FeOH^{-0.5}$ and $\equiv Fe_3O^{-0.5}$ groups, resulting in a net negative surface charge, whereas at sufficiently low pH, the mineral surface is mainly populated with $\equiv FeOH_2^{+0.5}$ and $\equiv Fe_3OH^{+0.5}$ groups, resulting in a net positive surface charge.

The salt dependency effect can only be described if ion pair formation is considered, and it is therefore necessary to include the equilibria between surface charged groups and electrolyte ions, as described extensively in the relevant literature (Tadanier & Eick, 2002; Antelo et al., 2005). In addition, charged surface groups may interact with ions or molecules to form inner- or outer-sphere complexes. These surface complexes are located differently in the electrical double layer. The effect of the ionic strength can be used to distinguish between inner- and outer-sphere complexes (Hayes et al., 1988; Sparks, 2003). Adsorption via outer-sphere complexes may be sensitive to changes in the ionic strength because: (i) the background electrolyte ion may compete for the available binding sites at the surface, and (ii) the activity of the adsorbing species may be influenced by the variation in the interfacial

potential due to changes in the ionic strength. Outer-sphere complexes are located on the same electrostatic plane in the solid-solution interface as the adsorbed background electrolyte ions. Inner-sphere complexation occurs in an electrostatic plane closer to the surface than the plane where the background electrolyte ions are located, and therefore is generally not affected by ionic strength.

The extended Stern model is used by the CD-MUSIC model to describe the solid-solution interface of the goethite particles and the location of the ionic charge in this electrostatic double layer is of great importance for calculating the energy involved in the electrostatic interaction. Protonated surface groups are located on the 0-plane, the charge of specific adsorbed ions or molecules is distributed between the 0-plane and the 1-plane, and supporting electrolyte ions are assumed to be single point charges located on the 1-plane or on the 2-plane. The diffuse layer starts at the 2-plane and extends toward the bulk solution. A schematic representation of the goethite-water interface and its double layer structure is shown in Figure 1.

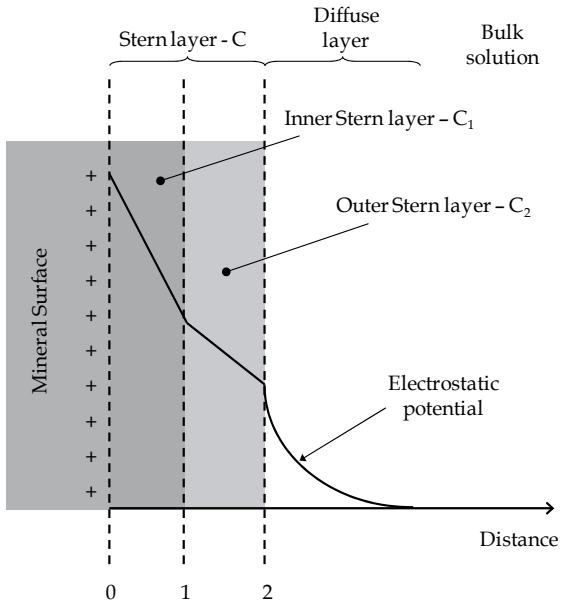

Fig. 1. Scheme of the extended Stern model for the solid-solution interface.

3. Case study: interaction between pesticides (Paraquat and MCPA) and model systems for soil fractions

3.1 Paraquat and MCPA interaction with soil organic matter

This section focuses on the interactions between SOM and ionic pesticides, PQ and MCPA, and presents binding results obtained with a dissolved humic acid sample and a peat soil. Humic acids (HA) constitute one of the most reactive fractions in the SOM. Binding studies on dissolved humic acid may be considered as representative of the processes occurring in the soil solution, in which dissolved organic matter molecules exhibit a less aggregated conformation. In contrast, a peat soil may be used as a reference material for the solid SOM comprising a complex aggregated mixture of diverse fractions.

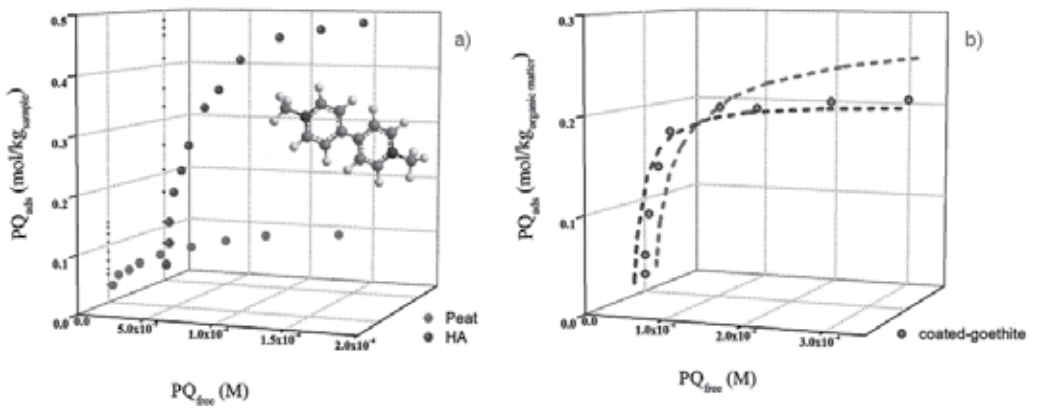

Fig. 2. a) Comparison of PQ bound to HA and peat soil samples. b) Simulation of PQ adsorbed on HA-coated goethite. Symbols: experimental data; blue dashed line: simulation with HA parameters; red dashed line: simulation with peat parameters.

The amount of MCPA bound to dissolved HA has been found to be almost negligible, even at the most favourable pH (Iglesias et al., 2009). In contrast, experimental studies of the interaction between PQ and dissolved HA (Iglesias et al., 2009) and with a peat soil (Gondar et al., unpublished results) have shown the high affinity of this cationic pesticide for the SOM, together with a significant effect of the pH and the ionic strength. This behaviour is indicative of the predominant role of the electrostatic interaction between SOM and ionic pesticides. The increase in PQ binding with pH is produced by the increase in the negative charge on the SOM resulting from the ionization of its acid groups. At the same time, the decrease in PQ binding produced as the ionic strength increases is a consequence of the screening effect produced by the inert electrolyte on the electrostatic interaction between species of opposite charge. Similar results have previously been reported (Narine and Guy, 1982; Pacheco et al., 2003; Brigante et al., 2010), suggesting that the binding of PQ to SOM samples may be explained by an electrostatic interaction.

A comparison of the behaviour of HA and peat soil as regards PQ binding is shown in Figure 2a. Under the same conditions of pH and ionic strength, the amount of PQ bound by dissolved HA is approximately four times greater than the amount bound by the peat soil. This can be explained by taking into account the electrostatic nature of the interaction and the charging properties of both sorbents in terms of the acid groups content (Table 1). The proton binding parameters show that the humic acid is richer in acid groups than the peat soil, and the content of carboxylic groups is approximately four times higher in HA than in the peat soil, whereas the content of phenolic groups is almost the same in both samples. Under acidic conditions the charge on the SOM is determined by the ionization of carboxylic groups, so that the HA will have a significantly higher charge than the peat, which will favour adsorption of the cationic herbicide.

Application of the Donnan model has enabled interpretation of the effect of pH and ionic strength on the PQ-HA interactions, so that the isotherms for experimental adsorption have been reproduced calculating the electrostatic effect with the parameters used to describe the proton binding of the dissolved HA (Iglesias et al., 2009).

ORGANIC FRACTION		
	Carboxylic groups *(mol kg^{-1})*	Phenolic groups *(mol kg^{-1})*
Humic acid *(dissolved organic matter)*	4.22	0.99
Peat *(solid organic matter)*	0.97	1.02
MINERAL FRACTION		
	Surface site density *(sites nm^{-2})*	Specific surface area *(m^2 g^{-1})*
Goethite	Singly coordinated: 3.45 Triply coordinated: 2.70	67.90

Table 1. General properties of the reference materials used for the soil fractions

It has recently been observed that the effect of the ionic strength on the ionization of acid groups is different for dissolved HA and for aggregated (solid) HA (Smith et al., 2001; Gustafsson & Kleja, 2005). Notably, the variation in the ionic strength of the medium has a much greater effect on the charge of the dissolved HA. Some authors (Smith et al., 2001) have proposed a modification of the Donnan model for solid SOM consisting of the use of fixed Donnan volume that is not dependent on the ionic strength. In order to interpret the contribution of the electrostatic effect on the PQ-peat interaction, we considered a value of 0.1 for parameter b (equation 3), obtained in a study of proton binding in peat soils of similar origin (Vasiliadis et al., 2007).

With the aim of comparing the affinity of both SOM samples for the cationic pesticide, and taking into account the difference in the amount adsorbed, the binding parameters were optimized, which enabled interpretation of the adsorption of PQ on the peat soil within a wide range of conditions (pH and ionic strength). Of the results obtained, the value of log K^{int} (0.36) was noteworthy as it was higher than previously observed for HA in solution (log K^{int} = -0.10), showing that the reference substances for the SOM behave differently in their interactions with PQ, as regards both the contribution of the electrostatic effect, and the nature of the groups involved in the adsorption.

3.2 Adsorption of Paraquat and MCPA on goethite

The herbicide MCPA (a phenoxyalkanoic acid herbicide) possesses a carboxylic group that is responsible for its high pH-dependent reactivity (Tunega et al., 2004) against humic substances or mineral oxides, due to changes in the percentage of ionization. Ionization of this weakly acid site on the molecule makes electrostatic interactions of great importance in describing the mechanism of adsorption.

As regards the negative charge of ionized MCPA molecules in solution, interaction with the goethite is only expected to be effective at pH values below PZC=9.4, at which the surface of the mineral oxide is positively charged. Experimental results (Iglesias et al., 2010a) indicate that the adsorption of MCPA on goethite decreases as the pH increases. At pH 4 there is a strong electrostatic interaction between both substances, but the adsorption decreases linearly with increasing pH because of the decrease in the positive surface charge of the goethite, so that at pH values close to the PZC, there is little interaction between the organic anions and the surface. This response is similar to that observed by Filius et al. (1997, 2001), who studied the adsorption of small organic acids on goethite and attributed the decrease in

adsorption at increasing pH to the fact that the sorbent-sorbate interaction is usually dominated by electrostatic effects. The present experiments involving MCPA adsorption on goethite did not show any significant adsorption of the herbicide at pH 7.0 or higher, at which the surface charge on the goethite is less positive or even negative. On the other hand, the adsorption of MCPA increased as the ionic strength of the system decreased (Figure 3a), an effect that is usually attributed to competition between the pesticide ions and the supporting electrolyte for adsorption sites.

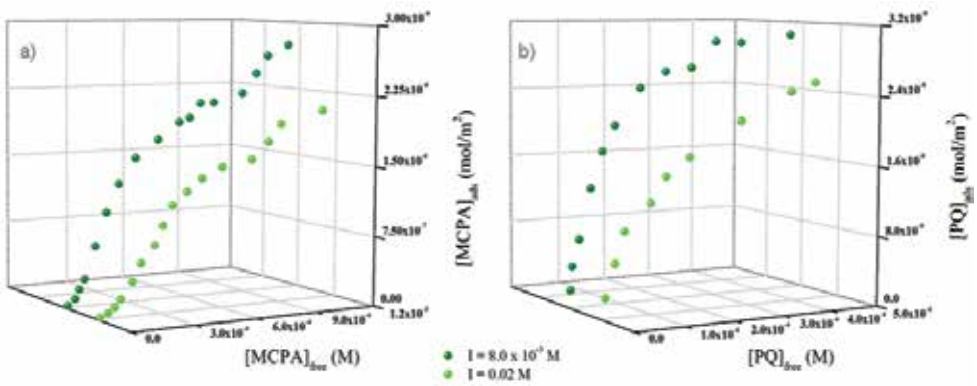

Fig. 3. Effect of ionic strength on the adsorption of ionic pesticides on goethite. a) MCPA at pH = 4.0 and b) PQ at pH = 10.5.

The adsorption isotherms for MCPA on goethite consist of two different regions (Figure 4a). The first corresponds to an S-shaped isotherm (Giles et al., 1960; Celis et al., 1999; Inacio et al., 2001; Iglesias et al., 2010a), which presents an initial region of low affinity between the solid surface and the herbicide, so that adsorption of a certain amount of adsorbate will be necessary to facilitate the adsorption process (Grant et al., 1998) and after this initial section, a Langmuir-shaped portion is observed in the isotherm. At acidic pH, MCPA may be sorbed through its -COO^- groups oriented toward the positively charged iron oxide surface, which favours lateral van der Waals interactions between the aromatic rings of the MCPA in the first adsorption layer (Celis et al., 1999). Pesticide-pesticide interaction can also be established between the first layer of adsorbed MCPA and the subsequent molecules of pesticide added, so that a second adsorption layer may be formed.

Among the experimental results that correspond to the interaction between the pesticide and the surface groups of goethite, the part of the isotherm which displays Langmuir behaviour (Figure 4b) has been interpreted with the CD-MUSIC model (Iglesias et al., 2010a). It can be assumed, as for other simple organic acids (Filius et al., 1997), that the carboxylic groups of the organic molecules can form inner-sphere and outer-sphere surface complexes with the singly coordinated surface groups (≡$FeOH^{-0.5}$), and that the triply coordinated surface groups (≡$Fe_3O^{-0.5}$) do not take part in the MCPA binding (Figure 5). In the inner-sphere complexes, one oxygen atom of the carboxylate group is exchanged for a surface water group. We can assume that the overall charge on the carboxylate group is equally divided over both carboxylate oxygens. One carboxylate oxygen, and its corresponding charge, is located on the surface plane (0-plane), and the charge on the second carboxylate oxygen is located on the 1-plane. On the other hand, outer-sphere complexes are linked to the protonated surface site by an H bond. The charge of the organic group is equally distributed between the 1-plane and the 2-plane, and the charge on the

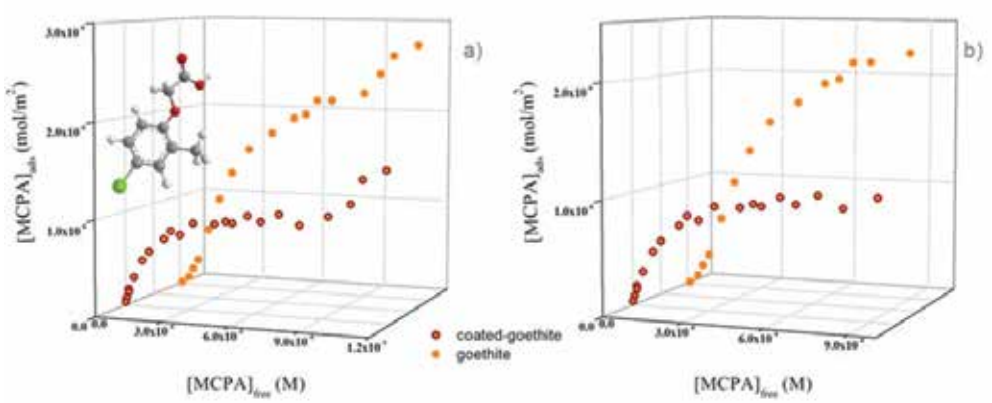

Fig. 4. a) Comparison of adsorption of MCPA on goethite and on HA-coated goethite. b) Section of the isotherms corresponding to the first adsorption layer (surface-pesticide interaction).

proton is distributed over the 0- and 1-planes. The affinity constants obtained for the formation of outer-sphere complexes are significantly larger than for inner sphere complexes, in accordance with the results obtained by Boily et al. (2000) and Filius et al. (2001) for the binding of benzenecarboxylates by goethite. Both authors indicated that carboxylates are predominantly adsorbed as hydrogen-bound outer-sphere complexes on mineral oxides. The assumption of the existence of these two types of complexes is consistent with the previously described effect of the ionic strength on adsorption of MCPA (Figure 3a).

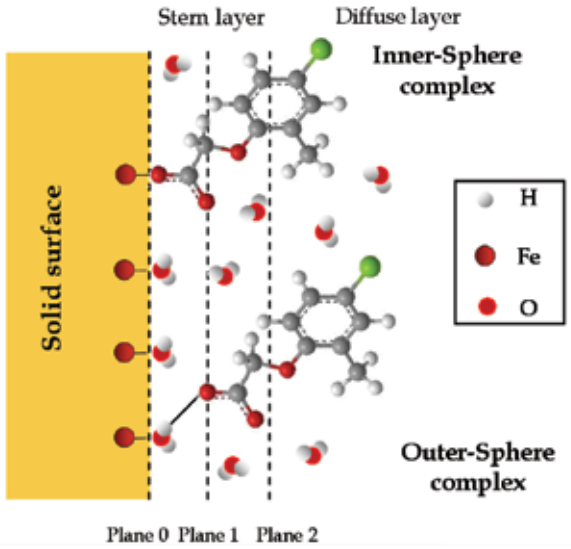

Fig. 5. Scheme of the solid-solution interface including inner- and outer-sphere surface complexes for MCPA

Adsorption of PQ on goethite occurred at pH values at which the mineral surface is negatively charged, i.e. above the PZC. On the other hand, no adsorption was observed on

goethite at any pH values below the PZC (Iglesias et al., 2010b). Electrostatic effects therefore play an important role in the interaction between PQ and the charged mineral surface. At pH below PZC, pesticide cations would be repelled, thus preventing their adsorption on the oxide. In contrast, at pH 10.5 some 90% of the surface groups would be ionized, thus favouring the adsorption process. Even under these most favourable conditions, the surface concentration of adsorbed PQ is rather low, 0.03 μmol m^{-2} at pH 10. The results for PQ adsorption on goethite show that the contribution of this iron oxide is negligible for retention of the pesticide. Nevertheless, Pateiro-Moure et al. (2010) recently found that quartz particles coated with fresh and aged iron oxide precipitates have a larger adsorption capacity for cationic pesticides like paraquat and diquat. In the latter study both adsorption surfaces showed lower PZC (around 8.0) than goethite particles (around 9.3). This again indicates the importance of the electrostatic interactions on the adsorption of cationic pesticides, since mineral surfaces with lower PZC will have a higher amount of negatively charged surface groups and therefore will favour interactions between the mineral surface and the pesticide molecules. On the other hand, the crystallinity of the mineral surface also has an important effect on the reactivity of iron oxides against cationic pesticides, with the most amorphous forms being the most reactive.

The adsorption of PQ depends on the ionic strength (Figure 3b) in the same way as MCPA, i.e. the cationic pesticide adsorption decreases as the electrolyte concentration increases. This effect indicates that outer-sphere surface complexes are involved in the adsorption of PQ onto goethite (Hayes et al., 1988; Sparks, 2003). So far, different types of surface complexes have been postulated for PQ adsorption on different clay mineral surfaces (Rytwo et al., 2002; Draoui et al., 1999), but there is not much data for adsorption on goethite or other types of iron oxides.

The PQ-goethite binding behaviour can be described with the CD-MUSIC model (Iglesias et al., 2010b), which considers the general charging parameters of goethite (Table 1), assuming that only singly coordinated surface groups, $\equiv FeOH^{-0.5}$, are responsible for PQ complexation. After considering the dimensions of a PQ molecule, 0.45 nm^2, its planar structure, and the density of the adsorption sites on the goethite surface (Table 1), it was assumed that the PQ forms surface complexes in a flat conformation by the interaction with two negative sites on the goethite surface, and that the charge of the organic cation is located on the 1-plane of the solid-solution interface. Previous simulations showed that each adsorbed PQ molecule interacts with two surface sites and at the same time, several other surface groups become blocked and unavailable for further interactions with other PQ molecules. Therefore good agreement between experimental isotherms and model predictions can be achieved considering the surface complexation constant as well as the density of singly coordinated surface groups bound to the molecules of adsorbed pesticide as adjustable parameters.

3.3 Adsorption of Paraquat and MCPA on HA-coated goethite

The interactions between anionic and cationic pesticides and well defined systems, i.e. HA, peat soil, and goethite, have been shown in previous sections. However, it is well known that in natural complex systems, associations between soil constituents occur, which may be particularly relevant in the highly reactive colloidal soil fraction. The interactions between SOM and mineral oxide particles may alter the chemical properties and the reactivity of each isolated fraction. In order to elucidate the effect of mineral oxide-SOM associations on the interaction with ionic pesticides, the adsorption of PQ and MCPA on HA-coated goethite was investigated (Iglesias et al., 2010a,b).

Previously discussed results have shown that significant adsorption of anionic pesticides on goethite takes place at acidic pH, when the net charge on the oxide surface is positive, while no adsorption was observed for cationic pesticides. Also, MCPA binding to dissolved HA is negligible under these conditions. It is therefore expected that at acidic pH, adsorption of PQ on HA-coated goethite is produced through the reactive sites of the adsorbed HA, while the adsorption of MCPA occurs through the available reactive sites at the goethite surface.

The MCPA adsorption isotherms on bare and coated goethite are compared in Figure 4. The adsorption isotherm for MCPA on HA-coated goethite also shows two plateaus, as already observed for the bare goethite (Figure 4a). However, there was a notable difference in the adsorption of MCPA on both surfaces, as reflected by the shape of the isotherms at low concentrations of MCPA. Unlike in the bare goethite, the adsorption isotherm for MCPA on HA-coated goethite does not exhibit an initial section of shallow slope, which suggests that the presence of HA on the goethite surface favours or activates MCPA adsorption at low concentrations of pesticide. Except at this lowest range of concentration of MCPA, the adsorption of MCPA on HA-coated goethite is significantly lower than on bare goethite. The adsorption of HA on goethite implies the formation of chemical bonds between reactive groups of HA and the mineral surface, so that it may be expected that there will be fewer goethite reactive sites available for the interaction with MCPA after HA adsorption. Weng et al. (2007) found that, under similar conditions of pH and ionic strength, less than 3% of the singly coordinated surface groups present in the goethite form inner-sphere surface complexes with HA. This small percentage of goethite sites that are specifically bound to HA, and thus, not available to MCPA, is not sufficient to explain the magnitude of the experimentally observed decrease in the adsorption of MCPA on HA-coated goethite. However, because of the relatively large size of HA molecules, more goethite surface reactive sites than those specifically bound to HA may become inaccessible. This screening effect produced by adsorbed HA molecules in the vicinity of the bound surface sites may hinder the adsorption of MCPA. In addition, the electrostatic properties of the mineral surface are expected to change after being coated by HA. Since the adsorption of negatively charged HA molecules on goethite surface reduces the magnitude of the positive charge on the oxide surface, and decreases the negative electrostatic potential near the goethite surface (van Riemsdijk et al. 2006), the presence of HA will make the electrostatic interaction between anionic pesticide and the mineral surface less favourable. According to the above, the interaction between MCPA and HA-coated goethite will occur via the available surface sites at the mineral oxide and the electrostatic properties of this coated surface.

A rigorous interpretation of this interaction, using the CD-MUSIC model, requires knowledge about the parameters that determine the electrostatic potential at the solid-solution interface of HA-coated goethite. Since this information is not available, interpretation of the adsorption of MCPA onto HA-coated goethite was based on parameters previously obtained for bare goethite, with some modifications (Iglesias et al., 2010a). As in the case of the bare goethite, the formation of inner- and outer-sphere complexes between MCPA and singly coordinated groups of the coated mineral surface was assumed for the modelling calculations. The main modification is that, together with the surface complexation constants, the site density of singly and triply coordinated surface groups were treated as adjustable parameters. Modelling results show that an adequate description of MCPA adsorption on HA-coated goethite can be achieved with surface complexation constants similar to those corresponding to bare goethite, along with a significant reduction in the density of surface sites (1.14 and 0.8 sites nm^{-2} for singly and

triply coordinated groups, respectively). Although there is a lack of experimental evidence for the availability of surface sites on the coated mineral oxide to support these results, it appears that the SOM coating on the goethite particles produces a significant reduction in the number of surface sites available to interact with MCPA.

As already mentioned, the interaction between PQ and bare goethite at acidic pH is almost negligible, whereas significant adsorption is observed on HA-coated goethite (Figure 2b), which is also consistent with the results reported by Brigante et al. (2010). Taking into account the favourable electrostatic interactions between SOM and cationic pesticides, the adsorption of PQ on HA-coated goethite appears to take place by direct binding to the HA molecules adsorbed onto goethite particles. Although the shape of the PQ adsorption isotherm on the HA-coated goethite is similar to that obtained for PQ binding on dissolved HA (Figure 2), the magnitude of the PQ adsorption, expressed in mol of adsorbed PQ per kg of organic matter adsorbed on mineral surface, is approximately 2.5 times lower. The HA-goethite association is produced by interactions between the negatively charged functional groups of HA and the positively charged goethite surface groups. The acid groups of HA are also involved in the PQ binding, and therefore a significant fraction of the ionized acid groups of the HA adsorbed on goethite become occupied and/or inaccessible for binding PQ.

The amount of PQ adsorbed by the HA covering the surface of the goethite was similar to the amount adsorbed by the same amount of organic matter in the peat soil. However, it is not known if changes in the distribution and/or conformation of the functional groups of the organic fraction occur during the process of formation of the goethite-HA aggregate, which would confer a more similar nature to that of the solid SOM represented by the peat soil.

In order to analyse whether the behaviour of the HA adsorbed on the goethite is more similar to that of the dissolved HA or of that of the peat soil, the amount of PQ adsorbed on the covered goethite was estimated from the individual behaviour of each of the reference SOM. The binding parameters corresponding to the HA and the peat soil were used for this purpose, and only a different concentration of the carboxylic groups accessible to the PQ was assumed. The results obtained in this simulation (Figure 2b) show how the form of the experimental isotherm for PQ-HA coated goethite was better represented by the behaviour of the dissolved HA. It is therefore possible to explain the adsorption of PQ on the goethite-HA aggregate by only taking into account a reduction in the available reactive sites on the HA, relative to the same amount of HA in solution.

However, as already indicated, rigorous interpretation of the interaction between PQ and the covered surface is more complex, and requires a more detailed knowledge of the properties of the surface, beyond the individual contributions of the component fractions. Given the electrostatic nature of the interaction between the ionic pesticides and binary goethite-HA systems, it is essential to know how the charge on the mineral surface is modified by the adsorption of negatively charged HA molecules.

4. Conclusions

An overall view of how PQ and MCPA interact with the HA and peat soil samples reveals the electrostatic nature of the interaction between ionic pesticides and SOM. The partial ionization of the acid groups in the SOM explains the high affinity of SOM for cationic species, such as PQ, and its low affinity for anionic compounds like MCPA. The difference in the amount of PQ bound to both types of SOM samples also appears to be directly related to their respective carboxylic group contents.

As a result of their anionic character, the MCPA molecules only interact with goethite at pH values below the PZC, at which the net surface charge of the goethite is positive. Adsorption of cationic pesticides on bare goethite was negligible at pH values below PZC, and was only observed at pH values at which the goethite particles are negatively charged. The experimental results observed for the interaction between pesticides and goethite revealed that these interactions are electrostatically controlled.

Adsorption of natural organic matter on the surface of goethite produces a clearly opposite effect on the subsequent interactions with anionic and cationic pesticides. While the interaction with PQ is significant under acidic conditions, and no interaction was observed on bare goethite, the adsorption of MCPA onto goethite was significantly lower when the goethite surface was coated with HA. In general, acceptable qualitative interpretation of the adsorption of cationic and anionic pesticides on these combined and complex systems can be achieved by taking into account the reduction in reactive sites on the fraction involved in the interaction.

5. Acknowledgements

The authors wish to acknowledge the Xunta de Galicia for financial support through projects PGIDIT06RAG50501PR and PGIDIT10PXIB209014PR and also to the Ministerio de Ciencia e Innovación for project CTM2008-03455.

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27

Behavior and Fate of Imidacloprid in Croatian Olive Orchard Soils Under Laboratory Conditions

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1. Introduction

Over the last two decades, the worldwide production and use of pesticides have greatly increased, causing great concern about their fate in the soil environment, as well as their adverse effects on nontarget organisms, including human beings. An important thing to realize it that only a small part of the pesticide doses used reaches its intended target (< 0.1%), while the major part (over 99%) of it is distributed into the ecosystem (Pimentel & Levitan, 1986), where it can cause difficulties through its toxicity to nontarget species, and cause serious environmental problems, such as groundwater contamination, food contamination, and air pollution (Larson et al., 1997; Mathys, 1994). There is also increasing interest in their transformation products, because they can be present at higher levels in the soil than the parent itself. In some instances, transformation products are more toxic, so they represent a greater risk to the environment than the parent molecule. Therefore, it is essential to study the residue and degradation pattern of pesticide in crop, soils and water systematically in order to generate meaningful data from the point of view of plant protection, public health and environmental safety.

In the past few decades, three major groups of insecticides have dominated the market: organophosphates, carbamates and pyrethroids. Nevertheless, pests resistance limited their use and caused a need for the synthesis of a new group that will be effective and nontoxic to the environment and to mammals. The results was "the birth" of neonicotinoids which exhibited high insecticidity and low toxicity to the environment (Maienfisch et al., 2001). But because neonicotinoids are becoming extensively used, both in agriculture and for home use, the chance of their polluting water is still present despite the low application rates.

Imidacloprid [1-(6-chloro-3pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine; IMI] was the first neonicotinoid registered by the United States Environmental Protection Agency (USEPA) for use as a pesticide through its actions as an agonist on the nicotinic acetylcholine receptor (nAChR) (Bai et al., 1991). The mode of action of IMI in the brain is shown in Figure 1. The toxicity of IMI is largely due to interference of the neurotransmission in the nicotinic cholinergic nervous system. Prolonged activation of the nAChR by IMI causes desensitization and blocking of the receptor, and leads to incoordination, tremors, decreased activity, reduced body temperature and death. IMI's favorable selective toxicity to insects versus mammals makes it safer for insect control than other neurotoxins (Tomizawa & Casida, 2003) and enables its diverse use in soil and foliar treatment in different crops, as

well as in non-agricultural practice. Thus, IMI is found in a variety of commercial insecticides. Its major manufacturer is Bayer Corporation that markets IMI products with the brand names Admire®, Confidor®, Gaucho®, Premier®, Premise®, Provado®, and Marathon®. However, one of the drawbacks of IMI's usage is a high toxicity in honeybees. In France, between 1994 and 1996, greatly increased mortality in honeybees was noticed when sunflowers were treated with a new pesticide Gaucho®. In addition Maxim & van der Sluijs (2007, 2010) and Suchail et al. (2004) found that IMI at very low doses causes bees mortality and adverse effects on laboratory-conditioned behavioral responses associated with feeding.

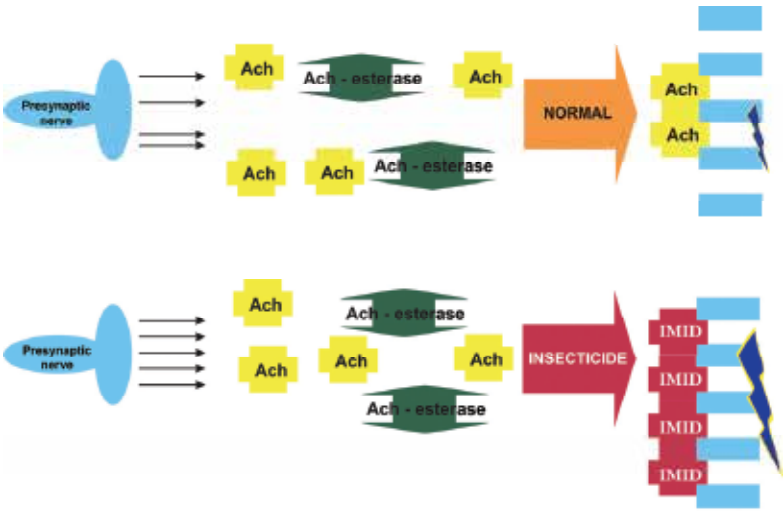


Fig. 1. IMI mode of action.

In Croatian coastal regions, IMI is increasingly being used in olive growing areas, including Istria and Kvarner islands, as an effective means of olive fruit fly infestation control. Although used at low dose rates (Capri et al., 2001), it is usually applied more than once during the growing season. Thus, intensive use of IMI, in addition to its high water solubility (510 mg/L, 20 °C) (Tomlin, 2001) might impose a great risk of water resources contamination which is consistent with USEPA statement regarding IMI's potential to leach to groundwater (USEPA, 1993). The review of literature revealed that few reports are available on leaching of IMI in soil (Carbo et al., 2008; Felsot et al., 1998; Gonzalez-Pradas et al., 2002; Gupta et al., 2002; Schmidt, 2010). For these reasons, there is a need for a more complete insight into IMI's fate in the soil (USEPA, 1993).

Among the processes that determine the behavior and fate of pesticides in the soil/water environment, sorption-desorption and degradation processes are the key processes affecting pesticide persistence, transport and bioavailability determining the amount of pesticide that can reach the target organism and that can be volatilized, or leached. Information regarding the sorption and desorption characteristics of IMI are essential for predicting its fate within the soil environment (Cox et al., 1998a; Cox et al., 1998b; Cox et al., 2001; Fernandez-Bayo et al., 2007; Liu et al., 2006; Ping et al., 2010). Capri et al. (2001) and Cox et al. (1998c) investigated the effect of selected soil properties on the sorption of IMI using the batch equilibrium technique. Their results indicated that IMI retention was highly dependent on the amount of the pesticide applied and on the physicochemical properties of the pesticide, but the key factor governing pesticide sorption-desorption were soil characteristics,

including soil texture, organic carbon content (OC), cation exchange capacity (CEC), pH and temperature. Field and laboratory studies have determined that IMI sorption to soil particles increases as the concentration of the insecticide decreases (Cox et al., 1998a; Kamble & Saran, 2005; Oi, 1999). Cox et al. (1997; 1998b; 1998c) have found that the potential for IMI to leach would decrease, as the OC levels and laminar silicate clay content in the soil increase. In contrast, IMI sorption in a calcerous soil was found to decrease with the addition of OC (obtained from peat and tannic acid) (Flores-Cespedes et al. 2002), which increased the mobility and leaching potential of IMI. In the study of Cox et al. (1998a) positive correlation between IMI sorption and CEC have observed, while the effect of soil pH did not significantly contributed to the sorption. The effect of temperature is of special importance in greenhouses, where higher temperatures are used for cultivation (Cox et al., 1997; Fernandez-Bayo et al., 2007; Gonzalez-Pradas et al., 2002; ten Hulscher & Cornelissen, 1996). On the other hand, desorption governs the release of IMI from the soil and several studies have reported irreversible sorption and the occurrence of hysteresis phenomena; i.e. less desorption than predicted by sorption isotherms (Cox et al., 1997; Fernandez-Bayo et al., 2007; Papiernik et al., 2006). This behavior can be attributed to a portion of the sorbed compound that is bound irreversibly to soil surfaces (Celis & Koskinen, 1999; Cox et al., 1997; Cox et al., 1998b). Numerous studies indicated that values for IMI half-lives (DT_{50}) are highly dependent on experimental conditions; namely field or laboratory, (Krohn & Hellpointner, 2002). In fact, DT_{50}s for IMI have been reported ranging from approximately 80 days to 2 years. Examples of laboratory experiments include Krohn & Hellpointner (2002) who reported a DT_{50} of 156 days, representing the geometric mean value of five studies. In a field experiment, Krohn & Hellpointner (2002) reported a DT_{50} of 96 days for the 11 bare soils in Northern and Southern Europe. However, lengthier DTs have also been determined from field studies. Mulye (1996) reviewed a two-year field investigation in Germany using IMI and from the study results calculated a DT_{50} of approximately 2 years, indicating that the compound would persist in soil.

Since a variability of pesticide sorption-desorption and persistence can occur among regions and even within the areas with the same geological and climatic characteristics, additional knowledge is needed to improve IMI's applicability in conditions which cover the Croatian climate. Consequently, the objective of this study is to analyze sorption-desorption characteristics as well as persistence of IMI in four soils, representative of northern Adriatic region, namely an island Krk and a coastal Istrian region. For a better understanding of factors governing IMI's behavior and fate in these regions, the relationship between selected soil properties, IMI's concentration and soil sorption-desorption coefficients as well as persistence was determined for the soils among and within regions. Additionally, applicability of mathematical models to predict IMI's sorption-desorption and degradation was tested.

2. The behavior and fate of pesticides in the soil

The behavior and fate of pesticides in the soil environment is controlled by their physico-chemical properties and by various complex dynamic physical, chemical and biological processes, among which are the most important sorption-desorption interactions of pesticide molecules with natural sorbents: soil organic matter and soil minerals, as well as degradation processes. These processes directly control the transport, retention and transformation of pesticides within the soil matrix and their transfer from the soil to other environmental compartments, and also determine the efficacy of pesticides in controlling target organisms and their potential for adverse effects on non target organisms (Pimentel &

Levitan, 1986). Once a pesticide molecule is bound to soil particles in the soil, the main processes in the soil cause its loss and transformation. Significant losses of pesticides can occur during application, with the amount of loss affected by the nature of the pesticide, formulation, atmospheric conditions, method of application, and application characteristics. High vapor pressure, photodegradability, and weak sorption by the soil contribute to losses of pesticides after application (Navarro et al., 2007). Retention does not affect the amount of pesticide present in the soil, but can decrease the amount available for transport; whereas transformation reduces the amount of pesticide present in the soil. Transport processes include leaching, surface runoff, volatilization, and uptake by plants. Figure 2 shows the main processes of pesticides inactivation in the soil.

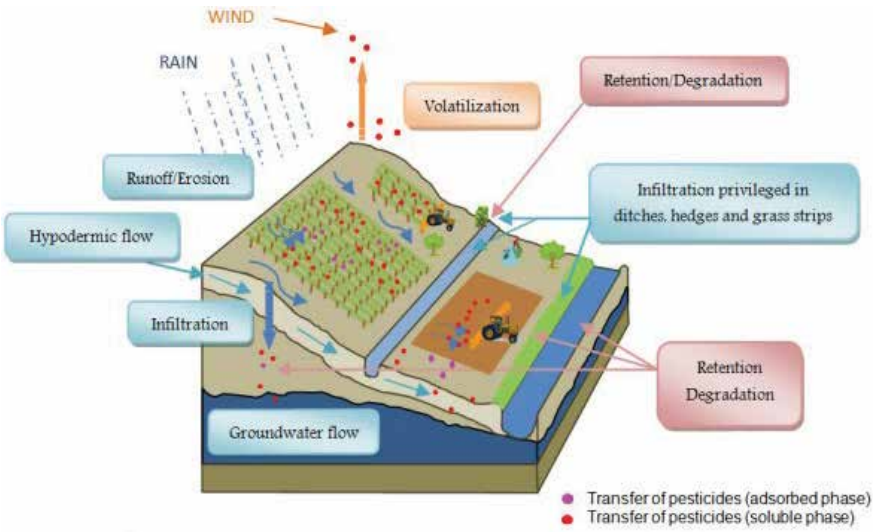


Fig. 2. The behavior and fate of pesticides in the environment.

2.1 Sorption-desorption processes

Sorption-desorption is a dynamic process in which molecules are continually transferred between the bulk liquid and solid surface (Koskinen & Harper, 1990). Sorption is the binding of the pesticide molecules by the surface of the treated soil, whereas desorption implies detachment of the molecules to the liquid medium. The ability of pesticides to sorb on soils and their tendency to desorb are the most important factors affecting soil and water contamination.

Several sorption models have been developed to describe, quantify and explain the sorptive process of pesticides on soils. The simplest one is the linear model depicted by the equation:

$$q_e^{sor/des} = K_D^{sor/des} \gamma_e \tag{1}$$

where $q_e^{sor/des}$ is the sorbed pesticide amount (mg/kg), γ_e is the equilibrium concentration in solution (mg/L) and $K_D^{sor/des}$ is the sorption-desorption partition coefficient (L/kg). Because $K_D^{sor/des}$ values for pesticides are soil specific and the $K_D^{sor/des}$ of one pesticide can differ considerably from soil to soil or with depth in a soil profile, the more widely accepted partition coefficient normalized to the fraction of OC content in the soil, K_{OC} was used. The K_{OC} was determined using the equation:

$$K_{OC} = \frac{K_D^{sor/des}}{f_{OC}} \times 100 \quad (2)$$

where f_{OC} represents the percentage of the OC content in the soil. High K_{OC} values (greater than 1000) indicate a tendency for the pesticide molecule to be sorbed by soil particles rather than remain in the soil solution (McCall et al., 1980). Since pesticide bond mainly to soil OC, the division by the percentage OC in soil makes the sorption coefficient a pesticide-specific property, independent of soil type. Sorption coefficients less than 500 indicate a considerable potential for losses through leaching.

This linear model is adequate if the sorption sites are of the same nature and in great amount to accommodate the chemical as the concentration increases. But in many cases, due to the heterogeneity of the soil, deviations from the linear sorption model are predictable and are effectively observed for pesticides (Delle Site, 2001; Wauchope et al., 2002). Two other nonlinear sorption isotherm models, the Freundlich and the Langmuir model, are frequently used when the amount of contaminant retained by the soil is abundant enough to impact the linear sorption.

The Freundlich isotherm is derived by assuming a heterogeneous surface with a non-uniform distribution of heat of sorption over the surface and it is represented by the equation:

$$q_e^{sor/des} = K_F^{sor/des} \gamma_e^{1/n} \quad (3)$$

where $K_F^{sor/des}$ is the Freundlich partition coefficient (coefficient of sorption-desorption capacity)(mg/kg)(mg/L)$^{1/n}$) and $1/n$ is the Freundlich's exponent (coefficient of nonlinearity), usually in the range $0<1/n\leq1$.

Sorption isotherm data could also be fitted to the Langmuir model given by Equation 4, with the assumption that soils have a finite number of sorption sites of uniform energy.

$$q_e^{sor/des} = \frac{q_{max}^{sor/des} K_L^{sor/des} \gamma_e}{1 + K_L^{sor/des} \gamma_e} \quad (4)$$

In Equation 4, $q_{max}^{sor/des}$ designates the maximum amount of sorbed IMI per unit mass of soil (mg/kg) to form a monolayer and $K_L^{sor/des}$ is the constant which depends on the enthalpy of sorption.

Although sorption processes of pesticides are usually characterized by a partition mechanism, in many cases, significant deviations between sorption and desorption isotherms have been observed. Typically, desorption is strongly delayed or hindered relative to the sorption (Huang & Weber, 1997; Lesan & Bhandari, 2003). In this phenomenon, called hysteresis, the Freundlich exponent $1/n_{sor}$ for desorption can be greater than the $1/n_{des}$ measured for desorption at a constant γ_e concentration (Huang et al., 2003). Sorption-desorption hysteresis can usually be explained by irreversible chemical binding, sequestration of a pesticide molecule into specific components of the organic matter, or entrapment of the pesticide into microporous structures or into the organic matter matrix (Pignatello & Xing, 1996). The extent of sorption-desorption hysteresis can be quantified for each pair of sorption and desorption isotherms using the hysteresis coefficient H (Cox et al., 1997). This coefficient is calculated using Freundlich exponent (coefficient of nonlinearity,

1/n) estimated from the sorption and desorption isotherms and it can be expressed by following equation:

$$H = \frac{1/n^{des}}{1/n^{sor}} \tag{5}$$

where, $1/n^{sor}$ and $1/n^{des}$ are Freundlich coefficients of nonlinearity for sorption and desorption, respectively. The lower the value of H is, the stronger the soil will sequestrate the pesticide molecule. Value H = 1 indicates that the hysteresis is insignificant and the sorption is reversible.

2.2 Degradation processes

Concern about the persistence of pesticides in soils has led to increased efforts to identify the nature, mechanisms, and factors affecting degradation processes, to identify the degradation products, and to predict persistence. Soil is an ideal medium for supporting degradation reactions of pesticides, which include photochemical, chemical, and biological reactions (Chen et al., 2005; Kuhad et al.; Ward & Singh).

Many published degradation studies assume that the degradation of pesticides follows simple first-order degradation kinetics (Baskaran et al., 1999; Calderon et al., 2004; Krohn & Hellpointner, 2002; Sarkar et al., 2001) which is represented with the following mathematical equation:

$$C(t) = ae^{-k_1 t} \tag{6}$$

where C is the amount of pesticide remaining at the time t (mg/kg), a is initial amount of pesticide degraded through one 1^{st} order process, t is the time (days) and k is degradation rate constant (1/days). First-order kinetics is advantageous for use in modeling as a constant degradation rate and allows estimation of a pesticide's half-life, DT_{50} (the time at which the concentration reaches half the initial concentration), which can be estimated according to equation:

$$DT_{50} = \frac{ln2}{k} \tag{7}$$

DT_{50} values are important in understanding the potential environmental impact of a pesticide. In fact, a molecule which degrades quickly has a low DT_{50} value and thus the impact of this species on the environment is reduced if the degradation products are harmless. On the contrary, the environmental impact of species with a high DT_{50} value can be substantial even if the molecule is only moderately toxic. Gavrilescu (2005) classified persistence of pesticides according to the DT_{50} value into three groups, where pesticides with $DT_{50} < 30$ days are non-persistent pesticides, compared to pesticides with $DT_{50} > 100$ days which are persistent.

However, deviations from the first-order degradation of pesticides have been reported. Typically, a fast initial degradation is followed by a gradual decrease in the degradation rate and eventually a very slow degradation. The gradual change in degradation rate may be better described by using two rate constants instead of one (Beulke & Brown, 2001; Henriksen et al., 2004; Ma et al., 2004; Sanchez et al., 2003). The two-compartment model (Equation 8) describes the degradation process as shared between two different compartments, where degradation proceeds at different rates (k_1 and k_2). The two constants,

a, and *b*, express the quantitative partition between the two compartments, where a + b is approximately equal to C_0 (mg/kg):

$$C(t) = ae^{-k_1 t} + be^{-k_2 t} \quad (8)$$

The fast degradation in the first compartment occurs when the pesticide is in the soil-water phase and readily available for microorganisms. In the second compartment the pesticide is sorbed to soil particles. Degradation is, therefore, controlled by the rate of desorption-diffusion into the soil-water phase. The partition between the two compartments depends on the pesticide sorption properties and soil characteristics. These characteristics suggest that a single DT_{50} may not be sufficient as an index of persistence. Beulke & Brown (2001) recomended using DT_{90} as a risk index to indicate the persistence, where the DT_{90} represents the time for 90% of the initial residues to dissipate; whereas Grover et al. (1997) and Wolt (1997) used both DT_{50} and DT_{90} as indices of persistence.

2.2.1 Metabolites

The proposed metabolic pathway of IMI in the soil is shown in Figure 3. Two main routes of metabolism responsible for the degradation of IMI were identified. The first step is hydroxylation of the imidazolidine ring leading to the mono- and dihydroxylated compounds, followed by loss of water to yield the olefinic compound. The second important degradation step starts with dehydrogenation of the imidazolidine ring to form desnitro-metabolit with further oxidation to 6-chloronicotinic acid (6-CNA).

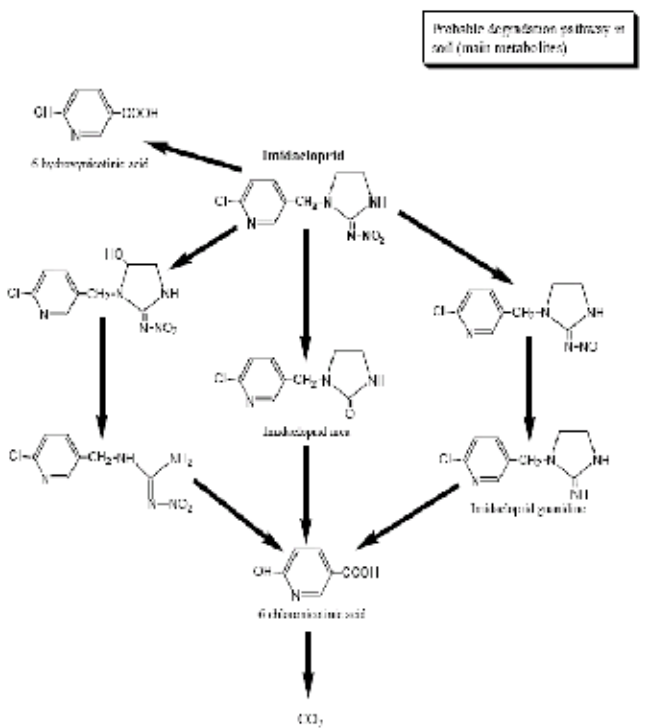


Fig. 3. The proposed metabolic pathway of IMI in the soil.

The main metabolites of IMI which have been identified in the soil include IMI-urea, 6-CNA, and 6-hydroxynicotinic acid (Rouchaud et al., 1996), which ultimately degrades to CO_2 (Scholz & Spiteller, 1992). For instance, depending on the soil type, IMI labeled with imidazolidin-^{14}C had a maximum mineralization to CO_2 of 8.8% or 14% after incubation for 12 weeks (Anderson, 1995; as reviewed in Mulye, 1996). In soils, when conditions were anaerobic and without light exposure, IMI was found to be readily decomposed, resulting in desnitro-IMI as the main transformation metabolite (Heim et al., 1996; as reviewed in Mulye, 1996). The desnitro-IMI produced under dark, anaerobic conditions has been found to be more persistent than its parent compound (Fritz & Hellpointner, 1991; as reviewed in Mulye, 1995). The major transformation products resulting from incubation under non-sterile, aerobic conditions and light exposure were desnitro-IMI, IMI-urea, 6-CNA and an unknown compound. Both desnitro-IMI and IMI-urea are highly water soluble, with solubility of 180 – 230 g/L and 9.3 g/L at 20°C, respectively (Krohn, 1996a, 1996b; as reviewed in Mulye, 1996), which is much higher than IMI's solubility, while 6-CNA has been found to be more toxic to honey bees than IMI itself.

3. Materials and methods

3.1 Chemicals

Analytical IMI at purity 99.9% (from Riedel-de Haën, Germany) and 6-CNA (chemical purity 99%, from Acros-Organics, USA) were used in this tudy. IMI's chemical structure and some selected physicochemical properties are shown in Figure 4 (Tomlin, 2001). Stock standard solutions of IMI and 6-CNA (1 mg/mL) were prepared by disolving the required amount in HPLC grade acetonitrile and stored at 4˚C. All other chemicals used were analytical grade, except acetonitrile which was of HPLC-grade (J.T.Baker, Holland). Sodium pyrophosphate, sulphuric acid, potassium dichromate, sodium hydroxide, sodium acetate and calcium chloride were purchased from Kemika (Croatia), while ammonium acetate, mercury chloride and methanol were from Alkaloid (Macedonia).

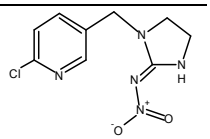
Structural formula	
Molecular formula	$C_9H_{10}ClN_5O_2$
Molecular weight (g/mol)	255.7
Water solubility, 20° C (g/L)	0.51
Log K_{OW}	0.57
Sorption coefficient, K_D (L/kg)	2.46
pK_a	11.2

Fig. 4. Chemical structure and physicochemical properties of IMI.

3.2 Experimental soils

Four agricultural soil samples, having different characteristics, from two coastal regions of Croatia, namely Istria and Kvarner, were used in this study. All soils were collected from the A horizon at depths of 0-30 cm following the standard methodology of soil sampling (USEPA, 2000), air-dried for 24 hours, ground (porcelain mortar + rubber pestle) and passed

through a 2-mm sieve prior to use. They were selected on the basis of their texture (mechanical composition), pH values, OC content and CEC. The soils have never been treated with IMI, as verified by analyzing its residues in the soil. Selected physicochemical properties of the tested soils are given in Table 1.

Soil	Textural classes	Clay (%)	pH	CEC[a] (cmol/kg)	HA[b] (cmol/kg)	OC[c] (%)
Krk I	sandy clay	21.12	7.12	25.67 (±0.74)	2.98 (±0.90)	0.55
Krk II	sandy loam	15.38	6.88	14.01 (±0.63)	1.93 (±0.23)	0.42
Istria I	clay loam	34.28	4.76	34.19 (±0.99)	5.65 (±0.45)	1.30
Istria II	clay	47.21	6.35	49.16 (±0.31)	16.21 (±0.09)	1.91

[a] cation exchange capacity; [b] hydrolitic acidity; [c] organic carbon content.

Table 1. Physicochemical properties of the tested soils.

The mechanical composition of the soil samples was determined by sedimentation using the "pipet method" (Kroetsch & Wang, 2007). Soil samples pH values were measured in a soil + deionised water and in a soil + 0.01 M calcium chloride suspension (1:2.5, w/v). The MP 220 laboratory pH meter (Metler Toledo, Germany) was used for pH determination in aqueous phase. Hydrolitic acidity (HA) was determined by the Kappen method (Hendershot et al., 2007), CEC was measured using ammonium replacement (Sumner & Miller, 1996), while Na, K, Mg and Ca were analyzed by Atomic Absorption Spectrophotometer (Perkin Elmer Analyst, USA). The OC content of the soils was determined spectrophotometrically (Cary 100 Bio WINUV, Varian, Australia) by dichromate method (Darrel & Nelson, 1996).

3.3 Batch sorption-desorption experiments

In the present study, the IMI sorption by soils was quantified using the standard batch equilibrium method (OECD, 2000). The predetermined mass of each soil (5 g), in triplicate, was equilibrated with 25 mL of aqueous solutions of IMI by shaking in an rotary agitator (Unimax 1010, Heidolph, Germany) at 20 (±1)° C for 48 h to achieve equilibrium. The equilibrium time was determined according to previous sorption kinetics studies of the IMI sorption (Capri et al., 2001; Nemeth-Konda et al., 2002). Initial insecticide solutions, in the concentration range of 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 mg/L respectively, were prepared in the background 0.01 M calcium chloride and 100 mg/L mercury chloride solution from stock IMI solutions prepared in HPLC-grade acetonitrile. Calcium chloride solution was used as background electrolyte in order to minimize ionic strength changes and to promote flocculation. Mercury chloride was added to the pesticide solution as a biocide to prevent any microbial activity during the sorption experiment. After equilibration, the suspensions were centrifuged at 4000 rpm for 30 min at 20 (±1) °C (BR4i Multifunction, Thermo electron corporation, France) to separate the liquid and solid phases. After filtration through a polypropylene hydrophilic filter of 0.45 μm (Whatman, Puradisc 25 TF, USA) the aqueous phase was analyzed by High Performance Liquid Chromatography (HPLC) using a Thermo Separation Products (Spectra System, USA) liquid chromatographic system, as described in the section 3.6. Blank samples without soil were also prepared in the same way and used to account for possible losses due to the volatilization and sorption of IMI to the cuvette walls. The average system losses were shown to be consistently lower than 3.4% of the initial

solute concentrations, therefore no correction was required. Control samples, containing no IMI, only soil and 0.1 M calcium chloride, were used for each series of experiment. The amount of IMI sorbed to soil after equilibration was calculated from the difference between the initial and equilibrium solution concentration using the mass-balance equation:

$$q_e^{sor} = (\gamma_i - \gamma_e)\frac{V}{m} \tag{9}$$

where q_e^{sor} is the amount of IMI sorbed at equilibrium (mg/kg), m is the mass of soil (g), γ_i is the initial concentration of IMI (mg/L), γ_e is the equilibrium concentration of IMI (mg/L), V is the volume of the solution (L) from which sorption occurs.

Desorption experiments were conducted on triplicate soil samples immediately after the sorption experiments with the same initial concentrations of IMI. After completing the sorption process, the supernatant (25 mL) were removed and replaced with the same volume of 0.01 M calcium chloride and 100 mg/L mercury chloride solution. After shaking for 24 h, the suspensions were centrifuged under the conditions described previously, and the concentration of IMI was determined in the supernatants using the HPLC. This desorption procedure was repeated five times for each soil sample. The amount of pesticide remaining sorbed by the soil was calculated as the difference between the equilibrium sorbed and the desorbed amount by the following equation:

$$q_e^{des} = (\gamma_e^{sor} - \gamma_e^{des})\frac{V}{m} \tag{10}$$

where q_e^{des} is the amount of IMI remaining sorbed by the soil (mg/kg), γ_e^{sor} is the equilibrium sorption concentration of IMI (mg/L) and γ_e^{des} is the equilibrium desorption concentration of IMI (mg/L). The percentage of IMI desorbed was calculated as follows:

$$P^{des} = \frac{\sum_{a=1}^{5} q_e^{des}}{q_e^{sor}} \times 100 \tag{11}$$

3.4 Degradation experiments

The persistence of IMI in the tested soils was studied at two concentration levels, 0.5 and 5 mg/kg under laboratory conditions at room temperature (20±1˚C). For fortification of the soil at 50 mg/kg level, 100 g weighted, air-dried and sieved soil was taken in a baker and 10 mL of standard solution of IMI (1 mg/mL, in acetonitrile) was added. Additional methanol was added to dip the soil completely. The soil suspension was mixed well using a rotary agitator (Unimax 1010, Heidolph, Germany) for 1 hour and then left at room temperature for 24 hours to allow complete solvent evaporation. After the complete evaporation of the solvent, the fortified soil was again mixed and then serially diluted with untreated soil to get a 5 and 0.5 mg/kg level of fortification. For 5 mg/kg treatment, 1350 g untreated soil was taken in a polythene bag and 150 g of fortified soil (50 mg/kg) was added and thoroughly mixed for homogeneity. For the 0.5 mg/kg treatment, 5 mg/kg treated soil was mixed with untreated soil in the ratio 1:9. The treated soils were maintained at 60% of the maximum water holding capacity (WHC) and stored in a dark at room temperature (20±1˚C). Moisture contents were maintained at a constant level throughout the experiment by adding distilled

water as necessary. Three parallel soil samples (25 g) at both concentration level including unspiked controls were used for analysis of IMI residues and its metabolite (6-CNA) at intervals of 0, 7, 15, 30, 45, 60, 75, 90, 105, 120, 150 and 180 days after application.

3.5 Extraction of IMI and 6-CNA from soil samples

IMI and 6-CNA were extracted from soil samples according to the method of Baskaran et al. (1997). At each sampling time, a 25 g sample of spiked and homogenized soil was extracted with 40 mL of acetonitrile-water (80:20, v/v) and shaken vigorously for 2 h using a rotary agitator (Unimax 1010, Heidolph, Germany) at 20 (±1)°C. After this time every sample was centrifuged for 20 min at 6000 rpm (BR4i Multifunction, Thermo electron corporation, France) and filtered through a polypropylene hydrophilic filter of 0.45 µm (Whatman, Puradisc 25 TF, USA). The operation of shaking and filtration was repeated three times and supernatants from each extraction were pooled. The solution was evaporated to dryness on a rotary evaporator (Laborota 4002/03 Control, Heidolph, Germany). The residue was dissolved in 1 mL of mobile phase (acetonitrile-water, 20:80, v/v). Three replicates of both level, including unspiked controls, were extracted and analyzed by HPLC.

3.6 Analysis of IMI and 6-CNA by HPLC

The concentration of IMI and 6-CNA in aqueous solutions was determined using a reverse-phase HPLC system (Thermo Separation Products, Spectra System, USA) equipped with a UV/VIS detector. All analyzes were performed on a Supelco reverse phase C_{18} column (150 mm length, 46 mm ID, 5 µm particle size). The mobile phase of acetonitrile and water (20:80 v/v) was used under isocratic conditions at a flow rate of 1.2 mL/min. The analytes were analyzed at 270 nm wavelength. The injection volume and the column temperature were 20 µL and 25˚ C, respectively. Under these conditions the retention times of IMI and 6-CNA were 4.3 and 1.6 min, respectively.

Calibration curves for both of chemicals were linear from 0.05 to 10 mg/L with regression coefficients of $R^2 > 0.999$ (six calibration points, in triplicate). The detection limits of IMI and 6-CNA were 0.001 mg/L and 0.003 mg/L, while the lower limits of quantification (LOQ) were 0.005 mg/L and 0.01 mg/L. The mean recoveries for IMI and 6-CNA were 91.4% and 87.8 % with a relative standard deviation lover than 5%.

3.7 Statistical analysis

Relationship between soil properties and sorption as well as degradation behavior of IMI was tested by a nonparametric correlation test, Kendall-Tau. Except nonparametric tests, multiple linear regression analysis was used, which combines the relationship between different soil parameters and the sorption coefficients, as well as DT_{50}, allowing the assumption of linear models for these parameters (Boivin et al., 2005). Differences in the soil sorption capacity among and within regions were analyzed using Mann-Whitney U test, while the DT_{50} values were tested by one-way ANOVA test with *post hoc* comparison (Tukey HSD test) to determine the effect of initial concentration and soil on the DT_{50} of IMI. Data are reported as mean ± standard deviations. The results were considered statistically significant at $p < 0.05$. The data were analyzed using Statistica® software package Version 7.0 and Wolfram Research Mathematica® software package Version 7.0.

4. Results and discussion

Sorption-desorption processes have a significant effect on pesticide persistence, pesticide concentration level in the soil solution and on the transport of pesticides from agricultural field to other environmental compartments (Arias-Estevez et al., 2008). An understanding of the variability of pesticide persistence and sorption-desorption processes within and among regions can improve the accuracy of estimates of the behavior and fate of pesticide in the soil and provide an additional support in design of intervention strategies against groundwater pollution.

4.1 Sorption-desorption study

4.1.1 Kinetic study

Sorption-desorption kinetic of IMI on the selected Croatian soils were studied using the initial IMI concentration of 10 mg/L in order to estimate the time needed to achieve the sorption and desorption equilibrium. The results for the case of Istria II soil are presented in Figure 5. Similar trends were observed in all tested soils. Equilibrium time for sorption process was reached at 48h, while for desorption process equilibration was achieved within 144h.

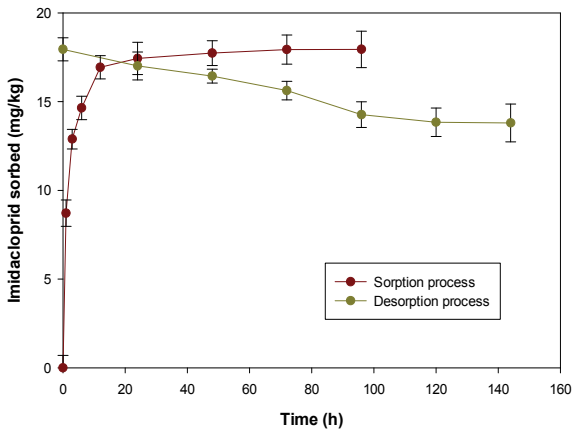


Fig. 5. Sorption-desorption kinetic of IMI on Istria II soil sample. The initial concentration of IMI was 10 mg/L.

4.1.2 Sorption equilibrium study

Figure 6a shows the sorption isotherms for IMI in the tested soils. All of the sorption isotherms are of L-type (Giles; et al., 1960) showing a convex initial curvature. This isotherm type indicates a decrease in specific sorption sites when the concentration of insecticide increases; however, in the case of IMI the curves did not reach the plateau of saturation.

In Table 2 the sorbed amount of IMI during the sorption processes in the tested soils is presented. The percentage of IMI sorbed on the Istria II soil was 35.96, 36.00, 37.13 and 37.88% at concentrations of 10, 5, 2.5 and 1 mg/L, respectively, whereas in the Istria I, Krk II and Krk I soil, the percentage sorbed ranged from: 30.41-33.01, 18.45-23.68 and 16.50-19.75% at the same respective concentrations. The percentage IMI sorbed was higher in Istria II soil than in the other soils.

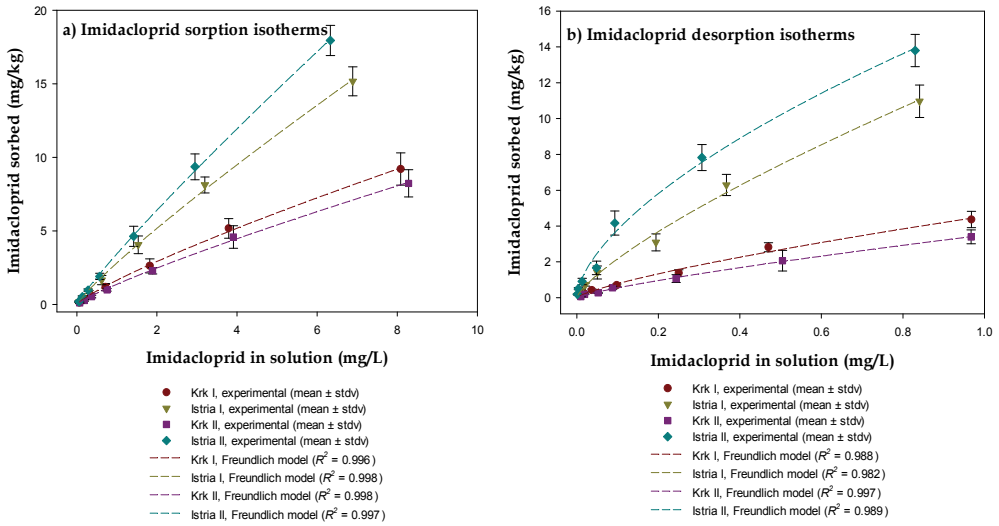


Fig. 6. a) Sorption and b) desorption isotherms of IMI in the tested soils represented by the Freundlich model. Values are means ± standard deviations. Symbols represent the experimental data, while lines represent the theoretical curves described by the Freundlich model.

Soil	Initial concentration, γ (mg/L)	Sorbed amount[a] (mg/kg)	Sorbed amount (%)	Desorbed amount[b] (mg/kg)	Desorbed amount (%)
Krk I	10	9.21	18.45	4.84	52.57
Istria I		15.17	30.41	4.20	27.71
Krk II		8.23	16.50	4.84	58.75
Istria II		17.94	35.96	4.15	23.12
Krk I	5	5.17	19.89	2.35	45.46
Istria I		8.13	31.26	1.84	22.58
Krk II		4.59	17.65	2.52	55.01
Istria II		9.36	36.00	1.53	16.39
Krk I	2.5	2.63	21.06	1.25	47.60
Istria I		4.06	32.54	0.97	23.90
Krk II		2.29	18.34	1.22	53.27
Istria II		4.63	37.13	0.47	10.04
Krk I	1	1.19	23.68	0.49	41.05
Istria I		1.66	33.01	0.25	15.12
Krk II		0.99	19.75	0.44	43.98
Istria II		1.91	37.88	0.24	12.70

[a] sorbed amount of IMI after 48 h of sorption reaction time; [b] desorbed amount of IMI after 144 h of desorption reaction time.

Table 2. The sorbed and desorbed amount of IMI in the tested soils in relation to the initial concentration.

All sorption data fit the Freundlich equation ($R^2 > 0.966$) and Table 3 summarizes the sorption capacity (K_F^{sor}) and intensity ($1/n_{sor}$) values. The K_F^{sor} values obtained from the Freundlich

isotherm model were 1.28, 1.53, 2.60 and 3.28 (mg/kg)/(mg/L)$^{1/n}$ for Krk II, Krk I, Istria I and Istria II soil, respectively. The highest K_F^{sor} value for IMI, is indicative of the strongest retention by the soil matrix. A primary consequence of strong retention of IMI is its limited mobility in the Istria II soil profile and thus lower risk of ground water contamination.

Soil	K_F^{sor} (mg/kg)/(mg/L)$^{1/n}$	$1/n_{sor}$	R^2	K_{OC}^{sor} (L/kg)	ΔG (kJ/mol)
Krk I	1.53 (± 0.06)	0.894 (± 0.019)	0.996	278.18	-13.72
Krk II	(1.28 ± 0.06)	0.907 (± 0.022)	0.998	304.76	-13.94
Istria I	(2.60 ± 0.10)	0.945 (± 0.021)	0.998	200.00	-12.91
Istria II	(3.28 ± 0.09)	0.937 (± 0.016)	0.997	171.73	-12.54

Table 3. The Freundlich sorption parameters, values of organic carbon/partition coefficient (K_{OC}) and Gibbs free energy (ΔG^0) for IMI in the tested soils.

In our study, the behavior of sorption was nonlinear. This is based on the best-fit estimated parameter *1/n* under the value of one (*1/n* < 1) (Table 3). In fact, for the Krk soils, *1/n* values were lower (0.894 and 0.907) than for the Istria soils (0.937 and 0.945). Nonlinear isotherm behavior is a measure of the extent of heterogeneity of retention reactions and the presence of sites having variable affinities for sorption of IMI by the soil matrix surface. Based on the estimated *1/n* values, an increased amount sorbed by soil is anticipated in all soils at low IMI concentration. A higher initial insecticide solution concentration led to the change of the affinity between insecticide molecules and soil, probably due to decreased accessibility to the free sorption sites (Kamble & Saran, 2005).

In the present study, K_F^{sor} values varied between the tested soils, indicating that the differences between the soils strongly influence the sorption. Several studies have shown that soil properties, particularly the soil organic matter and clay content play a key role in the performance of applied pesticides (Cox et al., 1998a; Fernandez-Bayo et al., 2007; Kamble & Saran, 2005; Liu et al., 2006; ten Hulscher & Cornelissen, 1996). In order to elucidate the factors that affect sorption of IMI on the soils, the K_F^{sor} values were correlated with the OC and clay content, CEC, and pH using a nonparametric Kendall-Tau correlation test. Correlation analysis between sorption coefficients (K_F^{sor}) and selected soil properties showed a significant correlation between K_F^{sor} and the OC content, CEC and clay, but the correlation between K_F^{sor} and pH was not significant (Table 4). It has been postulated that soil pH has an influence on pesticide sorption only when the pK_a or pK_b are within approximately two units of the soil pH (Farenhorst, 2006). As the pH value in examined soils ranged from 4.76 to 7.12, which was significantly below the pK_a value of IMI (pK_a =11.2), the effect of soil pH was not noticeable. Thus, these results suggest that the OC content and CEC had the major influence on the IMI sorption in these soils.

In addition to nonparametric test, multiple linear regression was used, which simultaneously compares various soil properties and sorption coefficients (K_F^{sor}), and leads to a linear predictive model for K_F^{sor} value (Golfinopoulos & Arhonditsis, 2002). These models may be useful for identifying areas (homogeneous soil types) where surface water resources could be threatened by pesticide contamination and for identification of pesticides which are more easily leached through the soil profile (Golfinopoulos & Arhonditsis, 2002). Multiple liner regression resulted in the following correlation:

$$K_F^{sor} = 0.4177\,OC + 0.0037\,CEC + 0.0398\,clay - 0.0866\,pH + 1.009 \left(R^2 = 0.989\right) \qquad (12)$$

	pH	clay	CEC	OC
pH	1.00	-0.21	-0.24	-0.15
clay	-0.21	1.00	**0.85**	**0.82**
CEC	-0.24	**0.85**	1.00	**0.79**
OC	-0.15	**0.82**	**0.79**	1.00
K_F^{sor}	-0.24	**0.79**	**0.82**	**0.85**
DT_{50}	0.21	**-0.67**	**-0.76**	**-0.64**

OC – organic carbon content; CEC – cation exchange capacity;
K_F^{sor}– Freundlich coefficient of sorption;
DT_{50}- time for 50% of the initial residue to degrade.

Table 4. Kendall-Tau correlation test for soil properties and sorption and degradation parameters of IMI, n = 12 (Bold typeface indicates significant correlations with $p < 0.05$).

Nonparametric regression showed that the amount of OC in the soil, the CEC and clay amount affected the sorption processes, but multiple linear regression equations suggested that the OC content predominantly influenced IMI sorption on the tested soils.

Given the difference between tested soils in the studied regions, statistically significant differences in soil sorption coefficients, K_F^{sor} were found among the studied regions, i.e. Krk and Istria region ($p = 0.004$). In addition, results of sorption study within the regions showed a statistically significant diffrence in K_F^{sor} values between the soils Istria I and Istria II ($p = 0.049$), as well as between soils Krk I and Krk II ($p = 0.050$).

The OC partition coefficient, K_{OC}^{sor} (Equation 2) usually illustrate the hydrophobicity of the pesticide and may be used to estimate or predict the migration and behavior of an organic pesticide in the environment (Xue et al., 2006). Thus, defined coefficient, normalized to the proportion of OC, should have a constant value for each pesticide molecule and the same values in the soils with different content of organic matter. However, variability in K_{OC}^{sor} values for the soils of different type and characteristics, and even for the soils with the same content of organic matter, indicated that not only organic matter content, but also its structure, aromaticity and polarity, affected the distribution of pesticide molecules in the soil/water system (Schwarzenbach et al., 2002). The values of K_{OC}^{sor} coefficient for IMI in the tested soils varied from 172 to 305 L/kg (Table 3), and they are consistent with reported K_{OC}^{sor} values (Cox et al., 1998b; Krohn & Hellpointner, 2002), especially for soils with similar textural characteristics. Estimated values of our study prove that, according to the McCall classification for the mobility of pesticides (McCall et al., 1980), IMI can be categorized as having a medium mobility (K_{OC}^{sor} 150 - 500 L/kg) (Comfort et al., 1994), showing less tendency to be sorbed by the examined soils. Therefore, these K_{OC}^{sor} values, together with reported K_{OW} values (3.7) (Krohn & Hellpointner, 2002) and a great water solubility (0.51g/L) (Tomlin, 2001) suggest a potential of IMI to leach to groundwater. However, the results of field studies have showed the lack of leaching for IMI, which could be due to a larger sorption potential at a lower concentration compared to higher concentration range (Kamble & Saran, 2005), or as a result of an increase in the sorption of IMI with time in the soil (Oi, 1999).

At equilibrium, the pesticide distribution between the solid and aqueous phases is ultimately governed by the sorption Gibbs free energy (ΔG°). The change in the Δ*G*° as a result of sorption process, was calculated from the thermodynamic relationship:

$$\Delta G^0 = - RTlnK_{OC} \tag{13}$$

where, Δ*G*° is the free energy change (kJ/mol), *T* is the absolute temperature (K), *R* is the universal gas constant (8.314 J/molK). Δ*G*° values of sorption processes ranged from -13.94 to -12.54 kJ/mol and are listed in Table 3. The Δ*G*° values obtained in the present study indicate that the sorption capacity of the soils would be in the order of Istria II soil > Istria I soil > Krk I soil > Krk II soil. The greater the absolute magnitude of Δ*G*° value, the greater is the extent to which the sorption reaction may take place. A small negative value of Δ*G*° indicated the exothermic nature of the reaction and a spontaneous process. In such cases, it can be inferred that the sorption of IMI takes place via physical processes involving weak attractive forces (ten Hulscher & Cornelissen, 1996), primarily by dissolution-like partition of IMI into soil organic matter (Sheng et al., 2001).

4.1.3 Desorption equilibrium study

Plot of the desorption isotherms for IMI are shown in Figure 6b. It can be seen that the slopes of the desorption isotherms are clearly different from those of the sorption isotherms. The characteristic steep slopes of all isotherms are observed at the low equilibrium concentrations of IMI corresponding to the low initial IMI content in solutions. With increase of the IMI concentrations in solutions the curve slopes become less steep.

The desorption data are also shown in Table 2, where desorbed amount was expressed as a percentage of the total amount sorbed. In all the tested soils significant differences of the amount desorbed between different concentrations and between the tested soils were observed. As the initial IMI concentration increased from 1 to 10 mg/L, the desorbed amount, as a percentage of the total sorbed, increased from 12.70 to 27.71% for the Istrian soils and from 41.05 to 58.75% for the Krk soils. The highest percentage of desorption was achieved for the Krk II soil, where actual amounts of recovery ranged from 43.98 to 58.75%of that sorbed by soil. This suggests that half of the amount sorbed was retained by the Krk II soil regardless of initial concentration. The lowest measured recovery of the amount of the desorbed IMI was observed for the Istria II soil. The higher release for Krk II than Istria II soil is likely due to difference in OC content. In a desorption study of IMI on a Hungarian soil, (Nemeth-Konda et al., 2002) found that the amount of IMI desorbed following three desorption steps (average the initial concentrations) was 62±15%. In their study, a similar background solution ($CaCl_2$) was used except that sorption was limited 24h.

The Freundlich desorption coefficient values (K_F^{des}) for the tested soils were higher than sorption values (K_F^{sor}), while desorption *1/n* values were lower than the Freundlich sorption equilibrium values (Table 5). K_F^{des} value was highest for the Istria II soil (clay soil with 1.91% OC) followed by the Istria I, Krk I and the Krk II soil (sandy loam soil with 0.42 % OC) which exhibited the lowest K_F^{des}. A higher K_F^{des} value indicated a stronger

affinity for the IMI. In our study, *1/n* constants ranged from 0.654 to 0.836 with deviations from the linear function ranging from 16.4% (Krk II soil) to 34.6% (Istria II soil). Fernandez-Bayo et al. (2007) made similar observations for IMI in Spain soils. This could be explained by a possible hysteresis effect taking place during desorption, involving various forces that caused the amount of IMI retained to be higher after desorption than after sorption at the unit equilibrium concentration. Hysteresis is manifested by an increase in the difference between the sorption and desorption isotherm slopes. Conceptually, the lack of similarity between sorption and desorption due to the hysteresis is likely a results of binding to organic matter and clay particles. Clay fraction is of great importance because it can enter into interactions with natural organic matter in the soil and it can control its structural configuration (Gunasekara & Xing, 2003). Particularly, in the interaction of organic matter with the clay fraction crystal-amorphous complexes are formed which can increase the nonlinearity of the sorption isotherm. Several studies have illustrated hysteretic behavior of IMI (Fernandez-Bayo et al., 2007; Papiernik et al., 2006).

Soil	K_F^{des} (mg/kg)/ (mg/L)$^{1/n}$	$1/n_{des}$	R^2	K_{OC}^{des} (L/kg)	H
Krk I	4.66 (± 0.16)	0.786 (± 0.049)	0.988	847.27	0.879
Krk II	3.65 (± 0.05)	0.836 (± 0.023)	0.982	869.05	0.922
Istria I	12.41 (±0.39)	0.749 (± 0.041)	0.997	954.61	0.793
Istria II	16.05 (±0.54)	0.654 (± 0.036)	0.989	840.31	0.698

Table 5. The Freundlich desorption parameters and hysteresis index (H) for IMI in the tested soils.

For the Krk II soil, the Freundlich K_F^{sor} was 1.28 (mg/kg)/(mg/L)$^{1/n}$ and the K_F^{des} was 3.65 (mg/kg)/(mg/L)$^{1/n}$, while the K_{OC}^{sor} and K_{OC}^{des} were 304.76 and 869.05 L/kg. These findings indicated that IMI was weakly sorbed, but slightly held by the soil. In contrast, the K_F^{sor} and K_F^{des} values for the Istria II soil were 3.28 and 16.05 (mg/kg)/(mg/L)$^{1/n}$, while the K_{OC}^{sor} and K_{OC}^{des} were 171.73 and 840.31 L/kg. The IMI was sorbed firmer by the Istria II soil and retained, though surprisingly the low K_{OC}^{sor} values of Istria II soil suggested that OC played less of role in sorption than with the lower OC content in Krk II soil. The difference may lie in the different pH values for the soils. The pH for the Krk II soil was 6.88 (nearly neutral) while the Istria II soil pH was 4.76 (acidic). Ping et al. (2010) found that IMI was sorbed more strongly at pH 4.5 than at pH 7.5. The effect of pH is probably due to the increased polarity of the humic material and the electrostatic interaction of the pesticide with soil particles at higher pH.

4.1.4 Sorption-desorption hysteresis

In order to estimate the discrepancies between sorption and desorption isotherms hysteresis coefficient *H* was calculated, and its values for the tested soils are presented in Table 5. When the value of *H* is lower, sorption-desorption hysteresis is more pronounced. We can see that the highest hystersis effect (the lowest *H*) was observed in the Istria II soil.

In Krk soils, no higher differences between sorption-desorption isotherm slopes were found, and therefore, hysteresis coefficients near to unit ($1/n_{des} \approx 1/n_{sor}$) indicates the high reversibility of IMI sorption by these two soils. Coefficient *H* was lower for Istrian soils than for Krk soils ($1/n_{des}<1/n_{sor}$). This indicates that a significant amount of the sorbed IMI is very difficult to desorb from Istrian soils which may have been caused by a higher amount of OC and clay content in Istria soils, leading to a higher sorption capacity than for Krk soils.

According to the dual model for the sorption of organic pesticides on the soil organic matter, sorption takes place through two sorption mechanisms: the partition and the sorption (Pignatello & Xing, 1996). Soil organic matter has not a uniform continuous phase and is rather represented as a three dimensional matrix, in which the condensed and amorphous phases form separated microenvironment. According to the proposed mechanism, at low concentrations of IMI, the sorption sites in the condensed aromatic area are occupied first, while at higher concentrations of IMI the sorption sites in the amorphous and aliphatic regions start to fill. This effect caused a pronounced hysteresis in the range of lower concentrations, which is consistent with the results obtained for the sorption of IMI. Since sorption area contains a limited number of high-energy sorption sites, molecules of sorbate occupy first these places at low concentrations, meaning that at low concentration the sorption mechanism dominates over the partition (Gunasekara et al., 2003). In addition, de Jonge & Mittelmejer-Hazeleger, 1996) showed that natural organic matter, has high microporosity, with a radius of pores <20 Å, and, therefore, the authors assumed that the observed sorption-desorption hysteresis can be the result of irreversible "trapping" of IMI molecules in the pores of natural organic matter. If we assume that the pore radius is 10 Å, than the calculated pore volume is about 4200 $Å^3$. Since the volume of one IMI molecule is 275 $Å^3$, it is possible that "irreversible entrapment" caused the observed sorption-desorption hysteresis.

4.2 Degradation study

4.2.1 Persistence of IMI

Results of degradation studies for IMI at two concentration levels (0.5 and 5 mg/kg) in four Croatian soils at various times are plotted in Figures 7 and 8. A visual examination of degradation pattern for the IMI in all tested soils suggests significant deviation from the first-order kinetic (R^2 range from 0.95 to 0.98). Consequently, alternative two-compartment model was used to describe the observed two-phase kinetic and to derive DT_{50} and DT_{90} (50 and 90 % degradation time) values. In our study, therefore, we presented the experimental data as a concentration of IMI degraded from the initial application on soil, and the corresponding values for DT_{50} and DT_{90}, were graphically estimated from Figures 7 and 8.

Following the treatment of soils at 0.5 and 5 mg/kg concentration level, the average initial concentration varied from 0.43 to 0.49 and 4.71 to 5.43 mg/kg (Figure 7 and 8). In all the tested soils, the residues persisted beyond 180 days at both levels and 4.7-13.8 % loss was recorded on day 7, 12.0-39.9 % on day 30, 32.7-65.2 % on day 90, and 55.0-82.6 % on day 180. The greatest loss of IMI was found in a clay soil with a higher OC content (Istria II soil, 75.4 -82.61 %) and the lowest in a sandy loam soil with a lower OC content (Krk II soil, 55.0-58.1 %).

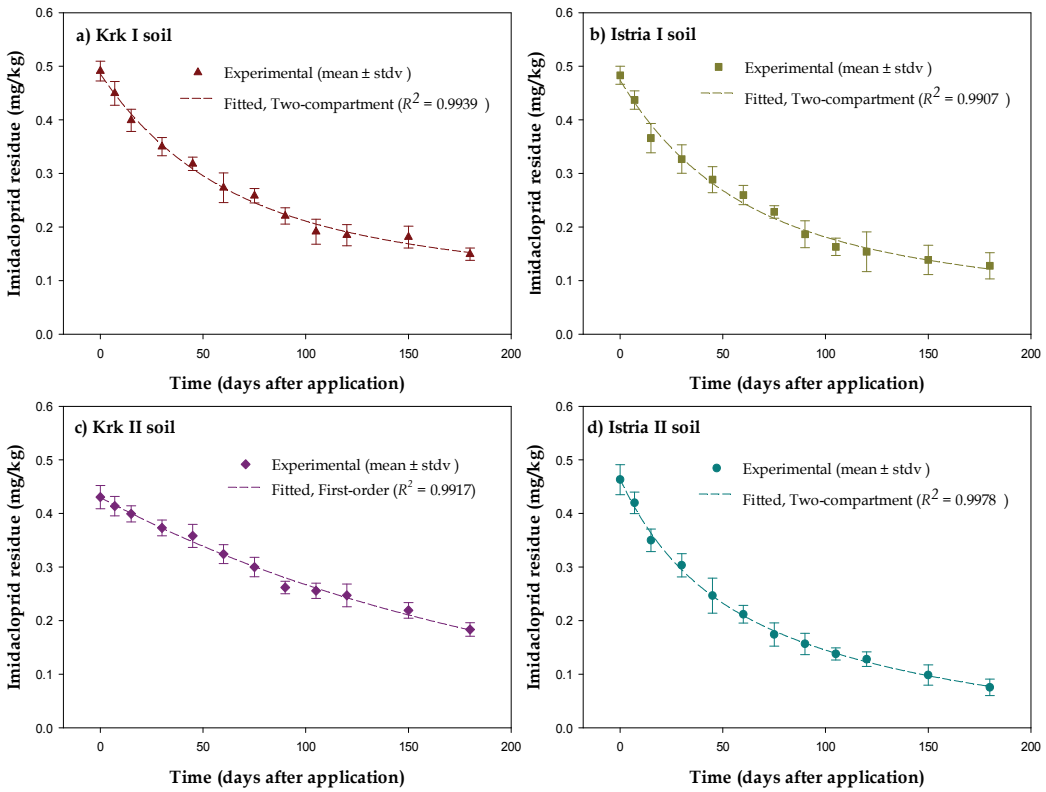


Fig. 7. Degradation of IMI in the tested soils at 0.5 mg/kg concentration level. Values are means ± standard deviations. Symbols represent the experimental data, while lines represent the theoretical curves fitted by the first-order kinetics model or two-compartment model.

The results from the curve fitting analysis are shown in Figure 7 and 8. In each figure, the measured data are shown together with the curves simulated by the first-order kinetic model or by two-compartment model. IMI degradation in all tested soils at the low concentration level appears to be adequately described by two-compartment model. In contrast, at the high concentration level the experimental data were better described using the first-order kinetic model rather than the two-compartment model, except for Istria II soil, where biphasic kinetic was observed (Figure 8d). In fitting two-compartment model, we assumed that the fast degradation phase occurred from 0 to 15 days after application and that the slow degradation phase occurred thereafter. This was visually determined based on the changes of the slopes of the degradation curves. Capri et al. (2001), who studied the degradation of IMI in Italian soils, found that IMI concentration decreased rapidly in the first 10 days followed by a slower decrease in the total amount recovered. The derived rate constants as well as DT_{50} and DT_{90} for each model are shown in Table 6 together with the correlation coefficient for each curve (R^2) and with root mean square error (RMSE). Both statistical indices (R^2 and RMSE) indicated that the first-order kinetic model better described IMI degradation at the high concentration level in all tested soils, except in Istria II soil, than at the low concentration level (Table 6). At the low concentration level, the more complex two-compartment model generated smaller RMSE.

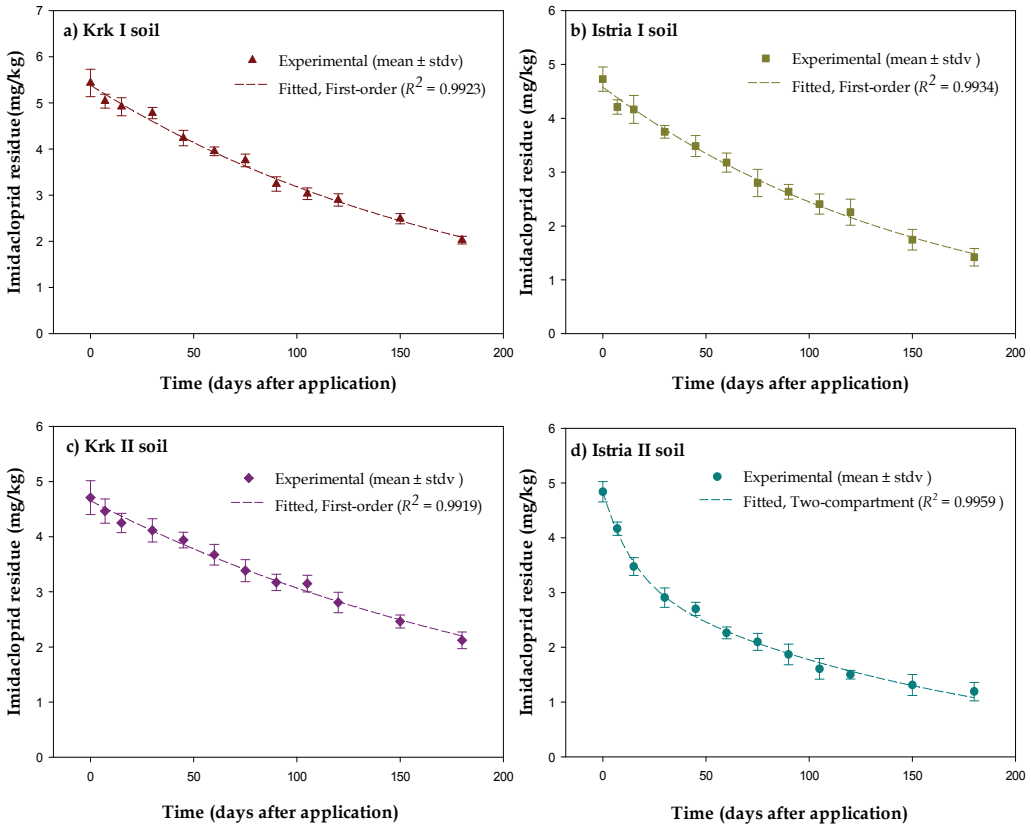


Fig. 8. Degradation of IMI in the tested soils at 5 mg/kg concentration level. Values are means ± standard deviations. Symbols represent the experimental data, while lines represent the theoretical curves fitted by the first-order kinetics model or two-compartment model.

The adequacy of the first-order kinetic model description at the high concentration level can be seen by comparing the fitted rate constants (Table 6). The fitted k_1 for the first-order kinetic model was equal (except the Istria II soil) to that in the rapid degradation pool of the two-compartment model. Moreover, the two-compartment model had the same rate constant in the rapid and slow degradation pools, which is also equal to k_1 of the first-order kinetic model. The rate of degradation was highest in the Istria II soil and the lowest in the Krk II soil. The higher degradation rate in Istria II soil than in Krk II soil could be due to the higher OC content of Istria II soil vs Krk II soil.

The calculated DT_{50} and DT_{90} values for the tested soils ranged from 50.18-145.97 and 247.38-914.97 days at the low concentration level respectively, and from 54.86-165.04 and 345.76-548.23 days at the high concentration level, respectively (Table 6). The DT_{50} and DT_{90} values were highest (for both concentration levels) for the Krk II soil, which were significantly higher than that in the other soils. The lowest DT_{50} and DT_{90} were observed in the Istria II soil. Estimated values for DT_{50} in our study prove that IMI can be categorized as moderately persistent pesticide (DT_{50} from 30 - 100 days) (Gavrilescu, 2005). Previous studies of IMI degradation at laboratory and at field conditions have reported DT_{50} values in

the range from 40 to 229 days (first-order kinetic) (Sarkar et al., 2001; Schad, 2001). However, high values of 156 days (Krohn & Hellpointner, 2002) and a greater than a year (Baskaran et al., 1999) have been measured. The DT_{50} values for IMI degradation in the present study are comparable with those reported under field conditions (Schad, 2001; 96 days) and degradation was slower than in other study at laboratory conditions (Sarkar et al., 2001; 40 days). If we compare the DT_{50} values derived from the used kinetic models between the two examined initial concentration levels, we can see that these values differed significantly. Higher persistence of IMI was observed at higher initial concentration level (mean DT_{50} = 118.46 days) compared to lower concentration (mean DT_{50} = 90.62 days), which was statistically significant ($p = 0.020$). This seems to lead to the conclusion that concentration level significantly affected IMI degradation.

Fitted parameter or index*	Model I				Model II			
	Krk I	Istria I	Krk II	Istria II	Krk I	Istria I	Krk II	Istria II
Concentration level 0.5 mg/kg								
k_1 (1/d)	0.0076	0.0090	0.0048	0.0114	0.0187	0.0210	0.0055	0.0353
k_2 (1/d)					0.0020	0.0031	$2.21*10^{-11}$	0.0075
a (mg/kg)	0.4613	0.4507	0.4297	0.4407	0.2798	0.2928	0.2209	0.2965
b (mg/kg)					0.2060	0.1814	0.2106	0.1675
DT_{50} (d)	91.20	77.02	144.41	60.80	82.39	63.60	145.97	50.18
DT_{90} (d)	302.97	255.84	479.71	201.98	722.10	466.64	914.97	247.38
R^2	0.9679	0.9695	0.9917	0.9844	0.9939	0.9907	0.9823	0.9978
RMSE	0.0212	0.0219	0.0077	0.0167	0.0103	0.0135	0.0084	0.0071
Concentration level 5 mg/kg								
k_1 (1/d)	0.0053	0.0063	0.0042	0.0093	0.0053	0.0063	0.0042	0.0356
k_2 (1/d)					0.0053	0.0063	0.0042	0.0051
a (mg/kg)	5.3848	4.5780	4.6514	4.3303	3.1555	2.9662	2.5149	3.4221
b (mg/kg)					2.2293	1.6118	2.1365	1.4170
DT_{50} (d)	130.78	110.02	165.04	74.53	130.78	110.02	165.04	54.86
DT_{90} (d)	434.45	365.49	548.23	247.59	434.45	365.49	548.23	345.76
R^2	0.9923	0.9934	0.9919	0.9483	0.9923	0.9934	0.9919	0.9959
RMSE	0.1010	0.0889	0.0769	0.2779	0.1130	0.0984	0.0859	0.0727

* DT_{50} and DT_{90} are times for 50 and 90% of the initial residues to degrade; k_1 and k_2 are first-order rate constants in the rapid and slow degradation pools; a and b are initial concentrations in the rapid and slow degradation pools; R^2 is the coefficient of determination; RMSE is the root mean square error

Table 6. Fitted parameters for the first-order kinetics model and two-compartment model for describing IMI degradation in the tested soils.

These results suggested that the persistence of IMI was significantly influenced by soil properties. Kendal-Tau correlation analysis between DT_{50} and selected soil properties demonstrated that IMI persistence in the tested soils was inversely connected to CEC, clay and OC content, with a strongest relationship between DT_{50} and CEC (Table 4). The analysis showed the positive, but very weak correlation between DT_{50} with soil pH. Other studies have found reasonable correlation between DT_{50} and pH. For example, Sarkar et al. (2001) showed that the persistence of IMI tended to increase as soil pH increased. In addition,

multiple liner regression confirmed that IMI persistence was primarily correlated with OC content, with a regression equation of:

$$DT_{50} = 72.7581\,OC - 4.9850\,CEC - 0.4689\,clay + 11.8127\,pH + 116.50 \left(R^2 = 0.825\right) \quad (14)$$

The DT_{50} values were further tested to determine the effects of soil type on the DT_{50} of IMI. Statistically significant differences in soil persistence, were found among the Krk (mean DT_{50} = 132.09 days) and Istria (mean DT_{50} = 77.00 days) region (p = 0.000002). In addition, results of degradation study within the regions showed a statistically significant difference in DT_{50} values between the soils Istria I (mean DT_{50} = 92.53 days) and Istria II (mean DT_{50} = 61.46 days) (p = 0.002), as well as between soils Krk I (mean DT_{50} = 108.16 days) and Krk II (mean DT_{50} = 156.02 days) (p = 0.00001).

Examining the soil properties reveals that the most contrasting difference between tested soils, with respect to IMI degradation, is soil OC content, which was in the range from 1.30 to 1.91% for Istrian soils and from 0.42 to 0.55% for Krk soils. Higher OC content in Istrian soils would cause more IMI sorption by the soil based on the concept proposed by (Park et al., 2003). An equilibrium sorption study, conducted to verify this hypothesis showed that IMI equilibrium sorption constants were higher for the Istrian soils than for the Krk soils (2.60 and 3.28 for the Istria I soil and Istria II soil; 1.28 and 1.53 for the Krk II soil and Krk I soil). Higher OC content in Istrian soils might have been accompanied by higher microbial population and activities that promoted biodegradation processes of IMI (Cox et al., 1997; Getenga et al., 2004; Park et al., 2003). In describing degradation of 2,4-D (2,4-dichlorpphenoxyacetic acid) Picton & Farenhorst (2004) hypothesized a mechanism according to which initially readily available chemical resulted in apparent rapid degradation, while subsequent increased binding to soil caused noticeable reduction in degradation rate. When comparing IMI degradation in all the tested soils, we observed that IMI degraded faster in Istrian soils than in Krk soils, although more IMI was sorbed in Istrian soils. Thus, it appears that sorption did not significantly inhibit IMI degradation in the soil. Otherwise, IMI should have degraded faster in the Krk soils than in the Istrian soils. Fitting two-compartment model to the measured data showed that the degradation rate constants in the rapid degradation pool in the Istrian soils were greater than those in the Krk soils at both concentration level (Table 6). Calculations using two compartment model based on the data in Table 6 revealed that readily available IMI amount in the rapid degradation pool initially represented 51-58 and 54-59% of the applied IMI in Krk soils and 62-64 and 65-71% in Istria soils at the low and high concentration level, respectively. These results suggested that IMI in the rapid degradation pool is not equivalent to the dissolved IMI molecule, as Wolt, (1997) proposed. Although pesticide molecule in soil solution is generally thought to be readily available to microorganisms for biodegradation, there is evidence that sorption can accelerate pesticide degradation (Park et al., 2003).

4.2.2 6-CNA formation

Metabolism of IMI was also studied in four Croatian soils at both concentration levels. The amount of 6-CNA, which was detected in all the tested soils as a metabolic product, varied irregularly with the time (Figure 9). The maximum concentration of 6-CNA in the tested soils was in the range from 280 to 720 μg/kg for the 5 mg/kg concentration level, while the corresponding concentration of 6-CNA was from 36.2 to 54.9 μg/kg for the 0.5 mg/kg

concentration level during the period of 150-180 days after application. Formation of the 6-CNA from IMI in soil has been reported earlier (Scholz, 1992). 6-CNA accounted for a maximum of about 15 and 10% of the initial concentration of IMI for the 5 and 0.5 mg/kg, respectively, in the Istria II soil. The corresponding minimum values for 6-CNA were 6 and 9%.

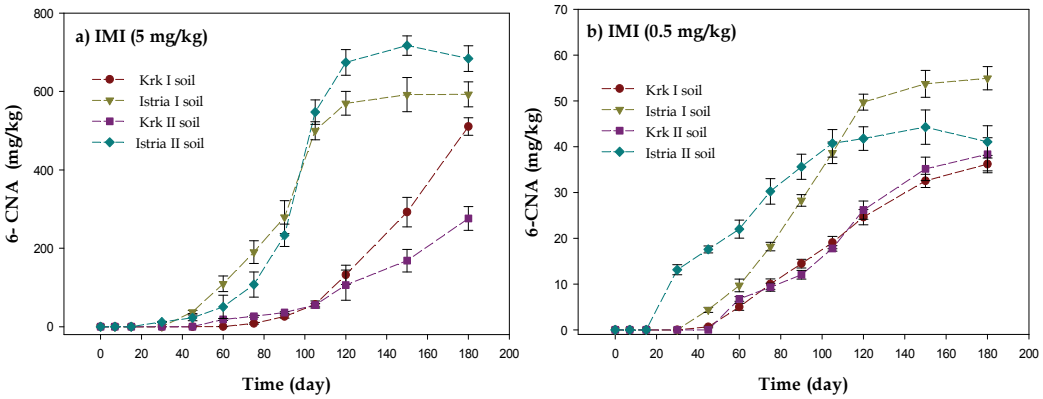


Fig. 9. Formation of 6-CNA in the tested soils at concentration level of: a) 5 and b) 0.5 mg/kg. Values are means ± standard deviations. Symbols represent the experimental data, while vertical bars represent the standard deviation in the triplicate samples.

5. Conclusions

The sorption-desorption and degradation of IMI was examined to understand the influence of concentration and soil properties on its behavior and fate in soils of Croatian coastal regions. The experimental data revealed that the sorption and desorption isotherms of IMI in the tested soils were nonlinear over the concentration range used, which can be best described by the Freundlich equation. Soil sorption capacity of IMI depended significantly on the soil properties. Especially, the sorption behavior of IMI was largely dependent on the soil OC content, where the soils with higher OC content (Istria soils) showed higher sorption capacity and less potential mobility of IMI. Given the spatial difference between tested soils, statistically significant differences in soil sorption capacity were found among and within soils of Istrian and Krk region. According to calculated K_{OC} values, IMI can be categorized as a medium mobility pesticide indicating that rational use of IMI entails little danger of the ground-water contamination. In all soils, a higher sorption capacity was observed at lower IMI concentrations, indicating that the percentage of desorbed amount of pesticide increased with increasing initial solution concentration. Desorption experimental data deviated significantly from the sorption data, indicating that these processes were distinctly different in tested soils. It can be assumed, that the desorption process appeared to be the result of a complex, time dependent interplay of several chemical and physical processes and irreversible binding of IMI to soil surfaces, leading to hysteresis. The negative and low values of the Gibbs free energy of the IMI sorption indicated exotermic characteristics of sorption reaction and corresponded to the physical process, suggesting that partitioning into soil organic matter was the main mechanism of IMI sorption in the soils used. IMI kinetic behavior in all tested soils at the high concentration level can be described by the first-order

kinetic degradation model, except for Istria II soil, where biphasic kinetics was observed. In contrast, at the low concentration level, the two-compartment kinetic model took place, characterized by the fast initial phase in the first 15 days of degradation followed by a slow degradation phase up to 180 days. According to the pesticide persistence classification, IMI can be categorized as moderately persistent pesticide (DT_{50} from 50 – 165 days), showing that the slow degradation of IMI in the tested soils further enlarges the danger of environmental damage. Concentration level significantly affected IMI's degradation, where higher persistence of IMI at higher initial concentration level was observed. In all tested soils, organic matter provided an accelerating effect on the degradation rate.

The study results emphasize the need for controlled IMI usage, especially in soils with low humus content (Krk soils), thus avoiding a risk of IMI leeching. Considering the abundant current use of IMI in the Croatian olive growing areas, regular monitoring is needed to evolve a strategy to manage the environmental hazards due to the IMI and its degradation products. Further research, aided also with the actual field data, will be directed to investigate the IMI's metabolism and binding mechanisms in order to better understand degradation pathway and the causes for hysteresis phenomena.

6. Acknowledgment

This work was supported by the Croatian Ministry of Science, Education and Sport (grant number 062-0621341-0061).

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Effects of Low-Molecular-Weight-Organic-Acids on the Release Kinetic of Organochlorine Pesticides from Red Soil

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1. Introduction

Organochlorine pesticides (OCPs) had been widely used in the world. Due to their strong persistence (The biological half-life period of DDT can reach 10 years in the soil (Li et al., 1999), the biological half-life period of DDE is longer than DDT((ATSDR), 1994)), high enrichment capability and amplification capability and potential high toxicity. They have been banned since the early 1970s in the global scope in succession, and China also banned these pesticides in 1983. After decades of biodegradation, its residual concentration in soil has been reduced significantly (Generally ranges from several to several hundred ng/g). But through the enrichment amplification of food chain, it still has high detection rate in soil, vegetables and human milk. DDE and DDD are the aerobic and anaerobic degradation products of DDT respectively, their insecticidal broad-spectrum and toxicity are higher than the DDT. As a kind of environmental estrogens, it may cause some damage to the health of human body and reproductive system (Maness *et al.*, 1998; Romieu *et al.*, 2000); hexachloroclohexanes (HCHs) have 8 kinds of isomers (*a-,β-,δ-,ε-,γ-,η-,θ-, and l* -HCH), and *γ*-HCH has obvious insecticidal efficacy (ATSDR), 1994). These two types of organochlorine pesticides are typically persistent organic pollutants (POPs).

The solubility of OCPs in water is low and the transference capability of OCPs in the soil is limited, but dissolved organic matter (DOM) in the environment could obviously change the transference behaviour of OCPs, and the predecessor's research mainly focuses on the influence of macromolecular DOM to the migration behaviour of OCPs, and the mechanism lies mainly on its chelation behaviour to OCPs (Chiou *et al.*, 1987; Chiou *et al.*, 2000; Chiou *et al.*, 1986; Hassett & Anderson, 1982; Landrum *et al.*, 1984), studies about the effect of low molecule weight DOM on the retained behaviour of OCPs have rarely seen. In the rhizosphere microenvironment, the existence of low molecular organic acids (LMWOA) of plant root exudates must have great influence on the migration of OCPs, the experiment conducted by White (2003) showed that the seven kinds of low molecular weight organic acid could significantly increase the desorption of p,p'- DDE, the increment could reach at the range of 19%-80% (White & Kottler, 2002; White *et al.*, 2003).

The object of this article is to discusses the dynamic release behavior of several organochlorine pesticides like DDT isomer (DDTs) and HCH isomer (HCHs) with LMWOA from variable charge soil (red soil) with self-designed dynamics device, and provide some reference to the migration and fate of these kinds of substances and also the phytoremediation and ecological risk assess ment of organic pollutants in the environment .

2. Research advance

Their source include: root exudates, microorganisms, animals, and the degradation of organic matters. The LMWOA, as one of the typical rhizosphere active components presenting in soil and a key molecule in the solubilization of phosphorus-containing crystalline or amorphous minerals or as a desorbing agent for orthophosphate adsorbed on other soil minerals, is sure to have an influence on the existing state of organochlorine pesticides in soil and on the interaction between organochlorine pesticides and soil colloids.

2.1 Source of LMWOA

Low-molecular-weight organic acids (LMWOAs) occur widely in soils and primarily originate from root exudation (Gao *et al*., 2010; Ling *et al*., 2009; Lu *et al*., 2007; White *et al*., 2003). Moreover, microorganisms, animals, and the degradation of organic matters can also produce LMWOA (Jones, 1998). People have noted the role of root exudates in the early 18th century, and then have a more profound understanding of the composition and the role of plant root exudates in latest 30 years. The composition and transportation of root exudates and its function in soil structure formation, soil mineral weathering, soil nutrient activation, promoting the nutrient absorption and rhizospheric soil nutrient movement and poison resistance (Al, acid) have been reported (Oburger *et al*., 2009; Strom *et al*., 2001; van Hees *et al*., 2002; van Hees *et al*., 2003). Root exudates are lubricant of the root - soil interface and microbial energy source, and they can improve the rhizosphere environment. They are the key materials for plants to adapt to nutritional stress (Strom *et al*., 2002; Strom *et al*., 2005) and environmental stress (do Nascimento *et al*., 2006; Gao *et al*., 2010; Guo *et al*., 2007; Jones *et al*., 2001; Liao *et al*., 2006; Luo *et al*., 2006; Toyama *et al*., 2011; Zhu *et al*., 2009). There are over 200 kinds of root exudates, including saccharides, organic acids, amino acids and allelochemicals, as well as small amount of fatty acids and steroids and trace growth substances and enzymes (Baudoin *et al*., 2003; Muratova *et al*., 2009). Their types and numbers will vary with the plant types and rhizosphere environment. Among them, the LMWOA are the main component of root exudates, which with one to several carboxyl and the most common LMWOAs identified in soils include citric acid, tartaric acid, maleic acid, malic acid, formic acid, acetic acid, oxalic acid, succinic acid, fumaric acid, propionic acid and so on (Baudoin *et al*., 2003; Kpomblekou-A & Tabatabai, 2003). Most of them are the intermediate products of tricarboxylic acid cycle. In special cases, plant secretes specific organic acids, such as mugineic acid which is secreted by rice and wheat when they lack iron (Inoue *et al*., 2009; Kobayashi *et al*., 2010). Molecular structure and charged characteristics of LMWOA can affect many processes in the soil, some stresses of adversity factor can induce plant roots to secrete a large number of organic acids, and this is an adaptive reaction of plant to ecological environment (Inoue *et al*., 2009; Kobayashi *et al*., 2010).

2.2 Residual characteristic of organochlorine pesticides in soil and rhizosphere

Organochlorine pesticides (OCPs) could quickly be adsorbed or bound to the soil or soil-organic matter due to their high hydrophobicity and low water solubility after they were introduced into the soil. With time, the diminishes of these OCPs's bioavailability due to an "aging" effect and the formation of "bound-residues", which takes place during processes of decomposition and humification of organic matter (Alexander, 2000). The residual charactaristics of OCPs in rehizosphere and bulk soil maybe related to the properties of OCPs (water solubility, degradability, volatility,etc.), characteristic of soil mineral (the type and content of organic matter, diameter composition of soil mineral particle, content of oxidation-reduction materials, moisture content, etc.), properties of plant (root system characteristic, kind and quantity of root exudates, lipoprotein content, specific surface, etc.) (Alexander, 2000; Calvelo Pereira *et al.*, 2006; Chen *et al.*, 2007; Gonzalez *et al.*, 2005; Inui *et al.*, 2008; Mikes *et al.*, 2009; Mo *et al.*, 2008; Skaates *et al.*, 2005; White *et al.*, 2002; Yang *et al.*, 2008; Yao *et al.*, 2007). For the OCPs with high volatility and relative easy degradation, the concentrations in rehizosphere are generally lower than that in bulk soil, and the results generally appear in the indoor simulation experments. For the OCPs with low volatility and relative hard-degradation, the concentrations in rehizosphere are generally higher than that in bulk soil, and the results generally appear in the field experments. The results of pot experiment maybe also differ with field experiment for the same compounds. This kind of contradictory results is mainly due to the pollution sources can be repeatedly inputted by irrigation and dry and wet deposition in field, pollutants would be enrich in rehizosphere with water flow; and the pollution source is single input in indoor simulation experiment, plant absorption or biodegradation result in the concentration of OCPs in rhizosphere soil is lower then in the bulk soil.

For example, Chen et al. (2007) showed that the measured DDXs in the rhizosphere soils were significantly higher than those in the bulk soils. p,p′-DDT, p,p′-DDD, and p,p′-DDE in the soil accounted for 38%, 47% and 15% of the total. For total DDXs, approximately one third remained on the outer surface of the roots. The partition of DDXs between rhizosphere soil and root surface depended on contaminant affinity to soil organic matter, soil organic matter content and root specific area (Chen *et al.*, 2007), Calvelo Pereira et al. (2006) reported that the roots of *Avena sativa L., Chenopodium spp., Solanum nigrum L., Cytisus striatus (Hill) Roth, and Vicia sativa L.* tended to reduce levels of the HCH isomers in the rhizosphere (Calvelo Pereira *et al.*, 2006),white et al. (2002) found that the chlordane concentration in the rhizosphere (soil attached to roots) was significantly less than that in the bulk soil. However, the enantiomeric ratio of the chiral components and overall component ratios had changed little in the rhizosphere relative to the bulk soil. (White *et al.*, 2002).

2.3 Interaction mechanism between LMWOA and pesticides in soil

LMWOAs have been shown to disrupt the sequestering soil matrix, thereby enhancing the desorption of organic pollutants in soil (White *et al.*, 2003). Consequently, it is expected that LMWOAs, in theory, may affect OCPs availability in soil environment. However, to date, few research has been conducted in this area, and there is limited information on the availability and sorption–desorption behaviour of OCPs from natural soils by organic acids.

Gonzalez et al. (2010) showed that sodium citrate and oxalate, at levels usually exuded by plant roots, effectively enhanced desorption of p,p'-DDT, p,p'-DDE and α-cypermethrin, while no effects were observed for α-endosulfan and endosulfan sulfate, the non-ionic surfactant

Tween 80 behaved similarly to the acids, whereas the anionic sodium dodecyl sulfate enhanced desorption of all pesticides (Gonzalez et al., 2010). Luo et al. (2006) reported that soil organic carbon played a predominant role in the retention of DDT. Oxalate significantly increased the desorption of p,p'-DDT, with the largest increments ranging from 11% to 54% for different soils. Oxalate addition also resulted in the increased release of dissolved organic carbon and inorganic ions from soils. Root exudates had similar effects to those of oxalate and significantly increased DDT desorption from the soils. So, Low molecular weight dissolved organic carbon amendments caused partial dissolution of the soil structure, such as the organo-mineral linkages, resulting in the release of organic carbon and metal ions and thus the subsequent enhanced desorption of DDT from the soils (Luo *et al.*, 2006).

We can put forward some mechanisms about the LMWOA or root exudates with OCPs desorption: (1) Mechanism of minerals dissolution. LMWOA induced the dissolution of soil minerals and resulted in the desorption of OCPs adsorbed by soil minerals; (2) Mechanism of indirect release. Soil inherent organic matter is dissolved and released by LMWOA and resulted in the desorption of OCPs adsorbed by soil inherent organic matter. (3) Mechanism of direct release. The LMWOA directly chelated with OCPs and released it. (4) Mechanism of locking and unlocking. LMWOA interacted with soil inherent organic matter like a key and induced the change of its conformation and properties, resulted in the OCPs were locked or unlocked by soil inherent organic matter (Figure 1).

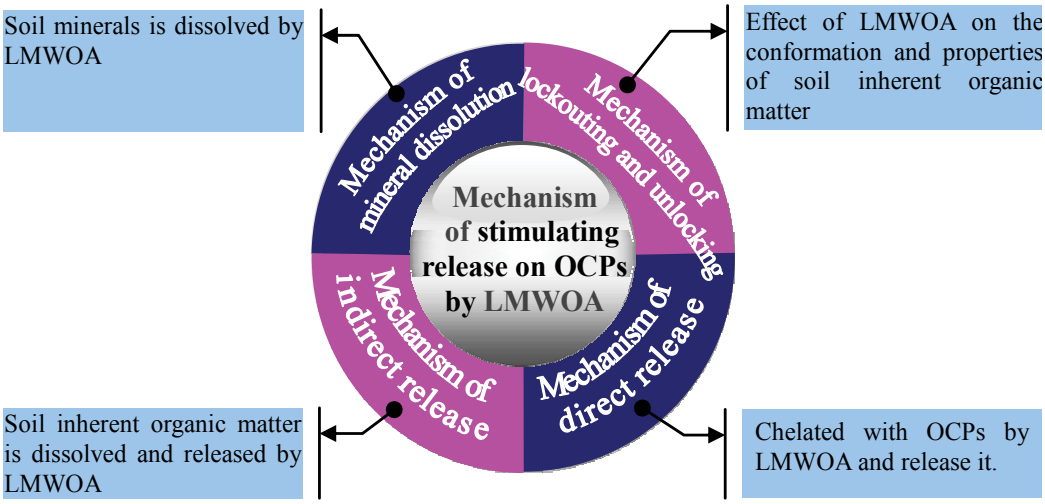

Fig. 1. Mechanism obout the stimulating release of OCPs by LMWOA

3. Release kinetic of organochlorine pesticides from soil in LMWOA system

3.1 Materials and methods

3.1.1 Instruments and reagents

n-Hexane (chromatographically pure, Tedia Company, USA); petroleum ether with boiling range of 60 °C to 90 °C (analytical reagent, Hangzhou Refinery, Zhejiang Province, P.R. China); methylene chloride, acetone and concentrated sulfuric acid (analytical reagent, Nanjing Chemical Reagent Plant, P.R. China); anhydrous sodium sulfate (analytical reagent, it was sieved by boult of 200 mesh, then treated at 225 °C for 4 h and stored in sealed

container before use); Organochlorine pesticides standard (o, p'-DDT, o, p'–DDE, p, p'-DDT, p, p'–DDD, p, p'-DDE, α-, β-, γ- and δ-HCH)were purchased from Dr. Ehrenstorfer Company, Germany. The characteristics of pesticides studied are listed in table 1.

DDTs	solubility	log*K*ow	HCHs	solubility	log*K*ow
p,p'-DDT	1.2~5.5[1)]	6.2~6.91	*α*-HCH	10	3.8
o,p'-DDT	1.2~5.5	6.76	*β*-HCH	5	3.78
p,p'-DDE	65	5.69~6.96	*γ*-HCH	7.3~17	3.61,3.72
o,p'-DDE	65	6.94	*δ*-HCH	1	4.14

[1)] Solubility in water at 24～25℃, μg/L

Table 1. Some properties of the organochlorine pesticides (Chiou et al., 1987; Harald et al., 2000)

Agilent-6890 GC/ECD gas chromatography and HP7683 automatic sampler with HP chemical workstation (Hewlet-Packard, USA) and HP-5 fused capillary column (30 m ×320 μm ×0.25μm) as chromatographic column; Sigma 2-16K high speed freezing centrifuge (Sigma, Germany); water bath rotary vacuum evaporator (Yarong Biochemical Instrument Plant, Shanghai, P.R.China); HS-10360D ultrasonic cleaning machine (Heng'ao Science and Technology Company, Tianjin, P.R.China); BS200S-WE1 electronic balance (1/10000, Sartorius Company, Germany); SPP cartridge and filter disc (Chemical and Physical Institute of National Chromatogram Center in Dalian, P.R.China).
Celite 545 (chromatographic grade, 0.020-0.045 mm, Serva Company) was dried in muffle furnace at 550 °C for 4 h, then treated at 200 °C in oven before addition of 3 % of deionized water to deactivate it, and stored in a sealed container before use; Purified SPE column was prepared by packing with a teflon filter disc + 1g celite 545 + 1g anhydrous sodium sulfate + a teflon filter disc.

3.1.2 Chromatogram conditions

Initial oven temperature was kept at 60 °C for 1 min. Then the temperature was increased to 140 °C at a speed of 20 °C min^{-1} and subsequently increased to 280 °C at a speed of 12 °C min^{-1} and kept at 280 °C for 4 min. The injector temperature was set as 220 °C, while the detector temperature was set as 280 °C. High purity N_2 (99.999%) was used as carrier gas at a flow rate of 2 mL min^{-1}. 2 μL of sample was injected in a splitless mode. Quantitative calculation was conducted with external standard method.

3.1.3 Dynamic experiment methods

Soil sample The red soil, Hydragric Acrisols - according to WRB (World Reference Base for soil resources) (ISSS/ISRIC/FAO, 1998), was sampled at depths of 5 cm to 20 cm from the Red Soil Ecologic Experimental Station of Chinese Academy of Science in Yingtan, Jiangxi province, P. R. China (28°12′34.1″N, 116°55′32.3″E), lyophilized and sieved (≤1 mm) for further analysis. The clay minerals of soil sample were mainly composed of kaolinite and hydroxy-aluminum vermiculite, and contained a spot of hydromica and a trace amount of gibbsite. The main physical and chemical characteristics of the soil were as follows: pH 5.07, clay content 32.3%, organic matter content 1.14 %, Cation Exchange Capacity (CEC) 10.10 Cmol (+) kg^{-1}.
Spiked soil Soil samples were sieved to <2mm and stored at room temperature until spiking procedure. Spiked soil samples were prepared by adding 500 μL of standard mixture of 13 kinds of OCPs (the concentration is 10 ng μL^{-1} for each compound dissolved

in n-hexane) to 20 g of soil according to the reference (Tor et al., 2006). This spike level corresponds to 250μg kg^{-1}. Then 20mL of acetone was added and suspension was mixed for 30 min with a mechanical shaker. After the bulk of the solvent was evaporated at room temperature, the samples were stored at 4 ◦C in stoppered glass bottle for six month in the dark. Then the extractions were carried out.

Preparation of eluent 3 kinds of low molecular weight organic acid eluent solutions (oxalic acid, tartaric acid and citric acid) were all prepared as 10mmol/L solutions by the analytical reagents, and their pH were adjusted to 5.5 by NaOH or HNO_3. The eluent's pH selected was based on the common pH of southern variable charge soil in China.

The dynamic devices are as follows: ①Storage Bottles; ②P200Ⅱtype high performance liquid chromatography pump (Scientific Instruments Co., Ltd. YiLite, Dalian, P. R. China); ③dynamic reaction cell made of PTFE to provide reaction space; ④SBS-100 automatic fraction collector (Huxi Analytical Instrument Factory Co., Ltd, Shanghai, P. R. China).

Dynamics experiment methods Weigh 7g of spiked soil, put it into the dynamic reaction cell, and seal the cell tightly after wetting the sample with distilled water. The upper and lower ends of dynamic reaction cell, respectively, connected with the automatic fraction collector and high performance liquid chromatography pump. The leaching velocity was set for 1mL/min, the collection time of each glass tube was 10 minutes and continuously collected 100 glass tubes of leacheate. The experimental temperature of the dynamic reaction cell was controlled at 298±0.5K by using thermostatic waterbath. When the samples were determined, two glass tubes were combined as one test sample point.Transferred the collected liquid into separating funnel, and add 10μL of internal standard (Five chlorine nitrobenzene methanol solution, 5ng/μL). After homogeneous mixing, added 10 mL petroleum ether and 0.5g NaCl in the separating funnel, and oscillated for liquid-liquid extraction. After adding 0.5 mL of acetone to eliminate stubborn emulsification phenomenon, transferred organic extraction phase into pear-shaped bottle, and then added 10 mL petroleum ether to repeat the extraction step. These two extract were combined and put it in pear-shaped bottle. The extract was concentrated to about 1mL by rotary evaporators, and translated it into purifying SPE column. The SPE column was eluted with 10mL of 10% dichloromethane / petroleum ether (V:V), and the leacheate was concentrated to about 1mL by rotary evaporators again and blew by nitrogen and metered volume to 1mL by n-hexane. Determined by GC-ECD, and quantified by external standard method. The results showed that, the recovery rate of this liquid-liquid extraction method was 80%~105% to different pesticides, and the relative standard deviation was 3%~8%, which meet the demands of the analysis of trace organic compounds.

3.1.4 Quality control and data analysis

Laboratory blank values for all the compounds were generally low and posed no problem to the analytical quantification. The overall reproducibility was evaluated using the replicate analyses (n = 3). The coefficient of variation (CV) was between 0.01 and 0.35 for the various compounds, and it was less than 0.3 in 90 % of the cases. Therefore, the reproducibility of the measurements was considered to be satisfactory.

3.2 Results and discussion

3.2.1 Effects of LMWOA on the release rate of organochlorine pesticides

The average releasing rate is calculated by the each pesticides quantity contained in leaching solution (20mL) divided by elution volume. From figure 2, the release rate of HCHs by

water and organic acids is much higher than that of DDTs, the difference is about 3 times. The release ability of water to DDTs is very low. This kind of pesticides is released by water at a certain concentration which practically is lower than its solubility, concentration, the elution volume does not have evident effects to its release rate. The release pattern of HCHs with high solubility by water follows a quick release at first, and then a slow release after an elution inflection point at which the elution volume is about 300mL when it achieves the elution inflection point.

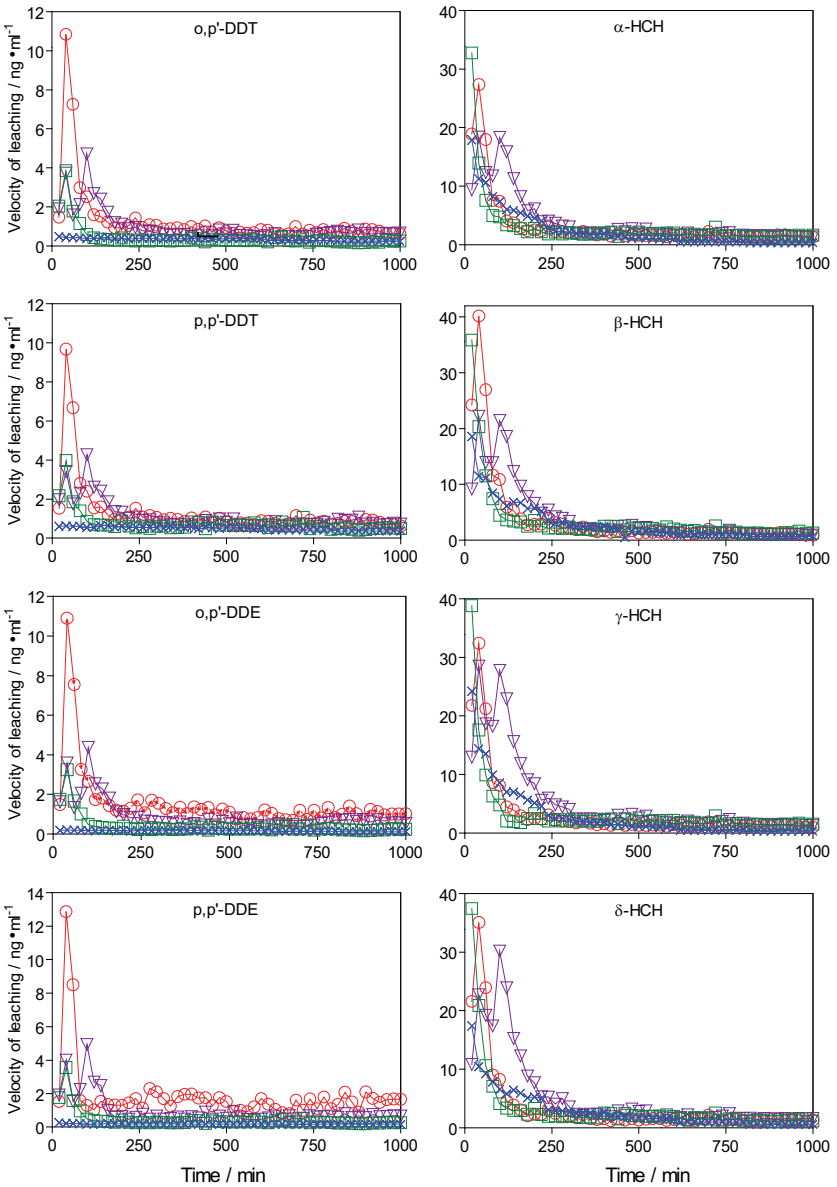

Fig. 2. Release velocity of organochlorine pesticides from red soil with LMWOA.-*-:water, -□- : oxalate, -▽- : tartrate, -○- : citrate

The release pattern of DDTs by oxalic acid is single-peak type curve. That means the release rate increases along with the elution volume increase, and it reaches the maximum elution rate when the elution volume is 40mL, after that the rate gradually decreases, and it is not stable until the volume reaches to 120mL. The release pattern of DDTs by oxalic acid is the same to that by water except that the elution inflection point is 120mL.

The release patterns of DDTs and HCHs by tartaric acid belong to bimodal curve. When the volume of leacheate was 40mL, the release rate of the two kinds of pesticides reached maximum, and then the rate slightly decreased. When the volume of leacheate increased to about 100mL, the rate reached another maximum. And it became stable till the volume reached 240mL. The release pattern of DDTs and HCHs by citric acid solution was also single-peak type curve, and their inflection point appeared at about 140mL.

The existence form of organochlorine pesticide in soil includes free form, loose bound form and tight bound form (for example aging residual form). When the leaching solution flowed through the soil, the free form and loose bound form would be released firstly, after loose bound form was eluted completely, the tight bound form OCPs were slowly dissolved out with approximately constant speed. The leaching pattern of OCPs by tartaric acid displayed the bimodal curve, it maybe relate to the comparatively weak elution ability to loose bound form pesticides. The first peak represents the release of free form pesticides, the second peak represents the release of loose bound form pesticides, and then the slow release of the tight bound form. This indicates that the release intensity of tartaric acid to the loose bound form pesticides is smaller than the citric acid. As the citric acid has stronger desorption ability to the free form and loose bound form pesticides, these two forms pesticides will be leached out the soil together and form a single peak.

3.2.2 The cumulative release of organochlorine pesticides from soil by LMWOA

Figure 2 shows the diagram of the cumulative release of organochlorine pesticides by several LMWOA from red soil. Table 2 lists the fitting results of the dynamic release data of organochlorine pesticides to several common kinetic equation, while t is the time, Qt is the cumulative release amount of pesticides, a and b are the parameters of the kinetic equation (with different meaning in different equations), k is the apparent speed constant in the first-level dynamic equation, q_{max} is the apparent equilibrium desorption amount. The multiple correlation coefficient (R^2) and standard error (Se) can be used to judge the degree of fitting. That is to say the larger R^2 and the smaller Se contribute to a better fitting degree.

$$s_e = \sqrt{\frac{\sum(s_t - \hat{s}_t)^2}{n-2}}, \quad R^2 = 1 - \frac{\sum(s_t - \hat{s}_t)^2}{\sum(s_t - \overline{s}_t)^2}$$

Where the S_t、$\hat{s}_t$、$\overline{s}_t$ and n are the measured value, the predictive value, the average value and sample number, respectively.

Figure 3 shows that the introduction of LMWOA strengthens the release of organochlorine pesticides to a certain extent (compared with water, it increases by 15~18 percentage for DDTs, while the HCHs increases by 7~25 percentage). The release ability of LMWOA for DDTs is: citric acid (18~26%) > tartaric acid (14~20%) > oxalic acid (6~10%) > water (3~8%). On the other hand, the release ability of LMWOA for HCHs is: tartaric acid (60%) > citric acid (49~55%) > oxalic acid (41~48%) > water (35~41%). The results match the experiment results conducted by White who used batch method and pot experiment to study the effect of 7 kinds of LMWOA to p, p' – DDE (White *et al.*, 2003).

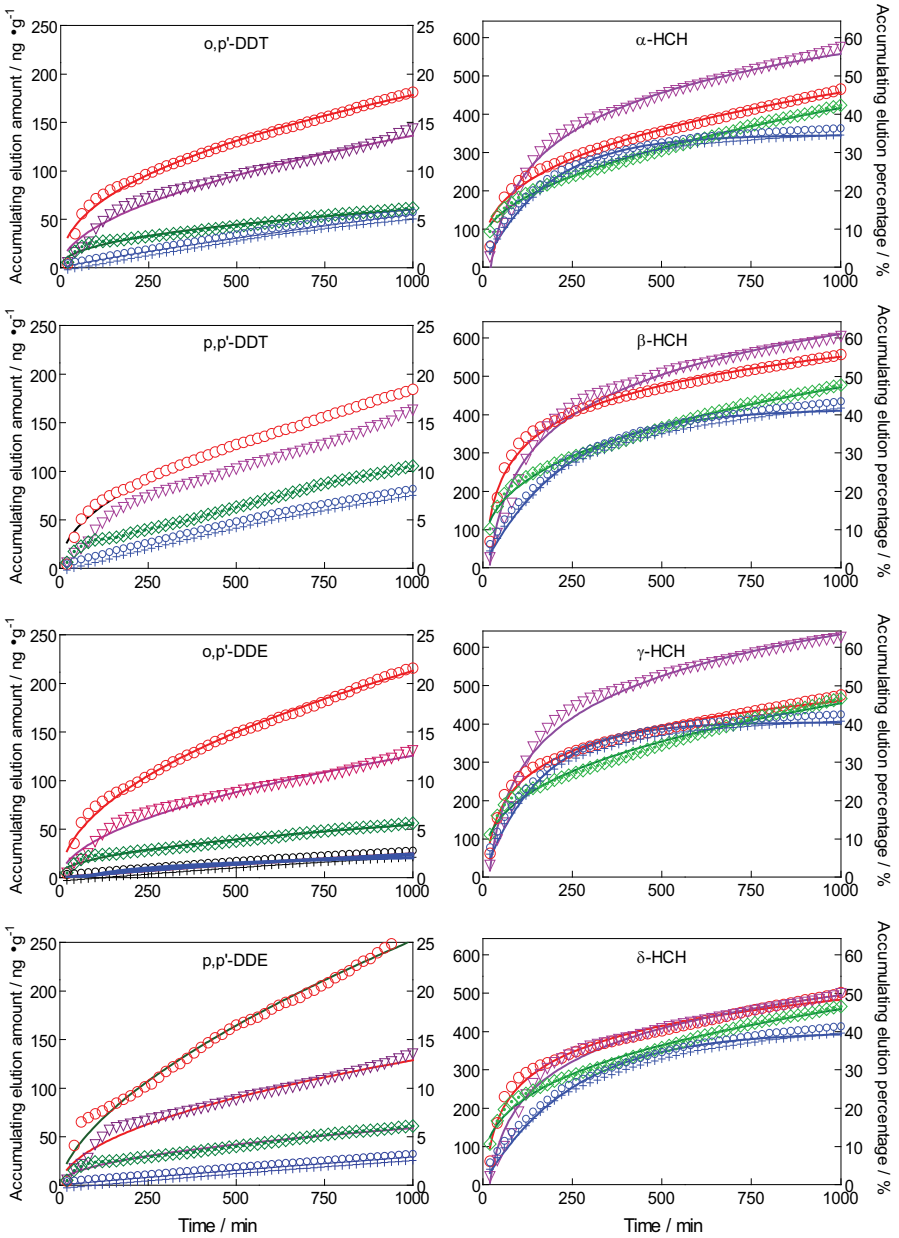

Fig. 3. Accumulative release kinetic of organochlorine pesticides from red soil with LMWOA leaching. -*-:water, -+- : oxalate, -▽- : tartrate, -○- : citrate

Table 2 shows that the kinetic release of organochlorine pesticides in water system is basically accord with the first-order kinetic equation (R^2: 0.99 - 0.9999, p<0.0001), p, p'-DDE appears to be more consistent with two-constant equation (its *Se* is lower than that of the

	pesticides	First-order kinetic: $ln(1-q_t/q_{max})=-kt$			Parabolic diffusion: $Q=a+bt^{1/2}$				Elovich equation: $Q=a+blnt$				Double constant equation: $Q=at^b$			
		q_{max}	k	R^2	Se	a	b	R^2	Se	a	b	R^2	Se	A	b	R^2	Se
water	o,p'-DDE	64.3	0.0005	0.9998	0.11	-7.1	0.97	0.9834	0.93	-31.06	7.47	0.8635	2.67	0.06	0.126	0.9986	0.27
	p,p'-DDE	116.6	0.0003	0.9999	0.09	-8.6	1.12	0.9753	1.33	36.06	8.63	0.8449	3.32	0.06	0.132	0.9999	0.07
	o,p'-DDT	123.9	0.0006	0.9995	0.36	-15.3	2.13	0.9851	1.95	-68.5	16.56	0.8686	5.79	0.14	0.123	0.9977	0.77
	p,p'-DDT	189.6	0.0005	0.9997	0.39	-22.6	3.10	0.9851	2.83	-99.79	24.06	0.8680	8.43	0.2	0.124	0.9982	0.98
oxalate	o,p'-DDE	54.9	0.0028	0.8852	4.14	3.1	1.63	0.9791	1.77	-39.4	12.95	0.9078	3.71	2.29	0.066	0.9769	1.86
	p,p'-DDE	61.84	0.0025	0.8978	4.36	1.5	1.82	0.9776	2.04	45.36	14.36	0.8943	4.43	1.99	0.070	0.9765	2.09
	o,p'-DDT	59.19	0.0032	0.8802	4.56	5.1	1.76	0.9813	1.80	-41.33	14.08	0.9220	3.68	3.04	0.062	0.9792	1.90
	p,p'-DDT	141.9	0.0013	0.9684	4.79	-13.1	3.59	0.9767	4.12	-102.61	27.86	0.8631	9.97	1.03	0.096	0.9894	2.77
tartrate	o,p'-DDE	124.4	0.0028	0.9583	6.23	-2.8	4.06	0.9783	4.49	-114.44	33.29	0.9615	5.98	3.86	0.072	0.9774	4.58
	p,p'-DDE	130.8	0.0026	0.9447	7.33	-2.8	4.16	0.9804	4.36	115.31	33.79	0.9465	7.22	3.77	0.073	0.9799	4.42
	o,p'-DDT	137.4	0.0027	0.9587	6.80	-4.2	4.46	0.9811	4.60	-126.16	36.44	0.9573	6.91	3.91	0.074	0.9798	4.76
	p,p'-DDT	172.1	0.0020	0.9705	6.83	-14.8	5.33	0.9903	3.91	-156.29	42.81	0.9360	10.06	2.69	0.084	0.9887	4.23
citrate	o,p'-DDE	218.1	0.0025	0.9555	10.74	-3.1	6.83	0.9919	4.60	-186.14	55.19	0.9477	11.7	6.34	0.073	0.9916	4.66
	p,p'-DDE	299.7	0.0017	0.9655	12.2	-24.0	8.54	0.9862	7.96	243.14	67.34	0.8988	20.8	3.69	0.087	0.9908	6.48
	o,p'-DDT	172.6	0.0033	0.9330	10.43	9.9	5.38	0.9829	5.27	-137.71	44.06	0.9641	7.64	8.33	0.063	0.9856	4.83
	p,p'-DDT	182	0.0027	0.9464	9.84	1.4	5.70	0.9898	4.29	-152.14	46.16	0.9510	9.41	6.21	0.069	0.9900	4.25
water	α-HCH	346.1	0.006	0.9908	7.38	77.5	9.8	0.9026	24	-219.6	85.1	0.9874	8.63	39.0	0.329	0.9408	18.7
	β-HCH	414.9	0.005	0.9905	9.45	56.5	12.7	0.9445	22.9	-313.3	247.6	0.9906	9.40	32.4	0.381	0.9641	18.4
	γ-HCH	404.6	0.006	0.9901	9.37	114.4	10.8	0.8777	29.9	-218.1	94.5	0.9818	11.6	56.4	0.299	0.9286	22.9
	δ-HCH	399.7	0.004	0.9907	9.15	40.2	12.5	0.9598	19.0	-317.7	241.4	0.9877	10.5	26.3	0.404	0.9729	15.6
oxalate	α-HCH	392	0.004	0.86	31.4	59.7	11.3	0.9954	5.66	-236.4	90.0	0.9305	22.1	28.6	0.386	0.9909	7.99
	β-HCH	438.6	0.005	0.8536	34.9	92.8	12.2	0.9923	8.01	-238.3	228.9	0.9578	18.7	43.3	0.346	0.994	7.05
	γ-HCH	427.4	0.004	0.8261	37.1	83.3	11.9	0.9938	7.02	-231.1	95.5	0.9315	23.3	38.3	0.358	0.988	9.76
	δ-HCH	425	0.005	0.827	36.1	99.4	11.6	0.9914	8.03	-214.4	217.1	0.9536	18.7	45.9	0.333	0.9916	7.94
tartrate	α-HCH	541.4	0.004	0.9733	21.6	48.3	17.3	0.9441	31.3	-456.7	146.9	0.9912	12.4	34.8	0.409	0.958	27.1
	β-HCH	575.1	0.005	0.9834	17.9	86.3	17.7	0.8956	45.0	-451.9	354.1	0.9855	16.8	49.9	0.368	0.9247	38.2
	γ-HCH	726.6	0.005	0.9783	20.4	116.5	22.2	0.8984	45.9	-555.7	192.3	0.9863	16.9	65.3	0.363	0.9283	38.6
	δ-HCH	466.3	0.005	0.9737	18.4	67.4	14.5	0.897	36.4	-370.3	288.4	0.9833	14.7	39.5	0.371	0.9242	31.2
citrate	α-HCH	419	0.005	0.8814	30.9	88.9	11.9	0.9697	15.7	-246.4	98.9	0.9779	13.4	42.8	0.343	0.9825	11.9
	β-HCH	506.9	0.008	0.8968	31.7	179.6	12.6	0.8978	31.5	-201.4	251.0	0.9855	11.9	88.9	0.267	0.9464	22.8
	γ-HCH	426.9	0.007	0.8717	30.6	133.5	11.1	0.9341	21.9	-191.9	94.4	0.9868	9.8	64.7	0.288	0.966	15.8
	δ-HCH	447.9	0.007	0.8614	33.4	140.7	11.7	0.931	23.6	-200.3	227.9	0.9826	11.8	68.2	0.288	0.9621	17.5

Table 2. Fit values of parameters for different kinetic equations

first order equation), and it implies that the release kinetics of the organochlorine pesticides studied in water is still a surface diffusion on soil particles. The kinetic release of o, p' and p, p'-DDE, o, p'-DDT and HCHs by oxalic acid seems to be more consistent with the parabolic diffusion equation, and it indicates that the release is controlled by a number of diffusion mechanism, the outward diffusion process of the pesticides from soil particle interior is the limit step of the whole release process; but p, p' - DDT more conforms to the double constant equation, it may be related to the dissolution and the heterogeneity of energy of soil particles surface induced by oxalic acid (activation and inactivation function of granular surface). In tartaric acid system and citric acid system, the parabola diffusion equation (tartaric acid system) and the double constant equation (citric acid system) may be better to describe the kinetic release behaviours of DDE and DDT; Besides α-HCH conforms to the double constant equation, the kinetic release of β-, γ- and δ-HCH seem to be more consistent with the Elovich equation. And it tells us that in the tartaric acid leaching system. So the release of DDTs is mainly characterized by several diffusion mechanisms in the tartaric acid leaching system and characterized by the release mechanism of different energy position in citric acid system. It may involve some more complex release mechanisms for the release of the HCHs, which own a larger solubility in water.

Overall, the release of organochlorine pesticides in water system is consistent with the first-order kinetic equation which is good at describing a simple diffusion mechanism. The release of DDTs by the oxalic acid and citric acid system can be well described by double-constant equation which is good at describing a uniform energy distribution; that in tartaric acid system can be describe by parabolic diffusion equation which is controlled by a number of diffusion mechanism. For HCHs, their release behaviour in oxalic acid system conforms to parabolic diffusion equation, and that in tartaric acid and citric acid system are more consistent with Elovich equation .

3.2.3 Discussion on the release mechanism of organochlorine pesticides by LMWOA

The difference of organochlorine pesticides release pattern in different LMWOA systems may be related to the differences of the pesticides' three-dimensional structure and different action mechanism of LMWOA on different bound pesticides on the soil surface. Hydrophobic pesticides are adsorbed mainly through hydrophobic force, van der Waals force, hydrogen bonding and other in soil internal space systems, inorganic mineral surface (surface physical adsorption), amorphous organic matter (soft carbon, fast linear distribution) and aggregate organic matter (hard carbon, slow linear adsorption) four regions, especially in the latter two regions that the inherent soil organic matter (SOM) contributed the most, and the soft carbon-bound pesticide has not solute competition and hysteresis of sorption and desorption, which explains the fast and slow release process (Chiou *et al.*, 1986). LMWOA with Carboxyl and hydroxyl can affect the migration of pesticides by competitive adsorption, structural changes (such as the aggregate decentralized by chelating the metal ions that acting as cross-linking agent in the SOM) of soil and SOM, the release (Chiou *et al.*, 2000) and mineral dissolution (Landrum *et al.*, 1984), their release ability to pesticides related to their ability of the dissolution to soil minerals and multi-coordination ability (Yang et al., 2001). Oxalic acid with smaller molecule volume has two activity carboxyl functional groups, citric acid with larger molecule has three carboxyl and one hydroxyl, and tartaric acid have two carboxyl and two hydroxyl groups (Figure. 4). The effect of LMWOA on pesticides bound to soil organic matter includes unlocking action

and locking action, if the activated pesticides do not leave the reaction system, they may soon be locked and bound with SOM, and resulted in the pesticide level in the system to reduce. Locking and unlocking mechanism to pesticides is the dominant mechanism for oxalic acid with smaller molecule, and the action mechanism of tartaric acid and citric acid with larger molecules to pesticides dominated by the chelation is the same as to macromolecules DOM (Yang *et al.*, 2001) and unlocking mechanism, it can avoid free pesticides being locked again, Therefore, the release amount of pesticides by tartaric acid and citric acid is much higher than that by oxalic acid and water.

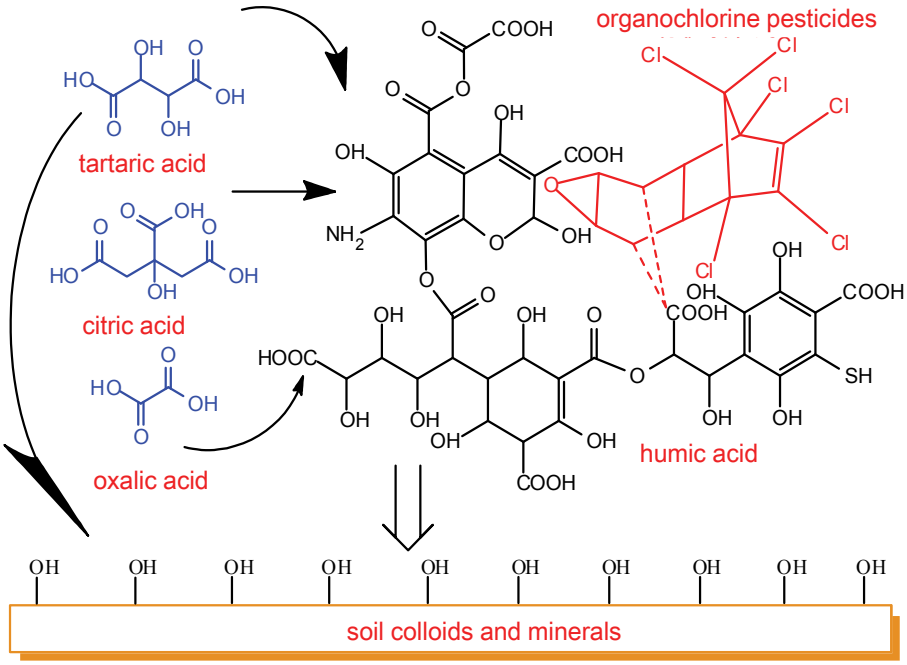

Fig. 4. Sketch about the interaction among pesticides, LMWOA, humic acids and soil mineral.

4. Conclusion

The results showed that the introduction of LMWOA could accelerate the release of the tested organochlorine pesticides (relative to the water, increased 15%~18% for DDTs, 7%~25% for HCHs). It implied that the LMWOA induced the complication of the kinetics release mechanisms of organochlorine pesticides (The best kinetics equation describing the release of pesticide changed from the first-order kinetic equation in water system to parabola diffuse equation, double constant equation or Elovich equation in LMWOA systems). It also indicated that the kinetics release mechanisms of OCPs by LMWOA involved not only the simple granular surface diffusion mechanism in water system, but also the outward diffusion mechanism of soil particles internal, activation and inactivation function of granular surface, the non-uniform mechanism of surface energy distribution induced by the solution of soil mineral and structure change of soil inherent organic matter coating onto the soil mineral surface by LMWOA.

The release velocity of HCHs was far higher than that of DDTs by water and LMWOA. Their difference was nearly 3 times. The variation amplitude of the release velocity and the influence of elution volume on release velocity for DDTs by water were all small and not obvious. The release velocity curves of OCPs from soil by LMWOA were all peak-type curve, and it included 2 stages which are rapid release and low release.

5. Acknowledgment

The authors would like to acknowledge National Science and Technology major program (2009ZX 07317-007), National Natural Science Foundation of China (50839002), Ministry of Education Key Laboratory of Integrated Regulation and Resource Development on Shallow Lakes foundation, Hohai University (2007KJ003) and the Fundamental Research Funds for the Central Universities (2009B17014).

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Fate of Pesticides in Soils: Toward an Integrated Approach of Influential Factors

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1. Introduction

Despite constraining legislation and increasing efficiency of pesticides (with a decrease in the applied amounts), their use still cause a contamination of environment (air, soil and water). To conciliate agricultural and environmental interests, a better understanding of the fate of pesticides is needed, in particular because it will determine the exposure and consequently the impact of pesticides on the target and non-target organisms. This goal requires new efforts of research at different scales (from molecular to field scale). Following application, most of the pesticides reach the soil either after direct application or after foliage wash-off. As a major interface between other environmental compartments, the soil plays a preponderant buffering role in the fate of pesticides. Apart volatilization, the main processes that control the fate of pesticides in soils are retention on soil particles and degradation (biotic and abiotic). These coupled bio-physico-chemical processes can lead to a transitory or permanent accumulation of pesticides in soils or, on the contrary, to their elimination from the environment. They determine the pesticide concentration in the soil solution, and have a large influence on pesticide transfer toward ground or surface waters and on their ecotoxicological impacts on soil organisms as well. The main difficulties in studying and predicting the retention and degradation of pesticides in soils are the diversity of chemical structures and reactivities of pesticides, the high diversity of soils and their heterogeneous composition and structure. In addition, the pedoclimatic conditions, in particular soil temperature and water content, have a strong influence on retention and degradation because of their effect on soil biological, chemical and physical properties. Therefore, the objective of this chapter is to provide an overview of the factors involved in the retention and degradation of pesticides in soils and to discuss and clarify the needs of new integrated approach. In particular, this work will examine (i) the pertinent scales (among elementary constituents, aggregates and mesoscopic scales) for both retention and degradation studies, (ii) the integrative properties that should be considered, such as hydrophobicity of the organo-clay granulometric fraction or soil structure, and (iii) the primordial role of water.

2. Retention

2.1 Definitions

The retention of pesticides in soils is mainly due to the adsorption, which is the passage of a solute from an aqueous phase to the surface of a solid adsorbent (Calvet, 1989). The solid

adsorbents are the different soil constituents. According to the properties of pesticides and adsorbents, several adsorption mechanisms are possible: hydrogen bindings, ion exchanges, interactions with metallic cations, polar interactions, charge transfers, London-Van der Waals dispersion forces and hydrophobic effects (Calvet et al., 2005). As the soil constituents contain polar and ionisable groups, the adsorption of pesticides possessing polar and non polar groups can involve several of these mechanisms. The reverse process of adsorption is desorption. In general, the desorption is inversely related to adsorption, being small when adsorption is great, and conversely (Mamy & Barriuso, 2007). For example, the adsorption of atrazine is fully reversible (Celis et al., 1998); on the contrary that of glyphosate is not reversible and hysteresis is observed (Mamy & Barriuso, 2007). The hysteresis can be due to irreversible adsorption, physical entrapment in organo-mineral aggregates or degradation.

2.2 Methods of measurement

Batch experiments. Most of the time, the retention of pesticides is measured with soil suspensions, known as batch experiments, according to the OECD 106 guideline (OECD, 2000a). A volume (generally 10 mL) of an aqueous solution of pesticide is added to a mass of dry sieved soil (generally 2 g) in glass centrifuge tubes. Soil suspensions are shaken mechanically for 24 hours in darkness and then centrifuged. The duration of 24 hours corresponds to the time needed to reach equilibrium between the adsorbed pesticide and the pesticide in solution. The degradation of the pesticide or the adsorption of the pesticide on the surface of the flask used for the experiment has to be determined (Calvet, 1989). The amounts of adsorbed pesticide in the soil are calculated as the difference between initial pesticide concentration in solution and centrifuged supernatant concentration. This experiment is done at several initial pesticide concentrations to determine the adsorption isotherm of the pesticide (adsorbed amounts as a function of the equilibrium concentration of pesticide). In general, the smaller the concentration, the greater the adsorbed amount per unit mass soil. From this isotherm, distribution coefficients between soil and soil solution can be determined according to the Freundlich (Kf) model (1):

$$Kf = Qs \ / \ Ce^{nf} \quad (1)$$

where Qs (mg kg^{-1}) is amount of adsorbed herbicide in soil at equilibrium concentration, Ce (mg L^{-1}) is pesticide concentration in supernatant solution, and nf is an empirical coefficient. When nf = 1, the isotherm is linear and Kf = Kd (L kg^{-1}). As organic carbon is a major adsorbent for pesticides (see 2.3.2.1), the Koc (L kg^{-1}) (2) coefficient is often calculated as:

$$Koc = (Kd \times 100) \ / \ Corg \quad (2)$$

where Corg is the percentage of organic carbon content in soil.

For a given pesticide, the Koc is generally less variable than the Kd among different soils (Calvet, 1989). However, the intensive shaking of soil-pesticide solution leads to dispersion of the soil structure, resulting in a higher availability of sorption sites. Therefore batch overestimates the sorption of pesticides (Walker & Jurado-Exposito, 1998).

Centrifugation. The soil sample is prepared at realistic soil moisture, treated with the pesticide, incubated, then centrifuged to collect the soil solution which is directly analysed for pesticide concentration (Benoit et al., 2007; Walker & Jurado-Exposito, 1998).

Filters. Gaillardon & Dur (1995) developed an original method using remoulded soil samples that are placed in Petri dishes to give a 3-4 mm thick soil layer at controlled water

content. The soil solution is sampled with glass microfibre filters laid on the soil surface. The volume of the soil solution and the dissolved pesticide retained in the filter are determined. This method could be adapted to undisturbed soil samples.

Soil columns. The soil columns allow the study of retention in dynamic conditions. The column is filled with disturbed (sieved or small aggregates) or undisturbed soil, a solution of pesticide is applied at the top of the column then water flow is imposed through rainfall simulation or pressure head control (Pot et al., 2005; 2011). Sorption coefficients can be estimated by inverse modelling of the elution curves and retardation factor calculation (Lennartz, 1999). Column experiments allow the possibility to determine the vertical distribution of the pesticide residues in the soil core if recovery in the leachates is incomplete (Benoit et al., 2000; Vincent et al., 2007). In a recent study, Vereecken et al. (2011) analysed the relationships between Koc derived from column data with more classical batch experimental data obtained on the same soils. The authors concluded that such relationships depended on pore water velocity and on the saturation status (saturated *vs* unsaturated) and packing status (disturbed *vs* undisturbed) of the soil column.

Lysimeters. A lysimeter consists of an undisturbed soil block or cylinder, embedded in an inert container with a bottom permeable to drainage water or leachate. An outstanding feature of lysimeters is the capability to monitor mass fluxes of water and chemicals under field climatic conditions and representative crop practices (Saison et al., 2008). The distribution of the chemical and of its metabolites in the soil along with their transformation rates can also be determined. Compared to laboratory experiments, outdoor lysimeter studies are closer to field environmental conditions, there is no significant disturbance of the soil structure, but the major limitation is the fact that certain variable experimental conditions such as environmental/climatic parameters are not controlled (OECD, 2000b).

2.3 Factors controlling the retention of pesticides in soils

2.3.1 Physico-chemical properties of pesticides

Surface, volume, and branching. In general, the adsorption of pesticides increases with the volume and with the degree of branching which is correlated to the surface area (Mamy & Barriuso, 2005; Sabljic et al., 1995). Indeed, the molecular volume is related to water solubility (see hydrophilic/hydrophobic balance) (Calvet, 1989), and the degree of branching encodes the intermolecular accessibility (Kier & Hall, 2000).

Electronic structure. The nature of atoms and of functional groups determines the electronic structure of the pesticides (therefore their permanent dipole moment and polarizability) that governs the type of interactions of pesticide with soils (donor-acceptor electron, hydrogen bonds) (Calvet, 1989). The different substitutions and their spatial arrangement in the molecule have an effect on the adsorption by influencing the reactivity of functional groups (carbonyl oxygen, amide nitrogen) participating in these bond interactions (Liu et al., 2000).

Ionization. It determines the charge of the pesticide and depends on its electronic structure. Strong bases always occur in cationic form in soils, but the ionization of weak bases and weak acids depends on the pH of the soil and on pKa or pKb values of molecules (Calvet, 1989). In general, the sorption of cations is strong on negatively charged surfaces like clays, oxides, hydroxides and humic substances. On the contrary, anions are not adsorbed on these surfaces, but their sorption is high in soils with positive charges, like tropical soils. For example, glyphosate has four pKa so that its sorption increases when the soil pH decreases because the number of negative charges of this herbicide decreases.

Hydrophilic/hydrophobic balance. The hydrophilicity of pesticides is defined by their water solubility and the hydrophobicity by their octanol/water partition coefficient. In general, the adsorption of pesticides decreases when their water solubility increases because of their high affinity for the water phase, and conversely, the adsorption increases with the hydrophobicity of pesticides. However, it also depends on the hydrophilic/hydrophobic balance of the soil adsorbents. The adsorption of polar compounds does not always decreases with increasing water solubility (Calvet, 1989). For example, glyphosate, a polar herbicide, is highly soluble in water (12 g L^{-1}) but is strongly sorbed to soils (Mamy & Barriuso, 2005). Indeed, the sorption of ionisable molecules and molecules with phospho-groups like glyphosate involve high-energy binding adsorption phenomena (ionic and coordination bindings, complex formation with metals in solution or at the solid–liquid interphase) that overbalance the effects of high solubility (Tao & Lu, 1999).

2.3.2 Soil properties

2.3.2.1 Elementary properties (minerals, organic matter, pH)

Minerals. The mineral adsorbents involved in the adsorption of pesticides are clays (as silicate minerals), oxides and hydroxides (Calvet, 1989). Their surfaces are mainly hydrophilic because of hydroxyl groups and exchangeable cations. The adsorption of pesticides on clay minerals is likely to occur on external surfaces of clay particles rather than in interlamellar space and increases with the specific surface of clays (Barriuso et al., 1994). Oxides and hydroxides are frequently associated to clays, they have a high surface activity and their charge depends on the soil pH (Calvet, 1989). For example, the adsorption of glyphosate increases as follows: kaolinite < illite < montmorillonite < nontronite (Mc Connell & Hossner, 1985). The adsorption of glyphosate on iron and aluminium oxides and hydroxides is high at intermediate pH and driven by ionic bindings between the positive surface sites of minerals and the negative acid groups of glyphosate (Morillo et al., 2000). However, sorption is much lower at very acid or very alkaline pH because oxides will bear the same charge as glyphosate.

Organic matter. Soil organic matter originates from crop residues, microbial biomass and organic amendments. It has very heterogeneous composition and contains both hydrophilic and hydrophobic groups (Calvet et al., 2005). Even if organic matter only represents few percents of the total dried material in soil, it is a major sorbent of pesticides in soil (Calvet, 1989). This is attributed to its high chemical reactivity towards both mineral surfaces and organic molecules, allowing various types of interaction with pesticides. The sorption capacities of organic matter are not only controlled by their chemical composition, but also by their size, due to a greater number of sorptive sites related to a greater surface area with decreasing particle-size (Benoit et al., 2008). In general, the adsorption of pesticides increases with organic matter, except for ionic molecules.

Soil pH. The soil pH plays an important role in particular for the adsorption of ionic pesticides like glyphosate or sulcotrione (Calvet, 1989; Mamy & Barriuso, 2005). Depending on the charge of the pesticide, the adsorption will increase (or decrease) with pH. For example, the retention of glyphosate increases when the soil pH decreases because the number of negative charges of the molecule decreases, allowing the adsorption on negatively charged adsorbents like clay or organic matter.

2.3.2.2 Soil structure

The soil structure is characterized by the bulk density and the pore geometry which depend on agricultural practices and on the climate. In addition, the bulk density depends on the size of the soil sample (from aggregate to macroscopic scale) due to the spatial variability of the soil structure (Alletto et al., 2010a). Pesticide movement through aggregated soils is mainly controlled by kinetic sorption and diffusion (Beulke et al., 2004). In static conditions, the rate of pesticide adsorption decreases when the density of soil aggregates increases (Chaplain et al., 2008). In dynamic conditions, retention depends on transport parameters such as pore water velocity and residence time (Pot et al., 2005; 2011). Compared to tilled soils, the no tilled or grassland soils are characterized by the presence of biopores (due to earthworm burrows, roots…) and high content of organic matter in the surface layers. The retention of pesticides is therefore generally higher in these soils (Benoit et al., 2000; Larsbo et al., 2009). However, the increase in retention can be counter-balanced by increased preferential transport because no tillage leads to enhanced macropore connectivity (Larsbo et al., 2009).

2.3.3 Effect of environmental conditions (water content, temperature)

Water content. The soil water content defines the specific exchange surface between solid and liquid phases. The adsorption of pesticides increases with water content as it facilitates pesticide diffusion to sorption sites. As water content increases, the organic matter also becomes more hydrophilic with greater sorption potential for hydrophilic pesticides (Roy et al., 2000). For hydrophobic pesticides like trifluralin, the adsorption decreases when the soil water content increases because the hydration of the surfaces of adsorbents decreases the accessibility to adsorption sites (Swann & Behrens, 1972). Low soil moisture content might also favour access to the hydrophobic regions of humus by generating more hydrophobic surfaces, thereby increasing the sorption of hydrophobic substances (Roy et al., 2000).

Temperature. In general, the adsorption of pesticides decreases when the temperature increases (Ten Hulscher & Cornelissen, 1996). However, fast sorption should be differentiated from slow sorption: the fast sorption increases with decreasing temperature, but the slow sorption is more rapid at higher temperature. This could explain why for some compounds, overall sorption with short equilibration times is nearly independent of temperature. The slow sorption is generally due to diffusion of the pesticide through the organic matter and increasing temperature decreases the density therefore increases the diffusion (Ten Hulscher & Cornelissen, 1996). For some pesticides that exhibit decreasing solubility at higher temperatures, an increase in the sorption with temperatures can be observed (Chiou et al., 1979, as cited in Ten Hulscher & Cornelissen, 1996).

2.3.4 Spatio-temporal variability of retention

Spatial variability. The retention of pesticides varies laterally and vertically. For example, at the scale of one watershed, the variation coefficients of the Koc of several pesticides in the soil surface can reach 30%. It seems mainly due to the variation of organic carbon content (Coquet & Barriuso, 2002). But, the variability of pesticides adsorption is high even at smaller scales (cm to m) (Mermoud et al., 2008, Vieublé-Gonod et al., 2009). The adsorption of pesticides also varies with soil depth: in general, the adsorption decreases because of a decrease in the organic carbon content (Mamy & Barriuso, 2005).

Temporal variability. The retention of pesticides is affected by their residence time in soil because of diffusion into soil micropores, physical entrapment or degradation (Koskinen et al., 2001). In the long term, the interactions responsible for retention evolve to the formation of pesticides non-extractable residues (Barriuso et al., 2008). Non-extractable (bound) residues are pesticides in soils which persist in the form of the parent substance or its metabolite(s) after extraction (Fürh et al., 1998, as cited in Barriuso et al., 2008). A large increase in retention with time is generally observed for weakly adsorbed herbicides, but for strongly adsorbed herbicides, adsorption decreases or remains stable (Mamy & Barriuso, 2007).

3. Degradation

3.1 Biodegradation

3.1.1 Definitions

Metabolism of pesticides. The metabolism of pesticides in living beings involves numerous enzymatic reactions grouped in three phases (Bollag & Liu, 1990; Mougin, 2002). Phase 1, probably the most important one, includes functionalization reactions (oxidation, reduction, hydrolysis) of the parent compound. By the introduction of additional functional groups such as OH, NH_2, SH and COOH, these processes often result in the formation of metabolites with modified physiological and biological properties, and a predisposition for further metabolism in the secondary phase. Phase 2 is a synthetic process known as conjugation that results in the formation of final metabolites by linkage of the activated metabolite with cell constituents. These metabolites are distributed and sequestered by the organisms, or excreted. Phase 3 involves synthetic reactions leading to the oligomerization of several units of the parent compound, or secondary conjugation of the parent compound with cellular components of the cells. They contribute to the formation of high-molecular weight compounds or bound compounds, which are incorporated and stabilized within the cells. In moving from their initial state to phase 2 or 3, metabolites become generally more hydrophilic, except in the case of insoluble polymers. Their initial mobility is also reduced, as well as their toxic or hazardous power.

Key actors responsible of pesticide metabolism. The degradation of pesticide through microbial metabolic processes is considered to be the primary mechanism of biological transformation. The different groups of microorganisms, mainly the prokaryotic bacteria, actinomycetes and eukaryotic fungi, can mediate an almost infinite number of biochemical transformations. The most numerous organisms in soil are bacteria, whereas fungi form the largest biomass. They are involved in other numerous functions such as mineralization and humification of organic matter, biogeochemical cycles, production of toxins and compounds of interest (antibiotics). Bacteria are the main biodegraders in soils. They are present in all soils with a competitive pH near neutrality. Despite some characteristics in common with bacteria, actinomycetes are similar to fungi. They are more tolerant to alkaline and acidic soil pH and low moisture content than bacteria.

Processes involved in the biotransformation of pesticides. The processes responsible of the biodegradation of pesticides include biodegradation, cometabolism and synthesis.

During biodegradation, one or several interacting organisms metabolize the pesticide into inorganic components (like CO_2). The organisms obtain their requirements for growth and energy by mineralizing the molecule. If microorganisms do not possess all the enzymatic equipment to mineralize pesticides, metabolites are excreted in soil and can be mineralized by other microorganisms.

Pesticide metabolism in the environment is also governed by cometabolism in which organisms grow at the expense of a cosubstrate to transform the pesticide without deriving any nutrient or energy for growth from the process. Cometabolism is a partial and fortuitous metabolism and enzymes involved in the initial reaction lack substrate specificity. Generally, it results only in minor modifications of the structure of the pesticide, but these modifications could greatly influence pollutant bioavailability and mobility in soil. Different organisms (mainly fungi) can transform a molecule by sequential attacks, or can use cometabolic products of one organism as a growth substrate. In addition, intermediate products with their own bio-physico-chemical properties can accumulate, thus causing some adverse effects on the environment. The metabolites are generally less toxic than the pesticides, but they can be more toxic in some cases (Tixier et al., 2002).

Synthesis includes conjugation and oligomerization. Pesticides are transformed into compounds with chemical structures more complex than those of the parent compounds. During conjugation, a pesticide (or one of its transformation products) is linked to hydrophilic endogenous substrates, resulting in the formation of methylated, acetylated, or alkylated compounds, glycosides, or amino acid conjugates. These compounds can be excreted from the living cells, or stored. During oligomerization, a pesticide combines with itself, or with other xenobiotic residues (proteins, soil organic residues). Consequently, they give high-molecular weight compounds, which are stable and often incorporated into cellular components (cell walls…) or soil constituents (soil organic matter). This biochemical process not only affects the activity and the biodegradability of a compound in limiting its bioavailability, but also raises concern about the environmental impact of the bound residues (Barriuso et al., 2008; Bollag & Liu, 1990).

3.1.2 Methods of measurement

Pure liquid cultures. Pesticide metabolism can be studied with pure liquid cultures supplemented with fungal or bacterial inocula. These cultures are potent tools to precise the transformation pathways of pesticides and the relevant metabolites. For example, white-rot basidiomycetes have been extensively considered because of their high potential for xenobiotics transformation. These filamentous fungi degrade pesticides using two types of enzymatic systems: intracellular (cytochromes P450) and exocellular (lignin-degrading system mainly consisting in peroxidases and laccases). Each of these systems could also be induced or inhibited by pesticides, thus able to modulate their metabolism.

Soil and inoculum. Studies can be performed in soils favouring microbial development and activity. Some experiments involved bacteria as an inoculum to remove pesticide residues from contaminated soils in laboratory conditions (Duquenne et al., 1996) or *in situ* (Qureshi et al., 2009). In some cases, carriers are developed to ensure fungal growth in the spiked soil. Lignocellulosic materials, that provide also nutrients and easily available carbon for the organisms, have been often retained. Nevertheless, fungal growth or activity are rarely assessed using biological descriptors in biotransformation experiments.

Soil incubation (disturbed or undisturbed soils). The degradation of pesticides is often studied using soil incubation. Soil samples are treated with the pesticide and incubated in the dark under controlled laboratory conditions (at constant temperature and soil moisture). After appropriate time intervals, soil samples are extracted and analysed for the parent substance and for metabolites. Volatile products are also collected for analysis. Using ^{13}C or ^{14}C-

labelled material, the mineralization rates of the pesticide (allowing the quantification of biodegradation) can be measured by trapping evolved $^{13}CO_2$ or $^{14}CO_2$, and a mass balance, including the formation of soil bound residues, can be established (OECD, 2002). From the degradation kinetics, the half-lives (DT50) of pesticides are determined. This method can use either disturbed soil samples (sieved) or undisturbed soil samples such as aggregates or cores (Benoit et al., 2000; Mamy et al., 2011; Monard et al., 2010; Vieublé-Gonod et al., 2009).
Field studies. In field conditions, soil temperature and moisture are highly variable with consequences on pesticides degrading microorganisms. Therefore, the measurement of pesticide dissipation in the field is fundamental to complete the data obtained in the laboratory. The pesticide is applied according to the intended use and, at appropriate dates, the soil is sampled and analysed for the pesticide and its metabolites. The DT50 can then be determined.

3.1.3 Factors controlling the degradation of pesticides in soils

3.1.3.1 Physico-chemical properties of pesticides

There is no clear relationship between the chemical properties of pesticides and their rates of degradation because several phenomena are simultaneously involved in the degradation and because of the high variety of structures of pesticides (Calvet et al., 2005). Nevertheless, some atoms (halogens, and chlorine in particular) are known to be toxic to microorganisms (therefore a decrease of biodegradation is observed), but there is no general rule (Naumann, 2000). For example, there is almost no biodegradation of chlordecone (organochlorine insecticide) because of its highly chlorinated cage-like structure that makes chlordecone a poor carbon source for bacteria (George & Claxton, 1988, as cited in Cabidoche et al., 2009).

3.1.3.2 Effect of soil structure on the degradation of pesticides in soils and spatio-temporal variability

Soil structure. The different practices such as soil tillage or organic amendments may affect soil structure by different mechanisms (aggregation of soil particles, creation of pore space) (Mapa et al., 1986), influence the hydraulic properties of the soil and hence pesticide transport through the soil (see 2.3.2.2). When an increase of leaching is observed in conventional tilled soil, this can be attributed to weaker sorption due to lower soil organic carbon content but also to slower microbial degradation (Gish et al., 1995 and Sadeghi et al., 1998 as cited in Alletto et al., 2010b). Tillage also affects soil organic carbon location and sequestration as well as water retention properties. This may lead to different sorption and degradation properties depending on tillage practices (Alletto et al., 2008; Stenrød et al., 2006). In agricultural soils, soil structure is affected by compaction under wheel tracks. Vieublé Gonod et al. (unpublished data) studied the impact of compaction on the fate of isoproturon in soil in laboratory. They did not observe any effect of compaction neither on isoproturon mineralization nor on its availability. The presence of fresh organic matter originating from urban waste compost did not modify the observed results. This was consistent with the results of Mamy et al. (2011) who showed that, in the conditions of the experiment, soil compaction did not modify significantly the degradation of isoproturon, neither the formation rates nor the nature of its metabolites.
Spatial localization of microorganisms. The spatial heterogeneity of microorganisms in heterogeneous soil structure plays an important role in microbial processes and in the persistence of pesticides (Strong et al., 1998). For example, Vieublé-Gonod et al. (2003)

observed that 2,4-D mineralization increased with the size of aggregates (inter classes heterogeneity) but varied also a lot in aggregates of a same size class (intra class heterogeneity). Authors explained this variability by an uneven distribution of the degrading microorganisms at this scale, and to a lesser extent, by an uneven distribution of carbon, necessary for cometabolism. Soil aggregates or small soil cubes enriched in 2,4-D degrading microorganisms were not distributed randomly in soil, but rather grouped into centimetric hot spots (Vieublé-Gonod et al., 2003; 2006). In a simulation, Vieublé-Gonod et al. (2006) showed that if soil cubes having a high potential of mineralization (hot spots of mineralization) were either not in direct contact with the pesticide (within soil clods, not exposed to preferential flow paths) or in direct contact with it (on the periphery of clods), 2,4-D mineralization was differently affected and was equal to 2% and 75% of added C, respectively. Cohen et al. (unpublished data) studied the impact of soil structure on isoproturon mineralization in small columns made of structured or sieved soil sampled in plots amended or not with a co-compost of green waste and sewage sludge or with a municipal solid waste. They showed that soil structure significantly affects isoproturon mineralization only in soil amended with compost (not in the control). But the effect also depended on type of compost. In soil amended with the co-compost of green waste and sewage sludge, isoproturon mineralization was stimulated in structured samples whereas in soil amended with a municipal solid waste compost, opposite results were observed. At a smaller scale, when comparing the mineralization of 2,4-D by intact aggregates to that of crushed ones, Vieublé Gonod et al. (2003) highlighted that disruption of the soil structure by crushing did not affect pesticide mineralization.

Effect of time. Soil structure can protect a fraction of pesticide from biodegradation and this phenomenon is emphasized when pesticides are "aged". Indeed, some works noticed that, over time, xenobiotics may become resistant to biodegradation (*e.g.* Nam et al., 1998) because pollutants may become physically segregated from degrading bacterial species. Hydrophobic pesticides could become inaccessible through their diffusion within the organic matter or micropore network (Shaw et al., 2002). Steinberg et al. (1987) demonstrated that degradation of aged 1,2-dibromomethane by indigenous microorganisms was negligible when compared to rapid mineralization of freshly added compounds and explained it by the entrapment of aged compounds in intraparticle micropores.

3.1.3.3 Effect of environmental conditions (water content, temperature)

Water content. Variations in soil moisture have effects on the diffusion of soluble substrates and on the mobility of microorganisms at lower soil water content, while at higher soil moisture diffusion of oxygen can become restricted, both of which affecting activity of soil microbial communities (Skopp et al., 1990). It is important to consider water content at a given pressure head rather than to compare gravimetric or volumetric water content (Alletto et al., 2006; 2008). Generally, aerobic microbial activity increases with soil water content up to a maximum point before decreasing (Linn & Doran, 1984). For example, Schroll et al. (2006) showed an optimum mineralization for isoproturon, benazolin-ethyl, and glyphosate at a soil water potential of -0.015 MPa, whereas, pesticide mineralization was considerably reduced when soil moisture approximated water holding capacity. Some fungi such as the white-rot *Trametes versicolor* may degrade triazines under low water availability conditions (Bastos & Magan, 2009). Because fungi often degrade pollutants using exocellular enzymes, these enzyme-catalyzed processes should be regulated in soils with respect to moisture for an optimal activity (Baldrian, 2009). Soil moisture affects not only the activity but also the

diversity of soil microbes (Bouseba et al., 2009). For example, fungi have an adaptative advantage with respect to bacteria to water fluctuations. The hyphal/mycelial growth form, present in fungi, facilitates organisms to cross water-poor or nutrient-poor spots (Yuste et al., 2011). As a consequence, fungi can tolerate a broad range of environmental conditions, including moisture content. The reduction of the microorganisms activity at lower soil water content could increase the contact times between the pesticide and the soil components, and so may favour long-term sorption (Cox & Walker, 1999). It could explain why some authors concluded that the amount of bound residues seemed to be inversely proportional to mineralization. These results also explain why pesticide degradation is reduced (with increase in persistence) in areas characterized by elevated temperature and limited rainfall (Bouseba et al., 2009).

Temperature. Alletto et al. (2006) demonstrated that isoproturon mineralization was largely affected by temperature in both surface and subsurface soils but degradation seemed more sensitive to water content variations than temperature ones. Pesticide biodegradation could also be affected in temperate regions if global warming lead to drastic changes (Bouseba et al., 2009). For example, Levy et al. (2007) showed that the harsh summer conditions of 2003 altered the structure of the microbial communities involved in isoproturon biodegradation and decreased severely isoproturon mineralization. In colder regions, slight increase of temperature in spring and fall and more frequent freeze-thaw cycles due to climate change modify herbicide degradation and can drastically affect the availability for leaching (Benoit et al., 2007; Stenrød et al., 2005).

3.2 Abiotic degradation

Abiotic degradation of pesticides in soil has mainly been studied through laboratory experiments performed under controlled conditions. Identification of the reaction mechanisms and pathways is essentially performed using simplified matrix such as pure or commercial soil components. However the processes are ascertained in soils, through pesticide and its by-products monitoring, and evaluated through kinetic and comparative studies. Chemical reactions are generally performed using large amount of water in front of soils or soil components, but sometimes at realistic soil water content; dry soil matrixes are only used in photodegradation studies. To distinguish abiotic processes from biodegradation, soils are sterilized through various technics: generally autoclaving, but also adding biocide chemical (NaN_3, $HgCl_2$) or γ-irradiating with ^{60}Co. The influence of temperature on abiotic degradation will not be treated in this chapter. Globally it plays a role through adsorption-desorption equilibrium and Arrhenius law (Calvet et al., 2005).

3.2.1 Hydrolysis

Hydrolysis is defined as a chemical transformation by nucleophilic displacement of an organic compound RX with water to form ROH and a leaving group X (3):

$$RX + H_2O \rightarrow ROH + X^- + H^+ \quad (3)$$

In addition to reaction (3), hydrolytic reactions can be acid- or base-catalyzed (*ie* by H_3O^+ or OH^- ions, respectively). Therefore, water availability and pH are key-factors for reaction occurrence and kinetics.

Hydrolysis may be favored by metal-ion catalysis, through two mechanisms: (i) direct polarization, where the metal coordinates the hydrolysable function (eventually through chelate formation) making it more electrophilic and thus more reactive and (ii) *in situ* generation of a reactive metal hydroxo species. Surface-bound metals may also catalyze hydrolysis according to those two mechanisms and a third one, where the electrostatic interactions enhance the OH^- concentration in the aqueous phase (Smolen & Stone, 1998). The most cited reactive metals are +II in solution (Cu(II), Pb(II), Mn(II), Fe(II),…) whereas +III or +IV as surface-bound or solid particulate species (goethite α-FeOOH, γ-Al_2O_3, TiO_2,…). Clay minerals also act as hydrolysis catalyzers, mainly because of their surface acidic pH. In the case of azinphosmethyl, cations involvement has been hypothesized to proceed from the formation of bidentate complex pesticide-clay interlayer cations, which increases P electrophilic character (Sánchez-Camazano & Sánchez-Martín, 1991). The role of soil organic matter on hydrolysis is complex, as the pesticides may be sorbed on the organic matter or dissolved in water: both states have to be considered but can hardly be distinguished experimentally. In the solution compartment, acid-catalyzed hydrolysis may be enhanced by acidic functional groups of the organic matter (Gamble & Khan, 1985). On the opposite, base-catalyzed processes may be inhibited because of the pollutant adsorbed status. This could be due to the negative charges associated with organic particles, which result in a decrease of the effective surface pH and/or stabilization of the transition species during reaction (Noblet et al., 1996). Organic matter effects may also be explained by a micellar catalysis model (Georgi et al., 2008). Furthermore, soil amendment with organic matter favors biodegradation and may hinder the effect of amendment on chemical hydrolysis reactions, as observed for triasulfuron by Said-Pullicino et al. (2004). However, those authors evidenced that this sulfonylurea degradation through (bio)hydrolysis was slightly reduced by compost amendment. Hydrolysis is a major degradation process for sulfonylureas in acidic soil (eventually more efficient than biodegradation), when their neutral form is prevalent: effect of soils parameters and potential pathways were reviewed by Sarmah & Sabadie (2002). Soil pH variation induces changes in sulfonylureas DT50 from less than 10 days to more than 100 days; soil moisture, clay or organic matter content act in a lesser extent on kinetics.

3.2.2 Oxidation and reduction

Oxidation and reduction correspond to reactions in which oxidation number (state) changes in a molecule (or ion, or atom): oxidation for its increase and reduction for its decrease. It can also be described by a loss or gain of electrons, respectively. Both types of reactions are systematically coupled, thus named redox reactions. Thus to oxidize (or reduce) a pesticide, there should be in the soil environment a chemical able to behave as oxidant/electron acceptor (or reductant/electron donor), *ie* presenting adequate redox potential.

Oxidation. Oxidative mechanisms in soils may be mediated by both oxidative enzymes (Dec & Bollag, 2000) and abiotic catalysts such as metal oxides. Among them, manganese oxides and hydroxides are major contributors because of their reactivity and frequency in soils (Li et al., 2003). Redox potentials of MnOOH and MnO_2 make those species able to oxidize organic contaminants with functionalities such as phenol (Lin et al., 2009), aniline (Laha & Luthy, 1990) or triazine (Shin & Cheney, 2004). In a lesser extent because of their lower potential, Fe(III) oxides or Fe(III) adsorbed on smectite clays may also act as oxidants (Li et al., 2003). The oxidation reaction begins with the formation of a precursor complex between

the pollutant and the surface-bound metallic species, followed by electron transfer within the complex and release of an organic cationic radical, that will evolve to an oxidized form or bind to a vicinal reactive species. Through the development of a mechanism-based kinetic model, Zhang et al. (2008) demonstrated that environmental factors may affect either the reaction kinetics of MnO_2-pollutant complex formation or the rate of electron transfer, directly or through action on the reactive surface sites. Among solutes, Mn(II) presents the strongest inhibitory effect, followed by other divalent cations as Ca^{2+} (Zhang et al., 2008). The pH plays a role by affecting ionizable pollutants and reactive surface sites, changing the speciation of MnO_2 surface. Organic matter may affect the reaction by reducing reactive surface sites through two processes: by contributing to the Mn-oxide reductive dissolution and by coating the mineral oxidant. Furthermore, it commonly reacts with the cationic radical and form covalent binding, resulting in bound residues (Li et al., 2000). Another abundant oxidizing agent in soil upper layers is oxygen, both in gaseous form and dissolved in liquid surface films. As a marginal pathway in soils, oxygen causes autooxidation or weathering through a radicalar mechanism involving $O_2^{-\bullet}$ production (Larson & Weber, 1994).

Reduction. In soils, pesticide reduction happens currently in suboxic and anoxic conditions encountered in poorly drained or groundwater-fed soils, riparian zones, wetlands or flooded areas and sediments. Nature of the reductants includes chemicals or "abiotic" reagents as reduced metal, sulfide ion or natural organic matter (Borch et al., 2010), but also extracellular biochemicals such as metal chelated in porphyrin or corrinoid or as transition-metal coenzymes (Kappler & Haderlein, 2003). Extensive studies using nitroaromatic contaminants as probe (such as trifluralin) showed that they are predominantly reduced by Fe(II) associated with iron minerals surfaces (Colon et al., 2006; Klupinski & Chin, 2003). Natural organic matter and particularly quinone moieties may also play a determinant role as an electron shuttle (Kappler & Haderlein, 2003). Furthermore, abiotic and biotic processes may be closely associated: Zhang & Weber (2009) demonstrated that reducible organic contaminants compete with iron oxides for the electron flow generated by the microbially mediated oxidation of organic carbon and subsequent reduction of quinone functional groups associated with dissolved organic matter.

3.2.3 Photodegradation

Photochemical reactions, *ie* reactions induced by UV or visible light, may degrade pesticides according to two types of processes, known as direct photolysis and indirect photodegradation. In direct photolysis, the pesticide itself absorbs light energy, becomes excited and, depending on the reaction activation energy, may undergo a transformation reaction. On the opposite, indirect photodegradation is defined as reaction of a ground-state pesticide with another photochemically produced species. This species can transfer energy, undergo an electron or hydrogen transfer, or lead to the formation of reactive entity (singlet oxygen, radical) which reacts with the pesticide. Reaction mechanisms and pathways were extensively reviewed in water and soil (Burrows et al., 2002; Katagi, 2004).

Light penetration in soil. The intensity and spectrum of sunlight reaching the soil depends on time of the day, season, latitude, altitude and state of the atmosphere (*eg* clouds or dust absorbing light), with shortest wavelength λ around 290 nm. In sunlight-exposed soils, photodegradation occurs within a shallow surface zone; its depth depends on soil characteristics and on the photodegradation mechanism. Hebert & Miller (1990) showed in

sandy loam soils that direct photolysis was restricted to 0.2-0.4 mm *ie* the photons penetration depth, while indirect process was deeper, 0.7 mm in mean but up to 2 mm or more due to the vertical migration by diffusion of active species and/or pesticide. Soil characteristics playing a role in photons penetration are essentially its light absorption and scattering by particles. Both vary with wavelength and moisture content (Ciani et al., 2005a). Through light absorption and scattering, soil texture plays a major role on light penetration: Gonçalves et al. (2006) found that higher porosity of a sandy soil in front of sandy loam ones may be responsible for the faster direct photolysis of quinalphos.

Direct photolysis. In direct photolysis, reaction kinetics (evaluated through degradation rate constant k) depends not only on the light intensity in the medium I(λ) as upper mentioned, but also on the pesticide molar absorption coefficient ε(λ) and on the quantum yield Φ(λ) of the chemical process (*ie* the efficiency by which photon energy is used for a chemical reaction) through equation (4):

$$k = \int_{\lambda} I(\lambda).\varepsilon(\lambda).\Phi(\lambda).d\lambda \quad (4)$$

The functions ε(λ) and Φ(λ) depend on the organic compound state, such as its ionization form and its sorption on soil components. Sorption may cause a bathochromic shift, reaching 45 nm for trifluralin on kaolinite or silica (Ciani et al., 2005b), and a hyperchromic shift is suspected (Ciani et al., 2005b; Menager et al., 2009). These modifications of ε(λ) favour the photolytic process. The effect of sorption on quantum yield Φ(λ) has been evaluated by Ciani et al. (2005c) through the determination of I(λ), ε(λ), the diffusion parameters and the knowledge of degradation pathways, which may change with irradiation wavelength. They demonstrated that the quantum yield of 4-nitroanisole on kaolinite was on the same order of magnitude as in water, whereas that of trifluralin was 10 times smaller than in water. Furthermore, for aromatic compounds, triplet lifetimes are extended when adsorbed on silica or alumina, which means they may be more susceptible to photodegradation (Larson & Weber, 1994).

After solar light absorption, excited singlet states convert to triplet states (PX*) and undergo homolysis ($\rightarrow P^{\bullet} + X^{\bullet}$), heterolysis ($\rightarrow P^{+} + X^{-}$ or $P^{-} + X^{+}$) or photoionization ($\rightarrow PX^{+\bullet} + e_{aq}^{-}$) (Burrows et al., 2002). The intermediate ions or radicals further evolve and lead to formation of by-products that may correspond to some also observed under other abiotic processes (*eg* photohydrolysis, photooxidation, deshalogenation) or to more specific by-products (*eg* photoisomerization, cyclization). According to reviews (Burrows et al., 2002; Katagi, 2004) the degradation schemes, although generally identical in water and soil, may present some singularities when the pesticide is adsorbed on soil. For instance, fipronil photodegradation scheme presents only one pathway in soil instead of two in water (Bobé et al., 1998a). Soil characteristics may play a different role according to the pesticide and its own properties: photodegradation efficiency was found to decrease when adsorption (Kf) increased for fipronil (Bobé et al., 1998a), or when pH increased for the sulfonylurea chlorimuron-ethyl (Choudhury & Dureja, 1997). Humic substances tend to decrease direct photolysis through light screening, static quenching (inhibition of the PX* formation through complexation of the ground-state molecule) and dynamic quenching (energy transfer from PX* to humic substance) (Walse et al., 2004). Specific study of direct photolysis in soil is complex because indirect processes may interfere, even if they can theoretically be distinguished through a two-steps kinetic model (Albanis et al., 2002; Hebert & Miller, 1990; Tajeddine et al., 2010).

Indirect photodegradation. In indirect photodegradation, soil components absorb sunlight and form a reactive species (^{3}HS* for humic substances HS) able to transfer energy, electron or hydrogen to the pesticide, or also to form a reactive intermediate that will degrade the pesticide (in the presence of water or oxygen: $^1O_2$, $^\bullet OH$, $O_2^{-\bullet}$ or its conjugate $HO_2^\bullet$ (pKa 4.8), $^\bullet OOR$, $^\bullet OOH$ and/or H_2O_2). HS may play all those roles, but may also reduce indirect photodegradation by light screening and by scavenging the reactive species. The resulting effect can be a photodegradation increase (Albanis et al., 2002; Besse et al., 2005; Xiaozhen et al., 2005) or decrease (Albanis et al. 2002; Tajeddine et al., 2010). Non-transition metal oxides such as aluminium oxide or magnesium oxide can photocatalyze the $^1O_2$ generation (Gohre & Miller, 1985). Transition metals such as iron and manganese, under oxide or hydroxide forms or included in clays, can act as quenchers or as catalytic sites for reactions, as electron mediators. In the presence of water, iron plays a great role in generating $^\bullet OH$, mostly through photo-Fenton reaction (Fe(II)/H_2O_2) or by reaction of a Fe(III)-HS complex. Clay minerals are also known to act on photodegradation. Existing literature tends to attribute their role to: 1) their sorption properties to retain pesticides at soil surface, 2) the bathochromic (and maybe hyperchromic) effects of sorption, 3) $^1O_2$, $O_2^{-\bullet}$ and $^\bullet OH$ active species formed by irradiation of clay in the presence of water and/or oxygen, 4) screen effect and steric constraint that photostabilize the sorbed pesticide, 5) charge or energy transfer of the photoexcited clay to the pesticide or of the photoexcited pesticide to the clay, and 6) clays content and coating in (by) reactive metallic species and HS, forming clay-(metal)-HS complexes. Points 1, 2 and 3 favour the photodegradation, 4 decreases it, whereas the two other may result in an increase or a decrease of the photoprocesses. Photodegradation enhancement by 6 is linked to $^\bullet OH$ generation by free and structural irons under Fe(II) and Fe(III) forms (Wu et al., 2008). In soil, clay-metal-HS complexes may also decrease photodegradation, because of Fe(III) efficiency as energy or charge acceptor (Rozen & Margulies, 1991; Mountacer et al., 2011). Furthermore, as HS, silicate surface may trap peroxy radicals and thus lessen the process on soil surfaces.

Effect of soil water content. Water plays a major role, directly as a reactant to form active species such as $^\bullet OH$ or indirectly as a solvent, favouring presence of precursor chemicals (for instance metallic ions) and diffusion. Therefore, if the soil is dry, there is far less possibility of indirect photodegradation. On the opposite, as light penetrates more deeply through the interstitial spaces of dry soil (Ciani et al., 2005a; Frank et al., 2002), direct photolysis could be favoured by dryness. However, the global resulting pesticide photodegradation has largely been demonstrated to be slower in dry soils, with half-lives multiplied by a 2-7 factor or more (Frank et al., 2002; Graebing et al., 2003). Relative importance of the degradation pathways can also be influenced by the presence of water, for instance if a photohydrolysis mechanism is involved (Tajeddine et al., 2010). Drying/rewetting cycles favour indirectly the photodegradation process, because of the movement of the pesticide through condensation and evaporation (Frank et al., 2002).

Using atrazine as a probe, Xiaozhen et al. (2005) showed that photodegradation on soil was enhanced by: presence of water (facilitating movements), acidic or alkaline soil pH (H^+ and OH^- catalyzing reactions), smaller particle size (higher catalytic surface area), higher humic acid content and presence of a surface-active agent that increase pesticide solubilization. Agricultural practices, such as addition of fertilizer or compost, may modify the photodegradation processes. However, even if nitrate ions are major photoinducers in natural waters (Nélieu et al., 2009 and references therein), their addition

in soil does not increase photodegradation (Graebing et al., 2002). Compared to surrounding soil, earthworm casts were found to favour the atrazine photodegradation and make it preeminent in front of biodegradation (Besse et al., 2005), maybe because of changes in organic matter and minerals in casts. When compared to other biotic and abiotic degradation processes, the photodegradation may be dominant on dry, sunlight-exposed surfaces while other dissipation pathways prevail in deeper layers (Hebert & Miller 1990; Dimou et al., 2004). *In situ* experiments showed that by-products issuing from fipronil appeared in the following order over time: photodegradates, products of oxidation and eventually reduction, and products resulting from (bio)-hydrolysis (Bobé et al., 1998b).

4. Relevant scale and influent integrated factors

Macromolecular scale. Humic substances are now seen as an assemblage of small molecular weight components. The structure of organic matter is governed by its macromolecular nature and the competition between polar and apolar interactions depending on the physico-chemical environment (Sutton & Sposito, 2005). Hydrophobic structures such as flexible micelle-like structure involve intra-chain interactions, and rigid condensed phase includes inter-chain interactions (Chassenieux et al., 2000). Micelle-like structure is rather associated to aliphatic carbon, and condensed phase involves preferentially aromatic carbons (Huang et al., 2003). The polarity of organic mater can be estimated by O+N/C atomic ratio and by its aromaticity H/C ratio (Xing et al., 1994). Structural descriptors, using ^{13}C NMR techniques complete this description by determining the percentages of aromatic-, aliphatic- carboxyl- and carbonyl-C (Ahangar et al., 2008; Xing & Pignatello, 1997). The interaction of less polar pollutants with the micelle-like structure is a partitioning process characterized by linear isotherm and reversible behaviour (Sierra et al., 2005). Polar contributions between aromatic rings and substituted monoaromatic π systems increase the energy, enhance the non linearity of isotherms and hysteresis (Keiluweit & Kleber, 2009; Schlautman & Morgan, 1993). But no significant correlation between aromaticity or aliphaticity and sorption affinity was established (Chefetz & Xing, 2009; Pan et al., 2008a). The local molecular organization has to be considered (Ahangar et al., 2008; Huang et al., 2003; Jung et al., 2010).

Organo-clay complex. The organo-clay complex refers to the granulometric fraction < 2 µm. In soils, the clay surface was found to be completely saturated in carbon (Séquaris et al., 2010). In this fraction, the organic matter is present in dissolved and sorbed phases. Sorption of organic matter on clay minerals can be seen as a fractionating process because high molecular weight polymers are preferentially adsorbed (Pan et al., 2008b). High molecular weight and high surface area confer to the organo-clay complex a major role towards the retention of pesticides in soils. For example, the wettability of the clay fraction, characterized by the contact angle θ of water drop deposed on a thin air dried film, is directly related to the retention of diuron by the soil (Chaplain et al., 2008). The cosθ is related to the liquid and solid interactions by the Young–Dupré equation and the polar and apolar components of these interactions can be measured using appropriate solvents (Van Oss, 1994). We suggest that diuron can be used as a reference pesticide to characterize the global hydrophobicity of soil due to its high chemical stability in a large range of pH (Chaplain et al., 2008). Finally, as drying is known to enhance soil hydrophobicity (Diehl et al., 2009), an increase in the sorption of pesticide by re-wetting

soils is expected but this is poorly described (Lennartz & Louchart, 2007). Biological processes are also affected by wettability (Braun et al., 2010).

Aggregate scale or micro-habitat. Soil characteristics measured on bulk soil samples poorly reflect the local environmental conditions experienced by microorganisms (Christensen et al., 1990). Microorganisms' sizes range from the micrometer (bacteria) to millimetres or centimetres (fungi). The spatial heterogeneity of soil conditions at this micro-habitat scale directly influences their activity but is rarely taken into account (Parkin, 1993). In particular, the rate of biodegradation depends on the presence of specific microorganisms, the bioavailability of the compound (adsorbed or not, physically accessible or not), and on favourable environmental conditions. Each of these factors may be heterogeneous at the microscale. For example, studies of the mineralization of 2,4-D in a silty soil at microbial habitat scale showed that mineralization was spatially heterogeneous and that its variability increased when the size of micro-habitats decreased (Vieublé-Gonod et al., 2006).

Mesoscopic scale. Mesoscopic scale (around 100 cm^3) appears as the pertinent transition scale between laboratory studies and field measurements. In any cases, the water status, including the water distribution into the heterogeneous soil structure, can be controlled by imposing a given water potential. The integrative measurable parameters at this scale are the bulk density and the water content of soil samples. Our recent results confirm that this scale is suited to study the effects of soil structure on the fate of pesticide (Mamy et al., 2011). This scale is also appropriate to measure, in an integrative manner, the fate and ecotoxicological impact of pesticides in conditions close to the field ones. Finally, this scale is also relevant to study the effect of agricultural practices on bulk density: contrasted effects of no-till were shown with various soils. As mechanical properties of soil are closely related to soil structure and water content, we also found that the variation of these properties with the initial bulk density of soil is a good tool to predict the long-term effect of no-till on bulk density for various soils (Chaplain et al., 2011).

Plot scale. At the plot scale, our results showed that 2,4-D mineralization variability decreased when compared to micro-habitat scale (Vieublé Gonod et al., 2006). At this scale, the location of the added organic matter in soil is an important controlling factor. Indeed, the incorporation of crop residues or organic amendments by ploughing induces a heterogeneous spatial distribution of the added organic matter in the tilled layer. The effects of this spatial variation on the fate of isoproturon have been assessed by Vieublé-Gonod et al. (2009). They showed that interfurrows, containing important quantities of fresh organic matter, constituted a special local environment with the highest level of microbial biomass and the highest isoproturon mineralizing capacities and respiration levels. The presence of compost in the interfurrows stimulated isoproturon biodegradation relative to controls. This effect was more pronounced for the municipal solid waste compost than for the co-compost of green waste and sewage sludge. Heterogeneity may persist several months after compost or stubble hiding depending on the nature of added organic matter. Another important driver of heterogeneity at the plot scale is linked to water retention properties as shown for the degradation of glyphosate (Stenrød et al., 2006) and isoxaflutole (Alletto et al., 2008).

5. Conclusion

The fate of pesticides in the environment is mainly regulated by their behaviour in soils, and in particular by their adsorption and biotic and abiotic degradation. The extent of these

processes depends on the physico-chemical properties of pesticides (electronic structure, solubility…) and on the soil properties (constituents, structure at different scales…). They also strongly depend on the environmental conditions like temperature and water content. To improve the understanding of the fate of pesticides in soils, there is a need to better take into account the soils heterogeneity and variability. Studies have to consider relevant integrative parameters describing the soil structure and interfacial properties at various water contents. Depending on the scale, integrative parameters are, in increasing order: hydrophobic structures of organic matter, wettability of clay fraction, soil hydrophobicity, bulk density related to water retention and physical properties like mechanical resistance, hydraulic conductivity and water retention, and spatial repartition of exogenous organic matter. The water content and its distribution in heterogeneous structures should be considered simultaneously. In the perspective of the reduction of pesticide use, the main options are the choice of pesticide and of its commercial formulation, and the improvement of agricultural practices. To evaluate their effects, we propose to use the mesoscopic scale as a link between molecular and field scales, because it allows measurements of coupled processes with any kind of materials, including structured soils. Finally, the effects of wet-dry cycles on the fate of pesticides are poorly known and deserve to be studied in view of climate change.

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Edited by Margarita Stoytcheva

This book is a compilation of 29 chapters focused on: pesticides and food production, environmental effects of pesticides, and pesticides mobility, transport and fate. The first book section addresses the benefits of the pest control for crop protection and food supply increasing, and the associated risks of food contamination. The second book section is dedicated to the effects of pesticides on the non-target organisms and the environment such as: effects involving pollinators, effects on nutrient cycling in ecosystems, effects on soil erosion, structure and fertility, effects on water quality, and pesticides resistance development. The third book section furnishes numerous data contributing to the better understanding of the pesticides mobility, transport and fate. The addressed in this book issues should attract the public concern to support rational decisions to pesticides use.

ISBN 978-953-51-4425-0

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