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Pesticides Use and Management

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# **PESTICIDES IN THE MODERN WORLD – PESTICIDES USE AND MANAGEMENT**

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Edited by **Margarita Stoytcheva**

## **Pesticides in the Modern World - Pesticides Use and Management**

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Edited by Margarita Stoytcheva

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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.



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## Preface

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Volume 4 of the book series “Pesticides in the Modern World” contains 24 chapters organized in three sections. It brings together issues on pesticides and biopesticides use with the related subjects of pesticides management and sustainable development.

The first book section (Chapters 1-8) provides an overview on the current use of pesticides, reporting data collected from all over the world. Chapter 1 is dedicated to the trends in the pesticides use, frequency of exposure, and health effects in Central Europe. Topics on pesticide utilisation, regulation and future prospects in small scale horticultural crop production systems in Uganda are discussed in Chapter 2. The objective of Chapter 3 is to provide an updated study on the patterns and impacts of pesticides use in Sahelian countries, and especially in Burkina Faso. Chapter 4 furnishes an exhaustive information on the application of pesticides registered in South Africa to control pests and disease vectors, on the regulatory status, on the levels of contamination, and on the pesticides management options. Chapter 5 supplies an analysis on the use of pesticides in the horticulture in Mexico, taking into account the existing regulations and the emerging during the last years problems such as environmental deterioration, usage of pesticides restricted and prohibited in Mexico, and a lack of training in the proper management of conventional and alternative products.

In Chapter 6 are summarized the recent studies on the use and the mode of action of the petroleum derived spray oils, found to be effective against orchard pests.

Pesticides application systems and techniques, aimed to produce uniform and fine droplets with better droplet adhesion and distribution, higher depositing efficiency, lower environmental contamination, and lower pesticide application rate are reported in Chapters 7 and 8.

The second book section (Chapters 9-14) is devoted to the advances in the evolving field of the biopesticides. Chapter 9 provides an actual information on the regulation of the plant protection products from natural origin in the European Union, on the associated organic farming practices, on the mode of action and the impacts of the widespread botanical pesticides rotenone, azadirachtin and pyrethrins, and on the current and future perspectives of the natural pesticides. Chapters 10 and 11 address

the application of neem (azadirachtin)-based pesticides, considered as a promising alternative to the synthetic pesticides, and on the techniques for the development of controlled-release systems. Chapter 12 comments on the use of wood pyrolysis liquids as plant protection products, and reveals the barriers in the commercialization of the biological control agents. Chapters 13 and 14 are axed on the utilization of bacillus-based products as biopesticides, on the mechanisms involved in the biocontrol of the plant diseases, and on the toxicity of the pesticides formulations.

The third book section (Chapters 15-24) covers various aspects of pesticides management practices in concert with pesticides degradation and contaminated sites remediation technologies, supporting the environmental sustainability. Chapters 15-19 call the attention on the ecological effects of the pesticides, provide information on pesticides management science and techniques, and suggest strategies for reducing the pesticides reliance and to develop integrated pest management knowledge. Chapters 20-24 fill the existing gap in data on pesticides degradation and biodegradation processes, and environment remediation.

The present book is an important reference work for anyone involved in pesticides issues, such as pesticides use and management. It was made possible due to the knowledge and the expertise of the contributing authors. They are thankfully acknowledged.

**Margarita Stoytcheva**  
Mexicali, Baja California  
Mexico

# **Part 1**

## **Pesticides: Current Use**



# Trends in the Exposures to Pesticides in Central Europe

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Rakovcova Hana and Fenclova Zdenka

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Czech Republic*

## 1. Introduction

The occupational exposures of the humans to pesticides may occur in the gardens, fields, and forests during their dissemination, but also in the course of the preparatory activities, repair of application equipment, and their re-entry, where most symptomatic exposures happened (Meulenbelt & de Vries, 1997). Agricultural workers in several countries in Africa, Asia and South America are at greater risk of pesticide poisoning than non-agricultural workers and there appears a need for heightened efforts to better protect farm workers from pesticide exposure (Litchfield, 2005, Dasgupta et al., 2006, Calvert et al., 2008, Mancini et al., 2009).

Pesticides also became a part of our households and are used for both outdoor and indoor domestic application. As they are considered to be toxic by the general population, they are frequently ingested with the intent to commit a suicide. Globally, agricultural pesticides account for at least 250,000 suicide deaths each year, making pesticides the single most common means of suicide worldwide (Gunnell et al., 2007, Nguyen et al., 2010, Lin et al. 2010, Roberts et al., 2010).

Additionally, children are frequently exposed to any type of pesticide, mostly by ingestion. The exposure of children to pesticides may lead to a different outcome depending on the type of pesticide, geographic location, healthcare system and other variables (Hruskovic et al., 1984, Sudakin & Power, 2009), in South Africa it represents an increasingly important problem and may lead to death (Balme et al., 2010).

Not surprisingly, both home pets and farm animals may come in touch with the pesticides, either accidentally or due to aggressive behavior of some humans, trying to poison them deliberately.

An amount of information has been written on the application of pesticides, their degradation and analyses (Fischer et al., 2011). However, relatively little has been published about the trends in the pesticides use, frequency of exposures and their changing impact on the health in Central Europe.

Therefore, the aim of this chapter is to describe the situation based on the inquiries to the Toxicological Information Centre (TIC) in Prague, which serves to the total Czech population of approximately 10 million inhabitants. Since our last detailed evaluation of the calls 20 years ago (Pelclova et al., 1991) the situation has substantially changed.

## 2. Methods

Data from the TIC database from the period 1991 to 2010 have been retrospectively evaluated. In all years, the information on the type of pesticide, dose, time, and way of exposure; age and gender of the patient, reason of ingestion, symptoms, and treatment recommended was available. As the electronic recording system started in 1997, the registration of the calls by the physicians – toxicologists in the first period of this study, i.e. in the years 1991-1996, was available only in the hard copy. Based on the electronic recording system in the years 1997-2010, the evaluation of the clinical severity, prognosis, and detailed recommendation for the case management were also available. All intoxications were classified in accordance with the Poisoning Severity Score (Persson et al. 1998).

Additionally, the number of calls due to occupational exposures was compared with the electronic Registry of Occupational Diseases (Urban et al., 2000), which collects all of occupational diseases that were acknowledged and compensated in the Czech Republic. The Registry records the information about the patient, exposure, job, diagnosis, noxious factors; on the other hand, it does not include the acute injuries. Occupational diseases are classified according to the chapters and intoxications with pesticides are recorded separately from skin disorders caused by pesticides.

## 3. Results

### 3.1 Total calls due to pesticides

The number of total calls to the TIC continuously increases, as can be seen in Figure 1. In the year 2010 it reached almost 12 thousand per year. Inquiries due to pesticides in the years 1991-2010 amounted total 8530 and accounted for 5.52% of total calls. As shown in Figure 1, the proportion of the calls concerning pesticides slowly decreased from 7.81% in 1991 to 3.75% in 2010.

### 3.2 Seasonal variation

The calls to the TIC due to pesticides do not have a symmetric distribution throughout the year and they display a seasonal variation, as can be seen in Figure 2. About 79.7% of all exposures occurred in the vegetation period from April to October, during which the occupational and home utilization of pesticides prevailed. Only 20.3% of inquiries were asked in the other months. In the non-vegetation period the proportion of exposures from other reasons, such as suicidal attempts and accidental ingestions of pesticides was more pronounced.

### 3.3 The population concerned

Among the inquiries concerning pesticides, 44.7% (from 38.0 to 59.4%) concerned adults, 43.1% (36.9-52.1%) children, and 12.0% (2.9-22.3%) animals. The proportion of adults, children, and animals during the years 1997-2010 did not exhibit a certain trend, as can be seen in Figure 3.

As to the gender of the subjects in human exposures, 56.6% of calls involved men; 41.8% women; and the gender was unknown in 1.6%.

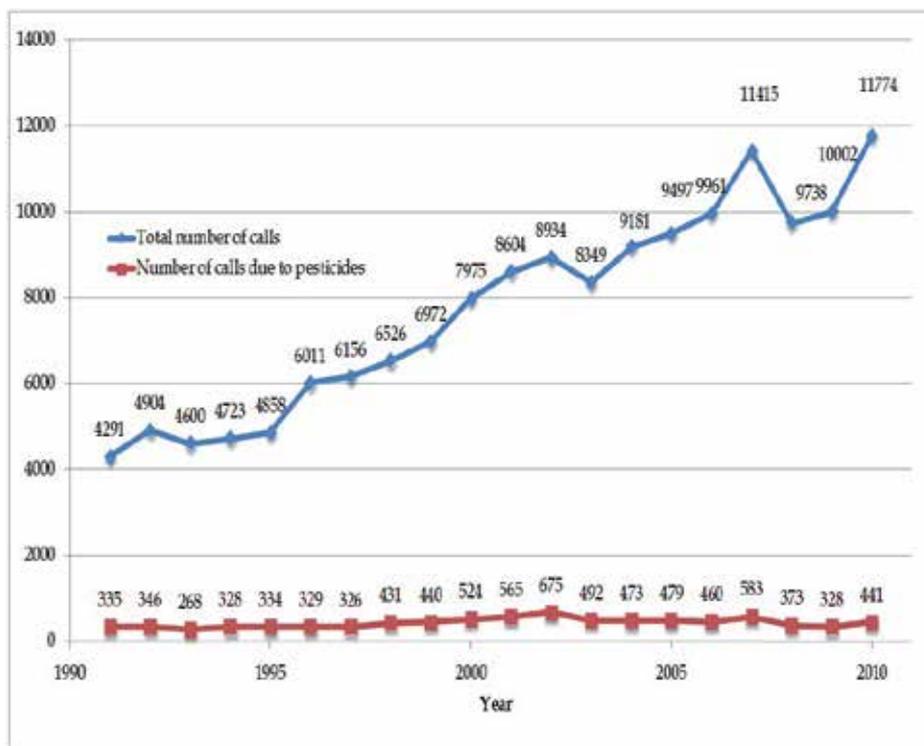


Fig. 1. Total number of calls to the TIC of the Czech Republic and the number of calls due to pesticides in the years 1991-2010

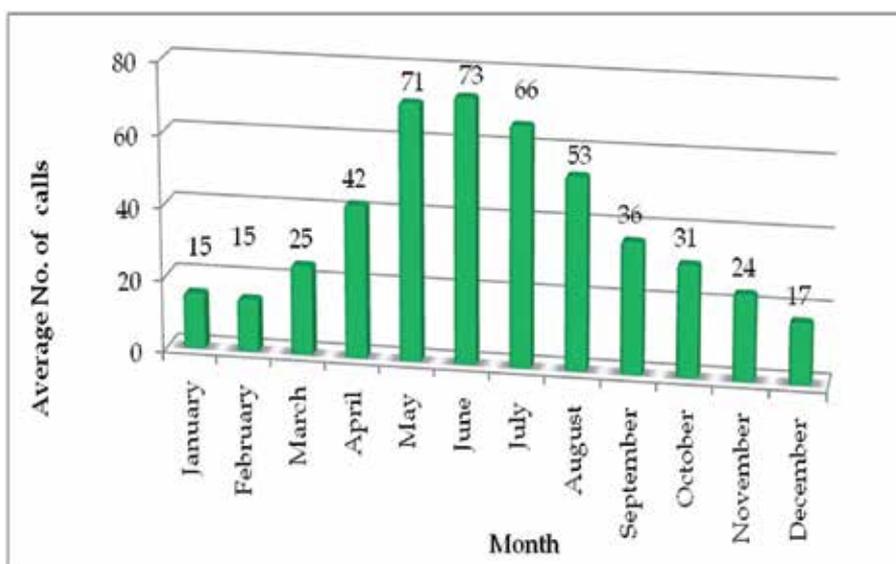


Fig. 2. Average number of calls to the TIC concerning pesticides throughout the months of the year.

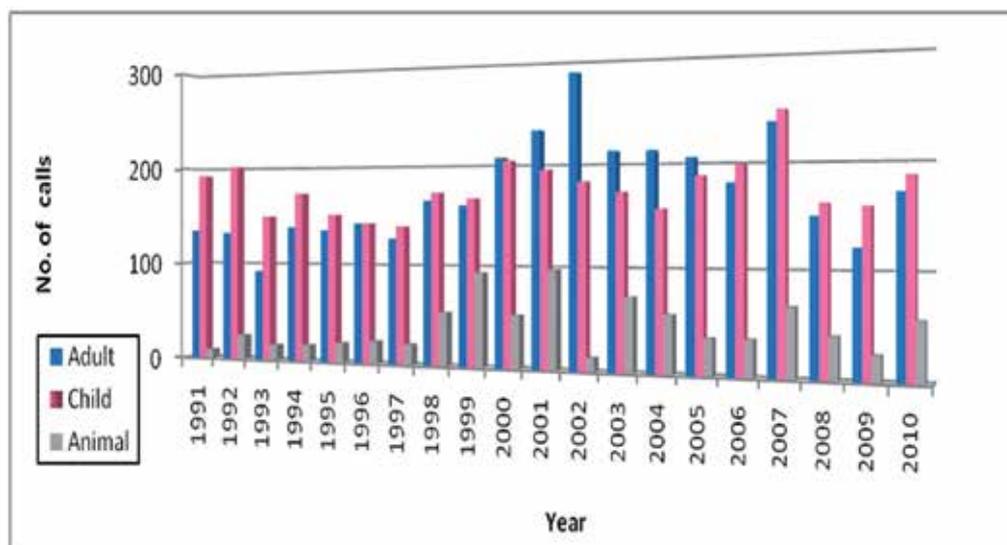


Fig. 3. The population in the calls to the TIC concerning pesticides in the years 1997-2010

### 3.4 The reason of exposure

Pesticide exposures occurred predominantly accidentally (including house work, wrong use and children exposures), which was the case in 90.5% of the calls due to pesticides in the years 1997-2010. In total, only 5.9% exposures were suicidal, 2.7% occupational and 0.7% due to aggressive behavior. The percentage of other or unknown reasons amounted to about 2.9%.

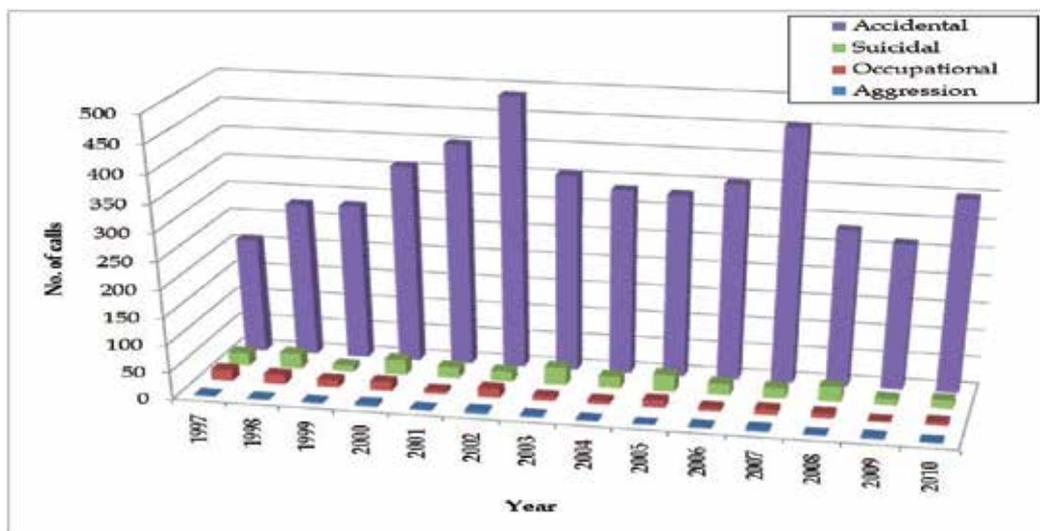


Fig. 4. The reason of exposure in the calls, concerning pesticides to the TIC in the years 1997-2010

A detailed analysis of the causes of exposure was possible in the past 6 years and can be seen in Figure 5. Again, most common was the unintentional exposure; the second most

frequent reason of exposure was the house work with pesticides. As can be seen, the suicidal exposure slightly decreased. Another new item, wrong use, appeared in the statistic.

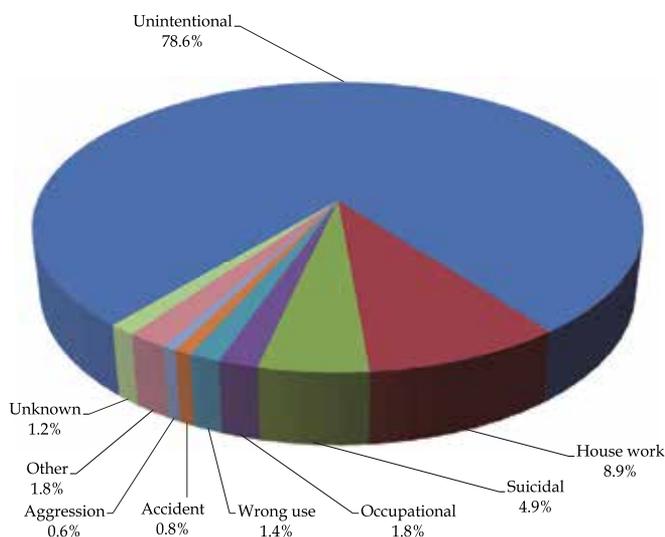


Fig. 5. Detailed reasons of exposure in the calls to the TIC in the years 2005-2010

The percentage of inquiries due to occupational exposures dropped in the last 6 years from 2.7% to 1.6%.

Accordingly, the data of the Registry of Occupational Diseases shows a low number of occupational diseases.

The poisonings with pesticides during past 20 years involved only 2 workers, occupationally exposed to synthetic pyrethrins, as can be seen in Figure 6.

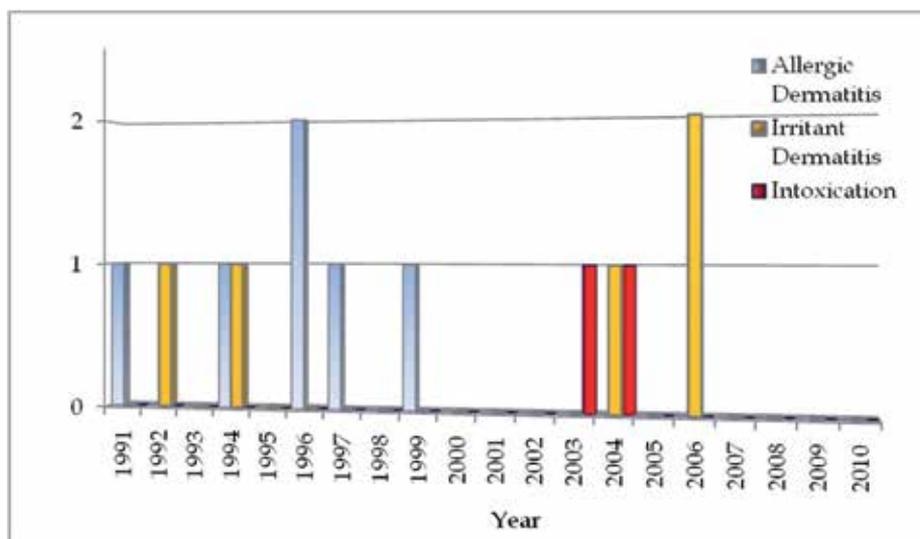


Fig. 6. Occupational diseases due to pesticides according to the Registry of Occupational Diseases in the Czech Republic in the years 1991-2010.

Occupational skin disorders were more frequent and involved 6 cases of contact allergic dermatitis. The diseases occurred due to the exposure to propoxur, cypermethrin, mancozeb. Other 5 subjects developed the contact irritant dermatitis (due to permethrin, triadimefon, metiram, sulfur, copper oxychloride, methyl and benzisothiazolinone). As can be seen in Figure 6, last occupational disorders were acknowledged in 2006.

### 3.5 The route of exposure

Ingestion was the most frequent scenario of exposure and accounted for 85.1%, inhalation for 12.4% and skin contamination for 2.5% of the calls due to pesticides.

Obviously, ingestion was the most common way of exposure in children and in adults committing suicide; on the other hand, the inhalation played a role in exposure almost exclusively in adults during their domestic or occupational work with pesticides in the house, garden or field.

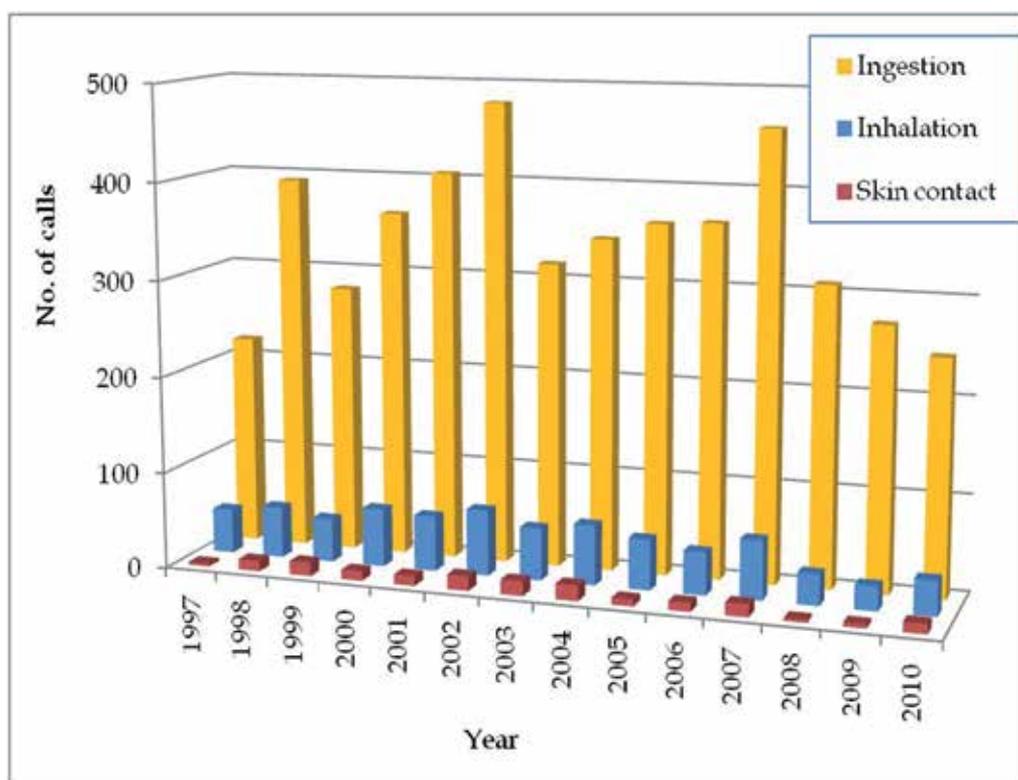


Fig. 7. The route of exposure to pesticides in the calls to the TIC in the years 1997-2010

### 3.6 The symptoms

The symptoms of intoxication in all inquiries due to all pesticides in the years 1997-2010 were absent in 69.7% of subjects, including the animals. About 23.6% of subjects had mild symptoms, 4.2% medium, and 0.6% severe; the symptoms at the time of the call were unknown in the rest of the cases. Consultation of a decease concerning a pesticide was the reason of the phone call in 0.1% of cases only.

The severity of symptoms was not the same in the group of cholinergic pesticides, in the group of rodenticides and other pesticides (fungicides, herbicides, molluscocides, etc.).

The differences in the frequency of symptoms due distinct groups of pesticides at the time of the inquiry can be seen in Figure 8 (for organophosphates and carbamates), Figure 9 (for rodenticides), and Figure 10 (for other pesticides).

No lethal case was recorded due to exposure to a rodenticide, inclusive the suicide attempts during the past 14 years. In humans, symptoms of bleeding developed after a repeated exposure, including skin exposure due to a contaminated bed in the hayloft. In other subjects, the exposure to rodenticides has not been proven and was merely considered in differential diagnosis. The symptoms occurred mostly in animals, especially dogs, where repeated exposures could not be excluded.

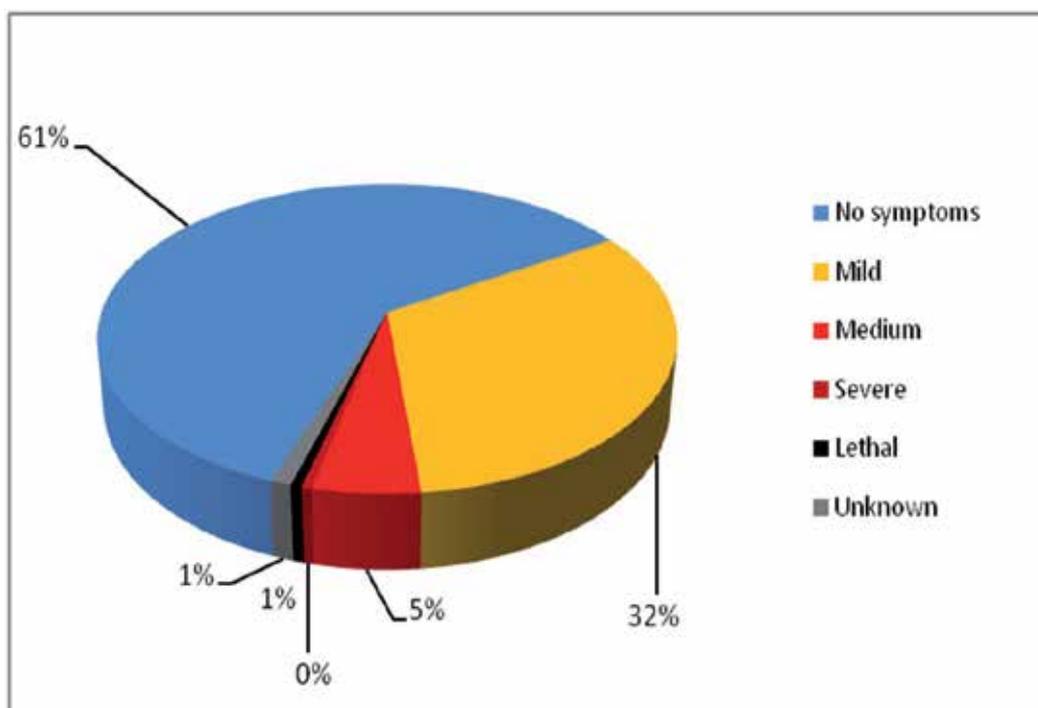


Fig. 8. Symptoms due to cholinergics (organophosphates and carbamates) during the call to the TIC in the years 1997-2010

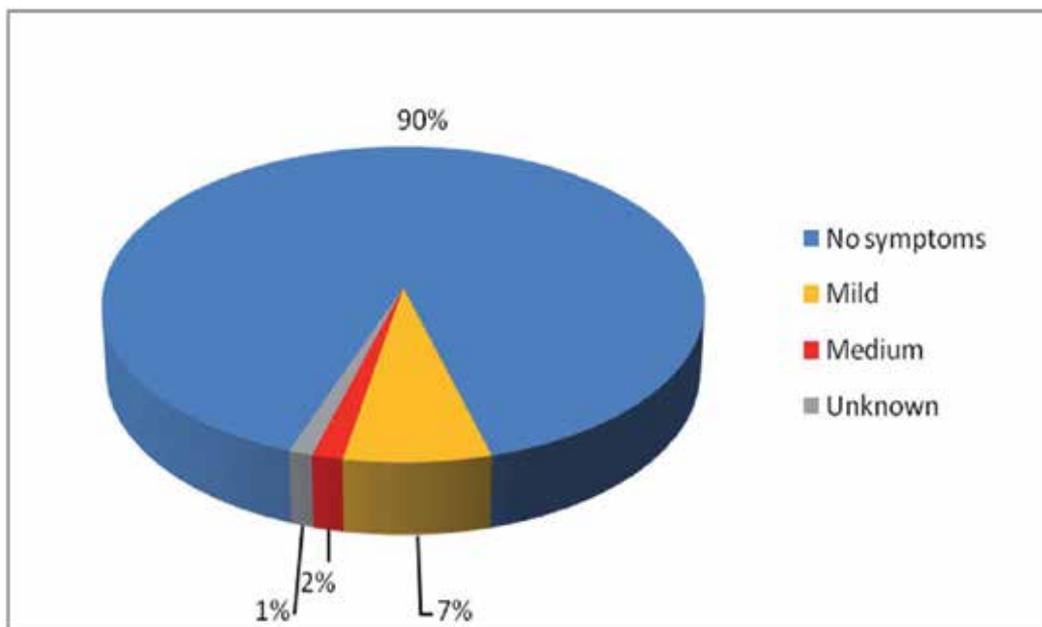


Fig. 9. Symptoms due to rodenticides during the call to the TIC in the years 1997-2010

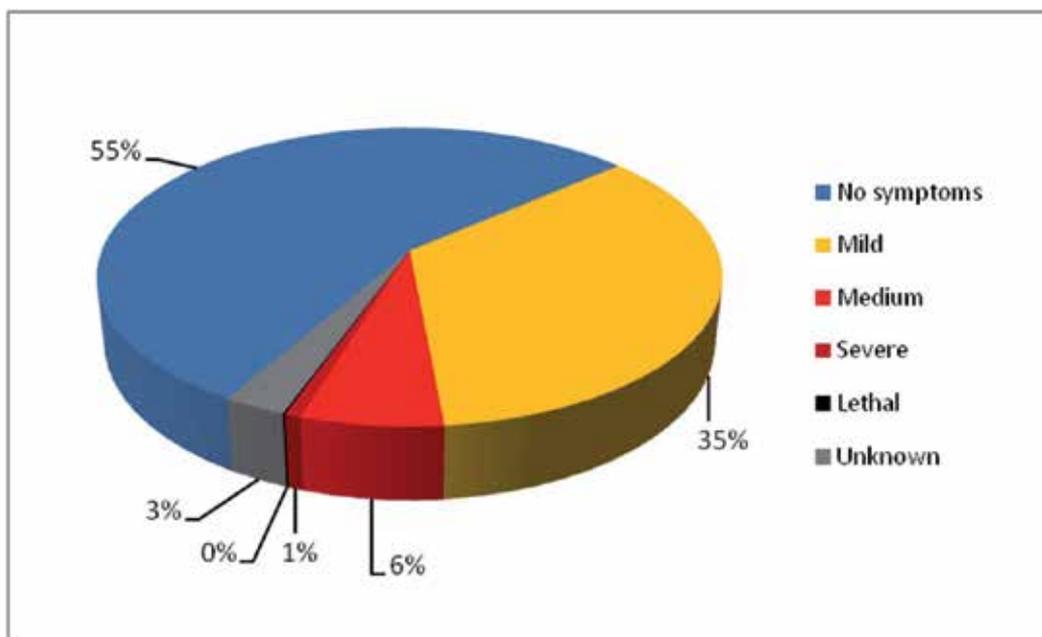


Fig. 10. Symptoms due to other pesticides during the call to the TIC in the years 1997-2010

Symptomatic exposures to other pesticides concerned most frequently the dogs, exposed to molluscocides.

### 3.7 The dose of the pesticide

The level of the pesticide exposure, evaluated by the physician at the time of the call, is shown in Figure 11; the most frequent evaluation was "Low".

The difficulty to make an exact estimate of the dose is seen in the high percentage of the "Unknown" classification, which includes especially oral exposures of the children and animals on one hand, and inhalational and skin exposures in all subjects at the other hand.

### 3.8 Prognosis of the exposure

Prognosis of the pesticide exposure, evaluated individually in every patient at the time of the call, is presented in Figure 11. The decision was based on several parameters, able to influence further development of the exposure/intoxication. To the most important parameters belonged especially the dose of the pesticide, time interval since the exposure, the symptoms of the patient, and the availability of the antidote or other efficient treatment.

It can be seen that the counts are relatively stable and prognosis is relatively favorable.

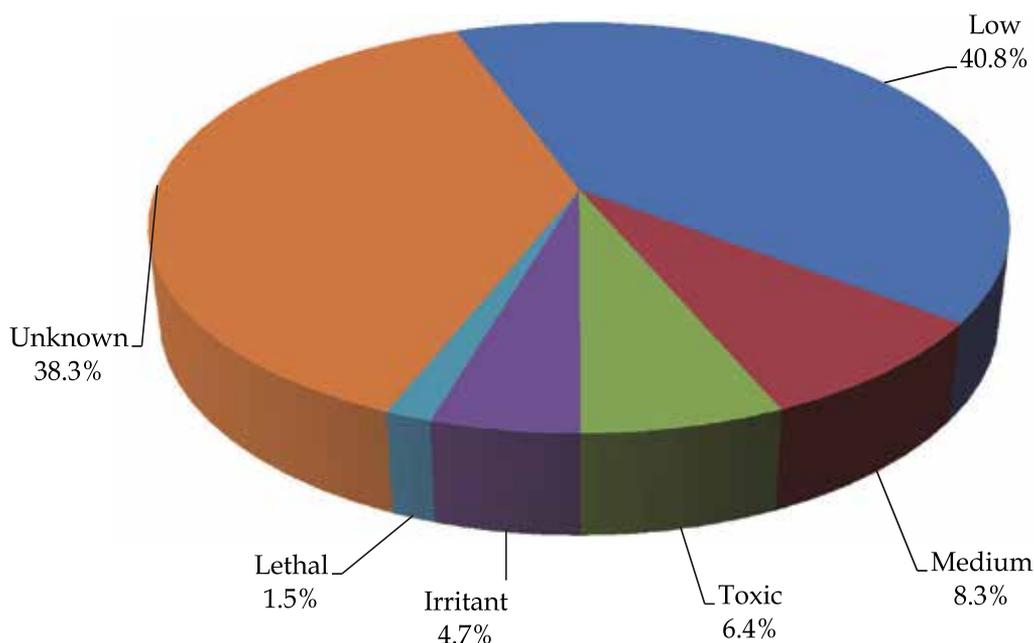


Fig. 11. Dose of the pesticide, evaluated at the time of the call to the TIC in the years 2005-2010

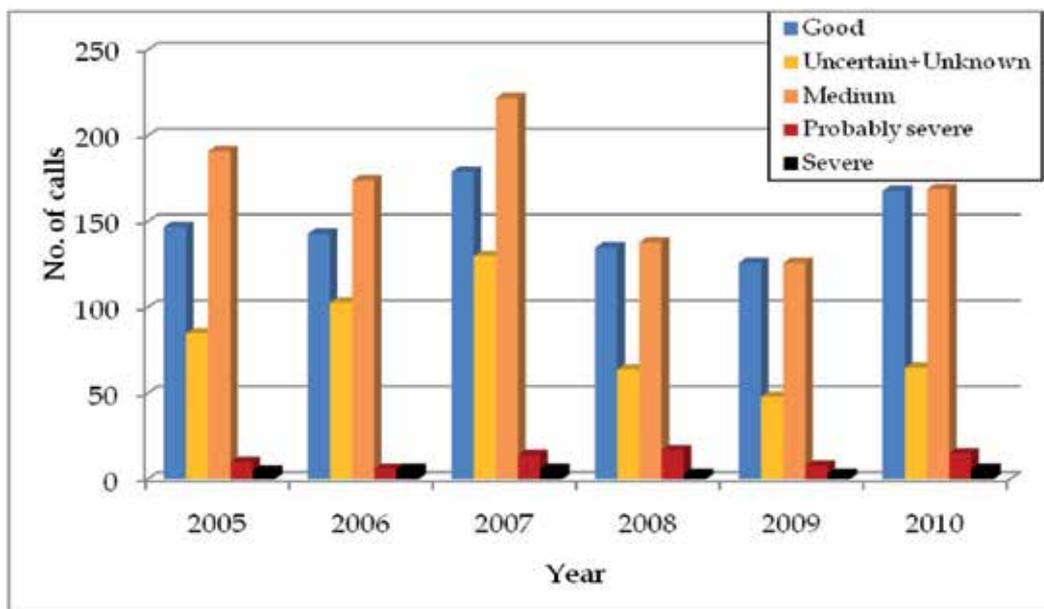


Fig. 12. Prognosis of the exposure, evaluated at the time of the call to the TIC in the years 2005-2010

### 3.9 Trends in the types of pesticides

The decrease in the number of calls since the 90ies can be seen especially in the group of insecticides. In spite of the increasing number of total calls, the number of inquiries due to pesticides remained mostly stable during the years, even with 10year's intervals, as can be seen in Table 1.

Year	1988	1989	1998	1999	2008	2009
Insecticides	180	128	180	189	134	132
Rodenticides	110	77	140	105	114	69
Herbicides	41	36	50	68	49	52
Fungicides	24	20	44	51	25	22
Other pesticides	20	14	17	27	50	49
<b>TOTAL</b>	<b>375</b>	<b>275</b>	<b>431</b>	<b>440</b>	<b>372</b>	<b>324</b>

Table 1. The types of pesticides in the calls to the TIC in selected years during two decades

In the electronic recording of the structured format of the calls in the past decade, the mean percentage of the calls due to rodenticides amounted 26.6%, cholinergic insecticides 13.0%, other insecticides, mostly pyrethrin-based 18.4%.

Herbicides reached 16.3%, among them, glyphosate took the first place with a slowly decrease from 12.2%, 10.2%, 10.8%, 10.8% to 7.2% in the years 2006, 2007, 2008, 2009 and 2010, respectively. The proportion of molluscocides and fungicides was 6.0% and 8.6%, respectively.

When the trends in the participation of different groups of pesticides were evaluated, only fungicides displayed a minor decrease of the calls since 2000, as can be seen in Figure 13.

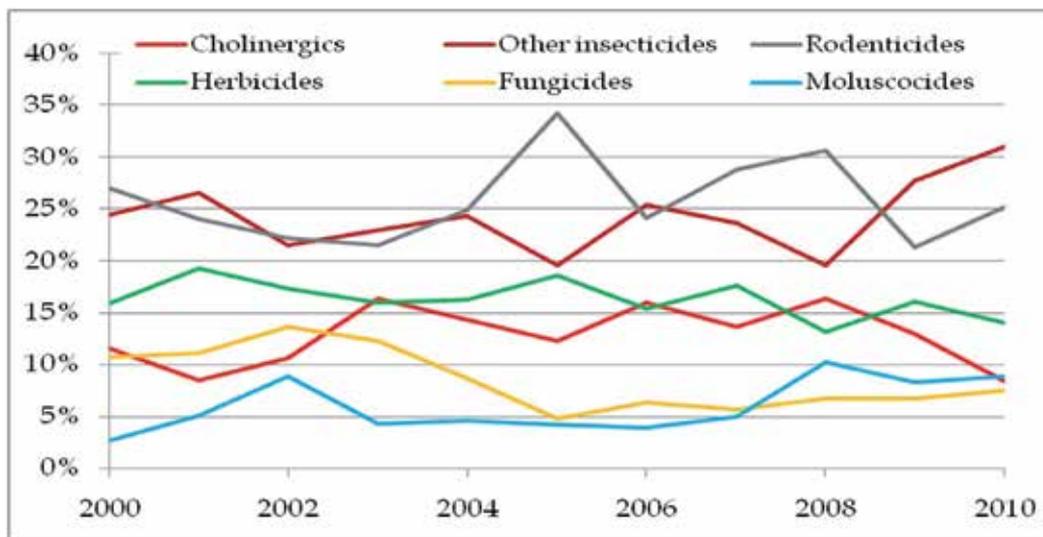


Fig. 13. Trends in the percentage of calls due to different groups of pesticides in the calls in the years 2000-2010

### 3.10 Exposures in animals

Veterinary calls in the 20 year's period represented 0.6% of the total calls to the TIC. In the last decade the percentage mildly increased and lies in the range of 0.8-1.9%. However, calls concerning animals reached 12.0% of the calls due pesticides, i.e. 2-3 times more than the humans. Exposures with a higher severity were registered for pyrethrins, wrongly used as insecticides in cats. On the other hand, carbofuran was given to the dogs or birds supposedly with the intent to damage or kill them by the neighbors. Moluscocide metaldehyde in pellets, appearing attractive to the dogs, carbofuran, and glyphosate belonged to the most frequent causes of symptomatic exposures.

### 3.11 Selected case reports of exposures

#### 3.11.1 Unintentional ingestions

2 years-old male drank organophosphate metathion from a bottle in the garden. His father immediately induced vomiting and brought him to the hospital, where the boy vomited again. Gastric lavage was performed and charcoal given. The patient received atropine; however, the symptoms appeared 3.5 hours later - fasciculations, diarrhea, and bradycardia. The condition slowly improved after repeated atropine injections and resolved within 20 hours without sequels (April 1991).

46 years-old female took by mistake 7 ml of liquid organophosphate diazinon instead of antitussic drops. Within few minutes she began to vomit spontaneously, experienced cholinergic symptoms, weakness and muscle fasciculations. She was treated with atropine in infusion. Cholinesterase on admission was 6.0  $\mu\text{kat/l}$ , further decreased to 0.5  $\mu\text{kat/l}$  and on discharge within 8 days it increased to 13.3  $\mu\text{kat/l}$ . Normal level is 87-190  $\mu\text{kat/l}$ . (September 1999).

### 3.11.2 Suicidal attempts

57 years-old female ingested 250 g of grey powder of warfarin rodenticide. Gastric lavage revealed rests of the rodenticide, she was given phytomenadione and transferred to psychiatry department. Coagulation parameters stayed within the normal range (December 1997).

30 years-old male ingested about 250 ml of pirimicarb with an unknown amount of alcohol beverage, then vomited repeatedly, had gastric lavage, was treated with atropin at the intensive care unit. He was anesthetized and cooled and completely improved within 24 hours. However, on the following day he took another unknown dose of pirimicarb at home and died (September 2005).

29 years-old male committed a suicide using intravenous injection of an unknown dose of pyrethrin. He was found dead 5 days later (January 2007).

### 3.11.3 Animal exposures

A young dog ingested metaldehyde granules in neighbor's garden and was found dead. During the autopsy, high amount of blue content, proven as metaldehyde was found. (August 2006).

Police was solving a problem of illegal bait containing carbofuran in eggs and a lethal poisoning of a fox, raven, and marsh harrier; all animals were discovered dead in one place in the forest. High concentrations of carbofuran were found both in the eggs and in the carcasses (July 2008).

## 4. Discussion

It can be concluded that the development of inquiries to the TIC in the Czech Republic in the past two decades concerning pesticides is favorable and there is a relative decline of calls due to pesticides comparing to the calls to other agents.

Interestingly, the proportion of different groups of pesticides was relatively stable and did not change very much especially in the past decade, only the proportion of fungicides slightly decreased.

Some poisonings with pesticides, such as cholinergic pesticides organophosphates and carbamates, display typical clinical signs and symptoms. Based on the symptoms in the relevant time interval after the suspected exposure, these intoxications may be confirmed or excluded. Less specific symptoms are caused by glyphosate. A prospective study focusing on the self-poisonings with glyphosate-containing herbicides was performed in Sri Lanka. Gastrointestinal symptoms, respiratory distress, hypotension, altered level of consciousness, and oliguria were observed in fatal cases. Death was strongly associated with higher age, larger ingestions, and high plasma glyphosate concentrations on admission ( $>734 \mu\text{g/ml}$ ). However despite treatment in rural hospitals with limited resources, the mortality was as low as 3.2% (Roberts et al., 2010), i.e. lower than that reported in previous case series. This is in agreement with our study, as the symptoms caused by glyphosate were relatively rare.

The decrease in the severity and prognosis of exposures can be explained by new formulas of pesticides, especially insecticides that have been substituted by synthetic pyrethrins with low toxicity for the mammals. The situation is even more favorable in the group of rodenticides. In the Czech Republic, solely warfarin or superwarfarin based rodenticides are

available on the market, which explains the asymptomatic course of most exposures after human ingestions of these products (Rakovcova et al., 2007).

The comparison with our data from the years 1988-1989 shows that the proportion of suicidal attempts using pesticides decreased from mean 11.5% to 3.8%. Additionally, also the severity of exposures substantially decreased. According to our survey (Pelcova et al., 1991), during two years 1988 and 1989, as much as 14 fatalities have been recorded. Among them, the reason for 7 persons was a suicide, and for 7 subjects a fatal mistake after drinking a pesticide from a soft-drink bottle.

In the past 20 years, on the other hand, death was recorded only exceptionally. The main difference is that the pesticides, which caused most deaths in the 80ies, such as metathion, paraquat, diquat and sodium chlorate, almost disappeared from the households. In phone inquiries to the TIC, suicide attempts were involved in less than in 1 case per year. Similarly in Poland, northern neighbor country of the Czech Republic, with the population over 38 million of inhabitants, 4 lethal cases were registered in 2002, among them 3 cases due to suicidal attempts (Przybylska, 2004).

The Slovak Republic, another neighbor country with the population of 5 million inhabitants, displays a similar spectrum of groups of pesticides in the calls to the National Toxicological Information Centre to Bratislava (Caganova et al., 2010). The proportion of calls due to insecticides is comparable; however the number of lethal exposures, 24 deaths in 15 years, is more than threefold than in the Czech Republic.

The proportion of suicide deaths attributable to pesticide self-poisoning varies considerably across the world: in Europe and the Americas fewer than 5% of suicide deaths involve pesticides; in the Eastern Mediterranean, African, and Southeast Asian regions 20%-25% involve pesticides; and in the Western Pacific region and in Sri Lanka, more than half of all suicides are pesticide related (Gunnell et al., 2007, Dawson et al., 2010). In aggregate, pesticide poisoning is involved in one-third of all suicides (Miller & Bhalla, 2010).

The low number of inquiries to the TIC in the Czech Republic due to occupational exposures is in agreement with the data from the Registry of Occupational Diseases and confirms the negligible incidence of occupational intoxications with pesticides. The situation is favorable also in Slovakia (Batora & Grellneth, 2008). In addition, the course of intoxication was frequently life-threatening, with severe symptoms or lethal. Ingestions of molluscocides led to more severe course of intoxication.

The efficiency of Czech legislation for pesticides appears satisfactory and there is no problem of the registration, labeling or unlicensed outlets. The production, import, handling, transportation, and storage of pesticides are under the control of public health institutions. No unregistered pesticides are sold on the black market, unlike the "street pesticides" in Africa or Brazil (Balme et al., 2010). Czech toxicologists nowadays mostly have solved situations, when the pesticide users did not follow the instructions on the label of the product or did not store the product safely to prevent children's and animals' accidental exposures.

## 5. Conclusion

It shows that acute human pesticide exposures in the Central Europe are mainly accidental and have favorable prognosis in general, due to a lower toxicity of commercial products used. The number of calls to the Czech TIC due to pesticides in the past two decades is

relatively stable, most important are the positive changes of the spectrum of pesticides, available on the market. During the past years, the number of calls concerning toxic pesticides, such as organophosphates and carbamates insecticides slowly decreased. It can be seen, that the number of calls due to rodenticides mildly increased, however no serious sequel has been recorded. The course of ingestion of pesticides is favorable and no harmful effects in children or successful suicides using pesticides have been registered at the Toxicological Information Centre.

Lower number of deaths is the most important difference from the situation in the late 80ies, both after suicidal and non-intentional ingestions.

## 6. Acknowledgment

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# Pesticide Utilisation, Regulation and Future Prospects in Small Scale Horticultural Crop Production Systems in a Developing Country

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## 1. Introduction

Uganda has a largely agrarian based economy with 85% of its nearly 35 million people living in rural areas and 80% of its labor force engaged in agricultural production as their primary form of livelihood. The agriculture sector also accounts for 40 percent of GDP and 85% of export earnings with 90% of this being generated by crop production. Horticultural production is one of the fastest growing agricultural sub-sectors with a growth rate of 20% per year. It contributes to value addition, income diversification and foreign exchange earnings through exports (UIA, 1999). Horticultural production in Uganda is dominated by small scale producers (2ha. or less) who produce for both local and export markets. The most important horticultural crops in the vegetable category include tomato, green beans, cowpea, pepper, onion, crucifers, and Amaranthas spp. Because of ravages of pests and diseases on these moderate to high value crops, pesticides are among the key inputs on these crops.

The increased use of chemical pesticides on horticultural crops has raised a number of economic, ecological and health concerns. Economic concerns arise from the over reliance and use of chemical pesticides which increase the costs of production. Indiscriminate use of pesticides has resulted in ecological problems of common pests developing resistance, elimination of natural enemies and other beneficial arthropods, and environmental pollution. Human health concerns focus on risks from shortcomings in protective clothing, large deviations from recommended doses/situations, and excessive run-off into the soil and water sources. These concerns are exacerbated by poorly regulated internal markets for pesticides that have fostered usage of banned or outdated products; creating a situation that if not stopped will negatively impact on horticultural exports to countries with more stringent regulatory requirements for fresh crop produce. Meeting these food safety requirements has become a major challenge for the fresh produce export sector of many African countries. To ensure and maintain export compliance, grower and consumer safety, and environmental integrity; farmers, government and development partners are developing programmes designed to improve pesticides usage, regulation and management on horticultural crops. In this chapter, three important horticultural crops grown in Uganda-

cowpea, hot pepper and tomato - have been selected to illustrate trends in pesticide usage and regulation, and the development and application of alternative pest management practices through farmer participation and training.

Cowpea (*Vigna unguiculata*) is an important legume in the north and north eastern parts of the country that receive little rainfall (800–1000mm per annum). Cowpea growers range from those who market all they produce (commercial) to those who consume all that they grow (subsistence). Many assert that it is not feasible to grow the crop commercially without the use of insecticide sprays (Jackai et al., 1985; Karungi et al., 2000). Hot pepper (*Capsicum chinense*) is an important fresh export crop. Uganda has been the market leader for supply of high quality hot peppers to EU market due to conducive production conditions in the country (UIA, 2009). It is mainly grown in the districts of central and western Uganda. Pests and diseases constitute a major limiting factor in hot pepper production. Insect pests alone account for about 20-40% yield losses (Asawalam et al, 2007). Farmers often apply chemical pesticides on a calendar basis to avoid risk of yield loss. However, as a fresh product export, mainly to the European Union, hot peppers need to meet international safety standards with regard to pesticide residue levels. Failure to comply with international food safety standards is a growing problem for many developing countries and an obstacle to accessing these lucrative international markets. Thus, if Ugandan hot peppers are to be marketed internationally, there is a need to ensure that their production complies with these pesticide usage standards. Tomato (*Solanum lycopersicum*) is the most important locally marketed vegetable in Uganda (Kasenge et al; 2002; Ssonko et al., 2005). Tomato production is a source of employment and income, and contributes to food security for large numbers of rural and peri-urban populations (Mwaule, 1995; Mukiibi, 2001). Yield losses due to pests and diseases are among the most important constraints resulting in excessive use of chemical pesticides by farmers.

## 2. Status of pesticide utilization – Case studies

In the last two decades, the Department of Crop Science of Makerere University and development partners have worked closely with horticultural crops farming communities in different parts of the country to develop integrated pest management systems conducive to local conditions. As rule of thumb, baseline studies collecting quantitative and qualitative data on priority pests and farmers' perceptions of pest control measures, including utilization of pesticides, from the farming communities have formed the first step in the mode of operation. Results from these studies have then provided the foundation for development and dissemination of specific interventions for the farming communities.

**Cowpea:** to answer questions regarding pesticide use and perceived efficacy on pests, a multiple cases study of 18 farmers categorised under subsistence, dual purpose and commercial was conducted in the growing districts of Pallisa, Kumi and Katakwi in eastern and north eastern Uganda over two consecutive years (Isubikalulu et al., 2000). Findings showed that pesticides were used in the districts of Pallisa and Kumi as the main pest control strategy because the varieties grown there were susceptible to pests. Katakwi had only the subsistence category which did not use pesticides. In Pallisa and Kumi, farmers were routinely using a variety of insecticides at varying rates and frequencies of application in cowpea production (Tables 1, 2). Most of the pesticides used on cowpea belonged to the organophosphate or synthetic pyrethroid chemical groups. Usage was found to be specific to the type of farmer (Table 2). Commercial cowpea growers sometimes applied as many as

8–10 sprays a growing season (70-80 days), and on occasion used tank-mixtures of different pesticides on cowpea (Tables 1, 2). Subsistence farmers had the lowest frequency of pesticide application (1–3 times), which was attributed to delayed application of first spraying and long spray intervals (14–20 days). Although poverty was a reason for the low frequency of pesticides application among subsistence farmers; high demand and need for pesticide-free young tender leaves, a local popular vegetable dish appeared to be the most important reason. Choice of pesticides depended on the farmer's perception of its efficacy on pests, type and intensity of pests, crop growth stage, and availability of the pesticide (Isubikalu et al., 2000).

Pesticide	Rates used	C	D	S	Total
Ripcord (Cypermethrin)	50ml in 15l of water	1	-	-	1
	100ml in 20l of water	-	-	1	1
	10ml in 5l of water		1	-	1
	50ml in 20l of water	-	1	-	1
Super ambush (Lamda cyhalothrin)	40ml in 20l of water	1	1	2	4
	40ml in 16l of water	1	-	-	1
	30ml in 16l of water	1	-	-	1
	50ml in 20l of water	1	-	-	1
	30ml in 20l of water	1	-	-	1
	25ml in 15l of water			1	1
Agrothoate (Dimethoate)	30ml in 20l of water	2	2	2	6
	30ml in 15l of water	1	-	-	1
Dimecron (Phosphamidon)	30ml in 20l of water	-	1	-	1
	40ml in 20l of water	-	-	1	1
Dursban (Chlorpyrifos)	40ml in 20l of water	1	-	-	1
Sumithion (Fenitrothion)	50ml in 20l of water	-	1	1	2
	40ml in 20l of water	-	1	-	1
	30ml in 15l of water	-	-	1	1
	30ml in 17l of water	1	-	-	1
	20ml in 20l of water	-	-	1	1
Agrocytrin (Cypermethrin)	50ml in 20l of water	-	1	1	2
	40ml in 20l of water	-	1	-	1
Super ambush+Dimecron	20ml ambush + 10 ml Dimecron in 20l of water	-	1	-	1
Sumithion+Agrocythrin	15ml Sumithion + 20 ml Agrocythrin in 20l of water	1	-	-	1
Sumithion +Thionex (Endosulfan)	20ml Sumithion + 20ml Thionex in 20l of water	1	-	-	1

Table 1. Types and rates of pesticides used by different types of cowpea growers  
Words in parentheses are names of the active ingredients; C = commercial farmer; D = dual purpose farmer; S = subsistence farmer; l = litres. Adopted from: Adipala et al., 2000.

Erbaugh et al (2003) followed the case studies with a gender assessment study on pesticide decision making and usage among farmers in Pallisa, Kumi and Iganga districts. They found that sources of information on pesticide usage varied by gender with men appearing

to have greater access than women to alternative and exogenous sources of information (Table 3). Decision making with regard to pesticide usage also varied with gender; men perceived pesticide decision making as largely a male affair, whereas women perceived pesticide decision making as a female or a household decision (Table 3). On the other hand, there was no relationship between gender and pesticides usage, as a matter of fact, pesticide usage was more related to the district than to gender, with both male and females in Kumi more likely to use pesticides than those in Iganga.

Stage of the crop (weeks after emergence)	Number of farmers (pesticide users only)			
	Commercial	Dual purpose	Subsistence	Total
1-3	5	3	1	9
3.5-5	1	3	1	5
5.5-8	-	-	2	2
Total	6	6	4	16
Frequency				
8-10	3	-	-	3
4-6	2	6	1	9
1-3	1	-	3	4
Total	6	6	4	16

Source: Isubikalu et al. 2000

Table 2. Frequency of insecticides usage and stage of crop sprayed in cowpea production

Pesticide usage					
All (N=200)	Male (N=96)	Female (N=104)	Total	X <sup>2</sup>	phi
Not using	37	37	74	.188	.031
Using	59	67	126		
By district					
All (N=200)	Iganga (N=100)	Kumi (N=100)	Total	X <sup>2</sup>	phi
Not using	56	18	74	30.97**	.394**
Using	44	82	126		
Person in the household who makes pesticide use decision (pesticide users only)					
All (N=200)	Male (N=59)	Female (N=67)	Total	X <sup>2</sup>	Cramer's V
Men	44	11	55	47.51**	.614**
Women	03	32	35		
Both	12	24	36		

Pesticide usage: degrees of Freedom = 1; \*p < 0.05; \*\*p < 0.01

Pesticide use decision: X<sup>2</sup> and Cramer's V, Degrees of Freedom = 2; \*p < 0.05; \*\*p < 0.01. Source: Erbaugh et al., 2003.

Table 3. Role of gender in pesticide decisions and usage in Iganga and Kumi districts

**Hot pepper:** a rapid rural appraisal (RRA) to learn when, why, and what pesticides farmers were applying was conducted in 2007 in four districts (Luwero, Mpigi, Wakiso and Mukono) that form the main growing areas in Uganda. Results of the RRA showed that of 50 farmers who participated in the study, 47 used pesticides as the main control strategy for pests on hot pepper (Table 4). Fenvalerate, Dimethoate and Cypermethrin were the most commonly used insecticides. Sulphur was used by some farmers to manage mites and

fungal diseases. In 2009, a more descriptive survey followed the RRA with one more district (Hoima) added to study area. A total of 84 farmers participated in the study, selected with the help of Sub County extension officers in the different districts.

District	Luwero	Mpigi	Wakiso	Mukono	Total
No. farmers sampled	10	15	10	15	50
No. farmers using pesticides	9	15	8	15	47
Pesticides being used					
Cypermethrin	3	2	5	8	18
Fenvalerate	5	13	2	7	27
Dimethoate	6	9	5	6	26
Sulfur	-	4	1	-	5
Chlorpyrifos	-	-	1	-	1
Mancozeb	2	-	1	-	3

Source: IPM CRSP, 2007.

Table 4. Status of pesticide usage among hot pepper farmers in 2007

Results of the survey showed a slight change in trend in pesticide usage; all the interviewed farmers used insecticides to manage pests on hot pepper. Fenitrothion, Malathion and Flubendiamide were the additions to the list of pesticides being used on hot pepper. Farmers reported using more than one pesticide in a growing season, sometimes tank mixing pesticides to improve on the effect. Farmers acquired pesticide information from different sources with the bulk of the information originating from agricultural extension workers and produce buyers (Table 5).

Source of information	Frequency	Percent (%)
Farmers' experiences	5	6.0
Pesticide providers	3	3.6
Extension workers	52	61.9
Produce buyers	24	28.6
<b>Total</b>	<b>84</b>	<b>100.0</b>

Source: Kwesiga et al (un published)

Table 5. Sources of information of pesticides among hot pepper growers

Farmers indicated that cost of pesticide was the main factor in deciding what product to use. From the information collected from the farmers, pesticides were estimated to cover between 18-21% of the total production cost of hot pepper.

**Tomato:** a baseline study in 1999 targeting peri-urban farmers in the districts of Wakiso and Mpigi to establish status of biotic constraints and prevailing management measures showed that control of the pests and diseases on tomato was mainly by synthetic pesticides. Farmers cited Ambush (Permethrin), Sumithion (Fenitrothion), Dimethoate, Nurelle-D (combination of Cypermethrin and Chlorpyrifos), Sherpa (Cypermethrin), Dursban (Chlorpyrifos), Salute (Trifluralin - herbicide), Zancor (Metribuzine - herbicide), Mancozeb (Dithane M45), Metalaxyl, and Ridomil (Mancozeb + Metalaxyl) as the pesticides used on the crop (Akemo et al., 2000). Fungicides were the most commonly used pesticides because fungal blights, especially *Phytophthora* are ever-present and if left unchecked result in crop losses greater

than 75% (Akemo et al., 2000). Farmers' response to this threat was to effect routine/calendar sprays with the fungicides with the majority of them spraying as often as twice a week throughout the tomato growing season. The majority of the farmers could not read labels to get the correct rates of application; the common practice was to use arbitrary measures like table spoons and bottle tops.

### 3. Regulation of pesticide usage on specified horticultural crops in Uganda

In all the presented crops, it was apparent that though necessary for increased productivity, the frequency of usage and handling aspects of pesticides left a lot to be desired. As a way to promote judicious pesticide usage, efforts have often taken the approach of developing and transfer of innovative techniques of minimising pesticide usage and/or promotion of alternative management options. Farmer training in judicious pesticide usage has gone hand in hand with IPM technology transfer. Another regulating mechanism has been through consumer demands and the need to comply to set standards with regard to fresh produce exports.

#### 3.1 Innovative spray schedules and alternative pest management options to reduce usage and impact of pesticides

**Cowpea:** to reduce and regulate pesticides usage on cowpea, efforts were put in determining the most yield reducing insect pests so that only those can be treated chemically with focused/targeted sprays. Findings by Karungi et al. (2000) indicated that pesticide usage could be reduced from the 8-10 sprays a season to only 3 well timed ones, with higher returns (Tables 6, 7). Targeting pests that attack the crop at the budding, flowering and podding stages (corresponding to a spray at 30, 45 and 55 days after planting) contributed most in increasing marginal returns (Tables 6, 7). When the spray schedule of the 3 targeted sprays was coupled with the cultural practices of timely planting and optimum plant density in an integrated pest management (IPM) strategy; the combination surpassed individual measures in terms of grain yield and marginal returns (Table 7). Moreover, this spray schedule would ensure that the young tender leaves that are normally picked for food in the vegetative stage of the crop are pesticide-free. The IPM package was duly recommended for transfer to farming communities.

Spraying schedule	Grain yield kg/ha	Yield gain kg/ha	Marginal returns <sup>a</sup>
No insecticide applied (control)	268.0	-	-
Weekly throughout the vegetative stage	590.1	322.0	0.66
Once at the vegetative, flowering, podding stages	983.7	715.8	2.18
Once at the budding, flowering, podding stages	1293.3	1025.3	3.12
Weekly throughout the growing season	1561.5	1293.5	1.77
SED	228.3	-	-

Source: Karungi et al., 2000; <sup>a</sup>Marginal returns > 1 are profitable.

Table 6. Grain yields and marginal returns for different insecticide spray schedules

	No control <sup>1</sup>	Cultural control <sup>2</sup>	Chemical control <sup>3</sup>	Combined control <sup>4</sup>	SED
Aphids score/plant	1.8	1.4	1.0	1.1	0.07
Thrips/20 flowers	70.5	92.0	19.8	14.0	7.8
Grain yield kg/ha	152.0	279.3	935.1	1135.5	130.0
Yield gain kg/ha	-	127.3	783.1	983.5	NA
Marginal returns	-	-	2.12	2.66	NA

NA - not applicable as they were derived from grain yield (kg/ha). Source: Karungi et al., 2000.

Table 7. Grain yield and marginal returns for three different pest management methods

**Hot pepper:** as a fresh export produce pesticides have to be used as judiciously as possible if the produce is to comply with internationally set standards of pesticide residues. Intervention efforts commenced with a study assessing the effect of a biological pesticide (Azadirachtin, commonly known as Neem), an inorganic pesticide (Sulphur), and prophylactic soil dressing treatments imposed on two different cropping systems of hot pepper (Karungi et al., 2010). Results from two growing seasons indicated that plants receiving the novel pesticide treatments yielded significantly better than the untreated control regardless of pesticide type (Table 8). In the first growing season of 2008, plants receiving applications of neem-only sprays had the highest fruit yield (Table 8). Further research on the crop is on-going to constitute a package that can be disseminated to farmers.

Season	Treatment	No. branches/plant	Fruit weight gm/fruit
2007B <sup>+</sup>	Prophylactic carbofuran treatment	3.50	11.16
	Weekly sprays of neem	3.83	11.85
	Prophylactic carbofuran + neem	3.83	11.91
	Untreated control	3.50	7.96
	<b>Mean</b>	<b>3.67</b>	<b>10.72</b>
	<b>SE</b>	<b>0.220</b>	<b>0.746</b>
2008A <sup>+</sup>	Prophylactic carbofuran treatment	4.80	9.27
	Weekly sprays of neem	5.07	10.22
	Prophylactic carbofuran + neem	5.03	9.47
	Sulphur sprays (every 10 days)	5.27	9.53
	Untreated control	4.07	8.27
	<b>Mean</b>	<b>4.85</b>	<b>9.35</b>
<b>SE</b>	<b>0.579</b>	<b>0.402</b>	

<sup>+</sup> 2007B denotes the second rainy season (August–November 2007) of 2007; <sup>+</sup> 2008A denotes the first rainy season (March–June) of 2008. Source: Karungi et al., 2010.

Table 8. Effect of pesticide treatment on branching level and fruit weight of hot pepper

<sup>1</sup> Cowpea at 60x20 cm (recommended);

<sup>2</sup> Cowpea at 30x20cm planted at on-set of rains (close spacing + early planting);

<sup>3</sup> Cowpea at 60x20 cm sprayed 8 times in a season (weekly, starting two weeks after emergence);

<sup>4</sup> Cowpea at 30x20cm, planted at onset of rains and sprayed once at budding, flowering and podding stages (3 sprays);

**Tomato:** a study examining effect of different pesticide spray schedules, cover cropping, and innovative technologies on incidence of fungal diseases and yield of tomato was the first research to be undertaken. A trial was laid out in a RCBD, with 4 replicates and 8 treatments i.e., treatment 1 = 2 spray applications of the fungicide Dithane M45 (Mancozeb) per week; 2 = one spray application of Dithane M45 per week; 3 = Pre-established cover crop (Siratro) mulch with no pesticide; 4 = one spray application of Dithane M45 per week + cover crop mulch; 5 = 2 spray applications of Dithane 45 per week + cover crop mulch; 6 = Bakers' yeast applications once a week + cover crop mulch; 7 = Bakers' yeast with no mulch; and 8 = Control (no spray and no cover crop). Results indicated that use of Dithane M45 significantly increased yields; one spray of Dithane M45 per week was found to be more effective than two sprays per week in increasing tomato yield; combining cover cropping with one spray of Dithane M45 per week gave the highest yields (Figure 1; Akemo, 2000). For the period 2000 to date, more alternative pest management options have been developed to help reduce pesticide usage on tomato notably use of the bacterial wilt resistant tomato variety MT56, staking, mulching and reduced pesticide usage. These components have been incorporated into an IPM 'basket' that has been disseminated to tomato farmers.

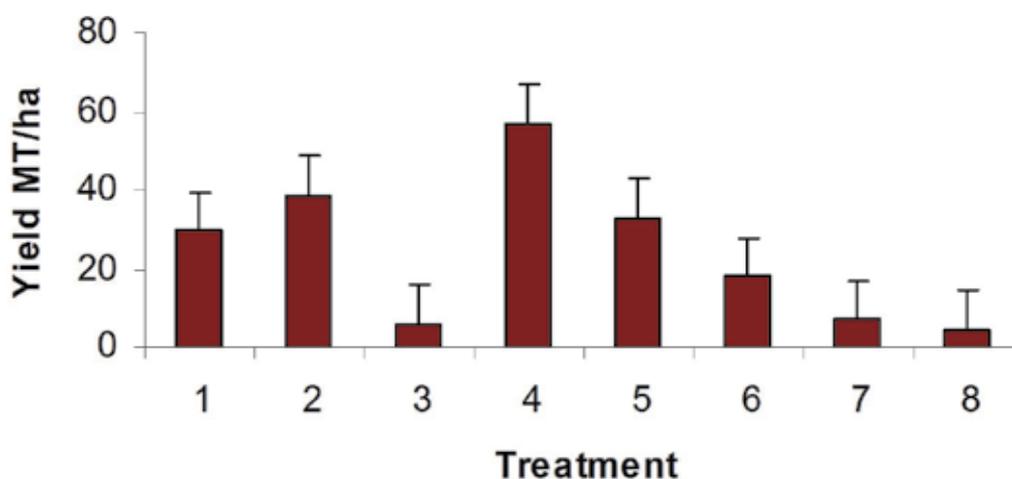


Fig. 1. Effect of different spray schedules of fungicide and/or alternative management options on tomato. Treatment 1 = 2 sprays of Dithane M45 per week; 2 = one spray of Dithane M45 per week; 3 = Pre-established cover crop (Siratro) mulch with no pesticide; 4 = one spray of Dithane M45 per week + cover crop mulch; 5 = 2 sprays of Dithane M45 per week + cover crop mulch; 6 = Bakers' yeast applications once a week + cover crop mulch; 7 = Bakers' yeast with no mulch; and 8 = Control (no spray and no cover crop). Source: Akemo et al., 2000.

### 3.2 Farmer training for pesticide regulation

IPM strategies though viable as presented above, are knowledge-based and effective implementation requires investments in farmer training and participation.

**Cowpea:** because of the great returns of the IPM package comprising of 3 targeted sprays in a growing season coupled with the cultural practices of timely planting and optimum plant density (Table 7), efforts were put in ensuring that cowpea growers get to experience it for

themselves. The farmer field school (FFS) approach was chosen for the technology transfer process in three districts in north eastern Uganda. The FFS approach takes into account farmers’ actual conditions and incorporates a ‘learning by doing’ approach, and had previously demonstrated that farmers can absorb IPM strategies, reduce their dependence on pesticides, and increase their ability to be decision-makers in their own fields (Chambers et al., 1989). The cowpea IPM FFS curriculum included sessions on safe pesticide usage and handling in addition to the IPM practices sessions. A total of 166 cowpea farmers were trained. When a post-test was done on the farmers that had participated in the FFS in Kumi and Pallisa districts, results showed that 76% and 50% of the farmers were using the recommended 3 targeted sprays schedule in Kumi and Pallisa, respectively (Figure 2). The success of the approach on cowpea led to the scaling up of the methodology to impart safe usage and handling procedures and IPM strategies to growers of cabbage (Slide 1), and groundnuts in other parts of the country.

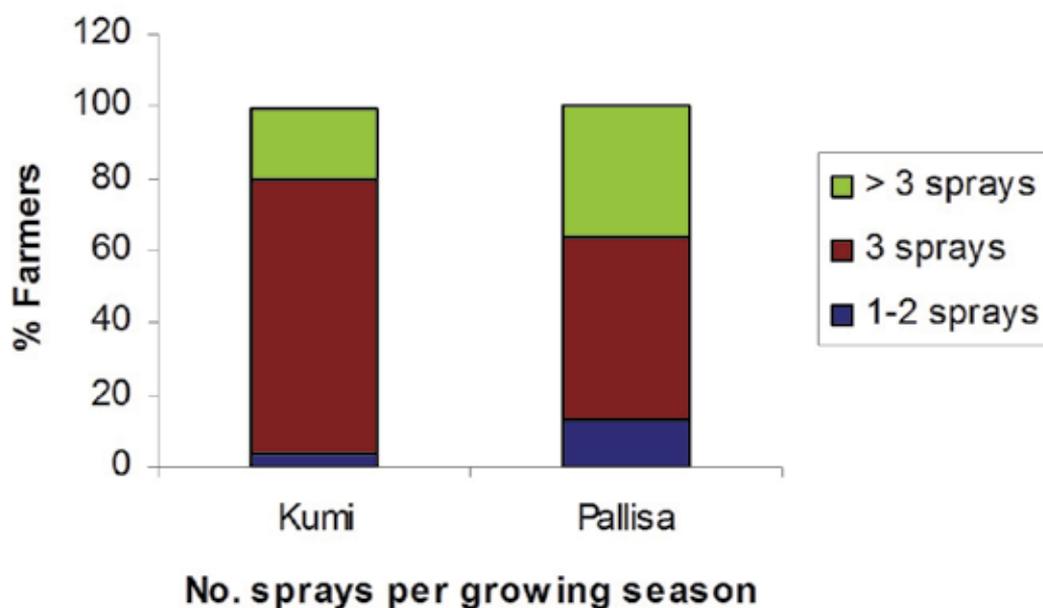


Fig. 2. Farmers’ pesticide spray schedules after technology transfer through FFS (Source: Karungi-Tumutegyereize & Adipala, 2004)

**Tomato** – an IPM package comprising of a bacterial resistant variety MT56, the cultural practices of mulching and staking, and reduced pesticide spray regimes was participatorily transferred to a pilot of 60 farmers in Busukuma Sub County, Wakiso district in Central Uganda over the period 2005-2008. Thereafter an impact assessment was executed and findings revealed that farmers only applied individual components of the IPM package; especially the bacterial wilt resistant variety MT 56. Surprisingly, only 6.7% of the respondents were practicing the recommended reduced pesticide usage schedule.

Component of the IPM package being used	% Farmers using the component
Resistant tomato variety MT56	73.3
Mulching	13.3
Reduced pesticide use (one spray a week)	6.7
Total	93.3

Source: IPM CRSP, 2009

Table 9. Assessment of adoption levels of transferred tomato IPM package

Results of the impact assessment also indicated that safety procedures were still not being followed as farmers did not wear protective clothing when mixing or applying pesticides; farmers did not read labels on the pesticides; and farmers did not observe the pre-harvesting waiting periods after applying pesticides (IPM CRSP, unpublished).



Slides 1. FFS beneficiaries demonstrating to fellow growers about safe pesticide usage

### 3.3 International markets' effort to regulate pesticide usage

In an endeavour to protect consumers of horticultural products from Africa and Uganda in particular, the European Union (EU), the main importer, constituted the Pesticides Initiative Programme (PIP) as a regulation strategy to ensure compliance of the private fresh produce sector through training in IPM and traceability. PIP helps rural producers in Africa, Caribbean and Latin American countries to stay competitive in the face of globalisation and supply chain integration and to cope with the present and future challenges imposed by food safety regulations and commercial requirements (Schiffers, 2007). In Uganda, PIP works with exporters (19 so far) and a selected number of out-growers (>1000) dealing mainly in pineapple, banana, green bean, okra, papaya, and hot pepper; in promoting IPM especially use of bio-pesticides and natural predators as an effective means of reducing pesticide residues in food, thus addressing the risks posed to human health and the environment as well as the costs brought about from the use of pesticides (PIP, 2006). PIP is engaged in capacity building through training for sustainable implementation and maintenance of food safety systems. By 2006, 30 trainers, and 67 participants selected from

service sectors had been trained and are currently working as service providers. In-company training sessions to train the members of Export companies' staff in supervisory position such as team leaders, field assistants and lead farmers had also been effected. The training usually takes place on a company's own premises and includes topics such as hygiene, safe use of pesticides, safe production practices, and record keeping. PIP is also currently supporting the national pesticides regulatory body (Agricultural Chemicals Board) in Uganda in its endeavour to improve registration procedures in the country especially upgrading of the pesticide approval process, and the skills of the registrar and registration officers in the evaluation of dossiers submitted for registration of pesticides as well as advising the Ministry of Agriculture to ensure that Uganda's pesticide policy and regulatory framework is harmonised with EU regulations.

#### 4. General discussion

High frequencies of pesticides application were reported on the presented horticultural crops in Uganda. Tomato was the heaviest consumer of pesticides at two routine sprays a week, a schedule that the majority of the farmers used even after involvement in IPM training programmes. This showed that farmers were reluctant to give up the 'insurance' against loss garnered from pesticide usage. Moreover, in Uganda, particular pesticides formulations are now within the purchasing power of more producers following the removal of the import tax on agricultural chemicals (Schaefers et al., 1999). Heavy usage of pesticides on tomato has also been reported from other countries in Africa; Ntow et al (2006) working on tomato in Ghana showed that farmers sprayed an average of 6–12 times a season, whereas it was 5–16 times or more per cropping season in Tanzania (Ngow et al., 2007). Such heavy use of pesticides results in frequent contact with pesticides, which can lead to significant health problems even though fungicides are the case in point. Fungicides are not easily observed to cause serious and acute damage to farmer's health but it has been reported that there is a long-term risk for cancer development and endocrine disruption resulting from farmer's exposure to fungicides containing mancozeb (Novikova et al., 2003). The dithiocarbamate family of fungicides are also suspected to have reproductive (Restrepo et al., 1990) and mutagenic effects in human cells (Puz-y-Mino et al., 2002).

Farmers were using arbitrary tank-mixtures of chemical pesticides as a way to increase effectiveness or save on labour. A similar situation was reported from Tanzania where a study on pesticides usage in small-scale vegetable farms revealed that a third of the interviewed farmers applied pesticides in tank-mixtures (Ngowi et al., 2007). In all cases, there were no specific instructions either from the labels or extension workers regarding these tank mixtures. Mixing of pesticides by inexperienced farmers is not encouraged because the combinations used are indiscriminate. The practice defies some of the basic principles of insecticide management. For instance, Metacalf (1980) in his recommendation of strategies for pesticide management, states that the use of mixtures of insecticides must be avoided, since mixtures of insecticides generally result in the simultaneous development of resistance. Biney (2001) working in Ghana attributed the increase in incidences of insect pest infestation of tomato after pesticide applications to indiscriminate combinations of pesticides, particularly of insecticides. Moreover, label instructions do not cover tank-mixtures of pesticides and give no information on the compatibility of inert ingredients such as emulsifiers and wetting agents. It is riskier to mix two different types of formulations for

example wettable powders with emulsifiable concentrates. Smit et al. (2002) observed that there was an interaction between fungicides, insecticides and water mineral content that influenced the efficacy of individual pesticide against fungal pathogens and insect mortality and some tank mixtures induced phytotoxicity on tomato. There is limited information on the reaction and effects of the mixtures being used in the case studies. In addition, farmers did not consider that unspecified tank mixing of pesticides could be less effective and cause adverse effects to their health or the environment. Instead, the tank mixing was carried out to save time, labour cost and with anticipation of high efficacy in pests and diseases control. Sherwood et al. (2005) also reported that potato farmers in Ecuador were mixing pesticides mainly to reduce costs associated with spraying.

Findings from the case studies and elsewhere in Sub Saharan Africa (Matthews et al., 2003; Ntow et al., 2006; Ngowi et al., 2007) show that internationally banned/restricted pesticides such as Carbofuran and Endosulfan are still being used by minimally educated farmers on horticultural crops. These pesticides pose a serious threat to human health and the environment. The problem is particularly widespread in sub-Saharan Africa, where the advent of liberalisation of agrochemical input markets has weakened quality control (FAO/WHO, 2001). Prior to liberalisation, there were relatively few actors involved in pesticide provision, which made regulation and control fairly simple. In Uganda, importation and distribution of pesticides and other agricultural inputs used to be conducted by the Government and its Parastatals, which had proper procedures for safe handling and distribution of pesticides. Entry of more firms into the market runs the risk of erosion of quality control and packaging standards, the breaching of national regulations and the unimpeded movement of banned or restricted chemicals across borders (Mudimu et al., 1995). It also raises concerns about the ability of regulatory agencies to control their activities, since it requires more vigorous scrutiny and screening of imports and monitoring of distribution and usage (Mudimu et al., 1995; Williamson, 2003), with huge financial and human resource implications for these agencies. Pesticide provision in a market-driven economy needs an effective regulatory framework in order to create full and fair competition, to protect the environment, to guarantee the quality of the products and to avoid the spread of pests and diseases (Shepherd and Farolfi, 1999). These are critical challenges for hard-pressed African regulators.

Most of the pesticides on the Ugandan market in particular are pesticides that have been around for a long time within a limited range. On record in 1999, 190 pesticide formulations had been registered (includes insecticide, fungicides, and herbicides), a pittance if compared to developed countries like the United States of America; where over 50,000 had been registered by that period (Schaefer et al., 1999). This is a great disadvantage to pesticide users who have limited choice for safe pesticides. This discrepancy in accessibility to safer pesticides between countries at different levels of economic development poses a challenge in developing IPM systems in some developing countries. Safer pesticides are either inaccessible or outside the income bracket of small scale farmers. For such countries to remain competitive in international export markets, the policy environment, particularly regarding registration of newer and safer agrochemicals has to be more conducive.

## **5. Conclusions, recommendations and future prospects**

Pesticide usage on horticultural crops in Uganda in particular and sub Saharan Africa in general, has seen a steady increase over the last decade, regulation and management of

pesticide usage is following the same trend albeit at a slower pace with more effort needed to ensure safety for users/handlers, consumers and the environment.

High levels of pesticide use in vegetables are not unavoidable and, whether producing for export or domestic markets, greater efforts must be made to help farmers grow vegetables economically, ecologically and without exposing themselves, workers or consumers to hazardous levels of pesticides. Pesticide provision and use can be made much safer and more rational when a concerted effort is made amongst exporters, pesticide companies and farmers to reduce hazardous and unnecessary use and to ensure cropping system sustainability and market acceptability of the produce. Safer, more sustainable methods of pest and crop management exist and are being used successfully by millions of small-scale farmers worldwide, delivering substantial yield, income and welfare benefits in some of the most challenging agro ecological environments. African governments and development partners should invest in adapting and refining these methods and in training and knowledge exchange as a priority in new programmes for smallholder intensification, in conjunction with crop varieties which do not lead to reliance on pesticides. Pesticide and pest management issues and policies must be considered in a more holistic context of crop management, marketing and better cost-benefit assessment, with opportunity for public participation in decision-making. The IPM/FFS approach is valuable on a project-by-project basis, but with government backing and supportive policies could help to transform production, and bring real benefits to small-scale vegetable producers.

Options for adapting existing pesticide channels to supply safer and more sustainable pest management products, such as biopesticides, chemicals based on insect pest behaviour, and insect growth regulators, need to be explored. Regulators can help by establishing fast-track systems for rapid registration of such products and enjoy better tax policies compared with chemical pesticides. Regional harmonization of pesticide registration is also an opportunity for overcoming registration problems. For example, this would encourage the adoption of a system of “reciprocity” in which the pesticide registration systems of the Great Lake countries would be mutually acceptable. In this manner, the costs of efficacy testing and quality testing could be shared, there would be less duplication of effort, and more resources would remain for the proper role of the private sector – enlightened enforcement.

Market signals, reinforced with well-informed consumer demand, better understanding and communication along the supply chain, and investment in capacity-building, provide the best options for tackling many of the factors which drive pesticide misuse and dependency and which under-resourced regulatory authorities have been unable to address. Consumer perception of pesticides and their residues in fresh produce as undesirable, can lead to stronger retailer controls over what producers may use, together with greater traceability requirements and the establishment of approved crop production protocols and their consequent uptake by growers, particularly with regard to pesticide requirement, choice and adherence to harvest intervals. If compliance to these stipulation yields better prices and/or preferential purchase from European importers, exporters and farmers would be hard-pressed to embrace the system.

## **6. Acknowledgements**

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# Pesticides in Burkina Faso: Overview of the Situation in a Sahelian African Country

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## 1. Introduction

Sahelian Africa is a transition zone which is located between the arid Sahara in the north and the humid tropical area from the south. The list of countries covering this area is such as follows; Senegal, Mauritania, Mali, Burkina Faso, Niger, Nigeria, Chad, Sudan as well as the so called "Horn" of Africa which is formed by Ethiopia, Eritrea, Djibouti, and Somalia. Among the most striking characteristic concerning the climate from this particular African area, we find its instability which means that either it may register heavy rainfalls in short periods, normally between June and September, or suffer from severe droughts.

Burkina Faso is an agricultural country with a large rural population. The total area in cultivation is estimated to be 2,900,000 hectares. Synthetic pesticides have been used in Burkina Faso for about eight decades. At a global level, some studies have been carried out on impacts of pesticide use (Eddleston et al., 2002, Konradsen, 2007, Lee and Cha, 2009). However, few updated studies have been carried out on patterns and impacts of pesticides use in Sahelian countries. The purpose of this paper is to provide an overview of pesticides in Burkina Faso, almost one century after the introduction of synthetic pesticides. Assuming that the other Sahelian countries have the same socio-economic level as Burkina Faso, their patterns of pesticides use may be not different.

This briefing summarizes the findings of studies regarding the situation of pesticides in Burkina Faso and the subsequent recommendations for taking action at policy and program levels.

## 2. Geographical description of Burkina Faso

### 2.1 Description of the physical environment

The country of Burkina Faso is located in the central area of West Africa (Fig. 1). It is 273 187 km<sup>2</sup> in area, and its altitude ranges from 150 to 750 m above sea level. It lies in the transitional areas between the Sahel in the north and the Sudano-Guinean zone in the south. The topography of Burkina Faso is mostly flat and eroded. The natural soil fertility is poor. It has been estimated that over 50% of Burkina is still covered with natural vegetation, which ranges from grass savannah in the north, to gallery forests in the south. Rainfall

ranges from 400 mm in the north to 1300 mm in the south-west. The monthly average temperatures vary between 12 and 42 °C. The country is drained to the south by the Black Volta (Mouhoun), Red Volta (Nazinon), and White Volta (Nakanbe) rivers and to the east by the Sirba, Gorki and Mahiou rivers connecting with the Niger. Other rivers include the Comoie, Léraba and Banifing, and the Pendjari on the frontier with Benin (FAO, 2010, INSD, 2010).

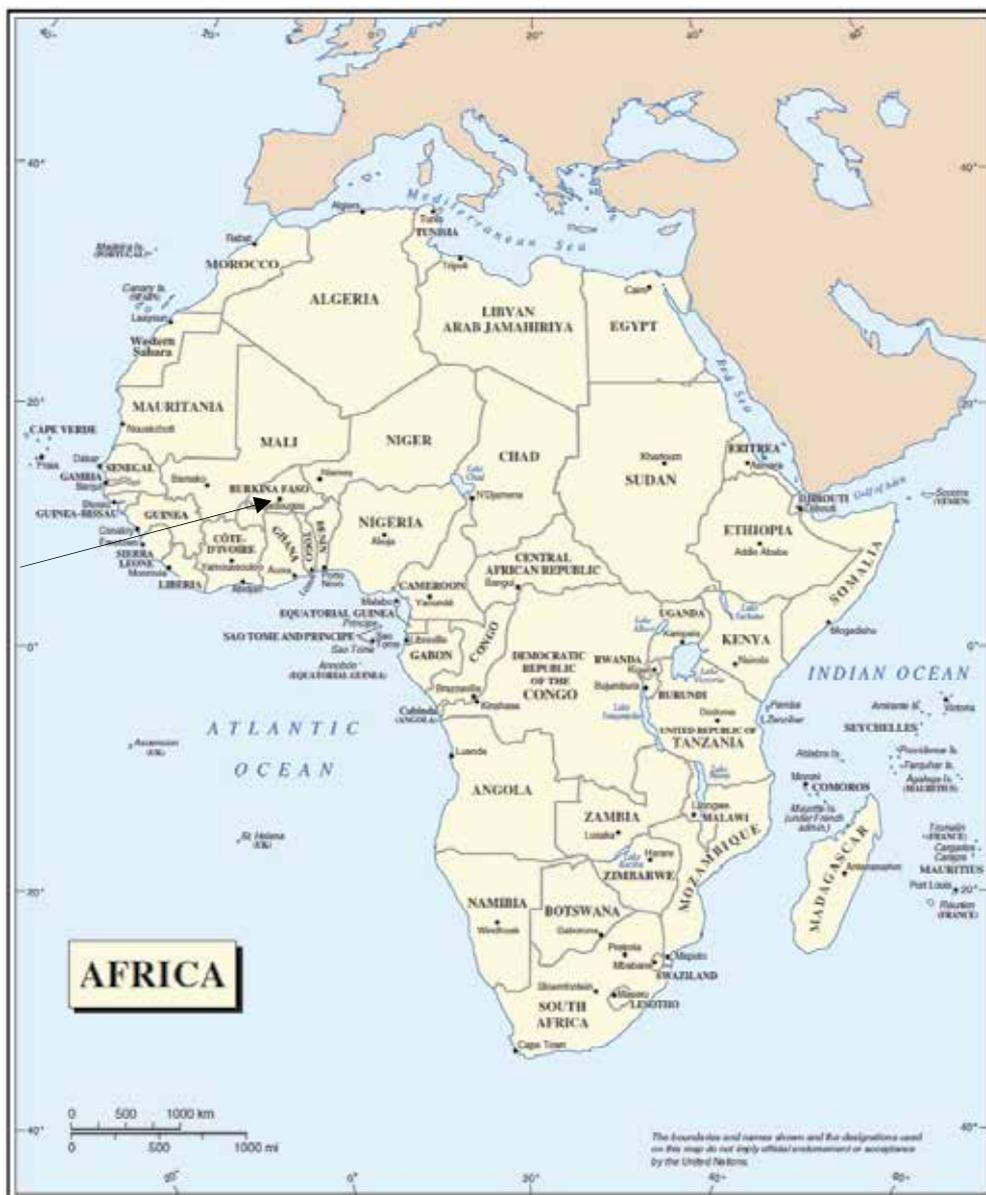


Fig. 1. No. 4045 Rev. 5 UNITED NATIONS Department of Field Support  
April 2009 Cartographic Section

## 2.2 Agricultural resources

The people of this landlocked Sahelian country are engaged primarily in agriculture. Agriculture plays a vital socio-economic role in Burkina Faso in term of export, employment opportunities, and food self-sufficiency. Burkina Faso is a country with agricultural vocation with 85 to 95% of the population which draws its subsistence from agriculture. The contribution of the agricultural sector to the GDP (gross domestic product) is 33.3%. The total area in cultivation is estimated to be 3.6 million hectares. It is dominated by the cereal cultures (approximately 82%) followed by market crops (15%), mainly the cotton and the groundnut which constitute 14%. The market gardening represents less than 1% of the total area in cultivation. The annual agricultural productions are dominated by the cereals (75%) followed by market crops, mainly cotton and the groundnut. The important crops in Burkina Faso are millet and sorghum. Other crops are grown in lesser quantities: corn, bean, rice, fonio, potatoes, vegetables, sesame (INSD, 2010).

During the crop year 2008-2009, the cereal production (sorghum, millet, corn, rice and fonio) was estimated at approximately 4,500,000 tons. An analysis of the composition of this production shows that the sorghum is the most produced cereal, followed by millet, corn and finally fonio. The production of the market crops was 1,100,000 tons in 2008. Cotton represents nearly 70% of this production. The production of bean always exceeds the 100,000 tons whereas those of the yam and potato don't reach 100,000 tons of production per annum. The diseases and insects of the cultures cause considerable damage, being able to generate in certain cases, losses in production rising more than 30% (CountrySTAT, 2010). The use of phytosanitary products is consequently necessary to dam up these enemies of cultures in particular those of the market crops: cotton, sugar cane, market gardening, etc.

## 3. Pesticides management in Burkina Faso

### 3.1 Pesticides production and distribution

The alone company producing pesticides in Burkina Faso is SAPHYTO. This company imports active ingredients, and fabricates products. While this company primarily serves the cotton industry, it also sells pesticides to other parties. The principal pesticides produced in term of weight and volume are diversified (Table 1).

The majority of pesticides used in Burkina Faso are imported from other countries. Points of origin include Senegal, Cote d'Ivoire, Nigeria, Mali, South Africa, Tunisia, Japan, Indonesia, China, Thailand, Europe and the United States. Once a pesticide arrives in Burkina Faso, it is distributed to the end-user through merchants. Import and in-country distribution is controlled by several governmental agencies. While a regulatory structure exists, it is not always followed by the distributors involved. From 1997 to 2001, more than thirteen million liters of liquid pesticides and 900000 kg solid pesticides were imported in Burkina Faso. The growth rate of the use of the pesticides per annum would reach 11% (Toé and Kinané, 2004). The obstacles to the performance of the distribution system of the pesticides in Burkina Faso are mainly: the absence of association of the professionals of phytosanitary products, the weak application of the existing lawful texts, the low technical level of the actors of this market, the insufficiency of the quality control of the pesticides.

### 3.2 Managing pesticides stocks

Only the cotton companies have suitable stores for the storage of the pesticides. The agricultural producers do not have in general suitable storerooms. Indeed it can happen that

the products are stored in the rooms, in the dwellings, in containers that are not labeled (Ouédraogo et al., 2009).

Trade name	Active ingredients	Pesticide type	Annual production
Calthio	lindane - thriname	Insecticide	20.8 tons
Durexa	chlorpriphos-ethyl	Insecticide	10 tons
Percal M	permethrin - malathion	Insecticide	4 tons
Cypocal 50 EC	cypermethrin	Insecticide	3.5 million liters
Cotodon plus	metolachlore - terbutryne	Herbicide	14 900 liters
Gramoxone	paraquat	Herbicide	14 262 liters
Primagram 500	atrozime - metolachlore	Herbicide	11 651 liters

Table 1. Principal pesticides formulated in Burkina Faso by SAPHYTO Company



Fig. 2. A pesticide box, kept under a traditional attic located in a dwelling

### 3.3 Pesticides labeling

Pesticides labeling is a way to give important information to the pesticide users. The label is the main and often only medium for instructing users in correct and safe use practices. The pesticide product label can be effectively used to communicate a number of important properties of the pesticide and precautions appropriate to its use. In addition to directions for use, the label should include needed protective measures, first aid measures, precautions recommending against use in certain environments, methods of container disposal, and application rates for particular pest species.

Pesticides labeling in Burkina Faso is quite variable. In general, pesticides in the original container carry a label with adequate information for application. Some labels, though not all, contained some information on first-aid or disposal. However, pesticides sold in the markets have either lost what labels did exist, or are illegible due to handling and exposure (Tankoano, 2008).

### 3.4 Obsolete pesticides and containers

The obsolete pesticides indicate products out-of-date, prohibited, or not identifiable. In Burkina Faso, the alone company producing pesticides set up a system of treatment and valorization of the obsolete pesticides: decontamination of empty packing, suitable storage of lost packing, crushing and decontamination of the container, incineration of biodegradable and decontaminated packing. Active ingredients are added to the expired, so they are usable at least for one year. The non-recoverable pesticides are eliminated after coagulation, flocculation, or decantation. The muds obtained in this case are stored in barrels. Then these barrels are sent to companies in Europe for ultimate elimination. Previous studies revealed the presence in Burkina Faso of enormous quantities of out-of-date pesticides going back to several years. These out-of-date pesticides are mainly organophosphates and pyrethrinoid insecticides (Toé and Kinané, 2004).

Once the pesticide has been used, the management operation is left with an empty container. This container can be either reused or destroyed. If reused it should be only be used for the same pesticide or to store fuel. It should never be used to store water or food. In Burkina Faso, the empty metal boxes and plastic cans are either abandoned in nature (48%), or are washed and reused (12%), or hidden in the fields (40%) by users (Tankoano, 2008). The majority of these modes of elimination of empty packing (abandon in nature, wash and re-use) increase the risks of intoxication of populations and pollution.

## 4. Pesticides usage in Burkina Faso

### 4.1 Socio-demographic characteristics of the users

The majority of the users of pesticides are male (more than 95%). The users are young (median age, 37 years). More than 80% of the users are illiterate and two users out of three are married. More than 50% of the users of pesticides have done it for at least 10 years (Konaté, 2010, Tankoano, 2008).

### 4.2 Pesticides commonly used in Burkina Faso

Pesticides are used in Burkina Faso mainly in agriculture for control of pests, weeds, rodents; in public health for control of malaria, and for control harmful domestic animals. Cotton is one crop which is grown only on 5% of the cultivated areas, but consumes more than 90% of total pesticides used in Burkina Faso. Thus, the use of pesticides is high intensive in parts of the country where cotton is cultivated i.e where soils are fertile. All the chemical classes of synthetic pesticides are used in Burkina Faso. The most used are organochlorines (endosulfan), organophosphates (profenofos), carbamates (carbofuran), pyridines (paraquat), and pyrethrinoids (cypermethrin). Among the pesticides used in Burkina Faso, they are PIC (Prior Informed Consent) Pesticides (Toé and Kinané, 2004).

The (PIC) procedure provides a framework for governments primarily aimed at developing countries to prohibit the import of certain pesticides which have been banned or severely restricted for health or environmental reasons. Convention on Prior Informed Consent (PIC) serves as an early warning system to notify developing countries of hazards and limit export of toxic pesticides by requiring exporting countries to receive prior approval from the recipient country (Konradsen et al., 2003). Among the PIC pesticides, lindane and methamidophos are found in Burkina Faso. The first is used in combined with thiram to treat cotton seeds, the second combined with cypermethrin to treat cotton plants.

Pesticides belonging to the class Ia or Ib (Classification WHO) are also used in Burkina Faso (Tables 2 and 3). However, the restrictions relating to their uses are generally ignored, thus not respected (Konaté, 2010, Tankoano, 2008).

Class	Restrictions
Ia Extremely hazardous	Usable only by applicators having licenses
Ib Highly hazardous	Usable by well trained applicators
II Moderately hazardous	Usable by applicators who strictly respect the precautions
III Slightly hazardous	Usable by applicators who respect the usual precautions

Table 2. WHO classification of pesticides by hazard (WHO/PCS)

Officially, no pesticide belonging to the list of Persistent Organic Pollutants (POPs) is used in Burkina Faso. However, it is possible that this class of pesticide is still used because some pesticides are marketed illegally and fraudulently in Burkina Faso. POPs are chemicals that persist in the environment, accumulate in high concentrations in fatty tissues and are bio-magnified through the food-chain. Hence they constitute a serious environmental hazard that comes to expression as important long-term risks to individual species, to ecosystems and to human health. POPs chemicals may cause cancer and disorders in the reproductive and immune systems as well as in the developmental process. They constitute a particular risk to infants and children who may be exposed to high levels through breast-milk and food (Mörner et al., 2002).

Pesticides Ia	Pesticides Ib	culture
	cypermethrin-triazophos-diméthoate endosulfan cyfuthrin-chlorpyrifos deltamethrine-triazophos carbosulfan cypermethrin-métamidophos	Cotton
Carbofuran		Sugar cane
	cyfutrín-chlorpyriphos cypermethrin -métamidophos	Tomato

Table 3. Some Pesticides Ia and Ib (WHO classification) used in agriculture in Burkina Faso

#### 4.3 Methods of pesticides application and factors affecting proper application

Pesticides application equipment used in Burkina Faso are mostly portable equipments which are manually operated. Most commonly used equipment is hand carried lever operated knapsack sprayer (Fig. 3) which is not very well designed. The applicators have experienced various problems with this equipment during spraying. The most common problems identified were replacement of the piston and clogging of the nozzles.

The major factors affecting the proper application of pesticides are operator knowledge, equipment design, and service conditions of equipment. Applicators still have wrong notion that high volumes, high pressure and high doses are the most appropriate ways of pesticides application. The quality of the equipments is low and they lack maintenance (Tankoano, 2008).



Fig. 3. Applicator operating in a cotton field with a knapsack sprayer

#### 4.4 Factors related to pesticide safety in Burkina Faso

Pesticides usage must be safe for environment, humans, and animals. However, in Burkina Faso, many factors make safety impossible. These factors are enumerated below (Konaté, 2010, Ouédraogo et al., 2009, Toé et al., 2004, Zeba, 2003):

- Poor literacy, which makes it impossible to read or follow complex label instructions. In Burkina Faso, approximately 80% of applicators are illiterate.
- Lack of training in pesticide use; few applicators receive an adapted formation.
- Ignorance about potential dangers of pesticides to health and environment.
- Inappropriate mixing (insecticides combinations i.e. mixing of two insecticides without technical advice) and application methods; different pesticides are frequently mixed together by farmers to make “more effective” pesticides, ensuring that subsequent medical management of poisoned patients is particularly complicated.
- Repeated application of pesticides and application of same pesticides at all stage of life cycle.
- Over application of pesticides.
- Wrong application practices, such as use of same concentration mixture for different sprayers and measurement of dosage by own convenient scale.
- Poor or faulty application equipment.

- Long hours of spraying and spraying during hot weather.
- Smoking or chewing while spraying.
- Poor regulation and easy availability of hazardous pesticides, including sales by untrained dealers.
- Lack of personal protective equipment (boots, gloves and glasses), which are costly. When they exist, they are not used because they are not adapted to a tropical climate.
- Lack of suitable washing facilities for workers.
- Demand for containers, leading to reuse of poorly cleansed pesticide bottles, barrels or cans.
- Reconditioning of the pesticides in non-labeled inappropriate containers.
- Poor household storage and disposal.
- Lack of health centers, medical facilities, antidotes and poison treatment centers, as well as confusion of symptoms of pesticide poisoning with common illness.
- The use of the pesticides apart from their indications, in occurrence on the parasites of the domestic animals, the termites and other insects in the dwelling houses.
- Residence in an agricultural area (Fig. 4).
- Presence of well or river near the treated fields.
- Presence of wild fruit trees in the treated fields (Fig. 5).
- The proximity between the treated fields and cereals non-treated fields (Fig. 5).

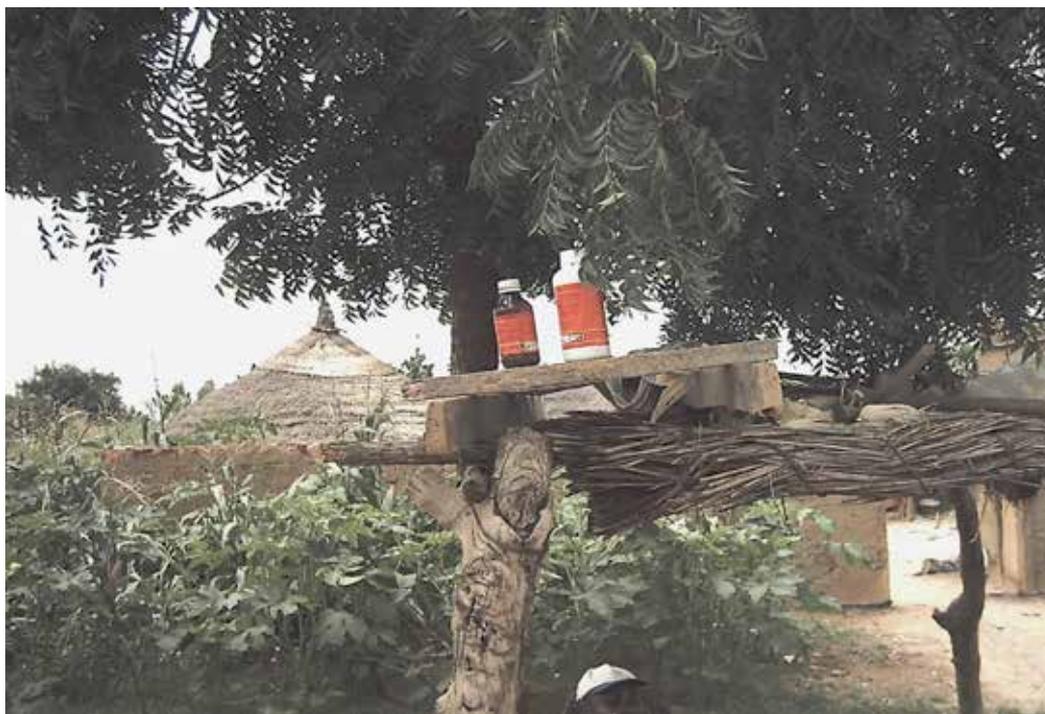


Fig. 4. A residence in the middle of fields probably treated by pesticides

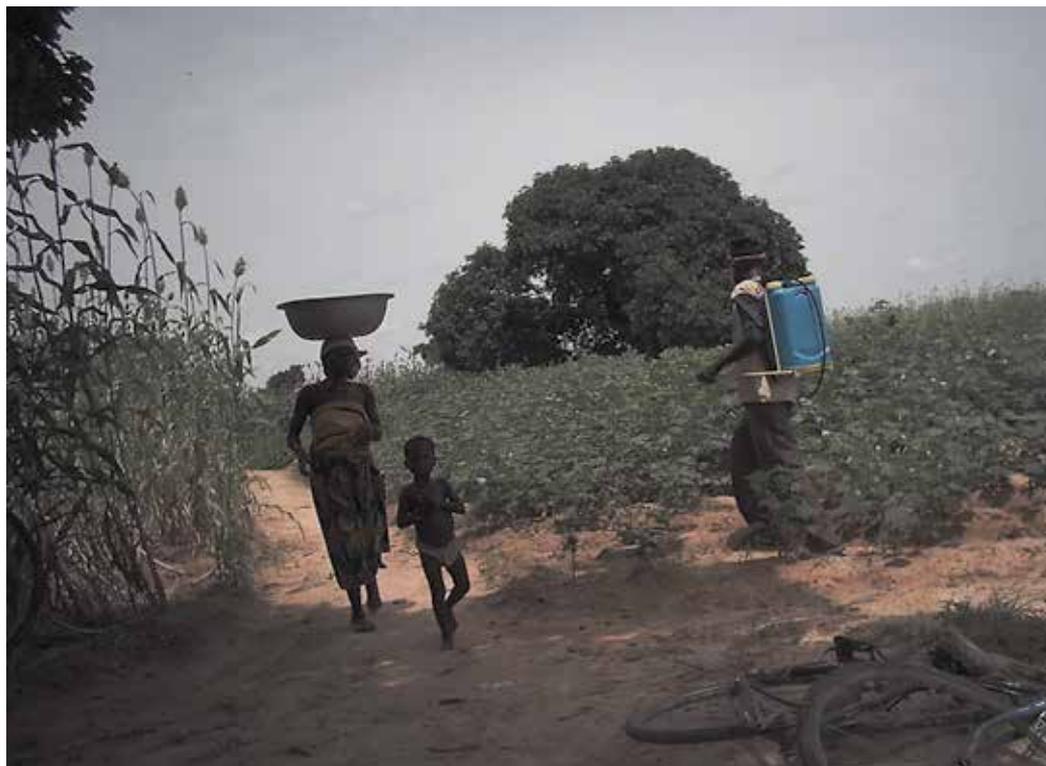


Fig. 5. Woman and child passing by a cotton field being sprayed with pesticides; on the left, a sorghum field.

## 5. Poisonings and pollutions due to pesticides in Burkina Faso

### 5.1 Acute poisoning

Acute pesticide poisoning has become a major public health problem worldwide, following the intensification of agriculture and the promotion of agrochemicals, with more than 300,000 deaths each year (Gunnell and Eddleston, 2003). The easy access to extremely toxic pesticides in the homes of the rural population has made pesticides the preferred means of suicide with an extremely high case fatality. The problem of acute pesticide poisoning in Burkina Faso is not well-documented though some preliminary epidemiologic studies were carried out in some parts of the country. The majority of the poisoned are over 15 years old, regardless of sex, possesses low levels of education and residing in rural areas. Etiologies are both accidental and deliberate self-poisoning (Toé et al., 2000). In two University Hospitals in Burkina Faso, agricultural pesticides are the chemicals the most implicated (29%) in acute poisoning after caustic agents (43%) in 2006 and 2007. The number of pesticides poisoning cases was the highest during the growing season from May to October (Yéré, 2007). The outcome of one-third of the acute intoxications is death (Toé et al., 2000). First aids given outside health centers to a minority (15%) of acute poisoned-patients are inadequate (e.g. drinking oil and/or milk). Drinking oil and/or milk is a worsening factor of intoxication because the majority of pesticides are soluble in fatty products. Evacuation, symptomatic or antidote treatment are often carried out in the health centers (Tankoano, 2008).

## 5.2 Chronic poisoning and pollution

The pesticide practices of farmers can lead to poisoning in long-term. These practices include spraying without any safety equipment for far longer than recommended periods, residing in agricultural area where pesticides are used (Fig. 4). The chronic exposure to the pesticides may trigger a variety of chronic health effects, such as cancers, neurologic effects and reproductive effects. The significant problems of human illness and death that follow chronic exposure to pesticides in Burkina Faso are not well-documented. Chronic poisoning could be common because pesticides applicators never have complete and adequate individual protective equipment (Ouédraogo et al., 2009). They think it is expensive and impractical to use safety equipment in Sahelian climate (Konaté, 2010). A study in the cotton-production region of Mouhoun in Burkina Faso revealed that more than 80% of pesticides applicators had lowered cholinesterase activity during at least two months after application (Toé et al., 2000).

The inadequate management of pesticides in Burkina Faso is a source of pollution of the environment (soils, waters, air, plants) i.e. the introduction of contaminants into a natural environment that causes instability, disorder, harm or discomfort to the ecosystem (physical systems or living organisms). Some studies confirmed the pollution of the waters and grounds of Burkina Faso, especially in the zones of cotton culture. Evaluation of soils pollution by the pesticides used in cotton production was carried out in seven sites of cotton culture area of Burkina Faso. Soils samples were taken in the cotton fields during the rainy season and the dry season in 2003 and 2004. Then, residues of pesticides were analyzed by gas chromatography. The results showed that the soils were contaminated by endosulfan (an organochlorine) in the level of 1 to 22  $\mu\text{g}/\text{kg}$ . A low pollution (1.7 to 5  $\mu\text{g}/\text{kg}$ ) by dimethoate (an organophosphate) was noted (Savadogo et al., 2006). That is not surprising because the organochlorines are known to be more persistent in the environment than organophosphates (Derkaoui et al., 2011). Another study revealed that in 2001, hundreds of fishers are found died on the edges of the main river that runs through the city of Bobo-Dioulasso in Burkina Faso. Chemical analysis indicated the presence of lindane and thiram in the organs of fishes, confirming the report that sacks containing the residues of pesticides had been washed in the river (Tarnagda et al., 2002). Such cases of pollution of waters could be frequent in the region of cotton culture where containers of pesticides are often washed in rivers or abandoned in the environment (Ouédraogo et al., 2009).

## 6. Alternatives to pesticides in Burkina Faso

### 6.1 Cultural, biological and traditional control

Sustainable pest management is a prerequisite to farming in semi-arid environments of Africa. Reliance on synthetic chemicals to control pests has also given rise to a number of problems such as destruction of beneficial non-target organisms (parasitoids and predators) thereby affecting the food chain and impacting on biological diversity. The injudicious use of synthetic pesticides can lead to secondary outbreaks of pests that are normally under natural control resulting in their rapid proliferation. There have also been cases of pests becoming tolerant to insecticides. In addition, due to other problems such as health hazards, undesirable side effects and environmental pollution caused by the continuous use of synthetic chemical pesticides there is renewed interest in the application of cultural and biological techniques, and in the use of botanical pesticides for crop protection. In Burkina Faso, researches have been undertaken to control pests by local plant materials. *Hyptis*

*spicigera* Lam., *Azadirachta indica* A. Juss. and *Euphorbia balsamifera* Ait. are investigated ; results from these studies are encouraging (Bambara and Tiemtoré, 2008). The use of such plant extracts to control pests is not a new innovation, as it has been widely used by small-scale subsistence farmers (Roy et al., 2005). Most of these botanical pesticides are non-selective poisons that target a broad range of pests. Botanical pesticides are biodegradable (Delvin and Zettel, 1999) and their use in crop protection is a practical sustainable alternative. They maintain biological diversity of predators (Grangen and Ahmed, 1988) and reduce environmental contamination and human health hazards. However, research on the active ingredients, pesticide preparations, application rates and environmental impact of botanical pesticides are a prerequisite (Buss and Park-Brown, 2002) for sustainable agriculture. The financial cost of these “biopesticides” could also be an obstacle to their development.

Research on field use of microbial agents in pests control is also an alternative to pesticides. In working with microbial pest control agents, attention must be given to handling and application techniques.

Numerous cultural and biological methods have been experimented by local populations for several years. For example, crop varieties which develop at different rates from the commonly planted varieties, or which show resistance to insect attack are often used by local populations. For example, sorghum is more resistant to attack by grasshoppers than millet.

## 6.2 Introduction of genetically modified organisms (GMO)

As cotton culture is the main market crop in Burkina Faso and consumes more than 90% of total pesticides used in Burkina Faso, development of genetically modified cotton (“Bt cotton”) has been undertaken by an American company in collaboration with Burkina Faso National Agricultural Research Institute. The American company has a gene derived from *Bacillus thuringiensis* (Bt) that protects the plants from specific lepidopteron insect pests. Bt cotton could reduce the need for costly pesticides and raising yields by around 30 percent. Two strains of Bt cotton, both developed from local varieties, have been approved for production and general sale in Burkina Faso. Bt cotton required only two pesticide treatments per season, compared with six or eight for non-modified cotton, according to its promoters. That cut pesticide use by at least 60 percent. 8,500 tons of Bt cotton were marketed in 2009. With its 115,000 hectares sown in 2009, a quarter of the surface area devoted to cotton, Burkina Faso will likely be into the top 10 producing countries of GMO cultivations with Bt cotton. Year 2010 marked the launch of large-scale genetically modified cotton on approximately 475,000 ha (Bonkougou, 2008, Tao, 2010) .

However, use of genetically modified crops has faced opposition from environmentalists who say the release of GMO could upset delicately balanced habitats or even lead to uncontrolled super species.

## 7. Recommendations

At the end of the overview of the situation of pesticides in Burkina Faso, the following recommendations could be suggested:

- Development of a system for dynamic inventory of pesticide chemical stocks.
- Pesticides used should be those with the minimum impact on non-target species.

- Appropriate labeling of all pesticides containers.
- Development of training courses for health personnel in areas where pesticides are used frequently.
- Development of training for pesticides storage and applications management, for empty containers and obsolete pesticides management intended to the users.
- It is also recommended to limit the fraudulent importation of not approved pesticides.
- A periodic evaluation of the environmental impact of pesticides in the areas where pesticides are used frequently.
- Epidemiological case-control studies should be implemented in areas of heavy human exposure to pesticides.
- Research on bio-pesticides must be encouraged.
- Research on the impact of genetically modified organisms (resisting to some pests) on environment and human health must be frequently carried out after their use at large-scale.

## 8. Conclusion

Pesticides are used in Burkina Faso mainly in agriculture for control of pests and weeds, especially in the cotton culture. All chemical families of pesticides are used but mainly organochlorines, organophosphates, carbamates, pyridines, and pyrethrinoids. Pesticides are not always handled carefully or tracked to insure correct use and disposal. So, acute, chronic poisoning and environment pollution were reported. Improvements in the system for managing pesticides must be implemented to protect human health and the environment. As alternatives to pesticides, cultural and biological techniques have been developed but their success is limited. In recent years, trends are to develop genetically modified organisms plants which could resist to pests. So, transgenic cotton production has become widespread in Burkina since 2010. However, use of transgenic has faced opposition from environmentalists. An evaluation of the environmental impact, even on human health of these GMO could be necessary after their use at large-scale.

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# Pesticide Use in South Africa: One of the Largest Importers of Pesticides in Africa

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## 1. Introduction

South Africa is a diverse country, with a diverse environment that is home to more than 49 000 000 people. Pesticide usage is very often necessary to maintain both agricultural productivity as well as human health. The climatic conditions range from semi-tropic to semi-arid regions. Although the majority of the country has summer rainfall, the south western coastal region is predominantly a winter rainfall area. These variations in climate allows for a wide variety of crops, from tropical fruit to maize and tree plantations. Each individual crop is susceptible to a unique host of pests that in-turn require a unique mixture of pesticides to ensure the best resulting turnover. Currently, South Africa has more than 500 registered pesticides (Pesticide Action Network (PAN), 2010) and is one of the four largest importers of pesticides in sub-Saharan Africa (Osbanjo et al., 2002). In 2006 the import of insecticides, fungicides and herbicides that were packaged for retail totalled \$ 170 056 000 the main import partners being Australia, China, Germany and the United States of America (USA) (International Trade Centre, 2011). These pesticides are used in almost every facet of our everyday lives; ensuring the quantity and quality of food we eat to managing the number of rodents and insects in our homes. Although it is evident that there is a vast amount of pesticides present in the South African environment, there is very limited data on the production of pesticides. The last published data indicates that in 2002 around 10 000 kℓ of liquid insecticides was produced exclusively for crop protection of which 43% consisted of organophosphates. During the same year 2 800-tonnes of solid insecticides were

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produced (Statistics South Africa, 2003). Although the usefulness of pesticides cannot be denied, the negative environmental and human health effects cannot be ignored. In South Africa, a number of environmental and anthropogenic factors have to be considered before the impact of large-scale pesticide use can be assessed.

South Africa is a water poor country, with water resources being utilised to their maximum capacity. As discussed by Dabrowski et al. (2009), the trade-off between the economic benefits of exporting agricultural products has to be measured against the loss of water, not only through crop irrigation but also through water quality degradation. The article highlighted this aspect through the calculation of virtual water volumes. These calculated volumes indicated that to ensure sufficient dilution of all agrochemicals, to an acceptable water quality level (used in a typical farming situation applying current-use pesticides), was greater than the amount of water needed for irrigation. The seriousness of these scenarios is highlighted in literature where a diverse array of agricultural chemicals has been measured during run-off events, by once-off sampling and by water monitoring during the growing seasons. Detectable levels of atrazine, terbuthylazine, simazine, acetochlor (Du Preez et al., 2005), DDT and its metabolites, endosulfan, hexachlorocyclohexane (HCH), heptachlor, aldrin, dieldrin, endrin, chlordane (Fatoki et al., 2003), azinophos-methyl, chloropyrifos (Schultz et al., 2001; Dabrowski et al., 2002) prothiofos (Schultz, 2001), malathion, zendoxsulfan (Thiere & Schultz, 2004), cypermethrin and fenvalerate (Bollmohr et al., 2007), to name a few, have all been measured in South African waters. Pesticides in the aquatic environment have the potential to affect all end-users, including both humans and wildlife.

South Africa has the distinction of being one of the countries with the most species richness in the world. To date more than 900 bird species as well as over 200 mammals, call South Africa home. Of these mammals, seven species are endangered and 30 are vulnerable according to the 2004 IUCN red data list (IUCN, 2010). These endangered species include bats, moles, shrews and mice that are often insectivorous, thus increasing their risk of unintentional exposure to pesticides. Within avian populations, 11 species are listed as critically endangered and 43 species as vulnerable. The sensitivity of avian species to pollutants has been widely reported. With this unique diversity of species, South Africans have a responsibility towards maintaining the viability of ecosystems and natural habitats to ensure the continued existence of these creatures. This objective is not only morally relevant but also economically relevant especially in a country where tourism creates over 400 000 jobs and contributes approximately 8% to the GDP. Few studies have reported the levels of insecticides in wildlife species. However, pesticides have been detected in wild bird species (Van Wyk et al., 2001; Bouwman et al., 2008), as well as in indigenous fish species (Barnhoorn et al., 2009), indicating pesticide contamination within various habitats. This is of particular concern due to the health risks associated with many pesticides.

## **2. Health effects associated with pesticide usage**

### **2.1 Biomagnification**

Depending on the chemical structure of the pesticides, they have a variety of detrimental effects that were not intended when first developed and used as an insecticides or herbicide. Two of the most common ecotoxicological effects almost all pesticides exhibit (to a greater or lesser extent), are their ability to be bioconcentrated by organisms and/or bio-magnified in food webs. Bio-concentration occurs when a compound has a higher concentration in the tissues of an organism, than in its surrounding environment. This often occurs in aquatic

organisms, since they are surrounded by the medium in which the insecticide/herbicide is distributed and the compound enters the aquatic organisms through their food and osmosis via the skin and gills. Bio-concentration is not limited to aquatic environments, but may occur in terrestrial environments as well. On the other hand, a compound is biomagnified if its concentration rises through the consecutive levels of a food web. This leads to the highest levels occurring in predators and these levels are much higher, than levels initially applied to land, crops or a water body. The one characteristic that probably contributes most to the extent to which a compound bio-concentrates or bio-accumulates, is its persistence. Pesticides are regarded as persistent if they are resistant to degradation through metabolic activity, ultraviolet radiation and extreme temperatures. Typical examples of pesticides with these characteristics are the organochlorine insecticides: DDT and its metabolites, as well as the cyclodiene insecticides (dieldrin, aldrin, heptachlor, endrin, telodrin and chlordane). Not only are they highly toxic, but they all share very low water solubility ( $\log K_{ow}$ : 3.78 – 6.36), are highly lipophilic and have low vapour pressures ( $4 \times 10^{-4} - 2 \times 10^{-7}$  mm Hg) (Walker, 2009). Due to their persistence in the environment, there is prolonged exposure to these pesticides. Therefore, they enter biota within the affected environment through all relevant routes of exposure.

As an example, when evaluating DDT, the most abundant and widespread residues found in the environment have been *p,p'*-DDE, *p,p'*-DDT and *p,p'*-DDD. All these compounds are highly persistent in soils, with half-lives of years once they have adsorbed onto the carbon content of soil. The longest half-lives have been recorded in temperate soils with a high abundance of organic matter (Cooke & Stringer, 1982). *p,p'*-DDE has the longest half-life in terrestrial animals and might explain its presence in terrestrial food webs years after bans on DDT were promulgated (Newton, 1986). Unchanged *p,p'*-DDT tends to decrease very slowly when absorbed/ingested by land vertebrates. In female mammals a certain amount is excreted into milk or across the placenta into the developing embryo or into eggs in the case of birds and reptiles (Walker, 2009), thus leading to detrimental teratogenic or reproductive effects. The same tendency is seen for the cyclodiene pesticides. In one example, dosing small female tortoiseshell butterflies (*Aglais urticae*) with dieldrin led to an increased number of deformed adults emerging from the pupae (Moriarty, 1968). Dieldrin, aldrin and heptachlor have half-lives in soil varying between 0.3 and 2.5 years (Edwards, 1973) and in vertebrates the half-lives vary between 12 and 369 days (Environmental Health Criteria 91). Biomagnification of highly lipophilic compounds, such as DDT metabolites and the cyclodienes, in the aquatic food web is due to bioaccumulation in the trophic levels, and through bio-concentration of the chemicals present in the ambient water. In a Pacific Ocean food web, zooplankton bioconcentrated total DDT residues 10 000 times to that found in the ambient water. The levels found in the striped dolphin (*Stenella coerulea alba*) were 100 times higher than that found in the zooplankton (Tanabe & Tatsukawa, 1992). *p,p'*-DDE can also undergo bioaccumulation in terrestrial food webs. Studies with earthworms and slugs showed the bioconcentration of total DDT residues, and dead blackbirds and song thrushes contained DDT residue levels 20 times higher than that found in the earthworms (Bailey et al., 1974). The bioaccumulation factor (BAF) of dieldrin was shown to be 63 in shag (*Phalacrocorax aristotelis*) as compared with its main prey, sand eel (Robinson et al., 1967). Although not all organometallic compounds are persistent, some free forms of methyl mercury ( $\text{CH}_3\text{HgCl}$  / MeHg) are highly lipophilic and undergo bioaccumulation and bioconcentration in the food web. According to a report from the US EPA (1980), fish bioconcentrated MeHg between 10 000 and 100 000 times its level in ambient water.

Additionally higher levels of MeHg were reported in predator compared to non-predator fish species (Environmental Health Criteria 101). Bio-accumulation of MeHg in birds was illustrated by the bioaccumulation factor of 2 in chickens that were fed dressed grain (a common application of MeHg) and a subsequent bioaccumulation factor of 4 in the goshawks that fed on the chickens (Borg et al., 1970). This provided further evidence that MeHg is slowly eliminated by vertebrates and, that predatory birds have weaker detoxifying capacity toward lipophilic xenobiotics as compared to non-predatory birds (Walker, 2009). Other forms of organometallic compounds containing mercury that have shown biomagnification, are the phenyl, alkoxy-alkyl or higher alkyl mercury compounds used as fungicides, although these mercury compounds biodegrade more easily and bioaccumulate less strongly than MeHg.

The second-generation anticoagulant rodenticides such as brodifacoum, difenacoum, flocoumafen and bromodiolone are also persistent and have very high cumulative toxicity that influences specifically the predators and scavengers of exposed rodents. They bind to proteins of the hepatic endoplasmic reticulum and therefore have long half-lives in vertebrates, often exceeding 100 days. The confounding factors that contribute to higher levels of rodenticides in predators and scavengers are:

- Rodents that consumed lethal doses of rodenticide may survive for 5 days or more before they die of haemorrhaging. In that time, they continue to feed, building up residues that finally exceed the levels needed to kill them;
- In addition, some resistant strains of rodents can tolerate relatively high levels of rodenticide and so act as more efficient vectors of the pesticide than susceptible strains;
- Poisoned rodents are likely to be more vulnerable and prone to be selected by the predator, increasing the possible dose to the predator (Walker, 2009).

Another example of a pesticide class which is not nearly as persistent as the organochlorine pesticides, but can undergo bioconcentration in the aquatic environment, is the organophosphorus pesticides (OPs). Chlorpyrifos bioconcentrated 225 fold in the eastern oyster (*Crassostrea virginica*) in comparison to its levels in ambient water (Woodburn et al., 2003). This bioconcentration was due to the very limited metabolic capacity of molluscs. OPs are easily metabolised by soil microorganisms and rapidly removed by soil animals so that these pesticides do not bioconcentrate in the soil (Walker, 2009). Although OPs do not biomagnify in the higher trophic levels, they have been implicated in the poisoning of predatory birds in the USA, UK and Canada (Mineau et al., 1999) as well as decreased earthworm numbers in South African orchards. The latter was due to chronic chlorpyrifos and intermittent azinphos methyl exposure (Reinecke & Reinecke, 2007). Pyrethroids are also lipophilic and can undergo bioconcentration in the lower trophic levels of the aquatic environment, but they are readily biodegradable by most organisms of higher trophic levels and do not biomagnify in either aquatic or terrestrial food webs. However, they strongly adsorb in soils and sediments where they become persistent.

## 2.2 Population decline of non-target organisms

Many pesticides can cause population declines of non-target organisms because of their persistence and modes of action. In this section examples of population declines are presented. Population decline of birds on the higher levels of the food web (such as the bald eagles, peregrines and double-breasted cormorant in North America) were explained by the two-fold effect of biomagnification and eggshell thinning (Peakall, 1993;

Wiemeyer et al., 1993; Walker et al., 2006). Eggshell thinning is possibly due to the inhibition of the  $\text{Ca}^{2+}$ -ATPase in the avian shell gland (Lundholm, 1987). A second possible mechanism for eggshell thinning is the evidence that *p,p'*-DDE can affect prostaglandin levels in the eggshell gland and thus contribute to eggshell thinning (Lundholm, 1997).

Population declines of bird species from England, Scotland, Canada and Norway have been reported because of DDT contamination of the environment (Walker, 2009). Another example of population decline of predatory birds such as the sparrowhawk (*Accipiter nisus*) and the peregrine falcon (*Falco peregrinus*), in Britain, coincided with the introduction of aldrin, dieldrin, and heptachlor in 1956 (Ratcliffe, 1993). Both these predatory birds preyed on seed-eating birds that fed on grain treated with these compounds. At the time when cyclodienes were widely used in Western Europe and North America, mammalian predators such as the fox (*Vulpes vulpes*) and badger (*Meles meles*) died due to lethal doses from their prey (Walker, 2009). Furthermore, terrestrial invertebrates such as honeybees are extremely susceptible to OPs.

Herbicides can be indirectly responsible for the population declines of animals by destroying their plant food source. An example of this is the decline of the grey partridge (*Perdix perdix*) in England. The chicks died due to lack of their insect food (sawflies) which in turn were limited because their food source, a weed, was destroyed by herbicides (Potts, 2000). However, a few are also toxic to animals. Dinitro-ortho-cresol and dinoseb act as uncouplers of oxidative phosphorylation in mitochondria, dissipating the energy that would otherwise have driven ATP synthesis. Paraquat and other bipyridyl herbicides have been implicated in the deaths of hares (Sheffield et al., 2001). Their toxicity to both plants and animals is believed to be due to cellular damage caused by oxyradicals (Hassall, 1990; Timbrell, 1999). Carbamate herbicides (chlorpropham) and sulphonylurea herbicides such as chlorsulfuron and sulfometuron have effects on cell division. In the next section two particular modes-of-action that contribute to population decline are presented. Both endocrine disruption and neurotoxic effects have specific methods through which they contribute to population decline.

### 2.3 Endocrine disrupting effects

Many pesticides (and herbicides) mimic hormones endogenous to animal bodies. In doing so, they can activate or inhibit the natural responses to the hormone causing disruption of the healthy process. This is described as endocrine disrupting (ED) effects. *o,p'*-DDT has been shown to have oestrogenic activity in birds (Bitman et al., 1978; Holm et al., 2006) and it is considered to be a more potent oestrogen than *p,p'*-DDE (Fry & Toone, 1981). The estrogenic effects seen in fish due to *p,p'*-DDE and dieldrin are attributed to three pathways:

- direct interactions with sex steroid receptors;
- changes in sex steroid biosynthesis;
- and changes in sex steroid metabolism (Garcia-Reyero et al., 2006).

The feminised effects seen in wildlife populations may result from chemicals blocking the androgen receptor (antiandrogenic) rather than as a consequence of exposure to (or in addition to) environmental oestrogens (Walker, 2009). Herbicides linuron and diuron and metabolites of the fungicide vinclozolin are antiandrogens as well (Gray et al., 1994).

Other organochlorine pesticides, methoxychlor and metabolites, and lindane and kepone can induce aberrant gonadal development, vitellogenin production, behavioural changes and disrupted ionic regulation in fish (Davy et al., 1973; Metcalfe et al., 2000). Another pesticide well known for its endocrine disruptive effects (an androgenic effect), is tributyltin (TBT). TBT compounds have been used as antifoulants on boats, as biocides for cooling systems, in paper and pulp mills, textile mills, breweries, leather plants and as molluscicides. Their toxicity is linked to two pathways:

- they act as inhibitors of oxidative phosphorylation in mitochondria (Aldridge & Street, 1964) causing disruption of the energy supply to the body;
- and they inhibit cytochrome P450 (Morcillo et al., 2004).

Cytochrome P450 enzymes are a large and diverse group of enzymes responsible for the oxidation of organic substances, including lipids, steroidal hormones, as well as drugs and toxic chemicals. It is this second toxic effect that most likely leads to TBT's hormone disrupting effect because TBT can inhibit cytochrome P450-based aromatase activity in both vertebrates and aquatic invertebrates (Morcillo et al., 2004; Oberdorster & McClellan-Green, 2002). Aromatase converts testosterone into oestrogen and when aromatase is inhibited, testosterone levels rise. The result of these inhibiting effects by TBT causes the masculinization of female gastropods (imposex) (Matthiessen & Gibbs, 1998). These females cannot reproduce, leading to decreasing population numbers. TBT also caused the masculinization of the Japanese flounder (Shimasaki et al., 2003). Of the pyrethroid pesticides, permethrin, fenvalerate, and cypermethrin have been reported to show (anti)oestrogenic and/or (anti)androgenic activity (Sun et al., 2007). Organophosphate pesticides (OPs) have been shown to have effects on the immune system of rodents (Galloway & Handy, 2003) as well as fish reproduction (Sebire et al., 2008). Blue death, a pesticide mixture consisting of carbaryl, carbufuran and camphechlor (although camphechlor has been banned in South Africa since 1970) indicated a positive correlation with birth defects of the male reproductive structures of babies born from mothers from the Eastern Cape in South Africa (Heeren, 2003). This might be due to endocrine disruption. The fungicide vinclozolin and the pyrethroid insecticides fenvalerate and permethrin have also been shown to interfere with progesterone function (Kim et al., 2005).

#### 2.4 Neurotoxic effects

*p,p'*-DDT and *p,p'*-DDD are persistent neurotoxins and may very well have caused behavioural effects in the field. *p,p'*-DDT binds reversibly to a site on axonal Na<sup>+</sup> channels, which are voltage dependent (Eldefrawi & Eldefrawi, 1990) and delays the usual quick closure of the channel and subsequent termination of the signal generated as a result of the Na<sup>+</sup> current. Pyrethroids have a similar effect. *p,p'*-DDT can also act on the K<sup>+</sup> channel, which is important for the repolarization of the axonal membrane after passage of the action potential. DDT and pyrethroids affect nerve transmission, and therefore, disruption of the regulation of the action potential occurs and this can lead to repetitive discharge. Pyrethroid show very marked selective toxicity. They are highly toxic to terrestrial and aquatic arthropods and to fish, but only moderately toxic to rodents and still less toxic still to birds (Walker, 2009). Some combinations of pyrethroids with ergosterol-biosynthesis-inhibiting (EBI) fungicides containing the active compound, prochloraz have synergistic effects and

become even more toxic to honey bees (Pilling & Jepson, 1993). This synergistic mechanism is largely attributed to prochloraz inhibiting cytochrome P450's detoxifying capacity (Pilling et al., 1995) and is of particular concern due to recent reductions in honeybee populations worldwide.

The cyclodienes, such as  $\gamma$ -HCH (lindane) are inhibitors of the gamma aminobutyric acid (GABA) receptor in the mammalian and insect brain as well as in insect muscles. The GABA receptors possess chloride channels that, when open, permit the flow of Cl<sup>-</sup> with consequent repolarization of nerves and reduction of excitability. They are particularly associated with inhibitory synapses and in vertebrates exposure to cyclodienes may lead to convulsions. Other symptoms include changes in the electroencephalogram (EEG) patterns, disorientation, loss of muscular coordination and vomiting (Hays & Laws, 1991). Dieldrin showed changes in the learning ability of squirrels (Van Gelder & Cunningham, 1975) and toxaphene caused changes in the behaviour of gold fish (Warner et al., 1966).

Mercury containing compounds are also detrimental to the nervous system, particularly the organic mercury compounds. They can cross the blood-brain barrier, but the inorganic mercury salts cannot. MeHg strongly binds to the -SH groups of amino acids, preventing normal protein function (Crosby, 1998). It binds to the cysteine groups (amino acids with -SH) of acetylcholine receptors and inhibits Na<sup>+</sup>/K<sup>+</sup> ATPase (Clarkson, 1987). This may cause extensive brain damage such as degeneration of small sensory neurons of the cerebral cortex leading to behavioural effects in mammals. Initially, the animals become anorexic and lethargic. As toxicity increases, muscle ataxia and blindness occur. At even higher levels, convulsions occur, which lead to death. Apart from its direct toxic effects, MeHg might have adverse interactive potentiation with other pollutants such as polychlorinated biphenyls, -dibenzo-*p*-dioxins, -dibenzofurans, *p,p'*-DDE, metals and selenium in specifically the aquatic environment specifically (Walker & Livingstone, 1992; Heinz & Hoffman, 1998).

OPs prevent the formation of the enzyme cholinesterase (ChE), which ensures that the chemical signal that causes a nerve impulse is stopped at the appropriate time and because of this, is neurotoxic to vertebrates and non-target invertebrates. They may cause behavioural effects. Symptoms of exposure include nausea, headaches, twitching, trembling, excessive salivation and tearing, inability to breath because of paralysis of the diaphragm and convulsions (Chopra et al., 2011). A few of these compounds (mipafox and leptophos) have been found to cause delayed neurotoxicity, but it was not caused by (ChE) inhibition. The target is neuropathy target esterase (NTE) (Johnson, 1992). No symptoms are seen immediately after phosphorylation of the enzyme but distal muscles become paralysed 2 to 3 weeks after the exposure and residues have disappeared from the body. Carbamates cause ChE inhibition poisoning by reversibly inactivating the enzyme acetylcholinesterase (Chopra et al., 2011). OPs and carbamates are readily biodegradable and do not bioaccumulate in the food web and are therefore regarded as "safer" than the more persistent organochlorine pesticides, but they have very high acute toxicity and some carbamates cause environmental problems because of their high vertebrate toxicity (Walker, 2009). In a study by Heeren et al. (2003) nervous system birth defects in babies born to mothers from the Eastern Cape in South Africa were positively correlated to the mother's exposure to agricultural chemicals that included OPs. Among the birth defects were nervous system defects.

The health risk posed by many pesticides was the driving force around the adoption of maximal residue levels (MRL) in agricultural products. MRLs have been initiated not only to ensure the quality of food imports, but also to ensure that the levels of pesticides that consumers are exposed to do not hold appreciable health risks. Of the 500 plus registered pesticides in South Africa, 229 have MRLs as listed in Table 1. Also listed in Table 1, are the chemical classifications of the pesticides as well the crops on which these pesticides are commonly used as related to the MRLs. The vast majority of these pesticides are carbamates, organophosphates and pyrethroid, that are additionally used on a wide variety of crops. There are also a number of alternative remedies registered for use in South African agricultural activities such as microbial, botanical and pheromone agents.

### **3. Agricultural activity in South Africa**

In 2009, South Africa was ranked 31<sup>st</sup> on the international gross domestic product (GDP) list, making South Africa the top producing country from the African continent. The agro-industrial sector contributes approximately 12% to the GDP and employs 8% of the formal workforce. However, estimates have been as high as 30% when non-registered farm workers and subsistence farmers in rural areas were included. This entire sector is dependent on agricultural yields for their livelihood. To maintain agricultural yields a host of crop protection methods are implemented. One of the most effective and widely accepted methods is the use of pesticides. The agricultural sector is an essential part of the South African economy, and is vital for food security. According to the World Bank (2011), 70% of the world's poor rely on agriculture as their main source of income and employment. This trend is evident in South Africa as well. Smallholdings and subsistence farming are prevalent in rural areas where weather conditions permit, although current trends indicate an increased drive to develop these small-scale agricultural activities into commercial farms. However, without the necessary training and management this often leads to farming practices predominantly reliant on the use of pesticides. In the economic domain, pesticides do not only assist but also hinder sales, through export restrictions. There is a universal trend to increase pesticide legislation with more stringent adherence to MRLs in the global market.

The agricultural sector is responsible for 8% of South Africa's total exports (South African Department of Agriculture (DAFF), 2009). In the year 2008/2009, the largest agricultural export products for South Africa were wine (\$922 million); maize (\$904 million); citrus fruit (\$814 million); apples, pears, quinces (\$465 million) and grapes (\$316 million) (DAFF, 2010). During this period the largest export trade partners were Zimbabwe (\$698 million), the Netherlands (\$695 million), the United Kingdom (\$689 million), Kenya (\$367 million) and Mozambique (\$333 million) (DAFF, 2010). Due to favourable climatic conditions, a surplus of maize was produced in 2008/2009. Also, the recent national crisis in Zimbabwe led to increased maize export from South Africa to Zimbabwe. The trend in previous years has seen African trade increase with both Zimbabwe and Mozambique as major export destinations (DAFF, 2009; DAFF, 2010). Agricultural production and trade does vary naturally. However, wine and fruit exports consistently dominate as the major export products for South Africa.

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Propyzamide	Amide herbicide	0.02 - 0.1: Apples, apricots, cherries, grapes, peaches, pears and plums
Diphenylamine	Amine fungicide, insecticide and plant growth regulator	10: Apples and pears
Boscalid	Anilide fungicide	5: Grapes
Fenhexamid	Anilide fungicide	5: Grapes
Clodinafop-propargyl	Aryloxyphenoxy propionic acid	0.05: Wheat
Fluazifop-P-butyl	Aryloxyphenoxy propionic acid herbicide	0.01 - 0.2: Apples, apricots, beans, carrots, coffee, grapes, nuts, peaches, pears, plums, potatoes, quinces, soya beans and sugar cane 0.05: Apples, apricots, citrus, grapes, peaches, pears, pineapples and plums; 0.1 - 0.5: Beans, beetroot, cotton seed, dry beans, peas, soya beans and sugar cane; 1 - 2: Groundnuts and lucerne
Haloxifop-R	Aryloxyphenoxy propionic acid herbicide	0: Milk; 0.05 - 0.2: peas, cucurbits and clover
Propaquizafop	Aryloxyphenoxy propionic acid herbicide	0.02 - 0.2: Apples, barley and wheat
Bromuconazole	Azole fungicide	0.02 - 0.1: Apples, barley, coffee, dry beans, grapes, pears, peas and wheat. 0.2 - 1: Cucurbits and oats
Cyproconazole	Azole fungicide	0.05 - 0.5: Apples, beans, citrus, grapes, groundnuts, pears, potatoes and tomatoes.
Difenoconazole	Azole fungicide	0.05 - 0.1: Apples, barley, pears, plums and wheat; 1: Apricots and peaches
Fenbuconazole	Azole fungicide	0.01 - 0.1: Apples, barley, dry beans, grapes, groundnuts, mangoes, pears, peas and wheat
Flusilazole	Azole fungicide	0.05 - 0.1: Apples, barley, dry beans, peaches, pears, soya beans and wheat
Flutriafol	Azole fungicide	0.01 - 0.05: Cucurbits, dry beans and mangoes; 0.1 - 1: Apples, grapes, peaches, pears, pineapples and pears
Hexaconazole	Azole fungicide	0.5: Cucurbits; 5: Citrus and musk melons
Imazalil	Azole fungicide	0.05 - 0.5 Cucurbits, dry beans, grapes and pears

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Penconazole	Azole fungicide	0.02 - 0.2: Apples, cucurbits, grapes, pears and peas 0.1 - 0.2: Barley, mushrooms, potatoes and wheat; 2- 10: Avocados, bananas, citrus, ginger and mangoes
Prochloraz	Azole fungicide	0.05 - 0.5: Bananas, barley, grapes, groundnuts, maize, peaches, nuts and wheat
Propiconazole	Azole fungicide	0.02 - 0.1: Barley, beans, citrus, groundnuts, mangoes, oats, onions, potatoes, soya beans, tomatoes and wheat; 2 - 5: grapes
Tebuconazole	Azole fungicide	0.5: Grapes
Tetraconazole	Azole fungicide	0.05 - 0.5: Apples, bananas, barley, cucurbits, mangoes, oats, peas and wheat; 2: Grapes
Triadimefon	Azole fungicide	0.05 - 0.5: cucurbits, peas, soya beans and apples; 1: Grapes
Triadimenol	Azole fungicide	0.05: Avocados, litchis, nuts, mangoes, peaches and plums
Paclobutrazol	Azole plant growth regulator	0.05: Potatoes and 2: grapes
Zoxamide	Benzamide fungicide	0.05 - 0.1: Maize, groundnuts, pears, sugar cane and wheat. 1 - 3: Apples, apricots, avocados, bananas, grapes, peaches, pears, peppers, plums and tomatoes. 5: Citrus and mangoes.
Benomyl	Benzimidazole fungicide	0.01 - 0.1: Avocados, chicory, dry beans, groundnuts, mangoes, maize, oats and potatoes. 0.2 - 1: Grapes, peas and tomatoes. 3 - 5: Apples, citrus and pears
Carbendazim	Benzimidazole fungicide	1 - 10: Apples, avocados, bananas, citrus, mushroom, musk melons, pears, pineapples and potatoes
Thiabendazole	Benzimidazole fungicide	0.01 - 0.05: Apples, cotton seed, canned peaches, pears and tomatoes
Novaluron	Benzolurea herbicide	0.02 - 0.1: Tomatoes and cabbage
Lufenuron	Benzolurea insecticides	0.2 - 0.5: Tomatoes and mangoes
Acibenzolar-S-methyl	Benzothiadiazole plant activator and fungicide	0.01: Potatoes, 0.1: mushrooms, 1: apples and pears.
Diflubenzuron	Benzoyl urea	0.05: Apples and pears
Flufenoxuron	Benzoyl urea insecticide	0.02 - 0.5: Citrus and litchis
Teflubenzuron	Benzoyl urea insecticide	

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Triflumuron	Benzoyl urea insecticide	0.1 - 0.5: Chicken fat, citrus, litchis, mangoes and peaches; 2: Apples and pears
Thiophanate-methyl	Bezimidazole precursor fungicide	0.1: Barley, groundnuts and wheat; 3 - 5: Apples, citrus and pears
Dicamba	Benzoic acid herbicide	0.1 - 0.2: Maize, sorghum, sugar cane and wheat.
Diquat dibromide	Bipyridylum desiccant and herbicide	0.05: Potatoes and 0.5: sunflower seed
Paraquat dichloride	Bipyridylum herbicide	0.02 - 0.5: Cotton seed, maize, sugar cane
Emamectin, benzoate	Botanical insecticide	0.01: Tomatoes
Pyrethrins	Botanical insecticide	1 - 2: Apples, apricots, beans, broccoli, Brussels sprouts, cabbage, cauliflower, cereal grains, citrus, cotton seed, cucurbits, dried fruit, dried nuts, dried vegetables, grapes, groundnuts, guavas, lettuce, oil seeds, peaches, plums, sunflower seed and tomatoes
Gibberellins	Botanical plant growth regulator	0.05- 0.2 Apples, citrus and grapes
Iprovalicarb	Carbamate fungicide	0.05 - 0.5: Grapes, potatoes and tomatoes
Maneb	Carbamate fungicide	0.01: all foodstuffs except cereal grains and grapes. 0.1: cereal grains. 180: grapes
Oxycarboxin	Carbamate fungicide	0.5: Beans
Propamocarb hydrochloride	Carbamate fungicide	0.5: Potatoes and 2: cucumbers
Thiram	Carbamate fungicide	3 - 5: Apples, apricots, grapes, peaches, pears and plums
Carbosulfan	Carbamate insecticide	0.05 - 0.2: Grapes and maize
Formetanate	Carbamate insecticide	0.02 - 0.5: Apples, citrus, grapes and peaches
Methiocarb	Carbamate insecticide	0.1 - 0.2: Apples, apricots, citrus, grapes, pears and plums
Methomyl	Carbamate insecticide	0.02 - 0.2: Beans, broccoli, Brussels sprouts, cabbage, cauliflower, citrus, maize, peaches, potatoes, sorghum, sunflower seed, tomatoes and wheat

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Pirimicarb	Carbamate insecticide	0.05 - 0.5: Apples, broccoli, Brussels sprouts, cabbage, cauliflower, citrus, cotton seed, groundnuts, oats, peaches, nuts, potatoes, sorghum and wheat
Propoxur	Carbamate insecticide	0.05: grapes
Thiodicarb	Carbamate insecticide	0.1 - 0.5 Cotton seed and maize
Carbofuran	Carbamate insecticide and nematocide	0.05 - 0.5: Broccoli, Brussels sprouts, cabbage, cauliflower, cotton seed, maize, potatoes, sorghum, sugar cane, sunflower seed and wheat. 0.1 - 0.5: Cactus pears, castor oil seed, cottonseed, maize, meat, eggs, milk and poultry. 2.5: Apples, apricots, beans, grapes, pears, sorghum and wheat
Carbaryl	Carbamate insecticide, nematocide and plant growth regulator	0.05: cottonseed, nuts, maize and pineapples. 0.2-0.5: Bananas, citrus, coffee, grapes, groundnuts, sweet potatoes and tomatoes. 1 -2: Fodder (hay), potatoes and hops (dry).
Aldicarb	Carbamate pesticide	0.1 - 0.2: Maize and sorghum
Bendiocarb	Carbamate pesticide	Beans, maize, potatoes, sugar cane, sunflower seed, sweet corn and sweet potatoes
EPTC	Carbamate pesticide	
Dichlorophene	Chlorinated phenol fungicide, herbicide, microbiocide	Pineapples, potatoes and tomatoes.
Alachlor	Chloroacetanilide	0.05 - 0.1: Broccoli, Brussels sprouts, cabbage, groundnuts, maize, pineapples, potatoes, soya beans, sugar cane and sunflower seed
Acetochlor	Chloroacetanilide herbicide	0.02- 0.05: Cotton seed, groundnuts, maize, sorghum and sugar cane
Metazachlor	Chloroacetanilide herbicide	0.05 - 0.1: Cabbage, dry beans, groundnuts, maize, potatoes, sugar cane, sunflower seed and sweet corn
Metolachlor	Chloroacetanilide herbicide	0.05: Beans, cotton seed, dry beans, groundnuts, maize, potatoes, sorghum, soya beans, sugar cane and sunflower seed
Propachlor	Chloroacetanilide herbicide	0.1 - 0.2: Maize, onions and sorghum

<b>Chemical</b>	<b>Pesticide notes</b>	<b>South African MRL (mg kg<sup>-1</sup>) and associated crops</b>
Diclofop-methyl	Chlorophenoxy acid or ester herbicide	0.05: Wheat
MCPA and its salts	Chlorophenoxy acid or ester herbicide	0.1: Barley, maize, potatoes, rye, sorghum, sugar cane and wheat
Triclopyr	Chloropyridinyl herbicide	0.1: Citrus
Cycloxydim	Cyclohexenone derivative herbicide	0.5: Beans, cottonseed, cucurbits, dry beans, grapes, groundnuts, onions, soya beans and tomatoes.
Tralkoxydim	Cyclohexenone derivative herbicide	0.05: Barley and wheat
Dimethipin	Defoliant and plant growth regulator	0.1: Cotton seed
Tebufenozide	Diacylhydrazine insecticide	1: Apples and pears
Iprodione	Dicarboximide fungicide	0.05 - 0.5: ginger, onions and canned peaches; 1 - 5: Apricots, apples, citrus, grapes, kiwifruit, peaches, pears, plums, raspberries, strawberries and tomatoes
Vinclozolin	Dicarboximide non-systemic general use pesticide and fungicide	1 - 3: Strawberries and grapes
Pendimethalin	Dinitroaniline herbicide	0.05: Potatoes
Trifluralin	Dinitroaniline herbicide	0.05: Cabbage, chillies, cowpeas, dry beans, groundnuts, kidney beans, soya beans, sunflower seeds and tomatoes; 1: Carrots
Dinocap	Dinitrophenol derivative fungicide and insecticide	1: Apples, broccoli, Brussels sprouts, cabbage, cauliflowers, cucurbits, grapes, peaches, pears and peas
Fomesafen	Diphenyl ether herbicide	0.05: Dry beans, groundnuts and soya beans
Oxyfluorfen	Diphenyl ether herbicide	0.05: Citrus and garlic
Zineb	Dithiocarbamate fungicide	0.05 - 0.5: Groundnuts, onions and potatoes; 3: Apples, apricots, bananas, beans, boysenberries, broccoli, Brussels sprouts, cabbage, cauliflower, citrus, cucurbits, grapes, guavas, mangoes, olives, papayas, peaches, pears, peppers, plums, quinces, tomatoes and youngberries
Furfural	Fumigant	Carrots, lettuce, onions, potatoes and sugar cane

<b>Chemical</b>	<b>Pesticide notes</b>	<b>South African MRL (mg kg<sup>-1</sup>) and associated crops</b>
Cymoxanil	Fungicide	0.01 - 0.2: Grapes, potatoes and tomatoes
Dithianon	Fungicide	2: Apples, apricots, peaches, pears and plums
Epoxiconazole	Fungicide	0.01 - < 0.05: Maize and barley
Famoxadone	Fungicide	0.01 - 0.02: Potatoes, 0.2: tomatoes, 1: grapes
Fludioxonil	Fungicide	0.5: Grapes
Fosetyl-Al	Fungicide	5 - 50: Avocados, boysenberries, citrus, cucumber, grapes, pineapples, potatoes and youngberries
Guazatine	Fungicide	2.5: Tomatoes and 5: Citrus
Spiroxamine	Fungicide	0.05: Barley and wheat; 0.1: peas and 1: grapes
Dodine	Guanidine fungicide and microbiocide	1: Apples, pears and quinces
Methyl bromide	Halogenated organic fumigant, herbicide, insecticide and nematicide	10- 100: Cereal grains, dried fruit, dried legumes, processed grain products and groundnuts
Cyhexatin	Heavy metal, organotin insecticide	2: Apples, citrus, peaches, pears, plums and tomatoes. 150: Hops (dry).
Fenbutatin-oxide	Heavy metal, organotin insecticide	0.2 - 2: Apples, beans, citrus, peaches, pears, peppers and tomatoes
2,4-D	Herbicide	0.5 - 2: Barley, citrus, maize, potatoes, rye, sorghum, sugar cane and wheat
Flurochloridone	Herbicide	0.02 - 0.05: Apples, carrots, grapes, nectarines, pears, plums, potatoes and sunflower seed
Mesotrione	Herbicide	0.01: Maize
Sulcotrione	Herbicide	0.05: Maize and sugar cane
Ioxynil	Hydroxybenzotrile herbicide	0.05: Sugar cane
Bromoxynil phenol	Hydroxybenzotrile insecticide	0.1: Barley, maize, oats, sorghum, sugar cane and wheat
Imazapyr	Imidazolinone herbicide	0.05: Dry beans, groundnuts and soya beans
Magnesium phosphide	Inorganic fumigant and rodenticide	0.01: all foodstuffs except cereal grains and grapes. 0.1: cereal grains. 180: grapes

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Phosphoric acid	Inorganic fungicide, herbicide, antimicrobial and pH adjuster	25 - 50: Grapes and citrus
Calcium arsenate	Inorganic heavy metal herbicide, insecticide and rodenticide	0.2: Citrus
Sulphur	Inorganic herbicide and insecticide	50 - 55: Apples, apricots, avocados, bananas, beans, boysenberries, citrus, cucurbits, grapes, litchis (pulp), mangoes, papaya, peaches, pears, peas, peppers, plums, tomatoes and youngberries; 1 000: litchis peel
Aluminum phosphide	Inorganic phosphide fumigant	0.01: all foodstuffs except cereal grains and grapes. 0.1: cereal grains. 180: grapes
Propineb	Inorganic -zinc carbamate antimicrobial and fungicide	0.5: Groundnuts and potatoes; 3: Boysenberries, grapes, tomatoes and youngberries
Mancozeb	Inorganic-zinc carbamate fungicide	0.01: all foodstuffs except cereal grains and grapes. 0.1: cereal grains. 180: grapes
Metiram	Inorganic-zinc carbamate fungicide	0.5: Potatoes; 3: Apples, apricots, beans, grapes, peaches, pears, plums and tomatoes
Buprofezin	Insect growth regulator	0.05: Avocados and peaches
Bromopropylate	Insecticide	0.2 - 3: Bananas, citrus, cotton seed and grapes
Etoxazole	Insecticide	0.1 - 0.2: Apples, pears and tomatoes
Fenazaquin	Insecticide	0.05 - 0.5: Apples, citrus, pears and tomatoes
Indoxacarb, S-isomer	Insecticide	0.01 - 0.05: Potatoes, cauliflower; 0.02 - 0.2: Tomatoes, beans, peaches and peas; 1: Apples, cabbage, broccoli, Brussels sprouts and pears
Propargite	Insecticide	0.05 - 0.5: Cotton seed and pears; 2 - 3: Apples, citrus, peaches, strawberries and tomatoes
Tetradifon	Insecticide	0.05: Cotton seed; 5 - 8: Apples, apricots, citrus, cotton seed, peaches, pears, plums and dry tea
Triforine	Insecticide and fungicide	0.1 - 0.5: Cucurbits and peas; 1 - 2: Apples, beans, peaches and plums
Spirodiclofen	Keto-enol insecticide	0.01: Peaches

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Ametryne	Methylthiotriazine herbicide	0.05 - 0.2: Bananas, maize, pineapples and sugar cane
Milbemectin	Microbial insecticide	0.01: Apples and tomatoes
Spinosad	Microbial insecticide	0.01 - 0.5: Apples, apricots, beans, citrus, cabbage, cucurbits, grapes, guavas, mangoes, olives, peaches, pears, peas, plums, potatoes and tomatoes
Dimethomorph	Morpholine fungicide	0.01: Potatoes, 0.1: tomatoes and 5: grapes
Tridemorph	Morpholine fungicide	0.1 - 0.2: Cucurbits and peas
Copper and its salts	Multiple forms and uses	1: Potatoes and nuts. 20: Apples, apricots, avocados, beans, boysenberries, broccoli, Brussels sprouts, cabbage, cauliflower, celery, cherries, citrus, coffee, cucurbits, granadillas, grapes, guavas, lettuce, mangoes, olives, peaches, pears, peppers, plums, strawberries, tomatoes and youngberries.
Acetamiprid	Neonicotinoid insecticide	0.2 - 0.50: Barley, canola, citrus, oats, cotton seed, tomatoes and wheat
Imidacloprid	Neonicotinoid insecticide	0.05 - 0.5: Apples, citrus, cotton seed, cucurbits, grapes, maize, sorghum, sunflower seed, tomatoes and wheat
Thiacloprid	Neonicotinoid insecticide	0.1: Peaches and 1: apples
Thiamethoxam	Neonicotinoid insecticide and fungicide	0.02 - 0.05: Apples and cotton seed
Cartap monohydrochloride	Nereistoxin insecticide	5: Onions, 10: tomatoes and 150: cabbage
MSMA	Organoarsenic defoliant and herbicide	0.05: Sugar cane
Dicofol	Organochlorine insecticide	Apples, apricots, bananas, beans, broccoli, Brussels sprouts, cabbage, cauliflower, cherries, citrus, cotton seed, cucurbits, granadillas, peaches, pears, peas, peppers, plums, quinces and tomatoes.

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Endosulfan	Organochlorine insecticide	0.05: Granadillas, nuts, pineapples and potatoes; 0.1 - 1: Apples, apricots, Boysenberries, broccoli, Brussels sprouts, cabbage, cauliflower, cherries, citrus, coffee, cotton seed, cucurbits, grapes, groundnuts, maize, onions, paprika, peaches, pears, peas, plums, quinces, sorghum, sugar cane, sunflower, tomatoes, wheat and youngberries; 20: Hops (dry)
Lindane	Organochlorine insecticide and rodenticide	0.01 - 0.02: Milk, cottonseed, onions, potatoes and sweet potatoes; 1: Apples, apricots, beans, broccoli, Brussels sprouts, cabbage, cauliflower, peaches, pears, plums.
Fenthion	Organophosphate acvicide and insecticide	0.1 - 1: Apples, apricots, coffee, cucurbits, grapes, guavas, kiwifruit, mangoes, peaches, pears, plums and quinces.
Acephate	Organophosphate insecticide	1 - 3: Apples, broccoli, Brussels sprouts, cabbage, cauliflower, grapes, peaches, pears, plums, potatoes and tomatoes
Azinphos-methyl	Organophosphate insecticide	0.05: Cottonseed, olives and potatoes. 0.04: Apples and pears. 0.1 - 0.2: Apricots, citrus, peaches and plums.
Cadusafos	Organophosphate insecticide	0.02 - 0.05: Bananas, citrus and potatoes
Chlorpyrifos-methyl	Organophosphate insecticide	8: Cereal grains
Malathion / Mercaptothion	Organophosphate insecticide	0.05: maize, peas, onions, sorghum and sugar cane; 1 - 8: Apples apricots, avocados, bananas, beans, broccoli, Brussels sprouts, cabbage, cauliflower, cereal grains, citrus clover, cotton seed, cucurbits, dried fruits, dried nuts, granadillas, grapes, groundnuts, guavas, litchis, mangoes, mushrooms, oil seeds, papayas, peaches, pears, peppers, pineapples, plums, quinces, sunflower seed and tomatoes

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Oxydemeton-methyl	Organophosphate insecticide	0.1 - 0.4: Apples, apricots, beans, broccoli, Brussels sprouts, cabbage, cauliflower, citrus, cotton seed, cucurbits, aubergine, groundnuts, maize, onions, peaches, pears, peas, peppers, plums, potatoes, Rooibos, sorghum, tomatoes and wheat.
Parathion	Organophosphate insecticide	0.05 - 0.5: Barley, beans, beetroot, broccoli, Brussels sprouts, cabbage, cactus pears, carrots, castor-oil seed, cauliflower, citrus, coffee, cotton seed, cucurbits, aubergine, groundnuts, mangoes, onions, peas, peppers, quinces, sorghum, spinach, sweet potatoes, tomatoes, turnips and wheat
Phenthoate	Organophosphate insecticide	0.1 - 0.2: Mangoes, onions and potatoes; 1: Broccoli, Brussels sprouts, cabbage, cauliflower and citrus
Phoxim	Organophosphate insecticide	0.2: Cereal grains and groundnuts
Pirimiphos-methyl	Organophosphate insecticide	3 - 10: Groundnuts, maize, sorghum, soya beans, stored wheat and sunflower seed
Procymidone	Organophosphate insecticide	0.05 - 0.5: Citrus, groundnuts, pears and potatoes; 1 - 10: Beans, grapes, peaches, plums and tomatoes
Prothiofos	Organophosphate insecticide	0.05: Apples, apricots, citrus, mangoes, pears and plums; 1: Grapes and guavas
Temephos	Organophosphate insecticide	1: Citrus
Trichlorfon	Organophosphate insecticide	0.05 - 0.2: Apples, apricots, broccoli, Brussels sprouts, cabbage, cauliflower, citrus, coffee, cucurbits, granadillas, grapes, guavas, litchis, maize, peaches, plums, quinces and sweet potatoes; 1: Beans and tomatoes

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Demeton-S-methyl (mixture) <sup>†</sup>	Organophosphate insecticide	0.1 - 0.5: Apples, apricots, barley, beans, broccoli, Brussels sprouts, cabbage, cauliflower, citrus, cotton seed, eggplant, groundnuts, maize, olives, onions, peaches, pears, peas, peppers, plums, potatoes, Rooibos, sorghum, tomatoes and wheat
Diazinon	Organophosphate insecticide	0.02: Milk, 0.2 - 0.7: apples, apricots, beans, broccoli, brussels sprouts, cabbage, meat, cauliflower, mushrooms, peaches, pears, pineapples, plums and tomatoes
Dimethoate	Organophosphate insecticide	Apples, barley, beans, broccoli, brussels sprouts, cabbage, cauliflower, citrus, cotton seed, cucurbits, grapes, groundnuts, peaches, pears, pineapples, plums, potatoes, sorghum, strawberries and wheat
Methamidophos	Organophosphate insecticide	0.05 - 0.5: Canola, citrus, potatoes and tomatoes; 1: Apples, apricots, broccoli, brussels sprouts, cabbage, mangoes, peaches, pears and plums
Methidathion	Organophosphate insecticide	0.02 - 0.3: Apples, apricots, cactus pears, cherries, grapes, peaches, pears, plums and potatoes; 2: Citrus
Mevinphos	Organophosphate insecticide	0.05: Potatoes; 0.1 - 0.2: Beans, broccoli, brussels sprouts, cabbage, cauliflower, citrus, cucurbits, grapes, lettuce, peas, peppers, spinach, tomatoes and wheat
Omethoate	Organophosphate insecticide	0.05 - 0.5: Barley, cotton seed, oats and onions; 1 - 1.5: Apples, grapes, pears, peas and wheat
Phosmet	Organophosphate insecticide	2 - 5: Apples and pears
Profenofos	Organophosphate insecticide	0.05: Onions and potatoes; 0.5 - 1: Brussels sprouts, cabbage, cauliflower, citrus and tomatoes

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Chlorpyrifos	Organophosphate insecticide and nematicide	0.05 - 1: Apples, apricots, bananas, broccoli, Brussels sprouts, cabbage, carrots, cauliflower, citrus, grapes, lettuce, mangoes, maize, wheat, peaches, pears, plums, potatoes and tomatoes.
Disulfoton	Organophosphate insecticide and nematicide	0.05 - 0.5: Cabbage, cauliflower, coffee, cotton seed, onions, potatoes, tomatoes and wheat
Ethoprop	Organophosphate insecticide and nematicide	0.01: Potatoes and 0.05: citrus
Fenamiphos	Organophosphate insecticide and nematicide	0.01 - 0.2: Bananas, citrus, cotton seed, ginger, grapes, groundnuts, guavas, litchis, onions, papaya, peaches, peas, nuts, pineapples, potatoes and tomatoes
Methyl parathion	Organophosphate insecticide and nematicide	0.05: Coffee and 1: citrus
Phorate	Organophosphate insecticide and nematicide	0.05: Apples, broccoli, Brussels sprouts, cabbage, cauliflower, cotton seed, maize, onions, potatoes and wheat
Terbufos	Organophosphate insecticide and nematicide	0.05 - 0.1: Citrus, dry beans, groundnuts, maize, potatoes, sorghum and sunflower seed
Fosthiazate	Organophosphate nematicide	0.05- 0.1: Bananas, citrus and potatoes
Ethephon	Organophosphate plant growth regulator	0.05: Maize and sugar cane. 1 - 5: Apples, cherries, citrus, cotton seed, grapes, peaches, pineapples, plums and wheat
Ortho-phenylphenol	Phenol antimicrobial	10: Citrus
Glyphosate and its salts	Phosphonoglycine herbicide	0.5: Sugar cane and 2: Maize
1-Naphthaleneacetic acid, methyl ester	Plant growth regulator	1: Apples and pears
Chlorfenapyr	Pyrazole insecticide	0.01 - 0.5: Apples, citrus, grapes, nectarines, pears, plums, potatoes and tomatoes.
Fipronil	Pyrazole insecticide	0.01 - 0.05: Broccoli, cabbage, cauliflower, citrus and mangoes
Pyraflufen-ethyl	Pyrazolyphenyl herbicide	0.01: Barley and wheat

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Bioresmethrin	Pyrethroid insecticide	0.05: Groundnuts. 0.1 - 1: Apples, apricots, beans, peaches, pears and plums.
Cyfluthrin	Pyrethroid insecticide	0.05: Cottonseed. 0.1 - 0.2: Apples, beans, broccoli, Brussels sprouts, cabbage, cauliflower, grapes, maize, pears, peas, sorghum and tomatoes. 1: Wheat
Cyfluthrin, beta	Pyrethroid insecticide	0.05 - 0.2: Apples, beans, broccoli, Brussels sprouts, cabbage, canola, cauliflower, cotton seed, grapes, nuts, maize, peaches, pears, peas, potatoes, sorghum, tomatoes and wheat.
Cyhalothrin, gamma	Pyrethroid insecticide	0.01 - 0.5: Apples, apricots, grapes, beans, cotton seed, cruciferae, groundnuts, nuts, maize, onions, peaches, pears, peas, plums, potatoes, sorghum, tomatoes and wheat.
Cyhalothrin, lambda	Pyrethroid insecticide	0.01 - 0.5: Apples, apricots, beans, broccoli, Brussels sprouts, cabbage, cauliflower, grapes, groundnuts, maize, onions, peaches, pears, peas, plums, potatoes, sorghum, tomatoes, wheat and nuts
Cypermethrin	Pyrethroid insecticide	0.05 - 0.1: Beans, broccoli, Brussels sprouts, cabbage, cauliflower, cottonseed, grapes, groundnuts, nuts, peas and plums. 0.2 - 1: Apples, citrus, maize, peaches, pears, green Rooibos tea, tomatoes and wheat. 2: Dried rooibos tea
Cypermethrin, alpha	Pyrethroid insecticide	0.02-0.05: groundnuts, cotton seed, grapes, nuts, potatoes, sugar cane and wheat. 0.1 - 0.5: Beans, broccoli, Brussels sprout, cabbage, cauliflower, maize, peaches, pears, peas and tomatoes
Cypermethrin, beta	Pyrethroid insecticide	0.05 - 0.5: Apples, beans, citrus, cruciferae, grapes, groundnuts, nuts, maize, peaches, pears, peas, plums, sorghum, tomatoes and wheat.

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Cypermethrin, zeta	Pyrethroid insecticide	0.05 - 0.5: Apples, beans, broccoli, Brussels sprouts, cabbage, cauliflower, cotton seed, grapes, nuts, maize, peaches, pears, peas, sorghum, tomatoes and wheat. 0.05: Cactus pears, groundnuts, mangoes, onions, potatoes, sweet potatoes and tomatoes. 0.1 - 0.2:
Deltamethrin	Pyrethroid insecticide	Apples, beans, broccoli, Brussels sprouts, cabbage, cauliflower, cotton seed, grapes, lettuce, maize, paprika, peaches, pears, plums, sorghum. 1 - 5: Hops (dry), oats, rye, stored grain and wheat.
Esfenvalerate	Pyrethroid insecticide	0.05 - 0.5: Apples, beans, cotton seed, grapes, mangoes, maize, pears, peas, potatoes, sorghum, sunflower seed, tomatoes and wheat, 15: hops (dry). 0.05 - 0.1: Grapes, mangoes, wheat, peas, potatoes, tomatoes; 0.5 -1:
Fenvalerate	Pyrethroid insecticide	apples, beans, cotton seed, maize, pears, sorghum, sunflower seed; 15: hops (dry)
Permethrin	Pyrethroid insecticide	0.05 - 0.5: Apples, beans, cotton seed, grapes, groundnuts, maize, pears, peas, potatoes, sorghum, soya beans and tomatoes; 2: Cereal grains
Tau-fluvalinate	Pyrethroid insecticide	0.05 - 0.2: Apples, canola, cotton seed, peaches, pears, tomatoes and wheat
Bifenthrin	Pyrethroid insecticide	0.05 - 0.2: Apples, cottonseed, maize, pears, potatoes and tomatoes.
Acrinathrin	Pyrethroid insecticide and acaricide	0.1: Apples, pears, tomatoes and hops with MRL of 10
Fluroxypyr	Pyridinecarboxylic acid	0.1 - 0.5: Fat, meat, milk and kidney
Bupirimate	Pyrimidine fungicide	0.05 - 0.5: Apples, cucurbits, mangoes and peaches
Cyprodinil	Pyrimidine fungicide	0.05 - 0.1: Apples, barley and grapes
Fenarimol	Pyrimidine fungicide	0.2: Apples and grapes
Mepiquat chloride	Quaternary ammonium plant growth regulator	1: Cotton seed
Quinoxifen	Quinoline fungicide	0.5 - 1: Cucurbits and grapes

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Azoxystrobin	Strobin fungicide	0.01 - 0.05: Brussels sprouts, cabbage, maize and potatoes. 0.2 - 1: Broccoli, cauliflower, citrus, grapes, mangoes and tomatoes.
Kresoxim-methyl	Strobin Fungicide	0.01 - 0.5: Apples, citrus, cucurbits, grapes, mangoes and pears
Pyraclostrobin	Strobin Fungicide	0.1 - 0.5: Citrus and grapes
Trifloxystrobin	Strobin fungicide	0.05 - 0.5: Apples, citrus, cucurbits, grapes, maize, pears and potatoes
Chlorsulfuron	Sulfonylurea herbicide	0.05: Oats and wheat
Iodosulfuron methyl, sodium salt	Sulfonylurea herbicide	0.05: Barley and wheat
Metsulfuron-methyl	Sulfonylurea herbicide	0.05: Barley and wheat
Nicosulfuron	Sulfonylurea herbicide	0.05: Maize
Thifensulfuron-methyl	Sulfonylurea herbicide	0.05: Barley and wheat
Triasulfuron	Sulfonylurea herbicide	0.05: Barley and wheat
Tribenuron methyl	Sulfonylurea herbicide	0.05: Barley and wheat 5 - 20: Apples, apricots, beans, broccoli, Brussels sprouts, cabbage, cauliflower, cereal grains, citrus, cotton seed, cucurbits, dried fruit, dried nuts, dried vegetables, grapes, groundnuts, guavas, lettuce, oil seeds, peaches, pears, plums, sunflower seed and tomatoes
Piperonyl butoxide	Synergist	dried nuts, dried vegetables, grapes, groundnuts, guavas, lettuce, oil seeds, peaches, pears, plums, sunflower seed and tomatoes
Clofentezine	Terazine insecticide	0.05- 0.2: Apples, pears and tomatoes 15: Apples, apricots, boysenberries, celery, grapes, guavas, olives, peaches, pears, plums, quinces, spinach, strawberries, tomatoes and youngberries.
Captan	Thiophthalimide	peaches, pears, plums, quinces, spinach, strawberries, tomatoes and youngberries.
Folpet	Thiophthalimide fungicide	0.5: Tomatoes; 15: grapes
Amitraz	Triazapentadien insecticide and acaricide	0.2 - 0.5: Apples, citrus, cotton seed, tomatoes
Simazine	Triazine herbicide	0.2: Apples, grapes, maize and pears; 10: Asparagus
Atrazine	Triazine herbicide	0.05: Maize, sorghum and sugar cane
Cyanazine	Triazine herbicide	0.05: Cottonseed, maize, sugar cane and sweet corn. 0.1 -1: Peas and rooibos
Prometryn	Triazine herbicide	0.05: Cottonseed and 0.5: carrots
Terbutryn	Triazine herbicide	0.05: Groundnuts and peas

Chemical	Pesticide notes	South African MRL (mg kg <sup>-1</sup> ) and associated crops
Terbuthylazine	Triazine herbicide, antimicrobial and algaecide	0.05: Maize, peas and sorghum
Cyromazine	Triazine insecticides	0.05: Potatoes, 0.5: tomatoes, 2: mushrooms and 5: green beans
Pymetrozine	Triazine insecticides	0.02 - 0.05: Cabbage and cotton seed
Hexazinone	Triazinone herbicide	1: Pineapples
Metribuzin	Triazinone herbicide	0.05: Asparagus and soya beans
Sulfentrazone	Triazolone herbicide	0: Sugar cane
Florasulam	Triazolopyrimidine herbicide	0.01: Wheat
Flumetsulam	Triazolopyrimidine herbicide	0.05: Wheat
Terbacil	Uracil herbicide	1: Peaches
Thidiazuron	Urea defoliant and plant regulator	0.5: Cotton seed
Pencycuron	Urea fungicide	0.05: Potatoes
Diuron	Urea herbicide	0.05 - 0.1: Asparagus and sugar cane
Benalaxyl	Xylylalanine fungicide	0.05: Potatoes and tomatoes. 2: Grapes 0.05 - 0.5: Avocados, broccoli, Brussels sprouts, cabbage,
Metalaxyl	Xylylalanine fungicide	cauliflower, pineapples, potatoes and tomatoes. 1 - 1.5: Boysenberries, citrus, grapes and youngberries

Table 1. Pesticides registered in South Africa, including information on chemical classification, application use and relevant crops as indicated by South African MRL levels (South African Department of Health (DOH), 2005; PAN, 2010).

The main agricultural exports in 2009/2010 were wine (\$846 million), citrus fruit (\$797 million), grapes (\$495 million), apples, pears and quinces (\$435 million) and cane sugar (\$377 million) (DAFF, 2010). During 2009/10, the Netherlands, the United Kingdom, Zimbabwe, Mozambique and Germany were the five largest trading partners. Approximately 20.7% of South Africa's total agricultural exports for the period July 2009 to June 2010 went to the Netherlands and the United Kingdom (DAFF, 2010). These are all crops requiring the responsible use of pesticides (including fungicides and herbicides). The EU, U.S.A and Japan, have stringent food safety requirements as compared to African countries that typically adhere to FAO/WHO CODEX Alimentarius recommendations. To ensure continued trade, South African exporters must continuously ensure that their export products may meet the EU food safety standards in addition to private standards set by large food retailers, e.g., Tesco, Marks & Spencer, Sainsbury's in the U.K (Urquhart, 1999; Frohberg, 2006; String Communication, 2007).

The financial impacts of providing fresh produce that meet the requirements of the importing country are significant. For example: should South Africa be suspended from trading citrus fruit due to exceeding an EU pesticide MRL (either through incorrect

pesticide use or incorrect measurements), South Africa would have lost an estimated income of \$ 54 million through trade (assuming a typical minimum four month period that is required to take corrective action and have the citrus trade re-instated). To avoid such losses, South Africa is obliged to meet export requirements in primarily two ways, namely through the responsible and correct use of pesticides and through the accurate, internationally recognised measurement and monitoring of pesticide residues (Frohberg et al., 2006; DAFF, 2009, 2010). The European Commission's (EC) Rapid Alert System for Food and Feed (RASFF) identifies risks in food and feed imported into the EU (EU, 2011). Depending on the risk level, the EC will either prohibit the consignment from entering the country (border rejection) or send out an alert notification or an information notification. The former requires immediate corrective action, i.e., withdraw/recall of the product, while the latter requires precautions to be taken in future to avoid further notifications (EU, 2011, 2009). Since 2000, South Africa has received only 4 information notifications and one alert notification for pesticide residues detected on fruit. The detected pesticides were omethoate, dimethoate and ethephon in grapes; methomyl in pears and prophenophos in peppers. The majority of South African border rejections and notifications are due to MRLs being exceeded for aflatoxins in groundnuts. In general the mycotoxin hazard category tends to dominate as the main cause for border rejection in the EU across all importers (EU, 2011, 2009).

In accordance with Article 12 of EU Regulation 882/2004 (EU, 2004), laboratories designated for official control of pesticide residues within the EU must be accredited to ISO/IEC 17025. Accreditation of laboratories is seen as the most effective way of defeating non-tariff technical barriers to trade (for example, eliminating doubt on the quality of test results from exporting countries). For export purposes, measurements performed by analytical laboratories who are accredited to ISO 17025, are internationally accepted, in terms of the International Laboratory Accreditation Cooperation Mutual Recognition Arrangement (ILAC MRA) for trade, of which South Africa is a signatory since November 2000 (ILAC, 2001). Although there are several laboratories analysing pesticide residues in South Africa (National Laboratory Association (NLA), 2010), according to the South African National Accreditation System (SANAS), (SANAS, 2010), there are only nine ISO 17025 accredited analytical laboratories in South Africa capable of analysing pesticide residues in food and feed. Of these laboratories, two are able to analyse the raw and finished products of pesticide formulations (SANAS, 2010). There are two government entities responsible for pesticide residue measurements in food and plant products in South Africa, namely the Department of Agriculture, Forestry and Fisheries and the Department of Health (DoH). Fresh produce for export is well monitored by the Perishable Products Export Control Board (PPECB). The PPECB is South Africa's official certification agency for the export of perishable products. It is mandated by the Department of Agriculture to deliver cold chain services in terms of the PPECB Act, No.9 of 1983 and delivers inspection and food safety services, under the APS Act, No.119 of 1990. The PPECB is also responsible for monitoring imported consignments of fresh produce. The PPECB is ISO 17025 accredited for the analysis of mycotoxins in various plant products (PPECB, 2011). The DoH Food Control Directorate has inspectors that regularly sample food items from the local trade industry; the analysis of which is outsourced to private accredited laboratories with the capacity to conduct extensive analyses.

The DoH, currently does not have the capacity to test a large number of samples for residues. As with several non-accredited facilities in South Africa, reasons for local laboratories not being able to obtain accreditation for residue monitoring include (Apps, 2007; Dlamini, 2007; Fernandes-Whaley, 2009):

- a high turnover of analysts in the laboratory,
- lack of skilled, competent technicians,
- poor financing for analytical equipment required to meet stringent MRLs,
- and lack of appropriate reference materials and the high cost of proficiency testing (PT) scheme participation.

There has been concern that the ever-decreasing MRLs for pesticide residues on food may be considered as technical barriers to trade, especially for developing countries in Africa (Urquhart, 1999; Frohberg, 2006; String Communication, 2007). This may be true, especially for countries in the regions without the necessary technical infrastructure, analytical capability and skills. However, in South Africa, recent proactive investment by government and industry has been implemented into improving the analytical infrastructure for pesticide residues on food (Aldrich & Street, 1964; SABS, 2011), although the lack of skilled human resources still remains a limitation (Apps, 2007; Dlamini, 2007; Fernandes-Whaley, 2009). South Africa currently has nine ISO 17025 accredited laboratories for pesticide residue analysis in plant products. Most of these accredited facilities have obtained technique accreditation for pesticide residues on processed foods and plant products using state-of-the-art gas chromatography mass spectrometry and liquid chromatography tandem mass spectrometry techniques, which are able to meet EU MRL requirements and thereby ensuring trade within the South African framework.

#### **4. Alternatives to the use of pesticides in agricultural pest control**

In recent years there have been fast developments in alternate pest control mechanisms to reduce environmental levels of organic and inorganic pesticides. One of these developments is genetically modified (GM) crops, such as GM maize and cotton. Sprays of the bacteria, *Bacillus thuringiensis* (Bt), have been used to control pests for decades. The crystalline (cry) protein produced by this bacteria kills certain insect species and was reported to have limited effects on most non-target species (Schnepf et al., 1998). The use of commercial Bt sprays have, however, been limited due to their relatively high cost, poor crop coverage, rapid environmental inactivation, and less than desirable level of pest control, especially when compared with less expensive conventional chemical insecticides (Benedict & Altman, 2001).

More recently, toxin-encoding genes from *B. thuringiensis* have been expressed in transgenic crop plants, providing protection from some key pests (Schnepf et al., 1998). In South Africa two of these key pests of maize are the lepidopteran stem borers, *Busseola fusca* (Lepidoptera: Noctuidae) and *Chilo partellus* (Lepidoptera: Crambidae) which are of economic importance throughout Southern and Eastern Africa. Large-scale planting of Bt crops to control these pests in South Africa commenced during 1998. Bt cotton for control of the boll worm complex, particularly the African bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae), was also introduced into South Africa during 1998. Adoption rates of Bt maize and cotton were high and in many areas of the country, more than 90% of

farmers plant GM maize or cotton. Based on surface area, South Africa is currently ranked 9<sup>th</sup> worldwide in planting GM crops (James, 2011). The benefits as well as possible disadvantages of planting Bt maize, from a South African farmers perspective was reported by Kruger et al. (2009, 2011) and that of Bt cotton by Mellet et al. (2003).

Although many benefits may be associated with the use of GM crops for pest control, there are also a number of disadvantages. From an environmental and human-health perspective, the use of genetically modified crops has several benefits.

- While many broad-spectrum insecticides reduce the impact of biological control agents that help to control insect and mite pests, studies indicated that Bt maize is compatible with biological control and has little effect on the natural enemies of pests (Bessin, 2010);
- Control of lepidopteran pests with Bt endotoxins provides several advantages from the grower's perspective. These benefits could be particularly important in subsistence agriculture where literacy levels of farmers are low and extension support is poor or lacking. Control is no longer affected by the weather. The crop is protected even if the field conditions are not suitable for aerial or ground application of insecticides (Meeusen & Warren, 1989);
- A related advantage is the protection of plant parts that are difficult to reach with insecticide spraying or the protection of new growth that emerges after spray applications like tillers and ears of maize (Meeusen & Warren, 1989);
- The crop is also protected continuously in the field and the laborious task of scouting to timeously detect pest infestations may be reduced;
- Finally, and most importantly, is the reduction in insecticide applications.

For example, reduced insecticide use was reported from the Makathini Flats region of Kwa-Zulu Natal, South Africa, where 95% of smallholder (1-3 hectares) cotton producers grew rain-fed Bt cotton. Farmers that adopted Bt cotton reported reduced insecticide use and a reduction in labour (Ismael et al., 2001). A typical farmer, often a woman, was spared 12 days of arduous spraying, saving more than a 1 000 litres of water that would have been used in pesticide application (Conway, 2004). Similar benefits have been reported in small-scale maize farming systems in South Africa. Between 16 and 62% higher yields were reported with Bt maize above the conventional iso-line (Gouse, 2005) due to improved stem borer control. Bt maize adopting-farmers were better off than farmers who planted conventional hybrids, despite the additional technology fee in terms of seed costs (Gouse, 2005). A reduction in pesticide application also reduces the potential pesticide drift onto other crops or environmentally sensitive areas (Meeusen & Warren, 1989). Because the active Bt toxin material is produced directly in the crop tissue, concerns such as spray drift and groundwater contamination are precluded (Meeusen & Warren, 1989). Therefore, the use of transgenic crops reduces the use of insecticides, minimizes the impact of these chemicals on non-target organisms, and has positive health consequences for farm workers themselves (Barton & Dracup, 2000).

Although GM crops have become a major component of insect control strategies, a proper perspective of its potential demands a close look at limitations and uncertainties that may reduce its future impact on agriculture. Since the first deployment of Bt crops there has been concern with regard to the development of resistance of target pests and potential non-target organism effects (Tabashnik, 1994; Gould, 1998). For this reason,

studies have been conducted to prioritise non-target Lepidoptera species for further research in South Africa (Van Wyk et al., 2007). Bt maize seed is more expensive than comparable non-Bt seed and is only an advantage when a specific insect pest is present. Wind-mediated gene-flow and cross-pollination of landraces (local variety of the plant species) of crops can result in “contamination” of maize fields within several hundred meters from GM crop fields. This may present a problem for organic farmers or in cases where it is important to keep the grain Bt-free. This could also be important in the development of target pest resistance since the uncontrolled presence of the Bt-gene in landraces results in reduced expression of the cry protein and subsequent increased survival of the target pest in crops.

Another potential disadvantage is that biotechnology is being pursued to repair the problems caused by previous agrochemical technologies. Since more than 500 species of pests have already developed resistance to conventional insecticides, pests can also evolve resistance to Bt toxin in GM crops (Altieri, 2004). Resistance of the target pest of Bt maize in South Africa, the African stem borer was reported by Van Rensburg (2007) and Kruger et al., (2009) who reported a further increase in the geographic spread of resistance. The most significant environmental threat of resistance development is the subsequent reversal to the use of broad-spectrum insecticides, which is currently the case in South Africa (Kruger et al., 2011).

The challenge for the sustained use of GM technology in South Africa and especially in developing agriculture throughout Africa is the management of these crops to delay resistance development and to prolong the usefulness of the technology. On its own, insecticidal GM crops will not be the solution to pest problems. Over reliance on this technology may be its downfall and only an integrated approach to pest management will be effective on a long-term basis.

One example of an effective alternative to Bt maize for the management of stem borers is the “push-pull” habitat management systems that has been adopted by between 20 000 – 30 000 farmers in East Africa. As part of a novel approach to control stem borers in cereal crops, the International Centre of Insect Physiology and Ecology (ICIPE) developed habitat management strategies for farmers in resource-poor regions of East Africa (Khan et al., 2000). In the push-pull system, trap and repellent plants are used to control populations of stem borers. Insects are trapped on highly susceptible trap plants (pull) and repelled from the main crop by repellent intercrops (push). Khan et al. (1997) observed that the two fodder crops, namely Napier grass (*Pennisetum purpureum*) and Sudan grass (*Sorghum sudanensis*) attract significantly more oviposition than maize. These grasses were consequently used in further developing habitat management strategies for stem borers. It was reported that planting Sudan grass as a border around maize plots reduced stem borer numbers in maize by 30% while only 20% of larvae on Napier grass survived, although this species was preferred by stem borers. The volatiles of non-hosts such as Molasses grass (*Melinis minutiflora*) can have a marked impact on reducing borer infestations on maize (Khan et al., 1997). It was discovered that these volatiles repel the stem borers, but attract their natural enemies (Khan et al., 2000). A push-pull system that combines the repellent *M. minutiflora* grass with a highly attractant plant like Napier grass would fit in well with farmers practising a mixed agricultural system. These alternatives in totality, if managed correctly, can help to reduce the widespread use of agricultural pesticides in modern farming practices.

## 5. Pesticides used to control disease vectors

In South Africa, as in many countries with temperate climates, pesticides are crucial not only in the agricultural setting but also in the management of disease vectors. For South Africa, malaria is the only disease currently managed in this manner. Since before the 2<sup>nd</sup> World War, malaria in South Africa has been controlled by means of ecological and biological control, as well as with oil and Paris green as a larvicide. Also, a weekly indoor application of pyrethrum has been used since 1943. Since the middle 1940s, the availability of DDT and hexachlorobenzene meant that Indoor Residual Spraying (IRS) with insecticides, a technique developed in South Africa by de Meillon in the 1930s (de Meillon, 1936), became the mainstay for effective malaria control worldwide (Sharp et al., 1988; Musawenkosi et al., 2004).

The insecticides are sprayed on indoor walls and roof beams, as well as outside under the eaves. The residual insecticide then kills female mosquitoes, interrupting transmission from a person with malaria to an uninfected person. The rates of malaria dropped significantly with the use of DDT, and many lives have been saved in this manner (Bouwman et al. 2011). However, in 1996, DDT was replaced with pyrethroids as IRS in South Africa. The rates of infections and deaths climbed dramatically, forcing the reintroduction of DDT in 2000 (Hargreaves et al., 2000; Maharaj et al., 2005). Infection and mortality rates have now returned to original levels, and pyrethroids are used in some places. In 2009 in South Africa, 6 072 people contracted malaria and 45 died (WHO, 2010). During the same year, an estimated 236 million people contracted malaria globally, resulting in nearly 781 000 deaths of which more than 90% were from Africa. This indicates a dire need for expanded and improved malaria control measures.

In South Africa, the registration of DDT for all purposes except malaria control was withdrawn in 1976, with all resale and use of hexachlorobenzene (HCB) and DDT prohibited since 1983 (Bouwman, 2003). DDT continues to be used in malaria control as indicated above, but this use often leads to exposure of both the inhabitants of the sprayed houses, as well as the immediate environments. At a rate of 2 g m<sup>-2</sup>, an average house receives anywhere between 80 – 200 g per year. Seen that this is applied mainly indoors, a continuously enriched DDT environment is created. Since DDT is also semi-volatile, it is present throughout the year in air in the homes, and probably redistributes itself to furniture and food inside (van Dyk et al., 2010). This continuous redistribution, adds to uptake opportunities that are reflected in the high levels of DDT found in inhabitants (Bouwman et al., 2006). The highest levels of DDT in blood were found in breast feeding infants of mothers living in sprayed houses (Bouwman et al., 1992). Van Dyk et al., (2010) suggests that a Total Homestead Environment Approach (THEA) should be followed when looking at exposure, uptake and exposure reduction opportunities.

Very little research has been conducted on the effects in people living in sprayed houses, but in 2009, Bornman et al. published convincing associations between living in DDT-sprayed villages and urogenital malformations in baby boys from the Limpopo Province. Eskenazi et al. (2009) reviewed 494 studies published between 2003 and 2008 and found that “...DDT and its breakdown product DDE may be associated with adverse health outcomes such as breast cancer, diabetes, decreased semen quality, spontaneous abortion, and impaired neurodevelopment in children.” Subsequently, Bouwman et al. (2011), based on further assessment of information,

strongly suggested that DDT (that is currently considered as safe for use in malaria control) should now be considered in terms of precaution, with an urgent recommendation that exposure reductions be implemented. DDT does not remain restricted to the homestead environment. It is transported to adjacent sites and can be found in water, sediment, birds and fish near and far away from sprayed areas (Barnhoorn et al., 2009). The findings of urogenital malformations in baby boys by Bornman et al. (2009) are a further implication of the endocrine disruptive activities of DDT, DDE and DDD all have some kind of endocrine disruptive activity (as discussed previously), as implied by the findings of urogenital malformations in baby boys by Bornman et al. (2009). Intersex in tilapia fish has also been found in the major river that flows through the same area where the malformations were found (Barnhoorn, 2010).

The alternative insecticides used in IRS should not be ignored. Most of them are pyrethroids such as deltamethrin and permethrin, but organophosphates such as malathion and primiphos-methyl, and bendiocarb, a carbamate, are also recommended by the WHO (2006). It has been noted though, that very little risk assessment of most of these compounds has been done with relevance to an IRS exposure scenario. Hopefully they are safe, but caution should be applied (Bouwman & Kylin, 2009), as pyrethroids have been found in the same breast milk as DDT (Bouwman et al., 2006), a potentially dangerous situation as toxic interactions have been detected in laboratory animals at relevant levels (Bouwman & Kylin, 2009).

These, often low-income, families are at an increased risk to DDT exposure and consequently the associated adverse health effects. People that are poverty stricken generally are at a higher risk to contract diseases due to malnutrition and lack of resources. When combined with the constant presence of HIV, these communities are more susceptible to health risks. Pesticide levels that would be of no real concern in healthy individuals could, theoretically be detrimental to people living in these communities. IRS using insecticides will undoubtedly remain in the arsenal of malaria control measures for a considerable period. However, as science and knowledge advances, urgent attention should be directed towards safer methods, exposure reductions, and better chemicals.

## **6. Pesticides levels in the South African environment**

Many studies have focused on both historic and current use pesticide levels in South Africa. As can be seen in table 2, these levels are highly variable depending on the activity of the area as well as the sampling media. As previously described, levels are typically higher in biotic matrices as compared to abiotic matrices due to the effect of biomagnification, specifically in OCPs. DDT is still a prevalent pesticide although use outside of the malaria regions is strictly prohibited.

Most of these studies have concentrated on areas where there were high levels of expected impacts. Therefore a pilot study was done in 2006 to assess the levels of persistent organic pollutants including OCPs in an industrial area of South Africa concurrently with areas mainly impacted by agriculture. The focus was on industrial pollutants but this study was also used to assess the occurrence and distribution of OCPs in areas with a large variety of land use.

This pilot study is presented here as a typical research approach to gather and interpret data concerning pesticides in the environment. It gives a brief overview of commonly used ratios

to differentiate between historic and current applications as well as the use of statistical tools to determine variation within datasets.

Land use or activity	Matrix	N	Pesticide and levels	Reference
DDT used in IRS programme	Water	8	$\sum$ DDT: LOD - 7 000	Barnhoorn et al., 2009
DDT used in IRS programme	Fat of indigenous fish	28	$\sum$ DDT: 360 - 24 000	Barnhoorn et al., 2009
Mixed: large city	Air	48	Pentachlorobenzene: 3.6 HCB: 4.5 Heptachlorepoide: 0.62 Toxaphene: 12	Batterman et al., 2007
Agriculturally impacted estuary (apple, pear and plum orchards)	Water	27	Chlorpyrifos: 0.085 Prothiofos: 0.032 Cypermethrin: LOD $\sum$ Endosulfan: 0.003 <i>p,p'</i> -DDE: 0.01	Bollmohr et al., 2006
Corn-production region	Water	5	Atrazine: 1.2 - 9.3 Terbuthylazine: 1.04 - 4.1	Du Preez et al., 2005
Various sampling areas across South Africa	Wild bird eggs	43	$\sum$ DDT: 1.9 - 710 $\sum$ HCH: 0.21 - 470 $\sum$ Chlordanes: 0.12 - 11 HCB: 0.29 - 7.8 Mirex: 0.21 - 1.8	Bouwman et al., 2008

Table 2. Pesticide levels ( $\text{pg m}^{-3}$  or  $\mu\text{g kg}^{-1}$  or  $\mu\text{g l}^{-1}$ ) reported in various publication from SA

## 7. Case study: A baseline of organochlorine pesticide residues in soil and sediment from the Vaal Triangle, South Africa

### 7.1 Introduction

OCPs have been used extensively throughout the world for the protection of crops as well as the control of disease vectors (Kumar et al., 2008). Because of their detrimental effects, many of the OCPs are banned, or restricted to the control of disease vectors. However, due to their highly persistent nature and potential for long-range transport, residues of these chemicals are still found in areas where use had been banned for decades (Gong et al., 2007; Hung et al., 2007). These residues may pose chronic toxicity to animals and humans via air, water, and food intake (Darko et al., 2008). Due to the general lack of data on organochlorine pollution in soils and sediments from South Africa, the objective of this baseline study was to investigate the presence and concentration of OCPs in soil and sediment from an industrialised in addition to agriculturally impacted areas of South Africa.

## 7.2 Materials and methods

### 7.2.1 Sampling area

The Vaal Triangle is a highly industrialised area in central South Africa (Figure 1) with a population of approximately 790 000. The main industries in the area include ferrous and non-ferrous metal production and petrochemical processes. There is also limited farming activity, mainly consisting of maize and cattle. Soil was collected in the industrial (Vdb-Ind-Soil and S-Ind-Soil) and low-income residential areas (Vdb-Inf-Soil and S-Inf-Soil) close to these industries. Sediment was collected from rivers and streams including the Vaal River (VaalRivV) and its tributaries; Riet Spruit (RietSpr), Klip River (KlipRiv), Suikerbosrand River (SkbrRiv3) and the Taaibos Spruit (TbosSpr) (Figure 1). Concurrently, sediment was collected from agricultural areas upstream and downstream of the Vaal Triangle: SkbrRiv1 and SkbrRiv2, east of the Vaal Triangle and VaalRivK, in the Vaal River approximately 70 km downstream of Vanderbijlpark inside the Vredefort Dome World Heritage Site, and a site in the Orange River (OrangeRiv) just after the confluence with the Vaal River. Agriculture in the Vredefort Dome is comparable to that of the Vaal Triangle while the Orange River catchment is impacted by the cultivation of a variety of other crops (wheat, cotton, potatoes, as well as fruit). There are no malaria control activities in this area; the closest malaria control activity is 260 km away towards the Mozambican border (Figure 1).

### 7.2.2 Sample extraction, clean up and analysis

Extraction and analysis for DDT, dicofol, endosulfan and hexachlorocyclohexane (HCH) was done under Norwegian accreditation at NILU in Norway. Analytical details are given in Bengtson Nash et al.(2008) and Knutzen (2003). In short: samples were spiked with  $^{13}\text{C}$ -labelled analogs of the analytes and extracted with cyclohexane. Interfering molecules were removed through size exclusion chromatography and sulphuric acid clean-up. Before quantification, the samples were spiked with a recovery control standard. Analytical quantification was using gas chromatography coupled with high resolution mass spectrometry (GC-HRMS; Agilent 6890N GC coupled to an Micromass Waters Autospec HRMS, Manchester UK). The HRMS was operated at a resolution of  $>10\,000$  using electron ionization. Two masses were monitored for each isotope cluster of both the analyte and the added  $^{13}\text{C}$ -labelled surrogate. The recoveries of the added internal standard compounds were also established. Procedural blanks were run throughout. The limit of quantification was set to the detection limit based on at least five consecutive blank samples plus three standard deviations. All concentrations are reported on a mass basis. The following conditions had to be met for an unequivocal identification and quantification of the analytes: (1) correct retention time (2) signal-to-noise ratio greater than 3:1, (3) correct recovery of the internal standard, and (4) acceptable blank values of the complete clean-up and quantification procedures.

### 7.2.3 Statistical analysis

The software package used for statistical analyses was STATISTICA (version 8). Normal distribution was tested using the Shapiro-Wilk test and groups were compared using the Mann-Whitney U test. Principal component analysis (PCA), using all of the congener data, with data transformations according to Howell (2007), was performed with CANOCO (version 4.5) to investigate the pollutant patterns at the sites, as well as possible differences in the matrices sampled.

### 7.2.4 Results and discussion

The concentration of OCPs at all the sites was relatively low when compared with literature (Table 3) from other countries.  $\Sigma$ OCPs measured ranged between 0.58 - 6.9 ng g<sup>-1</sup> with the highest values found in Vanderbijlpark soil (Vnd-Inf-Soil & Vnd-Ind-Soil), and sediment from the Klip River (KlipRiv) (Figure 2). Vanderbijlpark is a highly industrialised area with ferrous and non-ferrous industries, with some farming activities. The largest contributor of the OCPs in soil sites was *p,p'*-DDE measured in Vnd-Inf-Soil and Vnd-Ind-Soil (6.61 - 4.82 ng g<sup>-1</sup>), followed by  $\gamma$ -HCH (1.7 ng g<sup>-1</sup>) in S-Ind-Soil and *p,p'*-DDE (1.1 ng g<sup>-1</sup>) in Vnd-Inf-Soil. The Klip River (a tributary of the Vaal River) has a large catchment area that includes the industrial and residential areas of Soweto, Lenasia, Meyerton and Vereeniging. As with Vnd-Inf-Soil, the largest contribution to sediment was from *p,p'*-DDT (3.7 ng g<sup>-1</sup>) followed by  $\alpha$ -HCH (0.58 ng g<sup>-1</sup>).

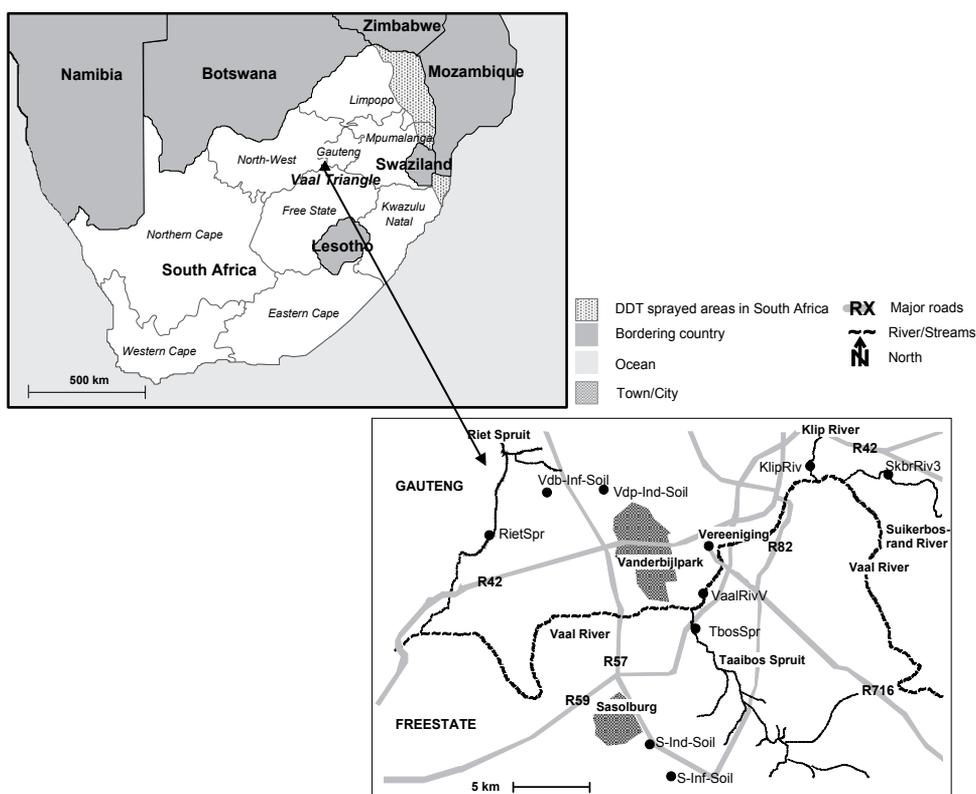


Fig. 1. Map of South Africa and its provinces; indication the location of the sampling sites in the areas assessed for OCPs.

Levels from South Africa and Brazil are in the same order of magnitude for DDT and HCH, with levels in Morocco being lower for these two compounds but higher for endosulfan (Table 3). It should be taken into account that China is currently the largest producer of DDT for vector control, as well as the pesticide dicofol (Cheung *et al.*, 2007) that may contain DDT as contaminant, this would explain the higher DDT levels (Table 3). On the other hand, the high average levels reported in the Canadian study were mainly attributed to a single site.

The lowest OCP levels for the South African study were measured in the Taaibos Spruit (TbosSpr), which receives run-off impacted by low-income residential areas as well as petrochemical industries. The OCP levels for sites primarily influenced by agricultural activities ranged between 2.3 - 0.59 ng/g (Figure 2). Of the four classes of OCPs ( $\Sigma$ DDT, dicofol,  $\Sigma$ HCH and  $\Sigma$ Endosulfan),  $\Sigma$ DDT was the highest at all sites, except for one sediment site in an agricultural area (OrangeRiv), and one soil site in an industrial area, S-Ind-Soil (Figure 2); in both  $\Sigma$ HCH was the highest. In general, the concentration of OCP classes decreased as follows:  $\Sigma$ DDT >  $\Sigma$ HCH >  $\Sigma$ Endosulfan > dicofol. The levels of OCP within the classes varied between sites as described in the next section.

Country	Land use	Matrix	N samples	$\Sigma$ Endosulfan	$\Sigma$ HCH	$\Sigma$ DDT	Reference
Canada	Mixed	Sediment	7	0.36	0.03 <sup>#</sup>	6.8	Wong et al., 2009
Canada	Mixed	Soil	7	0.13	0.04 <sup>#</sup>	68	Wong et al., 2009
China	Fallow	Soil			14 <sup>^</sup>	25	Wang et al., 2007
Brazil	Agricultural	Soil	29	0.52	0.1	1.4 <sup>*</sup>	Rissato et al., 2006
Morocco	Agricultural	Soil	5	1.5	0.02	0.03 <sup>*</sup>	Bakouri et al., 2008
Uganda	Mixed	Soil	8	0.4	NM	0.3	Ssebugere et al., 2010
SA	Industrial	Soil	4	0.09	0.70	3.5	Present study
SA	Mixed	Sediment	9	0.05	0.52	1.7	Present study

\* *o,p'*-DDT + *p,p'*-DDD + *p,p'*-DDT + *p,p'*-DDE, #  $\alpha,\gamma$  HCH, ^  $\alpha,\beta,\gamma,\delta$ -HCH, <sup>a</sup> I-Endosulfan + II-Endosulfan, NM: not measured, SA: South Africa

Table 3. Mean OCP levels (ng g<sup>-1</sup>) as reported in other international studies.

### 7.3 HCH

$\gamma$ -HCH, better known as lindane, was one of the most widely used pesticides in the world. However, on 9 May 2009 lindane was added to Annex A (chemicals cited for elimination) of the Stockholm Convention. Lindane was commonly used in the treatment of seed, livestock and as a household biocide (Osibanjo et al., 2002). Although relatively little literature has been published on the use and production of these chemicals in South Africa, it is known that HCH was produced until the early 1980s at a site in Kempton Park (Osibanjo et al., 2002). Kempton Park is situated approximately 80 km northeast of the Vaal Triangle. The main wind direction for that area is north and north-northwest, which could have contributed to dispersing lindane to the Vaal Triangle in the past. However, lindane is currently registered and used in the agricultural of cotton, maize and wheat crops, amongst others, as well as in domestic gardens (Nel et al., 2002, Table 1). It would be interesting to track possible changes in soil and sediment levels following eventual de-registration.

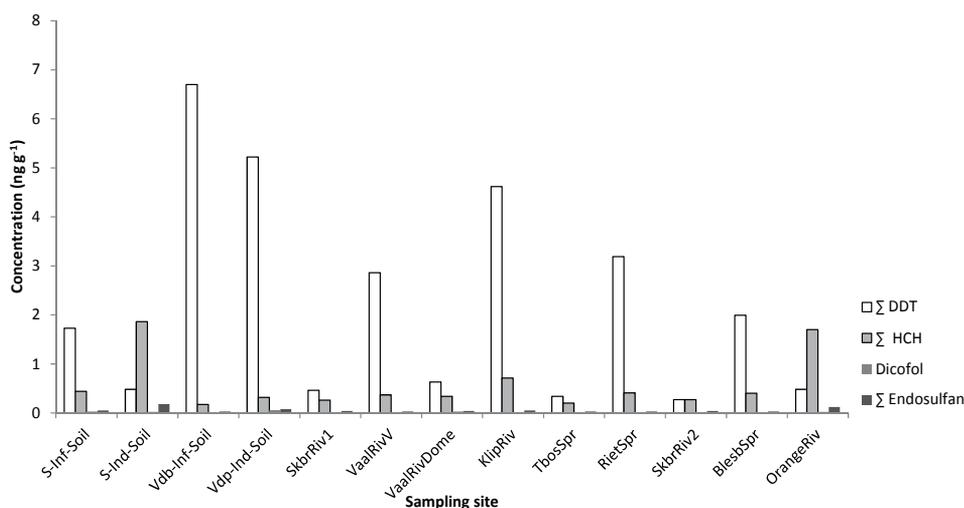


Fig. 2. Distribution patterns of different OCPs between sites

The  $\Sigma$ HCH concentration in soil varied between 0.17 - 1.86 ng g<sup>-1</sup> with the highest concentration measured at Sasolburg (S-Ind-Soil) in soil collected close to the petrochemical plant. The lowest level was measured in the low-income residential area in Vanderbijlpark (Vdb-Inf-Soil). In sediment, the concentration varied between 0.3 - 1.7 ng g<sup>-1</sup>, with the highest concentration measured in the predominantly agricultural area of the Orange River (OrangeRiv). The values for HCH are in the same order of magnitude as determined in other studies (Table 3).

Lindane consists of a mixture of all HCH isomers with approximately 90%  $\gamma$ -HCH (Wang et al., 2007). In technical HCH, the isomer ratio is different with  $\alpha$ -HCH representing 55 - 80% of the total mixture (Gong et al., 2007). In both soil and sediments,  $\gamma$ -HCH was the most prevalent with more than 80% contribution to  $\Sigma$ HCH levels (Table 4), followed by  $\beta$ -HCH and  $\alpha$ -HCH. This points to recent use of lindane rather than technical HCH or historic inputs, since  $\gamma$ -HCH is degraded more rapidly than  $\alpha$ -HCH both under aerobic and anaerobic conditions (Wu et al., 1997).

Matrix	Nr of samples	Mean $\Sigma$ HCH (ng g <sup>-1</sup> )	Concentration range $\Sigma$ HCH (ng g <sup>-1</sup> )	% $\alpha$ -HCH	% $\beta$ -HCH	% $\gamma$ -HCH
Soil	4	0.70	1.9 - 0.17	4	12	85
Sediment	9	0.52	0.2 - 1.7	5	6	89

Table 4. Summary of mean HCH values and percentage contribution of each isomer for soil and sediment

#### 7.4 DDT and dicofol

DDT, in use since the Second World War, was the first internationally used pesticide due to its highly effective insecticidal properties and ease of manufacture (Stenersen, 2004). DDT is often found in the environment even in areas where it is not currently applied due to its highly persistent nature and ability to be passively (wind and water) and actively (through biota)

transported. The current study area falls outside the endemic malaria regions of South Africa (Figure 1), the closest of which is 700 km away. As expected, the  $\Sigma$ DDT levels measured were relatively low, varying between 0.3 - 6.7 ng g<sup>-1</sup>, with the highest values measured in Vnd-Inf-Soil. The  $\Sigma$ DDT levels measured in soil were compared to those measured in sediment. The data was not normally distributed ( $p = 0.03$ , Shapiro-Wilk test) and the relationship was tested with the non-parametric Mann-Whitney U test. Although the levels in soil were higher than those in sediment, this difference was not statistically significant ( $p = 0.24$ ).

To assess when DDT was applied, the ratio of DDT/(DDE + DDD) was calculated. Values greater than one indicate recent applications, while values lower than one indicate historic use (Chen et al., 2005; Gong et al., 2007). The mean ratios were lower than 0.5 (Table 5) with the exception of S-Ind-Soil that had a ratio of 1.1. The mean ratio was also less in sediment than in soil. The ratio of *o,p'*-DDT to *p,p'*-DDT distinguishes between pollution caused by technical DDTs and pollution from other sources such as dicofol. (Fu et al., 2009). Since *o,p'*-DDT is less stable, it is expected to be less prevalent in the environment. Dicofol would produce a higher *o,p'*-DDT to *p,p'*-DDT ratio since there is generally more *o,p'*-DDT in dicofol than *p,p'*-DDT (Qiu et al., 2005). The ratio in this study was 0.24 for soil and 0.58 for sediment (Table 5). These values indicate that the DDT pollution detected is more likely from the use of technical DDT.

Matrix	Nr of samples	Mean $\Sigma$ DDT (ng g <sup>-1</sup> )	Concentration range $\Sigma$ DDT (ng g <sup>-1</sup> )	Mean ratio of DDT to DDT metabolites	Mean ratio of DDE to DDD	Mean ratio of <i>o,p'</i> DDT to <i>p,p'</i> DDT
Soil	4	3.5	0.5 - 6.7	0.42	120	0.24
Sediment	9	1.7	0.3 - 4.6	0.07	16	0.58

Table 5. Summary of mean DDT values and metabolite ratio's for soil and sediment

Once applied, DDT degrades to DDD and DDE. Degradation to DDD is through reductive dechlorination, while degradation to DDE is through dehydrochlorination processes (Wang et al., 2007). The ratio of DDE/DDD at all sites was greater than one. This indicates that the majority of DDT is degraded to DDE in both matrices, with the DDD levels playing a larger role in sediment than soil due to anaerobic conditions. This may show that a larger portion of DDT is degraded through reductive dechlorination in sediment than in soil.

Dicofol is a non-systemic acaricide used in the control of mites on numerous crops including cotton citrus and other fruit (Clark et al., 1990; Qiu et al., 2005). Dicofol is registered for use in South Africa, mainly for cultivation of fruit and garden use (Nel et al., 2002; Table 1). Due to the process of dicofol synthesis, it is often contaminated with DDT. Dicofol levels were low in all samples analysed, with soil and sediment levels in the same range. The highest dicofol level (0.06 ng g<sup>-1</sup>) was measured for Vnd-Ind-Soil. Considered together with the calculated DDT/(DDD + DDE) ratios (table 3), again indicates DDT rather than dicofol as source of the DDT breakdown products measured in soils and sediment.

## 7.5 Endosulfan

Endosulfan, a cyclodiene insecticide, is widely used in agricultural applications as both an insecticide and acaricide (Osibanjo et al., 2002). Endosulfan is a wide spectrum contact insecticide (Capkin et al., 2006) that consists of a mixture of endosulfan-I and II isomers

(Kumar et al., 2008), can moderately adsorb to soil, and is metabolised to endosulfan-sulfate, endosulfandiols and endosulfan ether, amongst other breakdown products. Endosulfan-I is the least persistent with a half-life of 35 days (Osibanjo et al., 2002) in contrast to endosulfan-II and endosulfan-sulfate, which may persist for years, depending on environmental conditions. Endosulfan is currently registered in South Africa for a wide variety of crops (Nel et al., 2002; Table 1).

Levels of endosulfan were low in all samples, ranging between 0.03 and 0.18 ng g<sup>-1</sup>. The highest values measured were in S-Ind-Soil (0.18 ng g<sup>-1</sup>) and in the sediment of the Orange River (OrangeRiv) (0.12 ng g<sup>-1</sup>). The mean concentration of endosulfan in soil and sediment was very similar (Table 6). Percentage contributions show the predominance of endosulfan-I when compared to endosulfan-II and endosulfan-sulfate (Table 6). This would usually indicate the current use of endosulfan. However, the low levels of this pesticide detected, makes it difficult to draw any clear-cut conclusions.

Matrix	Nr of samples	Mean concentration of $\Sigma$ Endosulfan	Concentration range $\Sigma$ Endosulfan	%Endosulfan I	%Endosulfan II	%Endosulfan -sulfate
Soil	4	0.09	0.03 – 0.18	61	21	18
Sediment	9	0.05	0.03 – 0.12	47	26	26

Table 6. Summary of mean  $\Sigma$ endosulfan values and compound ratio's for soil and sediment

## 7.6 Principle component analysis

Due to the restricted sample size, it was not possible to draw definitive conclusions from the PCA. However, the available data did shed some light on patterns in the data sets. In the PCA, factor 1 explained 40%, factor 2, 30% and factor 3, 16% of the variance in the data. The greatest contributors to factor 1 were endosulfan-I,  $\gamma$ -HCH and  $\alpha$ -HCH on the positive side and *p,p'*-DDE on the negative side (Figure 3). Factor 1 therefore contrasts OCPs still in use with DDT that is not in use in the study area. There is no clear clustering of sampled sites because of factor 1. Factor 2 is formed by a contrast between DDT isomers: *o,p'*-DDT and *p,p'*-DDT on the positive side and *p,p'*-DDD and *p,p'*-DDD on the negative side. This factor seems to have influenced the distribution of the sites on the biplot roughly into soil sites (on the positive side of factor 2) and the sediment sites on the negative side of factor 2 (Figure 3). Factor 3 consists of a contrast between DDT and its DDD metabolites on the positive side and the metabolites and isomers of endosulfan, *o,p'*-DDE and dicofol on the negative side. It is when factor 2 and 3 are plotted together that the clustering effect of the sites due to factor 2 is clearly observed (Figure 4). In a previous investigation by Nieuwoudt et al (2009) in the same sampling area, the distinction between soil and sediment concentrations was also made for dioxin-like chemicals. In summary, the pesticide residues measured in both soil and sediment in this study were generally low, with values ranging from below the detection limit to 6.6 ng g<sup>-1</sup>. The highest concentrations found did not coincide with agricultural land use, but with industry (Vnd-Ind-Soil). This site consisted of mainly industrial and residential areas in Vanderbijlpark. A PCA of the data showed a separation between sediment and soil profiles of the OCPs. Although agricultural use of DDT has been banned in South Africa since 1983, measurable levels were found in soil and sediment in areas outside the endemic malaria area where it is still sprayed.

The reasons for the differences between soils and sediment profiles and levels indicates the need for further studies, while exposure and accumulation in humans and biota needs scrutiny even at these low levels. A previous study by Bouwman et al (2008) found measurable levels of OCPs in wild bird eggs, including DDT and its metabolites as well as HCH. These levels ( $\Sigma$ DDT: 1.48 – 300 ng g<sup>-1</sup> ww) were also higher than what can be expected from the low levels measured in sediment and soil here ( $\Sigma$ DDT: 0.02 – 6.7 ng g<sup>-1</sup>dw). Although these interactions are influenced by other factors such as diet, bio-accumulation and collection area, they all play a crucial part. Very little is known about these aspects under African and South African conditions. This pilot study indicated that knowledge concerning background levels of pesticides is important. If background levels are not investigated, patterns of pesticide occurrence and distribution would be missed.

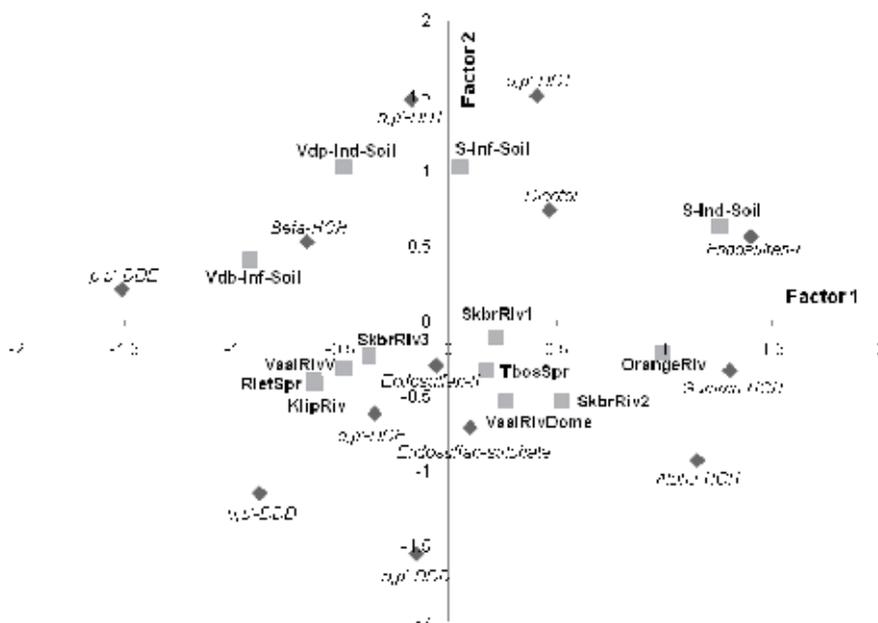


Fig. 3. A PCA bi-plot of factor 1 and factor 2, including all OCP data. Sites are indicated with a square and compounds with a triangle.

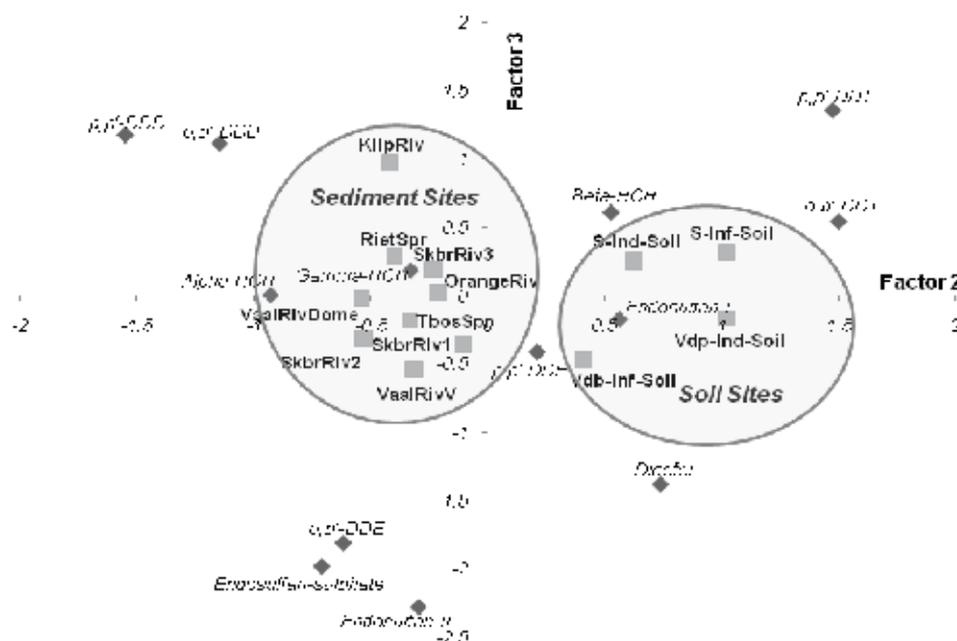


Fig. 4. A PCA bi-plot of factor 2 and factor 3, including all OCP data. Sites are indicated with a square and compounds with a triangle.

## 8. Conclusions and parting thoughts

Pesticide use is an integral part of every-day South African's life. It does not only impact populations at risk from malaria, but also the farmer, consumer, exporter and end-user of natural resources such as water. Therefore, the use of pesticides must be regarded as a serious management issue and not only at farm level, but, also at government level. Although pesticide research is well founded in South Africa, limitations exist within laboratory frameworks. Although pesticides have been detected in almost all conceivable media, there is still a lack of knowledge of background levels. These levels are needed to make realistic impact and health assessments when studying highly impacted areas. The serious health risks associated with certain pesticides are not only for occupational exposure, but also for end-user exposure. MRLs have been established to protect both the importer as well as the consumer. Table 1 indicates that South African MRLs are within the same range as those listed for the EU on the EU pesticide database (EU, 2010b). This ensures continued trade as well as protecting the health of the South African citizen. Aspects not specifically raised in this text that should be kept in mind within the South African context include the occurrence of accidental poisoning cases among farm workers as well as children. This occurs easily when the user did not receive proper training and does not understand how to use the pesticide. This is specifically a problem in areas with low literacy. There is also the question of obsolete pesticide stockpiles that can leach into the environment and have disastrous effects on ground water. These are challenges unique to developing economies, rarely seen in developed countries and should be considered in the international decision making process regarding the use and import of pesticides. In conclusion; although the need and usefulness of pesticides cannot be denied, the advantages of introducing these chemicals into the environment needs to be weighed against the possible negative side

effects. Therefore, serious consideration should be given to expanding the correct use of less harmful alternatives.

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# Use of Pesticides for Vegetable Crops in Mexico

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## 1. Introduction

The objective of this research was to analyze and compare the use of pesticides in Mexican horticulture, taking into account: the current Mexican regulation established by the Ministries of Agriculture, Livestock, Rural Development, Fishing and Feeding (SAGARPA), Environment, Natural Resources (SEMARNAT), and Health (SSA); the generalized recommendations according to different institutions linked to agricultural sector; farmers' organizations and companies; in correlation with the production and management practices of the farmers. It must also be considered that an important number of farmers have a limited knowledge of the regulations and lack training in the proper management of conventional and alternative products. Justification is based on the insufficient correspondence between the existing regulations and the emerging problems, registered in later years.

Given its geographic location and its diversity of climates, Mexico has the conditions for growing a great diversity of crops, and thus its agricultural activity is very dynamic. According to data from the INEGI (2007), the agricultural area of the country was 30,2 million hectares, of which 13,9 million were used for annual crops, 8,8 million for perennial crops, and 7,5 million hectares were not cultivated.

Among the agricultural activities, vegetable crop production is one of the most dynamic, due to their short cycle (1 to 5 months), which allows obtaining several harvests per year, and a great combination of them, or maintaining the same crop all year long. Some regions of the country have specialized on the production of certain vegetable crops, such as broccoli in Guanajuato, tomato in Sinaloa, chili pepper in Chihuahua, among others. This has allowed destining their products to the domestic as well as the international market. Albert (2005) mentions that in the northeast and central zones (Sinaloa, Sonora, Chihuahua, Baja California, Guanajuato, and Jalisco) important amounts of all kinds of pesticides are used to produce grains and a great variety of vegetable crops for exportation, among them, tomato, Curcubitaceae (squash, cucumber, etc), and chili pepper.

Cortina (2000) documented the states and zones with the greatest use of pesticides in Mexico: Sinaloa, Chiapas, Veracruz, Jalisco-Nayarit-Colima, Sonora-Baja California, Tamaulipas, Michoacan, Tabasco, State of Mexico, and Puebla-Oaxaca, being the greatest consumer of pesticides Sinaloa with 30% and the other states with 70%

The products destined to the export market are monitored for safe consumption, being very important the evaluation of the content of pesticide residues, in which case should not exceed the maximum permitted levels (LMRs), as well as of heavy metals and microbiological quality, so that there is no threat to human health. It is also important to monitor these parameters in the water used in the production of these products.

One of the main problems in intensive crop production, especially in the case of vegetable crops, is the control pest and diseases, which since the green revolution in Mexico, has been historically by means of chemical products. This has caused dependence on the use of agrochemicals, with the implications that it carries to health and ecological impacts. Some researchers point out this generalized problem in certain zones, for example Norzagaray *et al.* (2010) reports that in Sinaloa (the state with the greatest irrigated surface in Mexico) there is a certain environmental deterioration, which has its main effect on water use. Water is affected by over exploitation of the aquifers, percolation of pesticides and other wastes, causing damages to ecosystems and health. Another important factor of agricultural activities is their economic contribution.

Valdez *et al.* (2000) mentioned that due to the agrochemical control pest quickly, the farmers use them. The whitefly (*Bemisia argentifolli*) in the Mexicali Valley, Baja California, is one of the pests that causes great losses in cotton and vegetable crops. Around 800 tons of pesticides are applied in 150,000 hectares in one growing season. Likewise, the use of pesticides restricted and prohibited in Mexico due to their high toxicity has been detected.

There is no precise information on the amount of pesticides currently used in the country, but 6 years ago, it was indicated that around 50000 tons of active ingredient were used annually. The current market value is calculated to be between 400 and 600 million US dollars, although it is possible that this value is lower than it really is (Albert, 2005). In this context, it is important to document the use of pesticides on vegetable crops in Mexico, especially on those of greater importance, considering: regulations, management catalogues as technological reference, specific institutional and research results on pesticide residues, and the proposal of possible alternatives to improve the use of pesticides in vegetable crop production in Mexico.

A revision was done on the main databases of: the Ministry of Agriculture, Livestock, Rural Development Fishing and Feeding (SAGARPA), Service of Agro alimentary and Fishing Information (SIAP), National Institute of Ecology (INE), Inter secretarial Commission for Control and Use of Pesticides, Fertilizers, and Toxic Substances (CICOPLAFEST), National Reference Center of Pesticides and Contaminants (CNRPyC), World Health Organization (WHO), Pesticide Action Network (PAN), and FAO-STAT, among others.

The identification and analysis of technological systems for pest control was based on the gathering of data and creation of a database of the technological packages for each vegetable crop. The prospection universe was very wide, in the sense that the choice was to search for the necessary information, according to the matrix of the main vegetable crops, favoring the technological packages used and/or evaluated by: the National Institute of Agriculture and Livestock and Forestry Research (INIFAP), in its different experimental fields of the horticultural regions; the State Plant Health Administrations; Local farmers' organizations; and in some cases the theses and reports from local universities, among other sources.

A listing was made of the pests and products used to control them, the association between pests and the diversity of products to control them; as well as the listing of products and the spread of their cover among different crops and diverse pests. This information allowed creating a global database of the different vegetable crops. The universe of active ingredients used was classified according to SEMARNAT-WHO (2004).

Global data bases were analyzed from the National Reference Center of Pesticides and Contaminants (CNRPyC), which depends on the Ministry of Agriculture from 2005 to 2007, corresponding to the results of the national monitoring. There are programs for later years; however, they were not included since there was no access to laboratory results from those years.

From the annual databases, chilli pepper, tomato, squash and some samples of broccoli were select for this study.

The information was analyzed, allowing for the identification and analysis of the main residues from identified active ingredients, and their classification, considering the information provided by the CNRPyC, regarding to permitted, restricted, and prohibited materials, as well as the established LRM. The classification of the pesticides was based on the classification of the Pesticide Action Network (PAN), 2009.

## 2. Regulations on pesticides

Pesticides are regulated in Mexico by the Ministries of environmental, health, plant health, animal health, labor, and regulation for transportation. Also, indirectly, there are diverse customs and foreign trade regulations that must be observed in their handling (Table 1).

The laws involved are: The General Law of Ecological Balance and Environmental Protection, through its regulations on environmental impact regarding dangerous residues; The Federal Law of Plant Health; The Federal Law of Animal Health, Customs Law, General Health Law, through its regulations regarding health control of activities, establishment, products, and services; Federal Labor Law, through its regulations on safety and hygiene at workplaces; and the Law of Roads, Bridges, and Terrestrial Transportation. Specifically, the use of pesticides in Mexico is regulated by CICOPLAFEST (Inter-secretarial Commission for the Control and Use of Pesticides, Fertilizers, and Toxic Substances), constituted in 1987. This Commission includes the Ministries of Agriculture, Environment, Health, and Commerce and Industrial Foment (currently the Secretary of Economy), through a catalogue of registered products and their authorized uses. Its function is to carry out a uniform and integral procedure for the solution of registry requests, authorizations regarding pesticides, fertilizers, and toxic substances, as far as: exploitation, fabrication, formulation, mixing, conditioning, packaging, handling, transportation, distribution, application, storage, commercialization, keeping, use, and final disposal. Moreover, it contains the list of prohibited and restricted products in the country.

In the Official Catalogue of Pesticides, the chemical products are listed, per crop, the pesticides approved for control of plant health problems, safety intervals (days after application before to harvest), and maximum limits of residues authorized for each product (maximum amount of the active ingredient legally allowed in or on the agricultural product obtained, expressed in ppm). It also contains Technical information such as: Chemical name, synonyms, commercial name, formula (%), presentation, chemical structure, molecular weight, type of pesticide, classification, use, physical and chemical properties, hazard, and persistence. The official catalogue of pesticides constitutes an important reference to achieve a good use and management of pesticides. In conclusion, the only pesticides allowed to be imported, commercialized, and used in Mexico are those that have been registered by the CICOPLAFEST.

<b>Health</b>	NOM-044-SSA-1993, NOM-045-SSA-1993, NOM-046-SSA-1993, Projects for regulation NOM-058-SSA1-1993 y NOM-043-SSA1-1993
<b>Plant Health</b>	
NOM-0320FITO-1995	Health requirements and specifications to carry out studies on biological effectiveness of agricultural pesticides and their technical judgment.
NOM-033-FITO-1995	Health requirements and specifications to notify start of operations that physical or moral persons interested in commercializing agricultural pesticides must fulfill.
NOM-034-FITO-1995	Health requirements and specifications to notify start of operations that physical or moral person interested in making, formulating, and or working, or importing agricultural pesticides must fulfill.
NOM-052-FITO-1995.	Health requirements and specifications to notify start of operations those physical or moral persons that are dedicated to aerial application of agricultural pesticides must fulfill.
NOM-057-FITO-1995	Health requirements and specification to emit judgment on pesticide residue analysis.
<b>Ecological</b>	NOM-090-ECOL-1994 y NOM-052-ECOL-1993
<b>Animal Health</b>	NOM-023-STPS-1993
<b>Industrial Safety</b>	NOM-005-STPS-1993, NOM-006-STPS-1993, NOM-009-STPS-1993, NOM-010-STPS-1993.

Table 1. Health, Plant Health, Animal Health, and Industrial Safety regulations regarding pesticides

The Federal Law of Plant Health confers to the ministry of Agriculture, Livestock, Rural Development, Fishing and Alimentation the responsibility of regulating and promoting plant health through plant health regulations among which are included the vigilance of biological effectiveness, maximum residue limits, application, use, and management of pesticides.

Table 2 show the lists of prohibited and restricted products in Mexico as well as products prohibited in other countries but allowed in Mexico. It stands out that products such as Endosulfan, Methamidophos, Monocrotophos, among others, less frequent, are commonly used on vegetable crops in Mexico; therefore these products could present restrictions for the export market, depending on the plant health regulations in use. There would be no restrictions for the domestic market, as long as the registry and authorization regulations are respected for a specific crop, and most of all, not going over the PML of the products.

Prohibited pesticides	Restricted Pesticides	Pesticides prohibited in other countries but authorized in Mexico
Phenyl acetate	DDT	Alaclor
Mercury	BHC	Methamidophos
Acid 2,4,5-T	Aldicarb	Azinphos methyl
Aldrin	Dicofol	Monocrotophos
Cyanophos	Phorate	Captan
DBCP	Lindane	Oxyfluorfen
Dialifor	Methoxycloro	Methyl-parathion
Dieldrin	Mevinphos	Quintozene
Dinoseb	Paraquat,	Phosphamidon
Endrin	Pentachlorophenol	Tridemorph
Erbon	Quintozene	Maneb
Formothion	1,3 Dichloropropene	Methidathion
Sodium Fluoroacetate		Captofol
Fumisel		Mevinphos
Kepone/Clordecone		Omethoate
Mirex		Paraquat
Monuron		Diuron
Nitrofen		Phorate
Schradan		Triazophos
Triamiphos		Linuron
Thalium sulfate		2,4,D
Toxaphene		Endosulfan
		Sulfopros
		Kadethrin
		Carbaryl

Table 2. Prohibited and restricted pesticides in Mexico, and products prohibited in other countries but authorized in Mexico. Source: INE, 2010

## 2.1 Agricultural dynamics and vegetable crop production in Mexico

The cultivated agricultural surface area in Mexico, according to data from the INEGI (2007) is 22,7 million hectares. The most important crops are corn, beans, sorghum, wheat, barley, potatoes, and vegetables.

According to data from the Ministry of Agriculture (SIAP, 2011), the most important vegetables crops, by area cultivated, are 25, which sum up a total surface area of 451036 hectares, and they are: Chili pepper (*Capsicum annun*), tomato (*Lycopersicum sculentum*), green tomato (*Physalis exocarpa*), onion (*Allium cepa*), squash (*Cucurbita pepo*), broccoli (*Brassica oleraceae var Italica*), lettuce (*Lactuca sativa*), cucumber (*Cucumis sativus*), carrot (*Daucus carota*), and asparagus (*Asparagus officinalis*). From these, the first six were selected (chili pepper, tomato, green tomato, onion, squash, and broccoli), with areas ranging from 140439 and 24396 hectares, for chili pepper and broccoli, respectively. The total national area of the selected vegetable crops is 330487 hectares (Table 3), equivalent to 73,3%.

Product	Principal producing states	National Total surface (ha)	Total Surface in 5 States (ha)
Chili pepper	Chihuahua, San Luis P., Sinaloa, Durango, Chiapas	140439	63319,5
Tomato	Sinaloa, Michoacan, Baja California, Veracruz, Zacatecas	52383	28779,8
Green tomato	Sinaloa, Jalisco, Puebla, Mexico, Sonora	45704	26538,3
Onion	Baja California, Guanajuato, Tamaulipas, Chihuahua, Puebla	41725	24951,2
Squash	Sonora, Sinaloa, Puebla, Hidalgo, Michoacan	25840	16946,5
Broccoli	Guanajuato, Michoacan, Jalisco, Puebla y D.F.	24396	22445,5
Total		330487	

Table 3. Surface crops of the six principal vegetables in the five most important states producing. Source: Own elaboration with data from the SIAP, 2011.

### 3. Technological management for pest control in the main vegetable crops

The most important vegetable crops in Mexico, according to registries of cultivated surface and production values are: chili pepper, tomato, onion, green tomato, squash and broccoli. Information on other vegetable crops is very disperse, limited, and incomplete.

#### 3.1 Global analysis

The products used for pest control have been characterized into two great categories: 1) chemical groups. There is a variety of active ingredients within most of them, through which is commercialized a great diversity of “brands” in the national market, and 2) biological insecticides. Among the 6 main vegetable crops, grown and selected to illustrate the use of pesticides, a total of 97 active ingredients were identified, while only 6 (6,2%) biological insecticides were used.

With regard to registries of insect pests among the main vegetable crops, an important variability was registered, considering the greatest number in the chili pepper crop (19 pests) and the lowest for onion (8). The remaining crops registered: tomato 16 pests, squash 15, green tomato 11, and broccoli 10. On the other hand, the active ingredients used registered the highest number in squash (71), and the lowest in onion (11). Variability between these two crops registered the following totals: green tomato 33, tomato 24, chili pepper 23, and broccoli 22 active ingredients. The biological insecticides were used in all the crops, the greatest number of which was registered for squash, with a total of 4, and only one was registered for onion (Table 4).

Vegetable Crops	Pests	Active Ingredients	Biological Insecticides
Chili Pepper	19	23	3
Tomato	16	24	2
Onion	8	11	1
Green tomato	11	33	4
Squash	15	71	2
Broccoli	10	22	3

Table 4. Main vegetable crops and number of active ingredients and biological insecticides used to control pests. Source: Personal elaboration with data from the SIAP, 2011.

The toxicological classification of the active ingredients in the four typical groupings shows the following results: i/ Slightly toxic with 17 chemical groups and 22 active ingredients, ii/ Moderately toxic with 10 different chemical groups and 34 active ingredients, iii/ Highly toxic with a total of 6 chemical groups and 20 active ingredients, and finally iv/ Extremely toxic made up of 5 chemical groups and 15 active ingredients. Six (6) active ingredients were identified which are not classified (Table 5). The source of classification was CICOPLAFEST (2004), modified from WHO recommended classification of pesticide by hazard and guidelines to classification 2000-2002.

Toxicological Class	Extremely toxic	Highly toxic	Moderately toxic	Slightly toxic	Unclassified
Chemical groups	5	6	1015	17	0
Active ingredients	15	20	34	22	6

Table 5. Toxicological classification of active ingredients used in the main vegetable crops in Mexico.

According to information from the Pesticide Action Network (2009), the 97 active ingredients used to control pests in the reference vegetable crops were classified. The results show that 60,8% were classified as highly dangerous. Considering the total of highly dangerous active ingredients, the active ingredients used in all the crops were 3 (Diazinon, Malathion, and Methomyl). A biological insecticide (*Bacillus thuringiensis*) was registered for the same group of 6 crops. The use of 5 highly dangerous active ingredients (Abamectin, Chlorpyrifos, Endosulfan, Metamide, and Parathion Methyl), one unclassified ingredient (Azinphos-methyl), and no biological insecticides were reported for 5 vegetable crops. The use of 8 highly dangerous ingredients (Carbaryl, Cyromazine, Esfenvalerate, Fenvalerate, Imidacloprid, Lambda-cyhalothrin, Permethrin, and Trichlorfon) was identified in 4 crops. 6 highly dangerous ingredients were registered in 3 crops.

In 2 crops, the registry showed, similarly, 6 highly dangerous ingredients, and two unclassified ingredients. In this group was registered the greatest number of biological insecticides (4). Finally for only one crop, 31 highly dangerous ingredients were registered, 33 unclassified ingredients, and 1 biological insecticide (Table 6).

Groups		Number crops where the products were used					Total	
		6	5	4	3	2		1
Active ingredients	Highly dangerous (HD)	3	5	8	6	6	31	59
	Others, not HD	0	1	0	2	2	33	38
Biological insecticides		1	0	0	0	4	1	6
Total products		4	6	8	8	12	65	103

Table 6. Global classification of active ingredient considering highly dangerous classification products of PAN, 2009, and biological insecticides used in the vegetable crops studied.

### 3.2 Main vegetable crops in Mexico

**Chili pepper:** In the great diversity of regions and production systems destined to growing chili pepper, the existing report shows that 19 pest are controlled with 23 active ingredients and 3 with biological insecticides. The information corresponds to states: Chihuahua, San Luis Potosi, Sinaloa, Jalisco, Tamaulipas, and Baja California Sur.

According to the diversity of insect pests, the number of active ingredients used for their control, and the states where the production systems are registered, the use of 7 active ingredients or products is reported to control the pepper weevil (*Anthonomus eugenii* Cano), registered in the production systems of 3 states. To control whitefly (*Bemisia argentifolia* B&P and *B. tabaci* Genn), as well as flea beetles (*Epitrix spp.*), 6 active ingredients are reported for each, and which are registered in the production systems of 3 and 2 states, respectively.

The diversity of insecticide products, considering the active and biological ingredients, for the control of different pest is presented in Figure 1. The identified products were: Methamidophos is used to control 8 insect pests and its cover extends to 3 states. Endosulfan is used to control 7 pests and is reported in diverse production systems in 6 states. Permethrin is registered for the control of 6 pests and it is used in 3 states. Azinphos-methyl, Carbaryl, Chlorpyrifos, and Spinosad make up a group of active ingredients, each one for the control of 4 pests, in a diverse number of states. The greatest spatial cover (equal to or greater than 3 states) is seen in: Endosulfan: 6 states. Chlorpyrifos: 4 states. Cyromacyn, Methamidophos and Parathion are used in 3 states.

The biological insecticide *Bacillus thuringiensis* was used for the control of two pests (false inchworm and armyworm –*Spodoptera exigua*– in Baja California Sur and San Luis Potosí states; *Trichogramma* sp, registered to control hornworm in San Luis Potosí, and *Phaecilomyces fumosroseus*.

The toxicological classification of the ingredients and products used on chili pepper registers 4 toxicological classes, within which 9 chemical groups were registered. Also, the use of 3 biological insecticides (*Bacillus thuringiensis*, *Phaecilomyces fumosroseus*, and *Trichogramma*) was identified. The predominant classes correspond to moderately and extremely toxic, which concentrate, respectively, on 31% of the total active ingredients. On the other hand, slightly and highly toxic classes were each 19% of the total. The Extremely toxic chemical groups are: Avermectin, Carbamate, Organophosphorus and insect growth regulator. The Moderately toxic chemical groups are: Carbamate, Macrocylic lactone (Spinosad), Nicotinoid, Organophosphorus, and Pyrethroid. Three biological insecticides, one organophosphorus ingredient, and one antifeedants were registered in the Slightly toxic class.

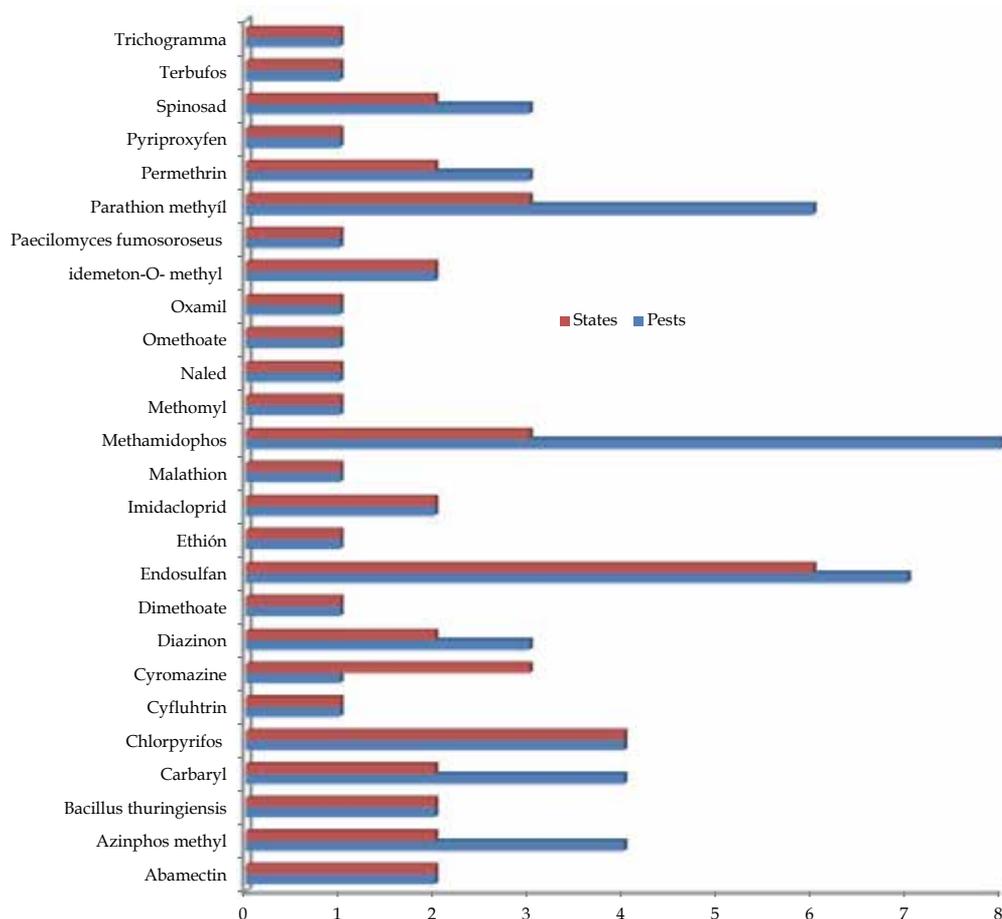


Fig. 1. Active ingredients and biological insecticides, number of pests controlled and total number of states where they are used in the chili pepper crop.

Tomato: There are 16 pests reported in tomato crops, where 24 active ingredients and 2 biological insecticides are used, in 5 main states: Sinaloa, Nayarit, Morelos, San Luis Potosí and Zacatecas. Among the most important insect pests are: the leaf miner (*Liriomyza spp.*) which is controlled with 12 active ingredients, in production systems of 4 states; secondly is the fruit worm (*Heliothis zea* and *H. virescens*), which are controlled with 11 active ingredients-products, in production systems in 5 states. Of similar importance is the whitefly (*Bemisia tabaci*), which is controlled with 9 active ingredients in production systems of 3 states.

The active ingredient with the most widespread use for pest control is Endosulfan, which is used against 9 pests in the production systems of 4 states. The active ingredients: Abamectin, Indoxacarb, Methamidophos, Permethrin, and Spinosad are used to control 4 different pests each one, with a cover varying from 1 to 3 states. With regard to biological insecticides, the cover is: *B. thuringiensis* is used to control 5 pests in two states, and *Paecilomyces fumosoroseu* is used to control whitefly (*Bemisia tabaci*) in one state.

The toxicological classification of the active ingredients and products used on tomato registers all 4 toxicological classes, within which 9 chemical groups were identified.

The Moderately toxic class is the most representative, within which 5 chemical groups were identified (Carbamate, Macrocylic lactone (Spinosad), Nicotinoid, Organophosphorus and Pyrethroid), among which 12 active ingredients were identified. The next most important class was Highly toxic, where 3 chemical groups were identified (Carbamate, Organochlorates and Organophosphorus), which 6 active ingredients. The Extremely toxic class registered two groups: Abamectin and Organophosphorus, including Methamidophos, Parathion, and Terbufos.

Green tomato. In the production of green tomato are reported 11 pests, for which 33 active ingredients and 4 biological insecticides are used, according to 3 management systems reported in the states of Morelos and Puebla. Among the main pests are: the whitefly (*Trialeurodes vaporariorum* West) which is controlled with 15 different active ingredients, the leaf miner (*Liriomyza trifolii* Burgess) and flea beetle (*Epitrix cucumeris* Harris), which are controlled with 12 active ingredients each, in different state production systems. Less important are: whitefly (*Bemisia* sp) and Diabrotica (*Diabrotica balteata* Le Conte), which are controlled with 9 and 7 active ingredients, respectively in different state production systems. Among the wide diversity of 37 products, there are 34 active ingredients and 3 biological insecticides. Among the most common is: Diazinon, with a wide spectrum of control and is used against 7 pests. On the other hand Monocrotophos, Methamidophos, and Fenvalerate make up a group of active ingredients with a cover of 5 pests controlled. Third in importance are Azinphos methyl, Dimethoate, Endosulfan, and Phosphamidon, which control 3 pests in different states (Figure 2).

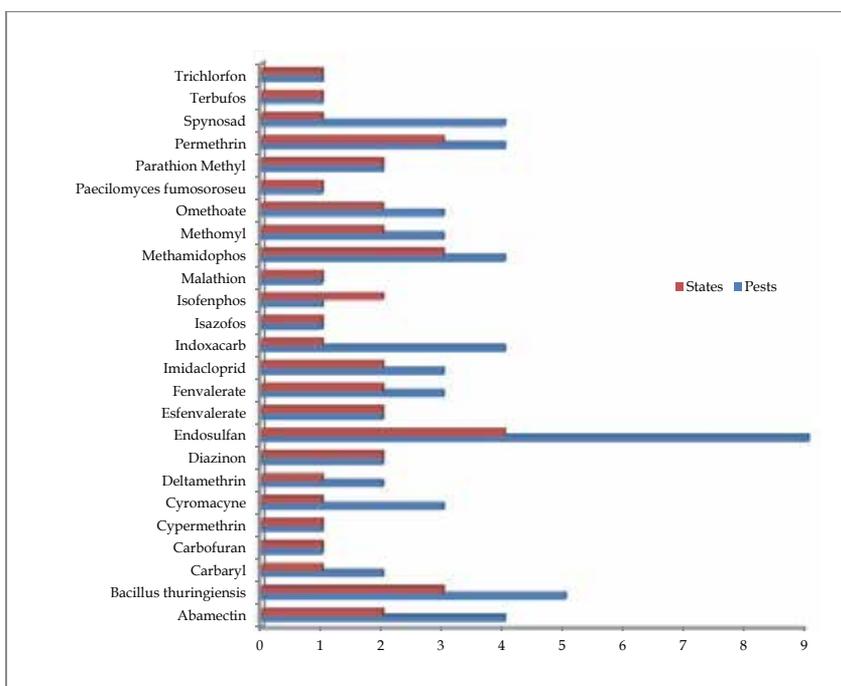


Fig. 2. Toxicological classification and chemical groups in tomato.

The biological insecticides used were: *Bacillus thuringiensis* to control fruit worm (*Heliothis suflexa* Genée); *Bauveria bassiana* to control whitefly (*Bemisia* sp), and *Trichoderma* spp.+ *Bacillus subtilis* used for plant production.

The toxicological classification of the ingredients and products used on green tomato registers all 4 toxicological classes, within which 14 chemical groups were identified. The most important class was Moderately toxic, with 5 chemical groups (Nicotinoid, Thiazole fungicides -Thiabendazole, Carbamate, Organophosphorus, and Pyrethroid), among which 12 active ingredients were identified. Secondly, the Extremely toxic class was identified with 4 chemical groups (Macrocyclic lactone -Abamectin-, Carbamate, Organophosphorus, and Pyrethroid), among which 10 active ingredients were registered.

Squash. In squash crop, 15 insect pests are reported, for which 71 active ingredients were used, as well as 19 mixtures of them and 2 biological insecticides, in Baja California Sur, Sinaloa and Oaxaca. Among the most important insect pests are: Diverse worm types (*Spodoptera exigua*, *Heliothis armigera*, *Chrysodesis chalcites*, and *Autographa gamma*), which are controlled with 31 active ingredients and their mixtures, indifferent production systems in 2 states; Greenfly (*Aphis gossypii* Sulzer), against which 28 active ingredients and mixtures were used, in different production systems in 2 states; Whitefly (*Trialeurodes vaporariorum*), against which 20 active ingredients and their mixtures are used, in different production systems in one state; Red spider (*Tetranychus urticae* Koch), against which 16 active ingredients and their mixtures are used, in different production systems in one state; and Trips (*Frankliniella occidentalis* Pergande), against which 10 active ingredients and their mixtures are used, in different production systems in one state.

The active ingredients with the most widespread use for pest control are: Bifenthrin and Deltamethrin, which are used to control 7 insect pests. Second in importance from their cover in controlling 6 pests are: Alpha-cypermethrin, Cypermethrin alone and with sulfur, Fenitrothion, Flucythrinate, Lambda-cyhalothrin, Malathion, and Pirimiphos-methyl. To control 5 pests: Acephate, Chlorpyrifos-methyl, Esfenvalerate, Etofenprox, Fenvalerate, Permethrin, Propoxur, and Tralomethrin. The biological insecticides used are: *Bacillus thuringiensis* 3,2% to control caterpillars (*Spodoptera exigua*), (*Heliothis armigera*), and (*Chrysodeisis chalcites*) and (*Autographa gamma*), false inchworm and armyworm and, *Bauveria bassiana* to control whitefly (*Trialeurodes vaporariorum*).

The toxicological classification of the ingredients and products used in squash registers all 4 toxicological classes, within which 25 chemical groups were identified. The most important class was moderately toxic, which includes 10 chemical groups, among which 29 active ingredients were identified. Secondly was identified the slightly toxic class with 11 chemical groups, among which 17 active ingredients and 4 unclassified products were registered. On the other hand, there were 5 highly toxic groups (Carbamate, Organochlorine, Organophosphorus, Organotin acaricides, and Pyrethroid), among which were 16 active ingredients. Finally, 4 chemical groups were registered from the extremely toxic class (Macrocyclic lactone -Avermectin-, Carbamate, organophosphorus and Pyrethroid), with 9 active ingredients.

Onion. In onion crop, 8 insect pests are reported, for which 11 active ingredients and one biological insecticide are used, in 6 states: Baja California Sur, Chihuahua, Guanajuato, Morelos, Tamaulipas, and Zacatecas. Among the most important insect pests are: Trips (*Thrips tabaci* Lindeman), which is controlled with 11 active ingredients in different production systems in 5 states; Leaf miner, against which 7 active ingredients are used in different production systems in 4 states; and armyworm (*Spodoptera exigua* Hubner), against which 4 active ingredients are used in different production systems in 4 states.

According to the widespread use of the active ingredients to control pests, there are: Azinphos-methyl and Diazinon, which control 5 insect pests, used in production systems of 5 states; second in importance are Malathion, which controls 4 pests in production systems of 5 states, and Methomil, which controls 3 insect pests in production systems in 4 states. The biological insecticide used was *Bacillus thuringiensis* to control armyworm (*Spodoptera exigua* Hubner) in two states.

The toxicological classification of the ingredients and products used in onion registers all 4 toxicological classes, within which 4 chemical groups were identified, as well as *Bacillus thuringiensis* as biological insecticide. The most important class was extremely toxic, which is made up of 2 chemical groups (Macrocyclic lactone and Organophosphorus), among which 4 active ingredients were identified. Secondly was the Moderately toxic class with two chemical groups (Pyrethroid and Organophosphorus), among which 3 active ingredients were registered. Then, there were 2 Highly toxic groups (Carbamate and Organophosphorus), with 2 active ingredients. Finally, there were 2 Slightly toxic groups with 2 chemical groups (Organophosphorus and Antifeedants –Pymetrozine), among which were 2 active ingredients

Broccoli. In broccoli, 10 insect pests are reported, for which 22 active ingredients are used, 1 mixture, and 3 biological insecticides, in different production systems in 4 states: Aguascalientes, Guanajuato, Morelos, and Sinaloa. Among the most important insect pests are: Imported cabbage worm, which is controlled with 11 active ingredients and 1 biological insecticide in different production systems in 3 states; Diamondback moth (*Plutella xylostella*), against which 9 active ingredients and 2 biological insecticides are used in different types of production systems in 2 states; Cabbage heart worm, which is controlled with 9 active ingredients and 1 biological insecticide in different types of production systems in 2 states. A group of 3 insect pests: Harlequin cabbage bug (*Murgantia histrionica* Hahn), False inchworm (*Trichoplusia ni*), and Armyworm (*Spodoptera exigua*), each controlled with 9 different products (active ingredients and biological insecticides), respectively, in production systems of 1, 2, and 1 states.

According to the importance of the cover of the active ingredients to control pests, there are: Methamidophos, used to control 7 insect pests in production systems in one state; secondly important are Azadirachtin, Fenvalerate, Parathion-methyl and Permethrin, registered to control 5 pests each, used in production systems in 1 state; finally in importance to control pests are: Azinphos-methyl, Endosulfan, Naled, and Trichlorfon, used in 1 and 2 states. The biological insecticides used were: *Bacillus thuringiensis* to combat 6 pests, *Diadegma* and *Trichogramma* to combat 4 pests.

The toxicological classification of the ingredients and products used in broccoli registers all 4 toxicological classes, within which 7 chemical groups were identified, and 3 biological insecticides. The most important class was that of Moderately toxic, made up of 4 chemical groups, among which 11 active ingredients were identified. Second in importance was Extremely toxic, which has only one chemical group (Organophosphorus), with 5 different ingredients (Azinphos-methyl, Methamidophos, Monocrotophos, Naled, and Parathion-methyl). There were 3 Highly toxic groups (Macrocyclic lactone, Carbamate, and Organochlorine), among which were 3 active ingredients. Lastly, there were Slightly toxic with 2 chemical groups (Distillates (Petroleum paraffinic petroleum oil- and Organophosphorus), among which are 3 active ingredients (Table 7)

Extremely	Highly	Moderately	Slightly
Organophosphorus (5)	Avermectin (1) Carbamate (1) Organochlorine (1)	Carbamato (1) Espinosina(1) Organophosphorus (4) Pyrethroid (5)	Alifatic (2) Organophosphorus (1)

Table 7. Toxicological classification and chemical groups: broccoli

#### 4. Pesticide residues in crops

In Mexico, there are no official antecedents on the monitoring of unauthorized pesticides in agricultural products. At institutional level, there were only studies, and the information generated came from countries importing agricultural products, which rejected shipments containing residues from pesticides without a LMR, or when these were greater than the allowed levels (CNRPyC, 2011). The National Center for Reference of Pesticides and Contaminants (CNRPyC) was established in 1991 as a normative reference center, for the development of training methods regarding pesticide residue analysis. It is part of the General Direction of Water and Fishing Agroalimentary Health (DGIAAP), which was created in 2003 with the responsibility of working on health safety of foodstuffs from land and sea, through health standards required by the domestic and international market.

The CNRPyC carries out an annual follow-through and evaluation program at national level in zones where there have been malpractices regarding the use and application of pesticides. This program has focused on establishing strategies that will allow designing operational plans, though which can be achieved awareness of the producers through training and assistance programs by the personnel from the State Delegations of the SAGARPA, where the good use and management of pesticides will be promoted. This will reflect on the production of harmless products, free of pesticide residues, which in turn will be reflected on the health of the consumers and farmers producers.

The role of the CNRPyC is to help in the coordination and application of the program with the State Delegations. The regions and crops to be considered in the monitoring were taken into account based on the presence of unauthorized pesticide residues detected and reported in the analyzed samples. Currently, there are results of pesticide residues from the samples analyzed from 2005-2007, and programming data from the 2008 monitoring.

##### 4.1 National monitoring 2005-2007

The pesticides products not authorized for some crops were detected by the CNRPyC, (2011), found 19, 15 y 17 for 2005, 2006 and 2007 respectively (Table 8). The products not authorized are: Permethrin, Omethoate, Monocrotophos, Chlorothalonil, Parathion methyl, Methamidophos, Acephate, Endosulfan, Dimethoate, Pentacloroaniline, Cypermethrin, Chlorpyrifos, Isozofos, Quintozene, Dichlorvos, Acetochlor, Dicrotophos, Pentachlorobencene, Diazinon, Folpet, Iprodione, Ethion, Lamda cyalotrine, Profenophos.

From the analyzed samples, approximately 12% of the products are destined to the domestic market. The main commercialization sites are supply depots in: Puebla, Cautla, Mexico City, Hermosillo, Chihuahua, Monterrey, Torreon, Guadalajara, Tamaulipas, and other local and regional markets. The destinations of the products of the exportation market to the United States (California and Arizona, among others) are equivalent to approximately 87%.

	2005	2006	2007	2008*
Total samples	335	337	323	308
States participating in the sampling	16 and one region	16 and one commercial center	12 and one region	19 and one region
Monitored crops	44	49	41	17
Samples for Exportation market	42	38	42	-
Samples for domestic market	290	299	281	-
Non authorized products	19	15	17	
Restricted	7	6	6	
Canceled samples	7			

Table 8. Participating states, crops and characteristics of the samples analyzed in the national Monitoring for the quantification of pesticide residues 2005-2007. Source: own elaboration with data available from the CNRPyC; \*=programmed

The results of the analysis of the 335 samples in 2005, in which 424 determinations were made, showed that 93 samples were free of pesticide residues, however 248 samples were found with the presence of at least one residue, 33 samples with two and 25 with 3 residues. Only a small proportion submitted more 3 pesticides residues. The most frequently product residues found were: Methamidophos, Endosulfan, Chlorpyrifos, Omethoate, Dimethoate and Acephate, Permethrin, Monocrotophos (Figure 3).

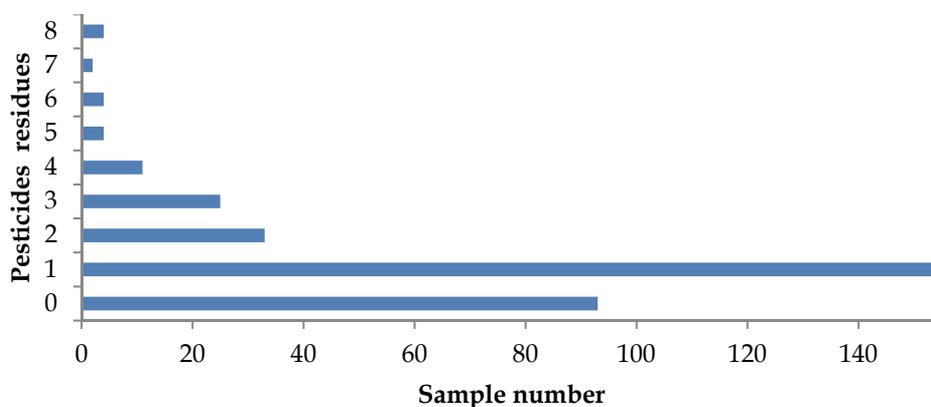


Fig. 3. Pesticides residues found in vegetable samples, 2005.

An important part to achieve a correct use and management of these products is to maintain the users and plant health professionals informed on the authorized pesticides, whose biological effectiveness has been positively evaluated in the field, as well as what the doses, time and number of applications, and safety intervals (days elapsed between the last application and harvest) that have to be observed in the use of pesticides. Likewise, the technicians from the SAGARPA must be updated in the knowledge of the products whose effectiveness in pest control has been proven, so they can carry out an adequate vigilance on their use and application.

According to the results found (Figure 4), almost 30% of the analyzed samples were free of pesticide residues. The pesticides more frequently found were Methamidophos, Endosulfan, Chlorpyrifos, Omethoate y Dimethoate. The different active ingredient found in the samples from 2005 to 2007 were 31.

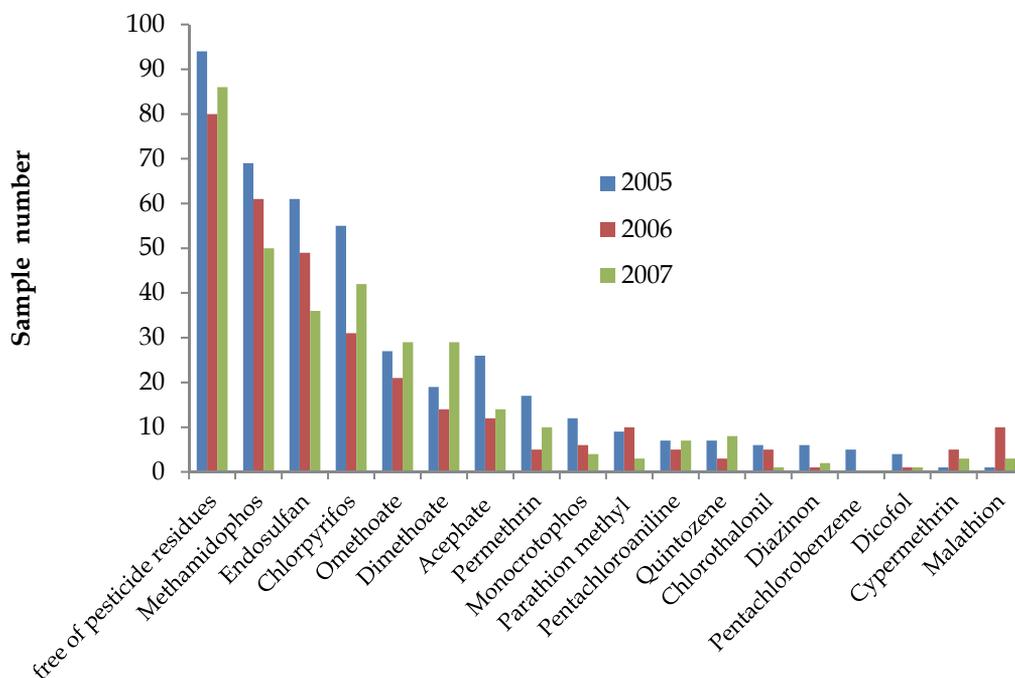


Fig. 4. Pesticides residues in diferents crops 2005-2007.

#### 4.2 National sampling for the main vegetable crops

The National Reference Center of Pesticides and Contaminants (CNRPyC), has carried out during 2005, 2006, and 2007 several samplings of pesticide residues in different agricultural systems and states in Mexico. Among the crops of economic interest for Mexican agriculture were selected: chili pepper, tomato, squash, and broccoli.

##### 4.2.1 Global multiannual analysis

During 2005-2007, 406 samples of vegetable crops of interest were analyzed, in which a total of 665 determinations were identified in the three years.

The analysis indicates that in 156 determinations of the 4 crops analyzed (23,5%) no residues were detected; in 391 (58,8%) residues from authorized active ingredients were detected; in 113 (17%) residues from unauthorized active ingredients were detected; in 5 of the determinations the active ingredients are over the LMR, established for the products; in 2 determinations residues were identified as restricted active ingredients, and in one of them a prohibited active ingredient was identified (Table 9).

	Samples number or determinations
Total samples	406
Total determinations	665
Active ingredients undetected	156
Authorized active ingredients	391
Unauthorized active ingredients	113
Active ingredients exceeding LMR	5
Restricted active ingredients	2
Prohibited active ingredients	1
Determination without data	5

Table 9. Global analysis of residues samples in chili pepper, tomato, squash, and broccoli, during 2005 to 2007

#### 4.2.2 Authorized active ingredients in the main vegetables

15 authorized active ingredients were identified: Bifenthrin, Clomazone, Chlorpyrifos, Chlortal-dimethyl (Dactal), Cyanofos, Diazinon, Dimethoate, Endosulfan, Ethion, Lamba-cyhalothrin, Malathion, Methamidophos, Mevinphos, and Quintozene. The total of authorized active ingredients per year was: 172, 114, and 105 in 2005, 2006, and 2007, respectively. The authorized active ingredients that were detected most frequently were: Methamidophos (extremely toxic organophosphorus) with 114 registries, representing 34%; Endosulfan (highly toxic organochlorine) with 99 registries (29,6%), and Chlorpyrifos (highly toxic organophosphorus) with 74 registries, equivalent to 22,1% of the total. In general, it can be seen that these three active ingredients make up 85,7% of the determinations of authorized ingredients.

#### 4.2.3 Unauthorized active ingredients

A total of 15 unauthorized active ingredients were identified: Acefate, Acetoclor, Cipermetrina, Cloratonil, Clorpirifos, Dimetoato, Folpet, Iprodione, Isazofos, Monocrotofos, Omethoate, Paration metílico, Pentacloroanilina, Permetrina, and Profenofos. The total of unauthorized ingredients found was 118: 52 determinations with different residues in 2005, 33 in 2006, and 28 in 2007.

The unauthorized active ingredients detected most frequently were: Omethoate (highly toxic organophosphorus) with 40 registries (35,4%); Monocrotofos (highly toxic organophosphorus) with 20 registries (17,7%); Acephate (slightly toxic organophosphorus) with 19 registries (16,8%); and Permethrin (slightly toxic pyrethroid) with 14 registries (12,4%). These four made up 82,3% of the total unauthorized ingredients

In squash, residues from 5 active ingredients were found, among which the most frequent were: Endosulfan with a total 7 samples in the states of Baja California, Puebla, and an unidentified site; Metamidophos with a total 5 samples in the states of Baja California, Oaxaca, and Puebla; and the group made up of Acephate, Dimethoate in different states.

In tomato, residues from 9 active ingredients were identified. The most frequent of these were: Endosulfan in 12 determinations in the states of Baja California, Oaxaca, Sonora, and the Laguna region, besides 3 unidentified sites; Metamidophos with 11 determinations in

the states of Chihuahua, Oaxaca, Sinaloa, and Sonora, besides 5 unidentified sites; Chlorpyrifos with 6 determinations in the states of Michoacan, Sinaloa, and the Laguna region. A group made up of Acephate and Omethoate, with 4 determinations each. The first of these was unidentified in 4 sites and the latter in the states of Michoacan and Sinaloa and Omethoate, each of which was reported in two different samples.

In chili pepper crops, residues from 11 active ingredients were registered, among which the most frequent were: Chlorpyrifos (slightly toxic organophosphorus) with a total 17 determinations in the states of Aguascalientes, Guanajuato, Michoacan, Sinaloa, and the Laguna region besides 5 unidentified sites; Methamidophos (extremely toxic organophosphorus) with a total 16 positive results in the states of Baja California, Chihuahua, Michoacan, San Luis Potosi, and the Laguna region; and a group made up of Endosulfan (highly toxic Organochlorine) and Omethoate (highly toxic Organochlorine), each one reported on 9 determinations; the first of these in the states of Baja California and San Luis Potosi, and the latter in three states: Guanajuato, Michoacan, and Zacatecas.

## 5. Final reflection

Bolognesi (2003) and Mansour (2004) mention that pesticides are the most common products used to control agricultural pests, specifying that a correct application is the most accepted and effective measure to achieve maximum production and best quality in the crops. However, in Mexico, the tendency to the specialization of certain crops in specific zones and/or states generates a predominance of monocrops growing and an increase in the frequency of incidence of typical pests. Consequently, there is a greater number of applications of agrochemicals to control the pests, an increase in the recommended doses, use of products successful in controlling a certain pest on crops on which they are not authorized, and there are even reports where the trust interval for the products is not respected. Such evidences converge to explain, as the main causes, the presence of pesticide residues in agricultural products, especially in vegetable crops. However, in most cases, the pesticides residue in the samples analyzed do not exceed the permitted maximum limits. Moreover, the CICOPAFEST (1998) and Bolognesi (2003) mention that there is a great diversity of products in the market, given that the industry of agrochemicals in the XX century has developed a great number of new compounds that are highly aggressive to human health, and which have caused noxious effects that have broken the balance of the ecosystem.

The Pesticide Action Network (PAN, International, 2009), on classifying pesticides according to their level of danger, even proposing the elimination of some of the more dangerous ones, has been the organization that has identified and integrated the factors that are dangerous to the ecosystem, and to human health in the long term, among others.

It is necessary the use of agricultural practices to reduce the use of pesticides. Some options are: incorporation of biological control measurement, the use of integrated pest management, respecting authorized products, doses, and trust intervals per crop, being among the most important (Martinez and Gomez, 2007; Perez *et al.*, 2009).

Is also of important to intensify efforts in training and permanent updating of the technical personnel, laborers, and farmers, as well as fortifying actions to prevent and educate the community (Martinez and Gomez, 2007). Moreover, the production processes of the producers or zones where products have been obtained free of pesticide residues must be valued and taken into account, in order to integrate proposals of technological packages that can be upgraded to regional levels.

Monitoring of agricultural products must be permanent, especially in the horticulture sector, which has the most intensive use of pesticides (Perez *et al.*, 2009) and monitoring of laborers and pesticide users must be considered as an integral part of a good medical vigilance in people, since it allows to take the necessary actions on early identification of genetic risk (Martinez and Gomez, 2007).

## 6. Conclusions

In Mexico, the use of pesticides in horticulture is regulated by the Mexican norms and regulations in use, as established by the Secretaries of: Agriculture and Livestock, Environment, and Natural Resources, which make up the Intersecretarial Commission for Control and Use of Pesticides, Fertilizers, and Toxic Substances. Its coordination and regulations are concreted in a national catalogue, which functions as the global reference framework to orient and achieve the good use and management of pesticides, considering that it integrates a list of pesticides whose importation, commercialization, and use are allowed in Mexico.

In the case of horticultural products, particularly for the exportation market, the importing countries have proven to be very rigorous in their regulations regarding residues from pesticides and other contaminants. Because of this, samplings are done to guarantee the quality of the vegetable crops that go into their countries, considering a zero tolerance in the cases of organic or ecological agriculture, and that the maximum allowed limits established for authorized pesticides for conventional crops are not exceeded. Consequently, the farmers who establish their crops for the exportation market have to use only those products that are authorized in the country where their products are intended to export. In some cases, there is even a contractual agriculture, where the farmer commits himself to applying a technological package that includes the products and technical assistance.

In the six vegetable crops analyzed, there are between 8 and 19 pests. The outlier values correspond to onion and chilli pepper, respectively. For the group of these vegetable crops, 97 active ingredients and 6 biological insecticides were identified. The use of active ingredients was greatest in squash (73,2%), and the lowest was in onion (11,3%). Biological insecticides represent 5,8% of the products used to control pests, with 1 to 4,1% in onions and green tomato, respectively.

The toxicological classification of the active ingredients in the corresponding categories is as follows: i/ Slightly toxic with 17 chemical groups and 22 active ingredients, ii/Moderately toxic with 10 chemical groups and 34 active ingredients, iii/Highly toxic with 6 chemical groups and 20 active ingredients, and iv/ Extremely toxic with 5 chemical groups and 15 active ingredients. There were also 6 unclassified active ingredients identified.

According to the classification by the PAN, 2009, the pesticides reported in the group of vegetable crops makes up 60,8% of the total identified active ingredients. Among them, Diazinon, Malathion, and Metomilo were used in all vegetable crops; Abamectina, Chlorpyrifos, Endosulfan, Metamidophos, and Parathion-methyl were used in 5 crops; and Carbarilo, Cyromazina, Esfenvalerato, Fenvalerato, Imidacloprid, Lambda-cihalotrina, Permetrina, and Triclorfon were all used in three crops.

The results from the national sampling of production systems during 2005, 2006, and 2007, in different vegetable producing states, showed for a cover of chili pepper, tomato, squash, and broccoli 4 of the vegetable crops identified as the most important ones- the following data: 406 samples, among which several residue analyses were done, giving off results from 665

analytical laboratory determinations. The total determination analysis proved that: i) in 23,5% of the samples no residues were found, ii) in 58,8% of the samples residues from authorized active ingredients were found, iii) in 17% of the samples residues from unauthorized active ingredients were found, for any of the 4 crops in study, iv) in 5 of the determinations, the active ingredients detected were over the established limit, and v) one of the determinations showed a prohibited active ingredient.

The authorized active ingredients that were detected registered a total 15 different molecules: Bifentrina, Clomazone, Clorpirifos, Clortal-dimetil (Dactal), Cyanofos, Diazinon, Dimetoato, Endosulfan, Etion, Lamba-cialotrina, Malation, Methamidophos, Mevinfos, and Quintozeno. The authorized active ingredients that were detected with the greatest spatial-temporal frequency were: Methamidophos, with 114 registries, representing 34% of the total; Endosulfan, with 99 registries (29,6%), and Clorpirifos, with a total 74 registries, equivalent to 22,1% of the total. The toxicological classification of these three active ingredients is: Metamidophos as an extremely toxic organofosforado, Endosulfan as a highly toxic Organochlorine, and Clorpirifos as a slightly toxic organofosforado. On the whole, it can be seen that these three active ingredients (Methamidophos, Endosulfan, and Clorpirifos) were detected in 85,7% of the determinations of authorized ingredients.

Grouping of the unauthorized active ingredients allowed to identify residues from a total of 15 different molecules, among them: Acefate, Acetoclor, Cipermetrina, Cloratonil, Clorpirifos, Dimetoato, Folpet, Iprodione, Isazofos, Monocrotofos, Omethoate, Paration metílico, Pentacloroanilina, Permetrina, and Profenofos.

The unauthorized active ingredients detected with the greatest spatial-temporal frequency were: Omethoate with 40 registries (35,4% of the total), Monocrotofos with 20 registries (17,79%), Acefate with 19 registries (16,8%), and Permetrina with 14 registries (12,4%). The toxicological classification of these 4 active ingredients is: Omethoate as a highly toxic organofosforado, Monocrotofos as an extremely toxic organofosforado, and Acefate and Permetrina both as slightly toxic, organofosforado and piretroide, respectively. On the whole, it can be seen that these four active ingredients (Omethoate, Monocrotofos, Acefate, and Permetrina) were detected in 82,3% of the total determinations of unauthorized ingredients.

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# A Review on the Mode of Action and Current Use of Petroleum Distilled Spray Oils

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## 1. Introduction

Petroleum based mineral oils have been used for insect pest control for over a century (Agnello, 2002). However, their use is as current today as it was before the advent of chemical insecticides, given that they are compatible with modern sustainable management practices. These products pose a number of advantages over conventional pesticides and they have very low mammalian toxicity, low residual activity, they have never been associated with development of insect resistance, and are less disruptive to natural enemies than broad spectrum insecticides (Beattie and Smith, 1993). Continuous studies on the efficacy and chemistry of petroleum based oils over the last sixty years, led to identification of the main factors related to their insecticidal activity as well as their phytotoxicity and allowed for the development of more refined and effective spray oils (Agnello, 2002). The efficacy of isoparaffinic petroleum distilled spray oils (PDSOs) typically increases as the molecular weight of their constituent oil molecules increases, but so does the risk of PDSO induced phytotoxicity (Riehl, 1969), which has been one of the main hindrances to the use of these products. Modern PDOs are highly refined, linear molecules with a range between 21 and 24 carbons, to combine good insecticidal efficacy with low phytotoxicity. The use of UV additives (e.g. sunscreens) to reduce the detrimental effect of the ultraviolet light on the breakdown of oil molecules has reduced the potential of some PDSOs to damage plants (Hodgkinson, 1999; Hodgkinson *et al.*, 2002). Thus, once limited to early season or dormant sprays to avoid oil injury to green plant tissue, newer narrow-range PDSOs are being reconsidered and assessed for incorporation into integrated pest management programs.

PDSOs have been found to be effective against numerous orchard pests including scales and mites (Beattie *et al.*, 1995; Beattie, 1990; Beattie and Smith, 1993), whiteflies (Larew and Locke, 1990; Liang and Liu, 2002), aphids (Najar-Rodríguez *et al.*, 2007), psylla (Zwick and Westgard, 1978; Weissling *et al.*, 1997), and fruit-feeding Lepidoptera (Davidson *et al.*, 1991; Al Dabel *et al.*, 2008). In apple orchards, the interest in PDSOs as part of integrated pest management programs has increased in the past years, particularly for the control of secondary pests (Fernandez *et al.*, 2005). This is in part due to better PDSOs formulations, but it also arises from a decline in the use of broad spectrum insecticides due to stricter regulations and to the widespread use of mating disruption (Fernandez *et al.*, 2005). Recent studies have also

suggested new uses of PDSOs against a wider range of pests, such as the European corn borer, *Ostrinia nubilalis* Hubner (Lepidoptera: Pyralidae) in maize (Mensah *et al.*, 2005a, 2005b; Al Dabel *et al.*, 2008), *Helicoverpa spp.* in cotton (Mensah *et al.*, 2002, 2001), and the obliquebanded leafroller *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) in orchards (Wins-Purdy *et al.*, 2009).

When overdosed with PDSOs, small insects die rapidly (Najar-Rodríguez *et al.*, 2007) while large insects are more tolerant and toxicity is often unpredictable (Zerba *et al.*, 2002; Mensah *et al.*, 2005b). As opposed to other synthetic insecticides, oils address multiple targets and do not bind to specific receptors since their toxic effects depend on the interaction between their physical and chemical properties and the anatomical, developmental, physiological and behavioural traits of the target insect. Smith (1952) summarized most of the theories regarding the mode of action of oils, which he categorized according to whether they were applied to eggs or motile forms. He proposed that when used as ovicides, they acted by preventing the normal exchange of gases through the outer coating, prevented hatching by hardening the outer covering, interfering with water balance of the egg, softened or dissolved the outer covering of the egg, penetrated the egg causing coagulation of the protoplasm, or interfered with enzyme or hormone activity. When used as insecticides, oils were thought to cause suffocation by blocking spiracles, to penetrate the tissue in the liquid phase and to 'corrode' it by breaking down tissue structure, and to contain toxic volatile components that act as fumigants. Although some of these hypotheses were tested, most of them were speculative and were not verified. More than fifty years have passed since Smith's (1952) review on action mechanisms of petroleum oils. During this time there have been remarkable advances on petroleum technology, as well as on scientific studies testing their efficacy and potential uses on a wide array of insect species, which have broadened the boundaries for the use of PDSOs. Therefore, we review the literature on the use and effect of PDSOs on various insect taxa and crops, and discuss the action mechanisms identified.

## 2. Mode of action and target sites of PDSOs

The modes of action of toxic chemicals are traditionally divided into two main categories: baseline toxicity or narcosis, and specific mode of action (Rand *et al.*, 1995). Narcosis can be broadly defined as a state of arrested activity of protoplasmic structures caused by a wide variety of organic chemicals with non-specific modes of toxic action (Veith *et al.*, 1983; Veith and Broderius, 1990), due to a physical action of the molecule and not to a chemical reaction (Ferguson, 1939). Narcosis is believed to be the result of reversible and non-specific disturbance of membrane integrity and function resulting from the partitioning of a given chemical into biological membranes (Escher and Hermens, 2002). Thus, the potency of a baseline toxicant is expected to correlate with its affinity to the cell membrane (Gunatilleka and Poole, 1999). On the other hand, specific toxicity refers to chemicals that interact with or disrupt the function of a defined receptor site (e.g. compounds acting as oxidative phosphorylation uncouplers, respiratory inhibitors, electrophiles, acetylcholinesterase inhibitors, and central nervous system seizure agents). Due to the non specific nature of narcosis, chemicals that meet structural requirements of specific modes of action (e.g. synthetic pesticides) should be excluded from narcosis. The non-specific nature of narcosis and chemicals that meet structural requirements of specific modes of action (e.g. synthetic pesticides) should be excluded from narcosis. However, most substances that are narcotics can be shown to be capable of producing both narcosis and toxicity, depending upon the

concentration used in the case of chemical agents and upon some measure of intensity in the case of physical agents (Mullins, 1954). Additionally, there are numerous mechanisms of narcosis as shown by the great variety of symptoms caused by (Veith and Broderius, 1990). For example, baseline toxicity can be categorized into polar and nonpolar narcosis based on the chemical structure and degree of toxicity of the xenobiotic (Russom *et al.*, 1997). Nonpolar narcosis results from hydrophobic bonding of the chemical to enzymes and/or membranes and polar narcosis may result from the presence of strong hydrogen binding group on the molecule (Veith and Broderius, 1990). However, more recent research proposes that there is no difference in membrane concentrations of the polar or non-polar chemicals, and thus equal intrinsic toxic potency is encountered for these two types (Escher and Schwarzenbach, 2002).

As opposed to traditional synthetic insecticides, oils target multiple sites and do not interact with specific receptors since their toxic effects or narcosis, depend on the interaction between their physical and chemical properties and those of the insect. Oils show affinity to the insect body surface and penetrate the insect cuticle (Stadler and Buteler, 2009), dissolve internal lipids (Taverner *et al.*, 1999) and eventually penetrate internal cell structures (Taverner *et al.*, 2001; Taverner, 2002) (Table 1).

## 2.1 Effects on insect eggs

Smith and Pearce (1948) studied the respiratory effects of oils on eggs of the oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) and found them to be responsible for decreased respiration rate, presumably through mechanical interference with normal gaseous exchange. They further concluded that the less reactive paraffinic oils showed greater ovicidal efficacy than did the more reactive unsaturated oils. A recent study by Al Dabel *et al.* (2008) showed a strong ovicidal effect of nC24 and nC27 PDSOs on *O. nubilalis* egg masses when applied at 3 - 10% (v/v). The PDSO treatments seemed to stop the embryonic development and killed the embryo in the eggs but the mechanism involved in the ovicidal action remains to be studied. Topical application of 2% Purespray Green Horticultural mineral oil (Petro-Canada) also led to almost complete egg mortality in the obliquebanded leafroller, *Choristoneura rosaceana* (Lepidoptera: Tortricidae) (Harris) through both contact toxicity and suffocation (Wins-Purdy *et al.*, 2009).

## 2.2 Effects on insect larvae and adults

### 2.2.1 Spiracle or tracheal blockage

Insect suffocation by spiracle blockage is usually held as the most accepted theory on the mode of action of mineral oils (Johnson, 1994). The tracheal inflow of oil was reported for the first time by Moore and Graham (1918), and addressed after that by several authors (Roy *et al.*, 1943; Stadler *et al.*, 1996; Taverner *et al.*, 2001). Stadler *et al.* (1996) found evidence of the inflow of PDSOs to the trachea of Lepidoptera larvae (*Anticarsia gemmatilis* Hub. (Lepidoptera: Noctuidae) by looking at the air-liquid interface inside the tracheae and tracheolar tubes. In *Blatella germanica* L. (Blattodea: Blattellidae), oils appeared to induce mortality due to asphyxia by occlusion of tracheae and tracheoles (Stadler *et al.*, 1996). No chronic damage was observed on *B. germanica* treated with sub-lethal doses of mineral or vegetable oils. Tracheal blockage by PDSOs was observed in living as well as dead insects, showing that the phenomenon is independent of insect metabolism and that it can be described by the Poiseuille equation (Tschapeck, 1961). By this equation, capillary pressure depends mainly on the viscosity of the oil, as well as on the radius of the cylinder (i.e. trachea). Taverner *et al.* (2001), observed that Ampol Citrus Postharvest Dip (CDP), an

EFFECT	SYMPTOM	PROBABLE CAUSES	AUTHOR	TAXON- STAGE
<b>Cuticle</b>				
Cuticle Softening	Mortality	Dehydration	Stadler et al. 2001	Coleoptera- adults
Disruption of cuticle waxes	Mortality	Dehydration	Ebeling 1945	Lepidoptera- larvae
Flaccid bodies, extended legs and dark cuticle	Mortality	Direct toxicity related to physical mode of action	Najar-Rodríguez et al. 2007	Hemiptera
Teratogenic effects on the epidermis and aberrant molts	Mortality	Integument damage. Corrosion	Stadler et al. 1996	Lepidoptera- larvae
<b>Respiratory system</b>				
Spiracle Blockage	Mortality	Suffocation	Davidson et al. 1991; de Ong et al. 1927; Stansly et al. 1996	Hemiptera
Trachea and tracheole blockage	Mortality	Suffocation	Stadler et al. 1996	Blattodea-nymph
Coating of tracheae	Reversible Knock down	CO <sub>2</sub> accumulation	Taverner et al. 2001	Lepidoptera- larvae
Disruption of tracheal waxes	Weight loss, Mortality	Desiccation	Taverner et al. 2002	Lepidoptera- larvae
<b>Behaviour – Receptors</b>				
Host location failures	Behavior abnormalities	Receptor coating	Simons 1982	Hemiptera
Repellence	Oviposition deterrent and reduced populations on treated leaves	Effect mediated by contact receptors	Stansly et al. 2002; Liu et al. 2001, 2002, 2006; Xue et al. 2002 a,b;	Hemiptera, Lepidoptera; Hemiptera; Tyssanoptera, Diptera
Oviposition deterrent	Reduced populations on treated leaves	Effect on host volatile compounds that mediate host location	Mensah et al. 2005	Lepidoptera
Repellence	Inhibition to attach on plants	Plant surface tearing	Trammel 1965	Hemiptera - nymph
Feeding deterrence	Starvation	Penetration and movement of oils within plant tissue	Beattie et al. 1995, Najar-Rodríguez et al. 2007	Lepidoptera - Hemiptera
Repellence	Antifeedant Starvation	Plant surface tearing	Baxendale and Johnson 1990	Lepidoptera- larvae

Table 1. Summary of effects of spray oils observed in insects, and probable causes.

EFFECT	SYMPTOM	PROBABLE CAUSES	AUTHOR	TAXON-STAGE
<b>Tissues</b>				
Accumulation in lipophilic tissue, particularly in fat bodies	In vitro death cell	Toxicity	Najar-Rodríguez et al. 2008	Hemiptera Lepidoptera larvae
Corrosive	Mortality	Histolysis	Stadler et al. 1996	Lepidoptera- larvae
<b>Nervous system</b>				
Neurotoxicity	Multiple nerve firing in peripheral nerves	Increased neuron membrane permeability to ion exchange	Richards and Weygandt, 1945; Taverner et al. 2001	Diptera, Lepidoptera- larvae
Accumulation in nerve cells	Muscular contraction, loss of coordination, death	Neurotoxicity, disrupt synaptic function and neurotransmission	Najar-Rodríguez et al. 2008	Hemiptera Lepidoptera- larvae
<b>Physiology - Metabolism</b>				
Colony growth rate	Failure to establish on plants	Feeding deterrence or toxicity due to ingestion	Najar-Rodríguez et al. 2007	Hemiptera - alates

Table 1 (Continued). Summary of effects of spray oils observed in insects, and probable causes.

emulsified C15 alkane used by Australian citrus packers to control surface pests, penetrates the tracheoles of lightbrown apple moth, *Epiphyas postvittana* Walker, (Lepidoptera: Tortricidae). Confocal microscopy showed that if larvae were dipped in oil and then exposed to the air, the oil penetrated the tracheal system, but the extent of penetration varied with the type of oil. An oil with a carbon number of 15 (CPD) was observed to penetrate deeper into the tracheal system than a narrow-range oil with a carbon number of 23 (Ampol D-C-Tron NR), presumably due to a lower interfacial tension between CPD and the tracheal lining. Figure 1 illustrates the tracheal blockage occurring when crickets (Orthoptera:Grillidae) are dipped in oil. Spiracle blockage has also been observed and documented when using other products with similar physical characteristics as insecticide oils. Richling and Böckeler (2008) observed a similar phenomenon when treating *Pediculus humanus* Haeckel, (Phthiraptera: Pediculidae) and *Acheta domestica* (Orthoptera: Gryllidae) with low viscosity silicone. When the insect was immersed in or coated with silicone, the fluid entered all spiracles equally and systematically flowed through the tracheal system and completely filled the trachea in the insect's head in less than one minute. Results from Burgess (2009) bioassays using low viscosity silicones on head lice *P. humanus* show that the most likely mode of action of these substances, when applied in great amounts, is the physical blockage of the outermost sections of the insect respiratory system. Contrary to the widespread opinion that physically acting pediculicides work by suffocation, Burgess (2009) concluded that spiracle blockage causes inhibition of water excretion and further physiological stress, which leads to death either through disruption of internal organs or due to prolonged immobilization.

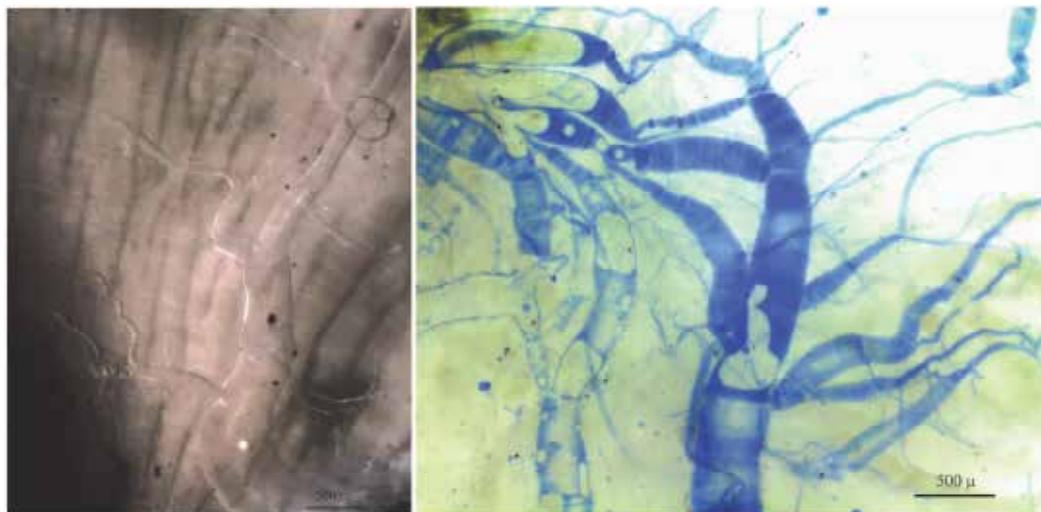


Fig. 1. Micrograph of tracheal branches from a cricket (Orthoptera:Grillidae) (untreated) [black background - left] and dipped in PDSO nC24 stained with aniline blue (lipophilic) [white background - right].

On the other hand, Najar-Rodriguez *et al.*, (2008) found that oil did not accumulate around the spiracular openings, inside the main trachea associated with the spiracles or in the small tracheoles associated with the gut and nerve ganglia in adult cotton aphids (*Aphis gossypii* Glover, (Hemiptera: Aphididae)) and cluster caterpillars (*Spodoptera litura* Fabricius (Lepidoptera: Noctuidae)). Unlike some of the other studies in which the insects were dipped in the oil, these authors applied topically only a small amount of a PDSO nC24, at a maximum concentration of 20 $\mu$ l.

Based on these results it appears that the extent of spiracle and tracheal blockage and toxicity depend on the relationship between physical properties of the oil such as its n-paraffin carbon and its viscosity, and the dimensions of insect tracheae. Regardless, this phenomenon is not as significant for insect pest control due the low affinity (wettability) of oil and the internal wall of insect trachea, which leads to a low penetration rate of the oil. Oil will pour through the spiracular valves into the atrium and flow into the trachea as long as spiracles are open and the insects are overdosed (i.e. dipped in oil) (Stadler and Buteler, 2009). In contrast, when small volumes of PDSO contact the insect body (i.e. when the insect moves through a treated substrate), the amount of oil reaching the spiracular valve will not flow into the trachea because of the low surface energy between oil and inner tracheal surface (Stadler and Buteler, 2009).

### 2.2.2 Effect on the integument

Symptoms observed on the integument after topical treatment with sub-lethal doses of oils include cell membrane disruption and darkening (Van Overbeek and Blondeau, 1954; Stadler *et al.*, 1996; Najar-Rodríguez *et al.*, 2007). Given their lipophilic nature, PDSOs accumulate in cell membranes and thus affect their structural and functional properties (Mazella *et al.* 2005). As shown by Najar-Rodríguez *et al.* (2007) *in vitro*, oils are able to penetrate the cell membranes, accumulate inside the cytoplasm and cause cell dehydration and DNA condensation inside the nucleus. Furthermore, findings of teratogenic effects to the insect epidermis and aberrant molts have been observed after topical application of oils at the site

where the oil had been applied (Stadler et al. 1996). However, the authors tested unrefined spray oils, containing residues of naftalenic and sulfonable compounds which could have been responsible for the “burn effects” on the insect integument.

Insects may lose water through respiration, excretion, secretion and transpiration through the cuticle. Data from inter-specific comparative studies reviewed by Chown (2002) showed that in 16 insect species belonging to diverse taxa (Blattodea, Orthoptera, Coleoptera and Hymenoptera), transpiration through the cuticle averaged 91.5% and constitutes the main cause of total water loss in insects. Thus, insect resistance to desiccation depends mainly on their epicuticular waterproof wax layer (Hadley, 1994). The insect cuticle is a complex passive barrier to evaporative water loss composed of a mixture of long-chain compounds which include hydrocarbons (saturated, unsaturated, branched), wax esters, free fatty acids, alcohols, ketones, aldehydes, and cyclic compounds, which may be present in very complex mixtures (Dekker et al., 2000). The quali- and quantitative arrangements of waxes on the cuticular surface are specific to each species in adaptation to its environment (Hadley, 1981) and its waterproof effectiveness is greater when they are in a solid rather than fluid state. Damage to the structural integrity of cuticular waxes or its removal by submersion in organic solvents (ether, chloroform, etc.) in insects (Hurst, 1940; Wigglesworth, 1941, 1942) and in millipedes (Cloudsley-Thompson, 1950), may lead to dehydration. Likewise, non-polar substances of low dielectric constant such as PDSOs may come into a competing equilibrium with some of the components of the cuticle wax layer, disrupting its continuity by interacting with the overall lipid mixture (Wigglesworth, 1945). This interaction leads to a decrease in the cuticle’s wax layer melting point, with either a broad melting point range or a narrow melting point at the eutectic temperature (Hägg, 1969). This in turn leads to changes in cuticle permeability and dehydration.

Whereas some non-polar hydrocarbons (e.g. ether, chloroform) cause a complete removal of the cuticle wax layer, as well as hardening and stiffening of the cuticle (Hayes and Smith, 1994; Barbakadze, 2005), mineral and vegetable oils can attain a competing equilibrium with some of the components of the insect cuticle wax layer and soften the cuticle. This was proposed by Stadler et al. (2002), who found that mineral oils caused a softening of the cuticle in adult cotton boll weevils *Anthonomus grandis* Boh. (Coleoptera: Curculionidae). The variation in cuticle hardness observed, suggests that oils induce structural changes in the cuticle. The authors also found a correlation between cuticle softening and oil toxicity in laboratory bioassays, where a greater softening was associated with increased mortality.

### 2.2.3 Effect on insect behaviour

Oils have a repellent effect that discourage egg deposition and feeding. The residual film may inhibit insects from attaching to plant surfaces (Trammel, 1965). Also, it should be noted that “arrested activity” in insects is one recurrent symptom caused by PDSOs that has been reported directly or indirectly by many authors in laboratory toxicity tests (Xie and Isman, 1995; Stadler et al., 1996; Taverner, 2001; Najjar-Rodríguez et al., 2007; Najjar-Rodríguez et al., 2008).

Antifeedant properties of oils and starvation through deterrence have also been documented (Baxendale and Johnson, 1988; Najjar-Rodríguez et al., 2007; Beattie et al., 1995). The deterrent effect of oil residues on oviposition has been observed in the citrus leafminer *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) (Beattie et al., 1995; Rae et al., 1996), codling moth (L.) (Lepidoptera: Tortricidae) (Riedl et al., 1995), white apple leafhopper (Hemiptera: Cicadellidae) (Fernandez et al., 2001), the pear psylla *Cacopsylla pyricola* (Homoptera: Psyllidae) Foerster (Zwick and Westigard, 1978; Weissling et al., 1997), and whiteflies (Larew and Locke, 1990; Larew, 1988; Liang and Liu, 2002). Studies by Mensah et al. (2001, 2002) have also shown

that application of 2% (v/v) rate of Canopy® oil (nC27) to cotton and 4-5% (v/v) Texaco® oil (nC24) to maize plants can reduce oviposition of *Helicoverpa* spp. and *O. nubilalis* on cotton and maize plants, respectively. Liu *et al.* (2001) demonstrated that efficacy of PDSOs as deterrents is also related to molecular weight increase, as reflected by *nCy* values, and, therefore, to the persistence of oil molecules on sprayed surfaces.

#### 2.2.4 Effect on the nervous system

Najar-Rodríguez *et al.* (2008) proposed that once the PDSOs penetrate the cuticle, they diffuse and accumulate within lipid-containing tissues, primarily the fat bodies. Results obtained by Schal *et al.* (2001) with *Musca domestica* (L.) (Diptera: Muscidae) suggest that lipophorin is involved in an active mechanism that selectively transports hydrocarbons from the haemolymph to individual tissues. These specific lipoproteic complexes are capable of sequestering hydrophobic core lipids from the hydrophilic environment of the haemolymph (Blacklock and Ryan, 1993) and of transporting lipids and hydrocarbons within the insect body (Schal *et al.*, 2001), which finally accumulate in lipid-containing tissues. Taverner *et al.* (2001) showed that treatment with Citrus Postharvest Dip (Ampol Research and Development Laboratories, Brisbane, Queensland), a formulated C15 alkane, affected neuron lipid membranes in *Epiphyas postvittana* Walker, (Lepidoptera: Tortricidae). Electrophysiology recordings showed that the alkane induced a rapid onset of multiple nerve firing in peripheral nerves of *E. postvittana* larvae. These authors suggested that nerve disruption was due to the displacement of protective neural lipids by solvent action of the alkane, affecting nerve activity by increasing membrane permeability to ion exchange. In another study Najar-Rodríguez *et al.* (2008) reported disruption of the synaptic transmission of nerve ganglia in *S. litura* treated with PDOs at concentrations equivalent to 0.1% v/v.

The effect of the absorption of hydrocarbons into phospholipid membranes is not clear, but is probably not site-specific, given the lack of any apparent structural complexity or stereoisometry of PDSOs (Najar-Rodríguez *et al.*, 2007; Taverner *et al.*, 2001). Thus, nervous disruption by PDSOs would not involve specific chemical binding to receptors or the active sites of enzymes, as is the case with traditional insecticides.

### 3. Discussion

Almost sixty years have gone by since the last review on the use of mineral oils for pest management. Since then over 50 scientific manuscripts and a book were published (Beattie *et al.*, 2002), as well as an international conference (Spray Oils Beyond 2000, Sydney Australia) on the subject of petroleum derived mineral oils in pest management. Oils have a long history of effective use on fruit trees, particularly in dormant sprays on fruit crops, with a good performance in those cases where the pest is small in size and restricted to a small area during its lifecycle (e.g adults and crawlers of scale insects, aphids, phytophagous mites and their eggs, and nymphs of pear psylla, nymphs of grape leafhopper and eggs of codling moth) (Davidson *et al.*, 1991; Northover and Timmer, 2002). Currently, spray oils are recommended to manage scales, *Psylla* sp., and leaf miners in some systems (Table 2), as well as mites (Agnello *et al.*, 1994; Girantet *et al.*, 1997; Nicetic *et al.*, 2001). Moreover, the recent studies reporting the effectiveness of PDSOs against other insect pests show the relevance of petroleum derived oils as a current topic in pest management (Table 2). These studies demonstrate that the mode of action of mineral oils is more complicated than it was originally thought, and that suffocation, which was the most accepted theory, may occur

Insect Species	Country	Crop	References	Level of testing or implementation
<i>Helicoverpa</i> spp. (Hubner) (Lepidoptera: Noctuidae)	Australia	Cotton <i>Gossypium</i> <i>hirsutum</i> (L.)	Mensah <i>et al.</i> , 1995, 2005b	Success in greenhouse and small field plot studies, success in field studies as a complement to other IPM tactics
Cotton aphid, <i>Aphis</i> <i>gossypii</i> Glover, (Hemiptera: Aphididae)	Australia	Cotton <i>Gossypium</i> <i>hirsutum</i> (L.)	Najar- Rodríguez <i>et</i> <i>al.</i> , 2007	Success in laboratory bioassays
Green peach aphid	Australia		Herron <i>et al.</i> , 1995	Success in Potter tower spray
<i>Ostrinia nubilalis</i> Hubner (Lepidoptera: Pyralidae)	Australia	Maize <i>Zea mais</i>	Mensah <i>et al.</i> , 2005a; Al Dabel <i>et al.</i> , 2008	Success in greenhouse studies
Obliquebanded Leafroller <i>Choristoneura</i> <i>rosaceana</i> (Harris) (Lepidoptera: Tortricidae)	Canada		Wins-Purdy <i>et</i> <i>al.</i> , 2009	Ovicide in laboratory bioassays
Citrus Leafminer, <i>Phyllocnistis citrella</i> Stainton (Lepidoptera: Phyllocnistinae)	USA	Citrus orchards	Grafton- Cardwell <i>et al.</i> , 2008	Recommended as a temporary oviposition deterrent and as an ovicide
Citrus Leafminer, <i>Phyllocnistis citrella</i> Stainton (Lepidoptera: Phyllocnistinae)	Australia	Citrus orchards	Beattie, 2004	Recommended practice for commercial orchards
White apple leafhopper <i>Typhlocyba pomaria</i> McAtee, (Hemiptera: Cicadellidae)	USA	Apple <i>Malus domestica</i> Borkhausen	Fernandez <i>et</i> <i>al.</i> , 2005, 2006	Success in field studies
Silverleaf whitefly, <i>Bemisia argentifolii</i> Bellows & Perring, (Hemiptera: Aleyrodidae)	USA	Melon <i>Cucumis melo</i> and tomatoes ( <i>Lycopersicum</i> <i>esculentum</i> Miller)	Liang and Liu, 2002; Liu and Stansly, 1995	Mortality and repellency in laboratory and or greenhouse bioassays

Table 2. Recent and most relevant attempts to use spray oils in insect pest management.

Insect Species	Country	Crop	References	Level of testing or implementation
Sweetpotato whitefly, <i>Bemisia tabaci</i> (Gennadius) (Hemiptera: Aleyrodidae)	United Kingdom	Poinsettia plants <i>Euphorbia pulcherrima</i>	Buxton and Clarke, 1994; Cuthbertson <i>et al.</i> , 2009	Success in greenhouse studies
Sweetpotato whitefly, <i>Bemisia tabaci</i> (Gennadius) (Hemiptera: Aleyrodidae)	USA	Tomato <i>Lycopersicum esculentum</i> Miller, cv. Lanai	Liu and Stansly, 1994, 2000, 2002	Success in greenhouse, field and in commercial crops.
Pear psylla <i>Cacopsylla pyricola</i> Foerster (Hemiptera: Psyllidae)	USA, Turkey	Pear <i>Pyrus communis</i> L.	Weissling <i>et al.</i> , 1997; Erler, 2004	Oviposition deterrent in laboratory and field trials
Asian citrus psylla, <i>Diaphorina citri</i> (Kuwayama) (Hemiptera: Psyllidae),	China	Calamondin trees, <i>Citrus madurensis</i>	Rae <i>et al.</i> , 1997	Success in field experiment in commercial orchards
Citrus Leafminer <i>Phyllocnistis citrella</i> (Lepidoptera: Gracillariidae)	China	Sweet orange ( <i>Citrus sinensis</i> (L.)) and pummelo ( <i>C. grandis</i> (L.))	Rae <i>et al.</i> , 2000; Chen <i>et al.</i> , 2009	Success in commercial orchards
Codling moth <i>Cydia pomonella</i> (L.) (Lepidoptera: Tortricidae) and secondary pests of pears	USA	Pear <i>Pyrus communis</i> L.	Van Buskirk <i>et al.</i> , 2002	Success in the field by reducing overall synthetic pesticide use in combination with mating disruption
Codling moth <i>Cydia pomonella</i> (L.) (Lepidoptera: Tortricidae)	USA	Apple <i>Malus domestica</i> Borkhausen	Rield <i>et al.</i> , 1995	Ovicidal activity in laboratory experiments
Codling moth <i>Cydia pomonella</i> (L.) (Lepidoptera: Tortricidae)	USA	Apple <i>Malus domestica</i> Borkhausen	Fernandez <i>et al.</i> , 2001, 2006	Unsuccessful in field trials
Scales (Hemiptera: Coccoidea)	USA, Iran, Australia	Fruit orchards	Davidson, <i>et al.</i> , 1991; Damavandian, 1993, 2003; Montazeri and Alavi, 2002; Beattie <i>et al.</i> , 2002	Recommended practice for commercial orchards

Table 2 (continued). Recent and most relevant attempts to use spray oils in insect pest management.

Insect Species	Country	Crop	References	Level of testing or implementation
Tomato thrips <i>Frankliniella schultzei</i> (Trybom)(Thysanoptera: Thripidae), greenhouse whitefly adults <i>Trialeurodes vaporariorum</i> (Westwood) (Hemiptera: Aleyrodidae), common brown leafhopper nymphs <i>Orosius orientalis</i> (Matsumura) (Hemiptera: Cicadellidae)	Australia	Tomato <i>Lycopersicum</i> <i>esculentum</i>	Kallianpur et al., 2002	Success in potter spray tower bioassays
Pine needle scale (Fitch) <i>Chionaspis pinifoliae</i> (Hemiptera: Diaspididae)	USA	Scots pine ( <i>Pinus sylvestris</i> )	Nielsen, 1990; Fondren and McCullough, 2005	Success in field trials
Euonymus scale <i>Unaspis</i> <i>euonymi</i> (Hemiptera: Diaprididae)	USA	Japanese pachysandra ( <i>Pachysandra</i> <i>terminalis</i> )	Sadof and Sclar, 2000	Success in field trials
Woolly apple aphid <i>Eriosoma lanigerum</i> (Hausmann) (Hemiptera: Aphididae)	USA	Apple <i>Malus domestica</i> Borkhausen	Fernandez et al., 2005	Success in field trials

Table 2 (continued). Recent and most relevant attempts to use spray oils in insect pest management.

only in particular cases of overdosing (Table 1). The paradigms describing the mechanism of action of PDSOs have been developed from the works of Smith and Pearce (1948), Van Overbeek and Blondeau (1954), Stadler *et al.* (2002), Taverner *et al.* (2001), Najar-Rodríguez *et al.* (2008) and Stadler and Buteler (2009), who provided evidence and description of the multiple target sites involved in PDSOs toxicity. These include the integument, nervous system, respiratory system and insect behavior.

As opposed to other synthetic insecticides, oils address multiple targets and their effects on the cuticle waxes, cuticle softening, epidermal teratogenicity, tracheal blockage, receptor coating, deterrence and neurotoxicity of spray-oils, are all concurrent phenomena once the oil contacts the insect body surface. The sum of these phenomena plays a leading role in the lethal effect of the oil. Hence, all of these factors can influence the kinetics of a compound and each one occurs with a different intensity on the action sites, depending on the insect species, its developmental stage, the oil type and dose.

It can be concluded that isoparaffinic spray-oils do not interact with specific receptors, showing a non polar narcosis in insects (non-specific mode of action), characterized by progressive lethargy and death without any specific sustained symptoms. Other insecticide oils, containing aromatic residues, would shift the syndrome to polar narcosis. PDSOs are

capable of coating the exterior as well as reaching the interior of the insect, targeting different structures and organs, depending on the oils physical and chemical properties and the insect species and physiology. The effects of PDSOs are variable as well, depending on the site reached, the dose and the affinity between the oil and the target site. Therefore, it is extremely difficult to determine the exact cause of insect death. Future research should explore structure-toxicity relationships for each oil type, and standardize assessment methodology and experimental design. Ideally future studies will provide comparable results across insect taxa and oil types providing a guide of recommended practices for PDSOs use against different pests that would also direct further observations and experimentation.

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# Design and Experiments on Droplet Charging Device for High-Range Electrostatic Sprayer

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## 1. Introduction

It presses a solution to apply high-range spray technique for controlling of forest diseases and insect pests. In recent years, there are more and more tall trees in China, as forest covering area becomes wider and wider, including defendable forest, shelter trees in urban areas, fast growth forest, field net forest and shelter trees on highways and etc. To improve the effective spraying range, decrease the droplets drift and enhance the penetration and deposition of droplets, it should be considered such features of sprayer as spraying height, water saving, low pollution and high efficiency. Nowadays, the application of high-range spraying technique mostly is high-pressure shoot gun and big-type spraying vehicle, but the spraying height of these sprayer can not reach 18m.

Electrostatic spray technique has been introduced into agricultural pesticide applications for many years. Law and Cooper (1988) investigated depositions of charged and uncharged droplets from orchard air carrier sprayer. They found the deposition charged droplets could increase from 1.5 to 2.4-fold over uncharged droplets from the same air-atomizing, inducting-charging nozzles. Combining Electrostatic spray technique with high-range spray technique, a trailer-mounted ULV sprayer was re-equipped with an electrostatic device (Zhu, 1990). A knapsack electrostatic sprayer was developed with a centrifugal fan driven by a gasoline engine. A rotary-cage electrostatic spraying nozzle was driven by air-stream with contact charging electrode (Zheng and Xian, 1990). A power-carried axial air-assisted electrostatic sprayer for locust control (Xian and Zheng et al, 1992) was developed. With the experiments in a wind tunnel, Almekinders et al (1993) indicated that charged sprays with additional air assistance could provide significantly improved deposition efficiency on targets for small charged droplets. The indoor and outdoor experiments of pest controls (Gao et al., 1994) verified that the electrostatic spray could improve deposition efficiency and coverage uniformity, accelerate the droplet settling speed, reduce the drift loss, and lower the pesticide application rate. A tractor mounted automatic target detecting air-assisted electrostatic orchard sprayer with low spray volume (He et al, 2003) has been developed.

The objectives of this research were to measure the droplet size, the spray breadth and the deposition characteristics with an invented air-assisted electrostatic sprayer so as to meet the forest pest control for tall trees, reduce the application expenses and improve the ecological environment.

## 2. Equipment and methods

### 2.1 Air-assisted electrostatic sprayer

Based on the theoretical analysis and practical experiments and the requirements for the forest pest control of tall trees, an axial-flow, air-assisted, ultra-low-volume, electrostatic sprayer was developed (China Patent No ZL03259151.9) as show in figure 1. Because adopting separating design with the sprayer from vehicle, the sprayer can be trailed by four-wheel or other type wheel tractor. A diesel engine mounted on the vehicle produced the power supply to drive axial-flow blower, pump, swing mechanism and generator (Figure 2). The featured parameters of the sprayer (not including vehicle) are listed as follows: configuration dimension of 1900x1400x1100 mm, net weight of 400 kg, and pesticide liquid tank of 400 L. an axial-flow blower (Model of Z50) with the outlet of 500 mm in diameter, 2920 rpm rotation speed and 25m/s maximum air flow rate, spraying height of 20 to 25 m. spraying breadth of 38 to 50m, flow rate of 40 to 460L/h. Droplet size could be regulated by adjusting liquid pressure, from 50 to 150 $\mu$ m. Bellows can swing at range of -15°to 85°in vertical plane.

An electrostatic generator was used to deliver voltage of 24-36V into a high-voltage generator with output voltage of 20 kV in common use and 30 kV in maximum. Both automatic control system and remote control system were used on the sprayer, so only operator was required.



Fig. 1. Electrostatic spray assembly.

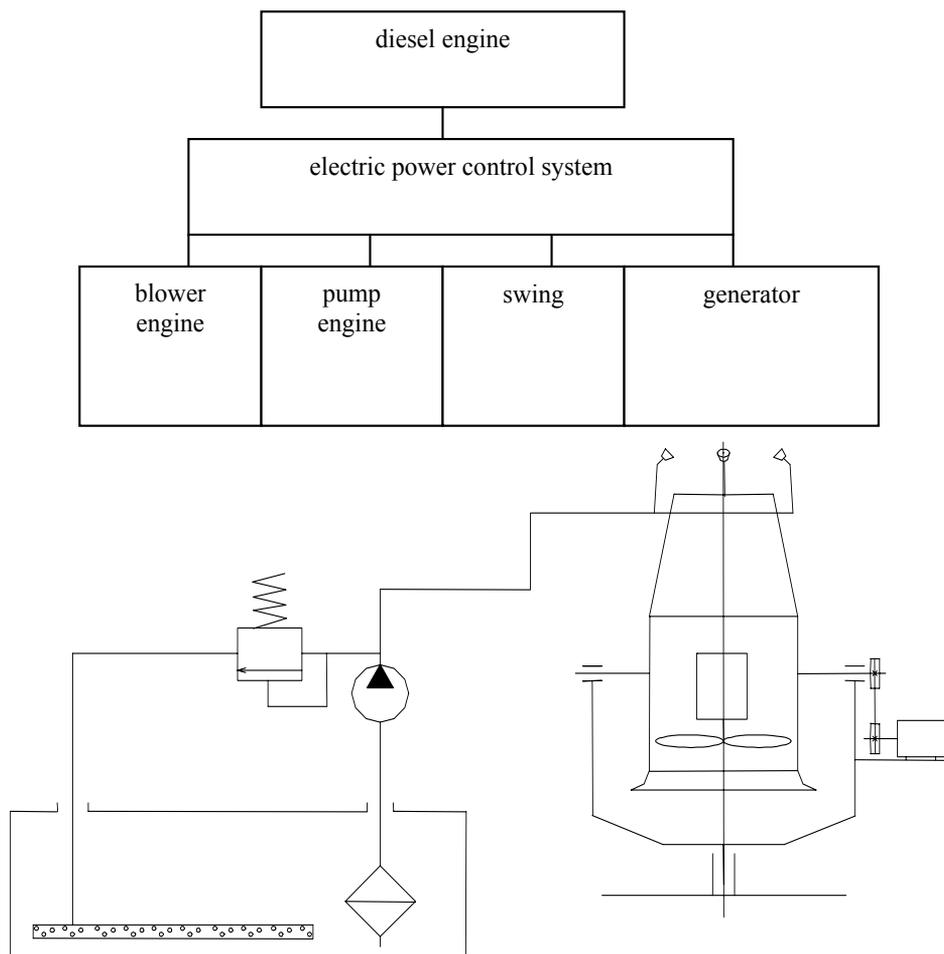
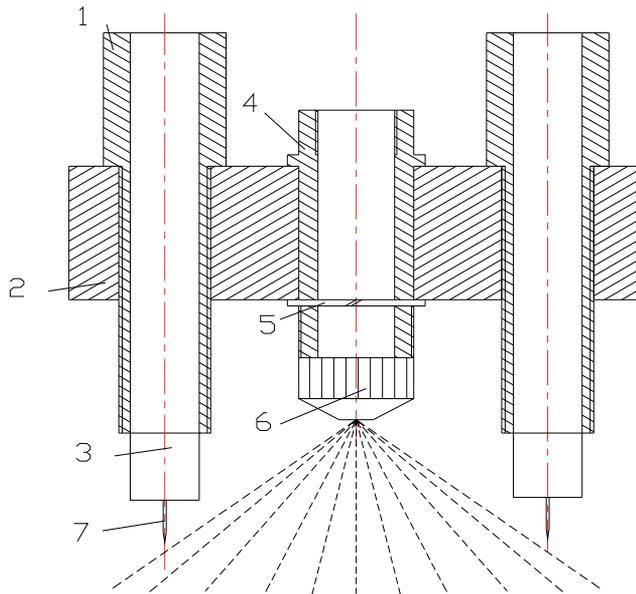


Fig. 2. Power distribution system for sprayer.

## 2.2 Electrostatic charging device

The charging device was designed using corona charging methods, supplying 20kV high voltage by double acicular electrostatic device (figure 3). Double acicular stainless steel electrodes were mounted on both sides around atomizing area for each nozzle. It charges droplets in the atomizing area, without intervening the droplet transporting and sub-atomization through the air-assisted system. High-voltage wires pass through the fixture and connect with stainless steel electrodes. The fixture is connected with support by screw thread in order to adjust the location between the electrode and the atomizing area. Nozzle passes through support and connects with spraying conduit. For desired charging effect, both fixture and support are made of nylon. The material for nozzles is copper as for better connection and not easy leak. There were six sets of electrostatic charging devices which were mounted around the 6HW-50 air-assisted electrostatic sprayer (Figure 4).



1. fixture, 2. Support, 3. high-voltage wire, 4. Conduit, 5. Gasket, 6. nozzle, 7. electrode

Fig. 3. Electrostatic Equipment Structure



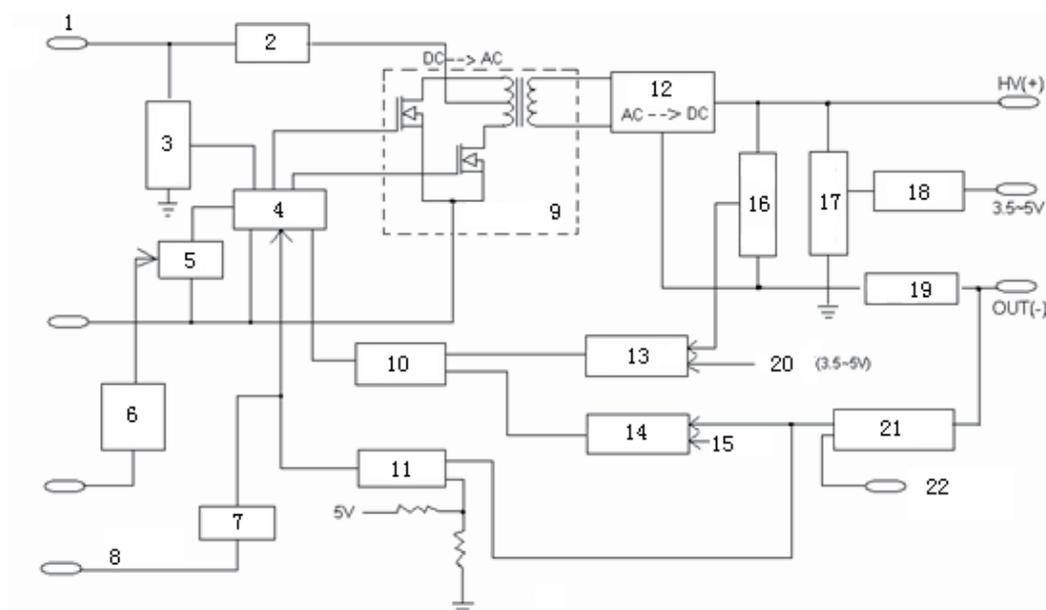
Fig. 4. Fixing of electrodes for sprayer

### 2.3 High-voltage generator

High-voltage electrode mounted on the nozzle produces high voltage corona which makes droplets charged. In order to ensure the formation of corona field, the high-voltage generator should be supplied with limited voltage and steady current. The highest voltage can be set by a regulating knob. Under steady power supply, stabilized current will decide current density of corona field. Voltage amplitude can be adjusted according to conditions of corona field. In this manner, output current keeps steady and charging equipment can work normally under wider variation range of corona field.

The nonlinear DC high-voltage power supply technique was introduced in the high-voltage power supply equipment, i.e. the typical DC high-voltage switch power supply technique in DC-DC mode (figure 5). The MOSFET, IGBT, quasi-resonant frequency conversion technique (the frequency of power supply can be 2500Hz) and the silicon voltage multiplier technique were introduced. Compared with the linear power supply, it has outstanding characteristics of high efficiency, small volume, low weight, fast reaction, low power storage and short period of designing and manufacturing.

The  $\pm 10 \sim \pm 30 \text{ kV} / 5 \text{ mA}$  DC high-voltage power was generated by the high voltage generator, the regulation and display systems were combined fixed in the same control box. The regulation switch · shunt supply insurance · trimmer pot · fault display LED · two  $\pm \text{HV}$  digital display and the power supply of the digital display were included in the control box. The control box has the significant advantage of small volume (only  $15 \text{ cm} \times 10 \text{ cm} \times 10 \text{ cm}$ ), low weight (only 250g), convenient moved and installed for monitoring and manual adjusting by driver.



1. power, 2. LC decoupling, 3. auxiliary power, 4. PWM, 5. slow starting, 6. starting control, 7. monostable circuit, 8. fault signal, 9. power converter, 10. control conversion, 11. short circuit protection, 12. double voltage rectification, 13. voltage comparator, 14. current comparator, 15. user setting, 16. voltage feedback network, 17. voltage divider, 18. voltage follower, 19. current sampling, 20. user setting, 21. Amplifier, 22. display the output current

Fig. 5. Work Principle of High-voltage Generator

Between August 10-12, 2009, a long trade tree of a fast growing poplar in Jiangsu Province were sprayed by high range sprayer with electrostatic spraying system to control *micromelalopha troglodyte*, a length of 10Km had been sprayed (figure 6). 10 kg raw pesticide was mixed with 200kg water for spraying. The vehicle speed was 8 km / h, each side lasted 1 hours. Totally 1000L working liquids had been carried out. Before the spraying, the mixed pesticide was added an appropriate amount of liquid fluorescent sodium till the light-green

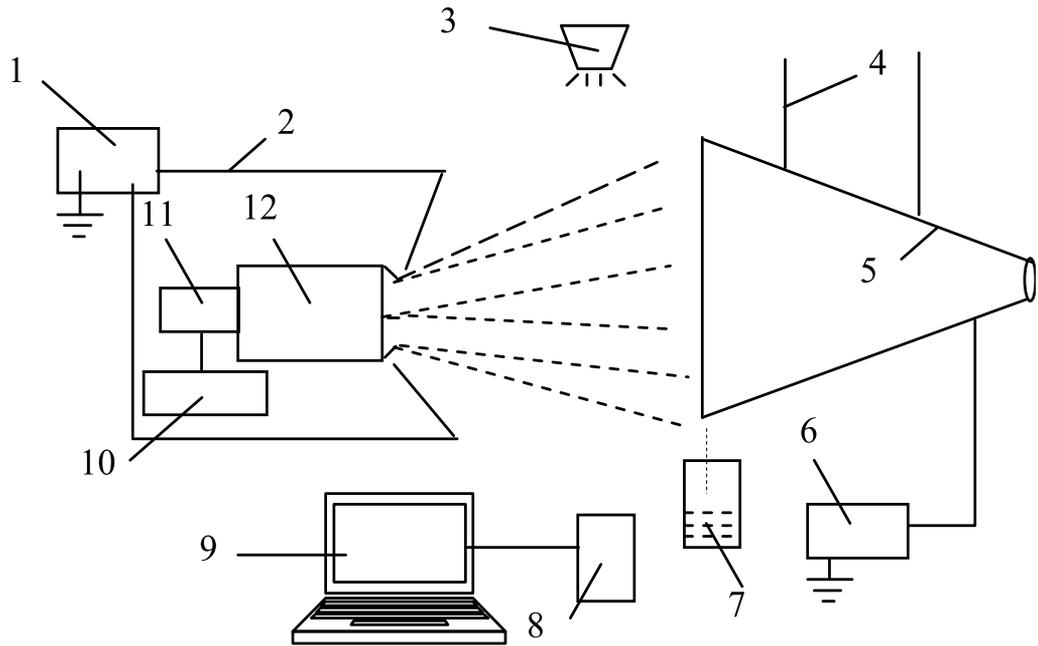
color appeared. After the forestry targets receive the color droplets, the light-green color was useful for observing and stating droplet deposition distribution and deposition density. After three days, the pest mortality or the viability of the pre-arranged standard plants (gauze was setup for observation) was investigated till the seventh day.



Fig. 6. Worksite of sprayer

## 2.4 Experiments

The experiment was performed in a void workshop with the area of 150m×80m. As measured, the ambient wind speed was 0.8m/s, the atmosphere temperature was 28°C, relative humidity was 60% and the wind speed at the outlet of the sprayer was 25m/s. An electrostatic spray experimental system was built for atomization tests, charge-mass ratio measurements and droplets deposit investigations. A charge-mass ratio measurement device was designed and applied to measure the charge attenuation of charged droplets along the spraying swath. A laser granularity-measuring system was used to evaluate distribution of droplets granularity on sectional planes along the spray jet (figure 7). Three stainless steel balls, mounted on metal pole for simulating tree, were well-distributed on metal pole from spray boundary to spray center from anywhere within the distance of 25m apart from sprayer, (figure 8). Away from 25m, stainless steel balls were distributed at the space out 0.8m on metal pole. To collect droplets, a number of white papers were attached to the balls. Red dye was filled in reagent for test so that red droplets can be seen distinctly on the paper. In practical operation, the spray vehicle moved at speed of 10km/h and collecting set was fixed on a wheelbarrow pushed through spray area at equal speed.



1. high-voltage generator, 2. high-voltage wire, 3. Emitter, 4. insulative hang frame, 5. cone-shaped cage, 6. Amperemeter, 7. calculation bucket, 8. Sink, 9. Computer, 10. liquid trunk, 11. Pump, 12. sprayer

Fig. 7. Electrostatic spray experimental system

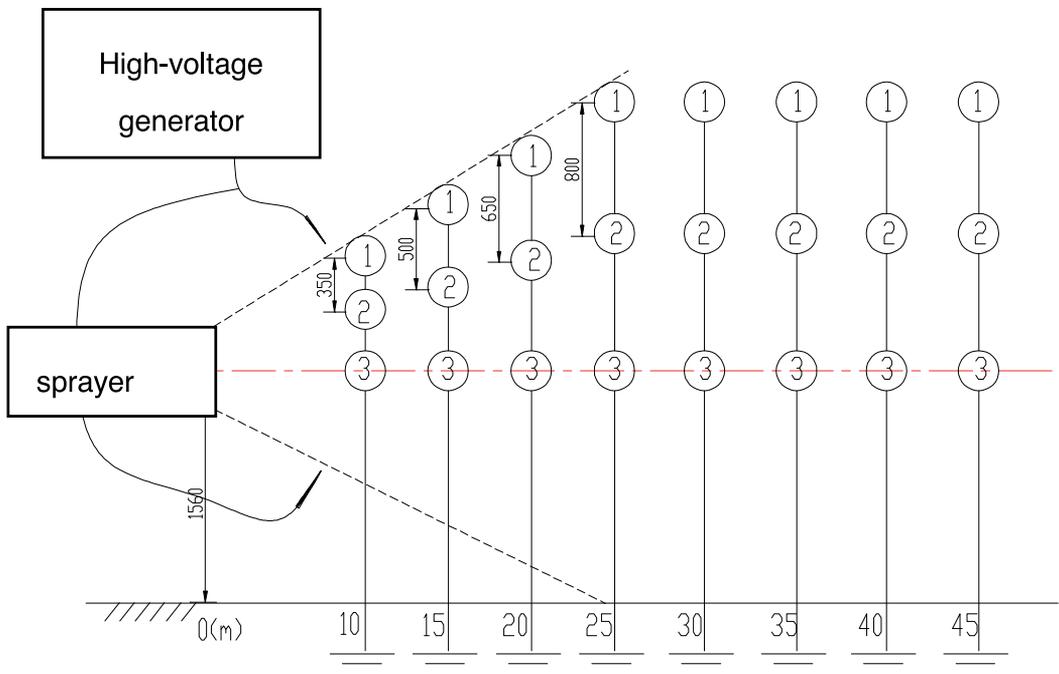


Fig. 8. Electrostatic spray simulation test system

### 3. Results and discussion

#### 3.1 Droplet size measurement

Droplet size can be measured in different positions and heights with different spraying condition and different charging voltages. The chart in figure 8 shows droplet size is measured in different charging voltages (15kV and 20kV) at the same positions (15m apart from sprayer) and in the same spraying condition. VMD is 108.4 $\mu\text{m}$  for non-electrostatic spraying, while it is 96.7 $\mu\text{m}$  at 15kV charging voltage and 80.6 $\mu\text{m}$  at 20kV charging voltage for electrostatic spraying. Comparing a with b in figure 9, curve of b was extend to small size direction, which showed more small size droplets were distinctly generated. So electrostatic spraying can make the droplets atomization better. Comparing b with c, curve of c was offset to small size direction, droplet were atomized deeply. Curve of c was steeper than curve of b which shows that droplet size was more uniformity when charging voltages increased.

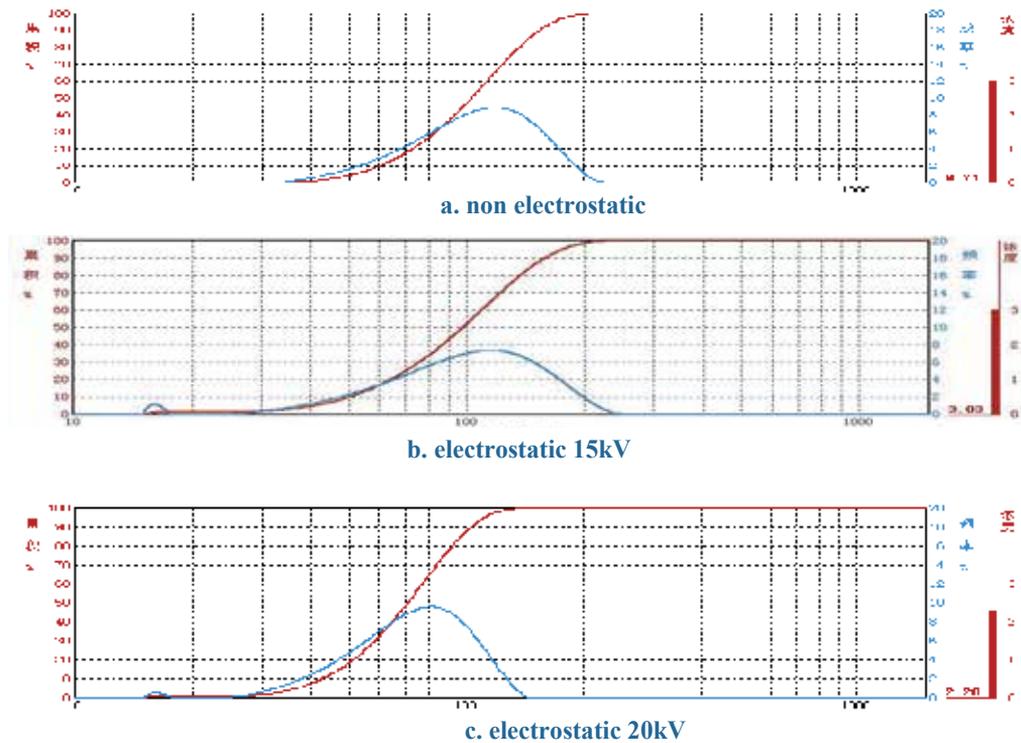


Fig. 9. Droplets granularity chart

#### 3.2 Charge-to-mass ratio test

One of the most important indexes to evaluate the effectiveness of electrostatic spraying is the charge-to-mass ratio. Generally speaking, the higher the charge-to-mass ratio, the more effective the electrostatic spraying should be. The test facility involves a bucket for collecting droplets, a micro-amperemeter for measuring the electric current and an insulated frame.

The charge could be determined by measuring the current with the micro-amperemeter and the mass rate was determined by collecting spray liquid for a specified time. The charge-to-mass ratio  $q_{cm}$  was calculated by dividing the current by the mass rate.

Charge-to-mass ratio was measured in three different position (10m, 20m, 30m) and different voltages (15kV to 25kV) with a certain spraying parameters. The figure.10 showed that the  $q_{cm}$  rises as the charging voltage increases and the droplet diameter decreases, and the  $q_{cm}$  goes down in a lower descent rate as the distance increases. This means that the charged droplet still carry charge at distant spots.

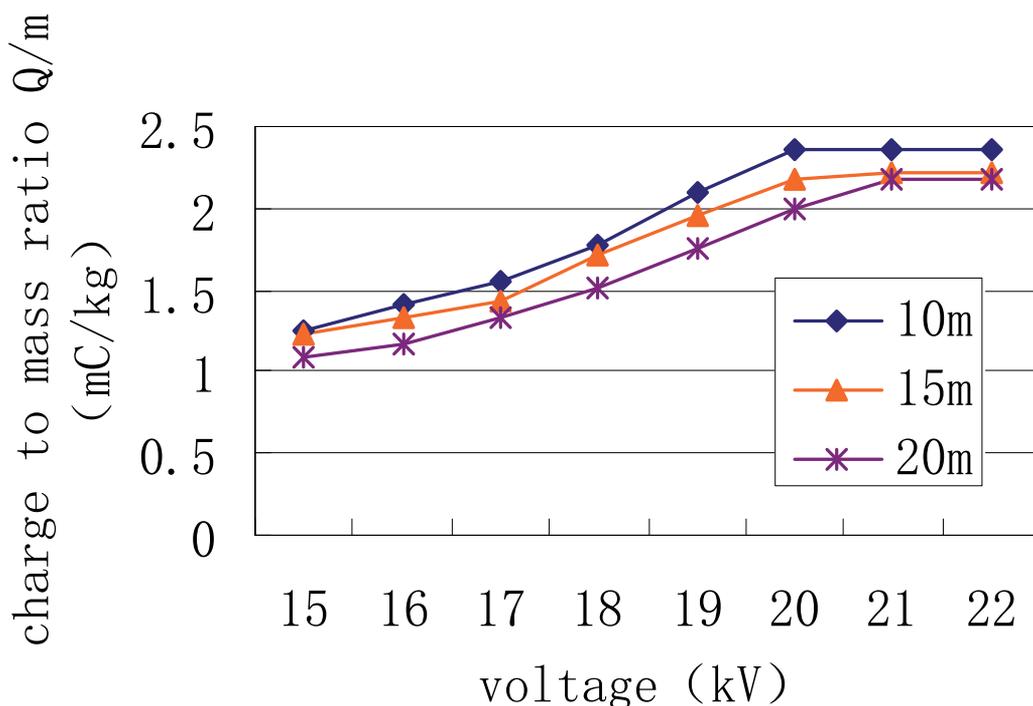


Fig. 10. Relation between charging voltage and charge-to-mass ratio

### 3.3 Droplet coverage rate

After collecting droplets, droplets volumes were estimated by a square window with area of  $1 \times 1 \text{ cm}^2$ . The test results were listed in table 1. As just as figure 7, number 1, number 2, number 3 display position of collecting ball. Table 1 shows test result for two mode of spraying such as non electrostatic spraying and electrostatic spraying. Because paper on front of collecting ball had been fully dyed into red within 15m, coverage rate had not been counted. Droplets volumes on the bottom collecting ball were more than others at the same location. Compared with non-electrostatic spraying, more droplets from electrostatic spraying deposited on the targets as far as 45m and fewer droplets drifted away. The electrostatic spraying can make coverage rate of target front increase 21 droplets per  $\text{cm}^2$ . Therefore, it can obviously decrease the probability of pesticide poisoning in other surface and the wastage of pesticide.

Mode of spraying	Ball number	Coverage ratio(droplets/ cm <sup>2</sup> )							
		10m	15m	20m	25m	30m	35m	40m	45m
Non electrostatic	1 front*			124	109	76	65	22	16
	2 front			177	128	87	71	41	19
	3 front			193	132	93	77	54	23
Electrostatic	1 front			158	136	102	81	63	34
	2 front			194	148	110	92	74	37
	3 front			208	165	115	97	79	41
Electrostatic	1 back*	72	67	58	49	39	31	19	7
	2 back	87	61	52	43	43	28	13	9
	3 back	90	76	72	52	47	35	17	11

\*front is the side face to sprayer on collecting ball, back is the side far away sprayer on collecting ball. Blank shows paper on front of collecting ball had been fully dyed into red within 15m, coverage rate had not been counted.

Table 1. Comparison with coverage rate in deferent conditions

### 3.4 Spraying breadth

The spraying breadth experimental results showed that electrostatic spraying could increase spraying breadth and averagely increase spraying breadth to 0.84m (Table 2). The reason is that charged droplets will repel each other exerted by the like charges.

Mode of spraying	Spray breadth(m)							
	2m	4m	6m	8m	10m	15m	20m	25m
Non electrostatic	1.04	1.53	1.92	2.32	2.77	4.26	6.15	7.65
Electrostatic	1.82	2.41	2.87	3.03	3.54	5.09	7.04	8.56

Table 2. Comparison with spray breadth in different conditions

### 3.5 Field test result

After field test, some result were received. with the electrostatic spraying, the average cumulative mortality of pest was 95.4% and with the non-electrostatic spraying, the average cumulative mortality of pest was 74.8%. The highest mortality of pest appeared on the fourth and the fifth day after spraying and the total number of dead pest reached up to 75% of total deaths. There was a sharp decline in deaths after the seventh day. It was shown in the tests that with the electrostatic spraying, the mortality of pest was significantly higher than that of the non-electrostatic spraying. It was positively correlated with the fact that the droplet deposition effect with electrostatic spraying was obviously better than that of non-electrostatic spraying. The reason was that with the electrostatic spraying, the droplet deposition density was larger, the distribution was more uniform and the larval had more chance to contact pesticide, so higher mortality; on the contrary, the droplet deposition density was smaller, less even distribution and the larvae had less chance to contact pesticide, so lower mortality. with the high-range electrostatic spraying for controlling *Micromelalopha troglodyte*, to achieve the desired effect, the dosage of raw pesticide was only 300g/mu. If controlling *Micromelalopha troglodyte* for 20,000ha, fund can be saved \$90,000, wood loss can be reduced 30,000m<sup>3</sup>, economical loss can be retrieved \$6,600,000, utilization ratio of patricide can be improved 60%.

It was shown that the effect of high-range electrostatic spraying was superior to conventional aerial spraying. It was beneficial for reducing spraying drift losses, improving the density of droplet deposition. Therefore, it had the outstanding advantages of high spraying efficiency and low spraying cost.

#### 4. Conclusions

Nanjing forestry University has started the research work on basic theory, testing, measurement and practical applications in electrostatic spray since 1990s. The test research result shows superiority for electrostatic spraying. The electrostatic spraying can make the front target coverage rate increase 21 droplets / cm<sup>2</sup>. Droplet can be found on front and back of target at far as 45m. When charging voltage is 20kV, electrostatic spray can averagely increase the spray breadth for 0.84m, improve the droplet distributing uniformity with the average volume medium diameter (VMD) of 80.8 $\mu$ m and obtain the maximum charge-to-mass ratio of 2.35mC/kg. The electrostatic spray could produce uniform and fine droplets with better droplet adhesion and spread, higher deposit efficiency, lower environmental contamination, lower application rate, less application expenses and longer residual action than conventional sprays.

On the bases of the experimental results and practical production examinations in the laboratory and field, it showed that combining high-range spray technique with electrostatic spray technique, the invented air-assisted high-range electrostatic sprayer was provided with scientific design, rational structure, convenient operation, high productivity and high efficiency. There was not droplet backward phenomenon during the electrostatic spray, and thus the possibility, of pesticide contamination by the sprayer was greatly reduced.

#### 5. Acknowledgements

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# Reducing Spray Drift

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## 1. Introduction

Spray drift can be defined as unwanted physical movement of spray droplets into non-target areas by air movements while application and after application (R. Frank, 1988). It can be divided into two main categories by type of occurrence.

### 1.1 Vapour drift

This kind of spray drift is occurring by non target movement of vaporize pesticide related to its evaporation and volatility characteristics . As it can be clearly seen, this kind of spray drift is much more depended to pesticide characteristics than used spraying techniques (Robert E. Wolf, 2000). Evaporation can happen during application or after application. But volatility caused drift can only happen after that pesticide dried on plant surface (R. Frank, 1988).

### 1.2 Particle drift

Spray droplets move on air from nozzle to target surface when they have pulverized. During this movement droplets are very open to environmental effects. Air movements and wind can cause to spray drift. This kind of spray drift is happening during application. (Robert E. Wolf, 2000).

## 2. Drift dynamics

Beside the wind, droplet size is another important factor which is increasing drift. There is an inverse proportion between droplet size and drift. Decreasing of droplet size will increase drift risk.

Generally micrometer is used for measurement of spray droplets. Studies showed droplets under 150 micrometer diameter cause a significant spray drift risk (Vern Hofman and Elton Solseng, 2001). Also droplets under 50 micrometer diameter vaporize before reaching to target surface (Ergin Dursun et al.,2005).

Decreasing of droplet size will also be decreased mass of droplet and because of these, droplets will travel on air longer time. Longer time of travel will increase risk of spray drift (R. Frank, 1976; Vern Hofman and Elton Solseng, 2001).

An equation to define the effect of evaporation on the change of the mass of the drop was described by (Pruppacher and Klett,1997) where:

$$\frac{dm}{dt} = \frac{4\pi r M_w D_{wa} f_w}{R} \left( \frac{p_{sat}(T_w)}{T_w} - \frac{f p_{sat}(T_a)}{T_a} \right)$$

**m** = mass of the drop (kg)

**t** = time(s)

**r**= radius (m)

**M<sub>w</sub>** = molecular mass water (kg.mol<sup>-1</sup>); value 18.015x10<sup>-3</sup>kg)

**D<sub>wa</sub>**= diffusivity of water vapur in air (m<sup>2</sup>s<sup>-1</sup>)

**f<sub>w</sub>**= mean ventilation coefficient for water vapour. This coefficient gives the ratio between the water vapur mass flux for a moving drop and the water vapur mass flux for a non-moving drop.

**R**= gas constant (8.314510 J mol<sup>-1</sup>K<sup>-1</sup>)

**p<sub>sat</sub>** = saturation pressure water (Nm<sup>-2</sup>)

**T<sub>w</sub>**= temperature water drop (K)

**f**= relative humidity divide

Smaller droplets will be on air, longer than bigger droplets and this will make smaller droplets weaker to spray drift. Droplet size's effect on travel time on the air is showed at Table 1.

Diameter(microns)	Time to fall in 3 meters
1 (fog)	28 hours
10 (fog)	17 minutes
100 (mist)	11 seconds
200 (fine spray)	4 seconds
400 (coarse spray)	2 seconds
1000 (coarse spray)	1 second

Table 1. Droplet size classification(Ross and Lembi, 1985)

Studies showed potential risk of spray drift is significantly decrease when the spray droplet is bigger than 150 and 200 micron.

Smaller droplets have bigger surface area when compared with bigger droplets. This is result of lossing mass. Less mass and bigger surface area caused to more viscosity and this situation will increase the time of reaching to target. So droplets can move off target easily. Table 2 showing effects of wind on different size of spray droplets.

As we mentioned before droplets which have 50 and lower micron diameter can not be controlled for drift. Although usually applications don't need very small droplets, we need small droplets at insecticide and fungicide applications for having better penetration into plant canopy and for better coverage. Beside these kind of advantages of smaller droplets, they will be very open to drift risk.

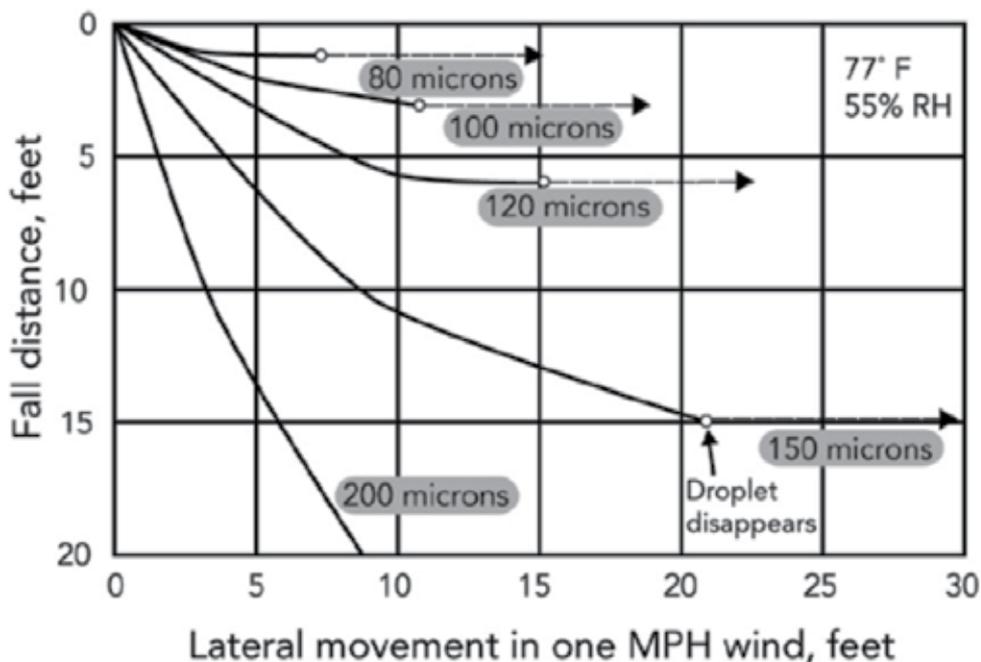


Table 2. Lateral movement of water droplets (Hofman et al., 1986)

### 3. Drift factors

Spray drift is being affected by environmental conditions and application features. Droplet size, wind speed, climatically conditions, application tools and methods are important factors to determine amount of droplets which can be sent to target surface (Robert E. Wolf, 2000). Adjustment of these factors will increase the efficiency of spraying and this also mean drift will be reduced. General reasons of spray drift can be listed like below :

- Characteristic features of pesticide solution (like viscosity and evaporation characteristics)
- Weather conditions (wind speed, wind directions, air stability)
- Droplet size
- Travel speed
- Nozzle type
- Boom height
- Spray pressure
- Nozzle spacing
- Attention and talent of operator

#### 3.1 Nozzle type

Spray droplets are produced from nozzles in different ways. **A flat-fan nozzle;** forces the liquid under pressure through an elliptical orifice, and the liquid spreads out into a thin sheet that breaks up into different-sized droplets. **A flood nozzle;** deflects a liquid stream off a plate that causes droplets to form. **A whirl-chamber;** nozzle swirls the liquid out of an orifice with a circular motion and aids the droplet formation with a spinning force.

Different types of nozzles have effect on drift because of their different orifice size and outputs. Nozzles must be choose professionally for different applications. Choosing right nozzle for right application is first step of reducing drift.

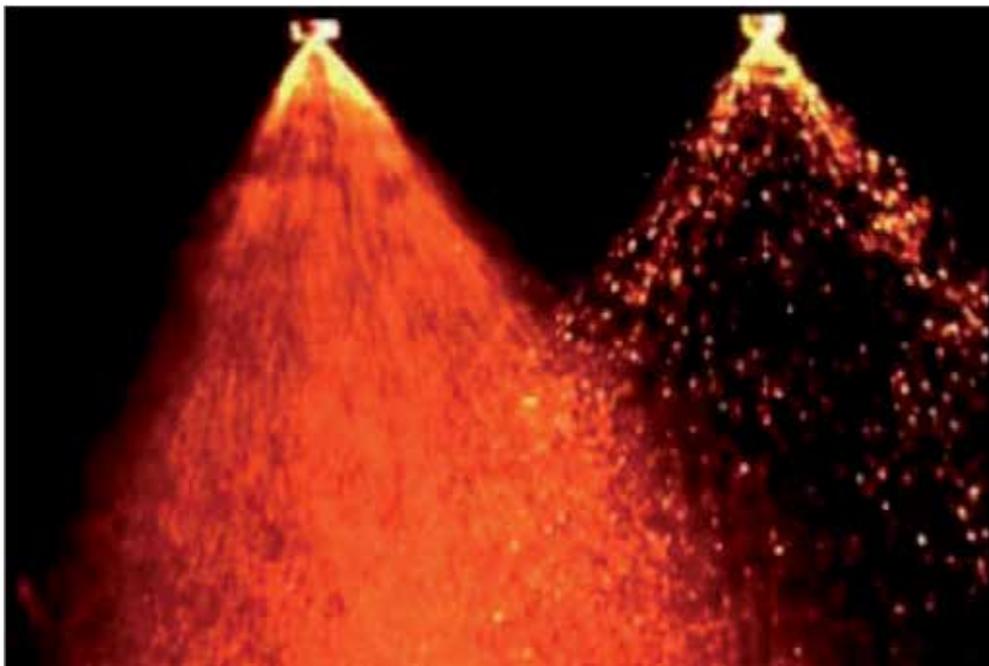


Fig. 1. Effects of nozzle type on the droplet size  
(<http://www.yorktonaircraft.com/documents/drift.pdf>, 2005)

### 3.2 Driving speed

Spraying usually performed by tractor mounted sprayers. Travel speed are usually between 8 and 10 km/h and trying to keep certain height. Faster travel speed have effect on drift occurrence. It will increase pressure of air on spraying nozzle and this pressure will cause finer droplets (T. Wolf, 1997).

### 3.3 Spray pressure

Spray pressure influences the size of droplets formed from the spray solution. The spray solution emerges from the nozzle in a sheet, and droplets form at the edge of the sheet. Increased nozzle pressure causes the sheet to be thinner, and this thinner sheet will break into smaller droplets than from a sheet produced at lower pressure. Also, larger orifice nozzles with high delivery rates produce a thicker sheet of spray solution and larger droplets than smaller nozzles.

### 3.4 Boom height

There is a direct relationship between drift and boom height. Boom height must be at optimum level related to nozzle characteristics for decreasing drift that is caused by wind. Higher boom height makes droplets very open to spray drift risk (Arvidsson, 1997; Miller, 1999).

### 3.5 Nozzle spacing

Nozzle spacing have a direct effect on spraying pattern with boom height. Spray angle is the angle formed between the edges of the spray pattern from a single nozzle (Fig.4). Nozzles with wider spray angles will produce a thinner sheet of spray solution, and smaller spray droplets than a nozzle with the same delivery rate but narrower spray angle. However, wide angle nozzles are placed closer to the target for proper overlap than narrow angle nozzles and the benefits of lower nozzle placement offsets the disadvantage of slightly smaller droplets for drift reduction.

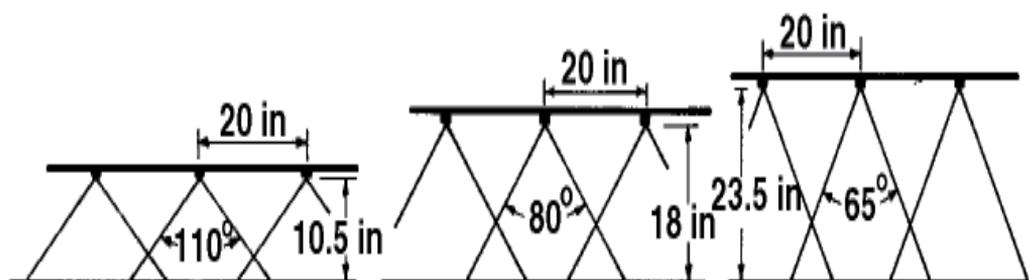


Fig. 2. Influence of nozzle spray angle on nozzle height for proper overlap to give uniform spray distribution.

The angle of nozzles relative to direction of travel can influence drift from aerial application. Because of greater wind shearing when nozzles are pointed into the wind, nozzles pointed toward the direction of travel will produce smaller droplets than nozzles pointed back. The smallest droplets are produced from nozzles 45 degrees forward of vertical, while the largest droplets are produced by a straight-back (90 degree) orientation. Droplet size becomes progressively larger as the nozzle is rotated back from 45 degrees forward to the straight-back position.

### 3.6 Weather conditions

Air condition have an critical effect on spray drift. Microclimatic features of spraying field and much more aerial factors can caused drift. These factors;

- Wind speed and direction
- Relative humidity and temperature
- Air stability

Factors that are mentioned above have especially important effect on drift of droplets under 150 microns. Elimination of fine droplets will decrease whether conditions effect on drift mechanism.

#### 3.6.1 Wind speed

Wind speed is most important meteorological factor on spray drift and it have a direct effect on drift. Increasing of wind speed will increase drift, proportionally. Drift will be uncontrollable at over 10-12 km/h wind speed and spraying must be finished over that wind speeds (R. Frank, 1988).

Wind speed will show some variability at different periods of day. Because of that spraying must be done at stable air conditions. At the other hand so lower wind speeds or no wind conditions can cause a spray drift too. As it can be understand from that, spraying must be

done at constant wind speed and direction conditions. Early time of morning and night will be proper choice for spraying (T. Wolf, 1997).

Also nozzle pressures must be adjust into values which are well-matched with wind speed. There is an inverse proportion between nozzle pressure and wind speed. When the wind speed increase, nozzle pressures must be decreased related to producers catalogs. When wind speed reaches 3 m/second, nozzle pressure must be decreased and beside that orifice of nozzle must be greater too. With these adjustments drift risk will be reduced. Wind measurements must be taken continuously during spraying application and needed adjustment must be performed immediately.

### 3.6.2 Wind direction

Wind direction must be considered as much as wind speed. Especially if there is sensitive plants around the spraying field, drift must be taken under control. As a result of that wind direction must be monitored continuously and there must be a minimum 30 meter buffer space. This buffer zone can be sprayed when the wind direction has changed.

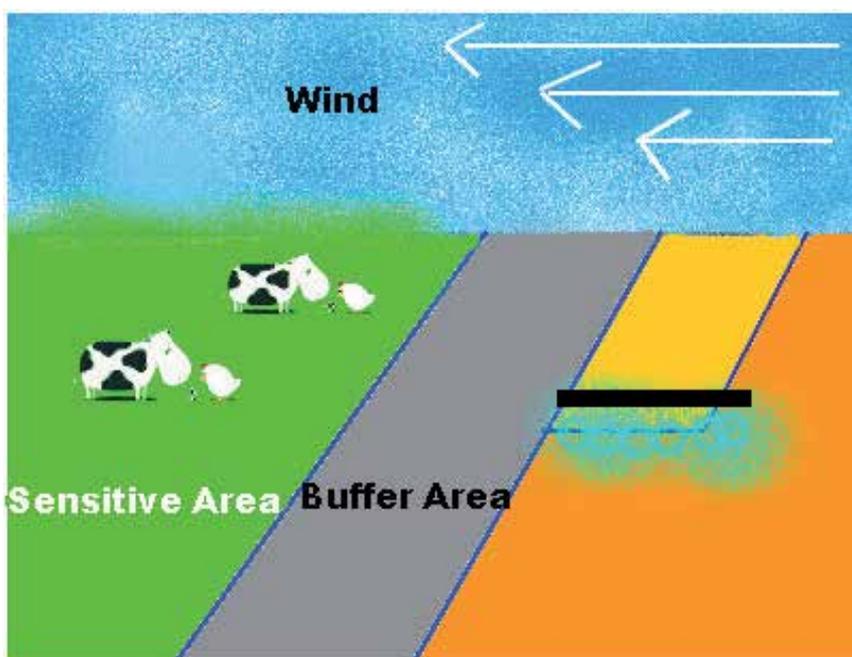


Fig. 3. Buffer area for protection of sensitive area

### 3.6.3 Relative humidity and temperature

Spray droplets start to dry and evaporate just right after sprayed by air temperature and relative humidity. Drying period is directly affected by air temperature and relative humidity. Studies showed drying period can be decrease four times. Faster drying period will decrease droplet size and mass during its travel at the air. This will increase risk of spray drift (T. Wolf, 1997).

Relative humidity and temperature are factors that affecting drift together. Usually they are not as important as wind speed, but at some geographical places and meteorological

conditions they became significantly important. Spray droplets are transported by air and because of that when water content of spray evaporated, this will cause to loss of mass with size and will make droplets tended to drift. Drift risk will be more when the size loss increased. Amount of evaporated water content of spray droplet is related to relative humidity and temperature.

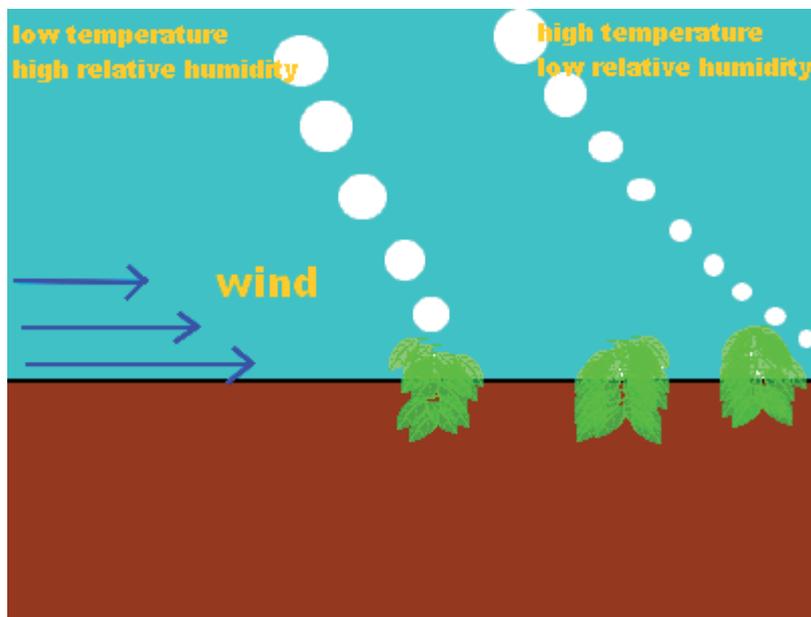


Fig. 4. Effects of temperature and relative humidity on drift  
(<http://www.yorktonaircraft.com/documents/drift.pdf>, 2005)

When the temperature increased, relative humidity will decrease and this will make easier evaporation of droplets.

When the evaporation happened, droplet size will decrease, air travel time and as a result of that drift risk will increase. This losses will decrease at early hours of night and day because of cooler air conditions. Temperature also can evaporate spray droplets on the target surface. Temperature have also indirect affect on air turbulence, air stability and inversion.

Studies showed fine droplets are tended to vaporize drift when the temperature is over 25 °C and relative humidity is low. Coarse droplets nozzles have to be choosed if it has to be sprayed at high temperature conditions.

### 3.6.4 Air stability

Air stability is another important factor for controlling drift. Air temperature is decreasing 12.2 °C at every 305 meter in normal meteorologic conditions. Cool air is tend to sink and this makes a vertical mix with hot air.

Athmosphere is not stable at sunny days because air is more hot at near to ground than higher layers of air. Hot air will rise in the cool air and this will make turbulence at unstable air conditions. Droplets in the air will move easily laterally and vertically and adjacent air layers will mix .This situation will caused to drift and also clean air will reduce concentration of pesticide (T. Wolf, 1997).

Under stable air conditions while ground becoming cool, warm air layer will take cool air layer underneath. At this condition droplets in cool air can move only laterally. This is called as temperature inversion. Spray cloud can keep density for a long time at inversion conditions.

However much stable air conditions are not fit for spraying too. Because when the wind speed is increased, high density spray cloud will be very opened to drift risk (T. Wolf, 1997) Atmospheric inversions are usual part of daily atmospheric cycle. Occurring in the early morning hours when the ground is cooler than the air layer just above it. Inversions tend to dissipate during the middle of the day when wind currents mixed the air layers. Because of that applicators must wait until late afternoon or early evening for spraying.

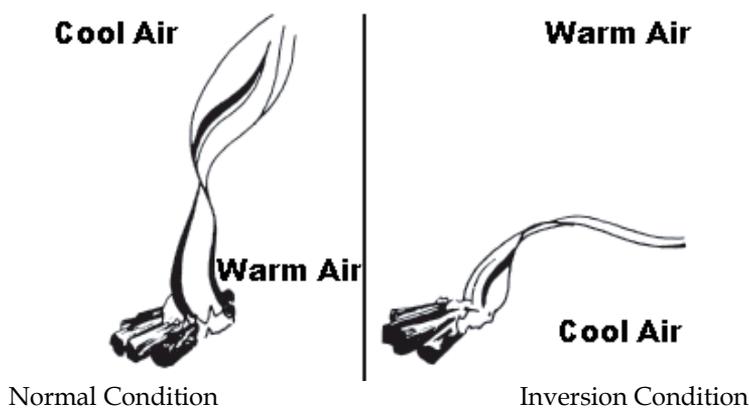


Fig. 5. Atmospheric Inversion dynamics

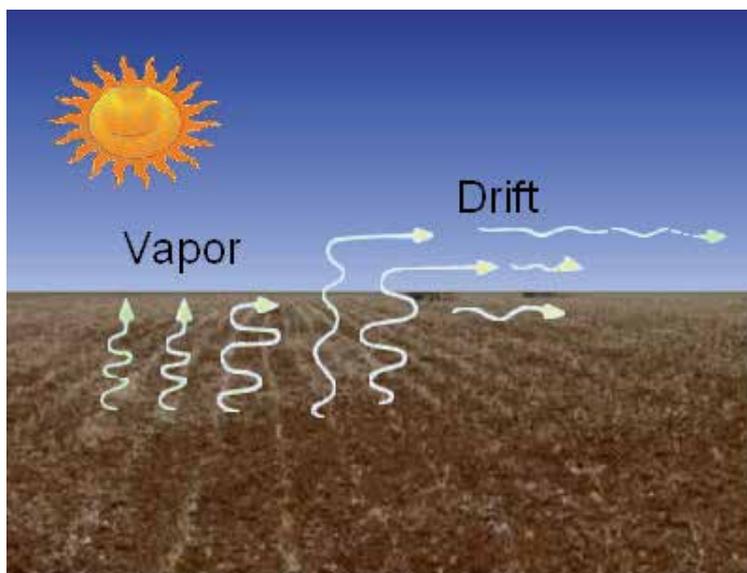


Fig. 6. Atmospheric Inversion

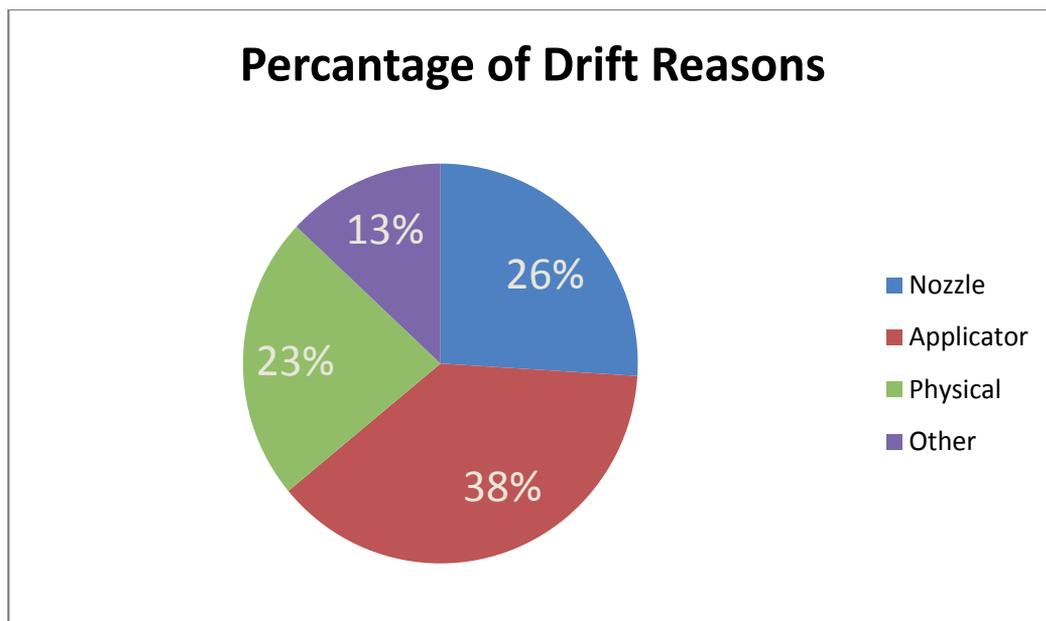


Table 3. Percentage of Drift Reasons (*agsafety.tamu.edu/Programs/Ag-Chemical/drift.ppt*)

#### 4. Drift measurement techniques

There are five types of spray drift sample are commonly used for measuring

- droplet sedimentation onto horizontal surfaces
- airborne concentrations at defined points downwind of an application site
- a total quantity of airborne spray passing through an imaginary frame at some distance downwind of an application site
- using sensitive plant species which are placed at defined distances at downwind
- laser based sampling instruments

**Ground sedimentation;** is very usual method for measuring spray drift. This method is based on measuring spray sedimentation on horizontal surfaces which are positioned at downwind of and application site. Some rules have to be done while using of this method:

- Sampling surfaces are horizontal and they must not be block by any of object like vegetation.
- Adequate size and spatial distribution of samples must be used. Minimum 1000 cm<sup>2</sup> total area is an international standard for field experiments.

Evaluation of drift can precisely controlled under laboratory conditions. Because all parameters can be controlled and this will be caused to a simple relationship between drop size/speed. But in practice field variables results a complex relationship. For reducing the complexity, all meteorological parameters have to be recorded at drift measurement field. To minimise the impact of field variables such as wind turbulence, the experiments are sited in open areas away from hedges/trees.

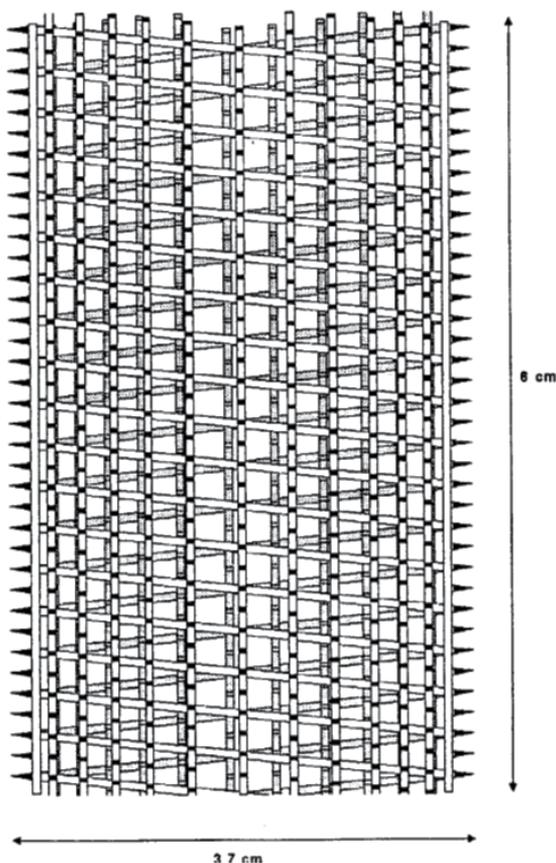
Results of sedimentation can be used at risk assessment for such as protect surface water sources and defining buffer zone sizes (Anonymous, 2003).

Airborne concentration measurements; can be performed with high volume air samplers, cascade impactors or rotary samplers. Small droplets can be collected efficiently with this method. But the main disadvantage is high power requirements and complexity of the system. (Anonymous, 2003)

**Airborne spray flux;** evaporation will reduce the size of spray droplets. Because of that very small droplets (10-100 micrometer) must be quantified with samplers. Airborne spray drift flux at downwind can be sampled by below methods:

- iso kinetic air sampling; at this method a thin-walled sampling tube can be aligned with the air flow direction.
- passive collection surfaces; at this method spray droplets are collected by some lines, rods, cotton and woollen threads, pipe cleaners etc. (Anonymous, 2003).

**Drift targets,** drift sampler targets have to be enough big to collect sufficient data and at the other hand have to be enough small to not block passage of air. Also it must be effective for trapping very small inflight drops. Targets are positioned at three dimensions to describe size and density of spray cloud at enough points and positions. Usually 10 m height masts are using at 200 m away from downwind for sampling. That masts are built up from 2mm wide plastic tubes, which are mounted vertically and horizontally, permit sampling at all points and make an imaginary line for sampling spray cloud. At some studies researchers use complex target shapes for increasing small drop impaction.



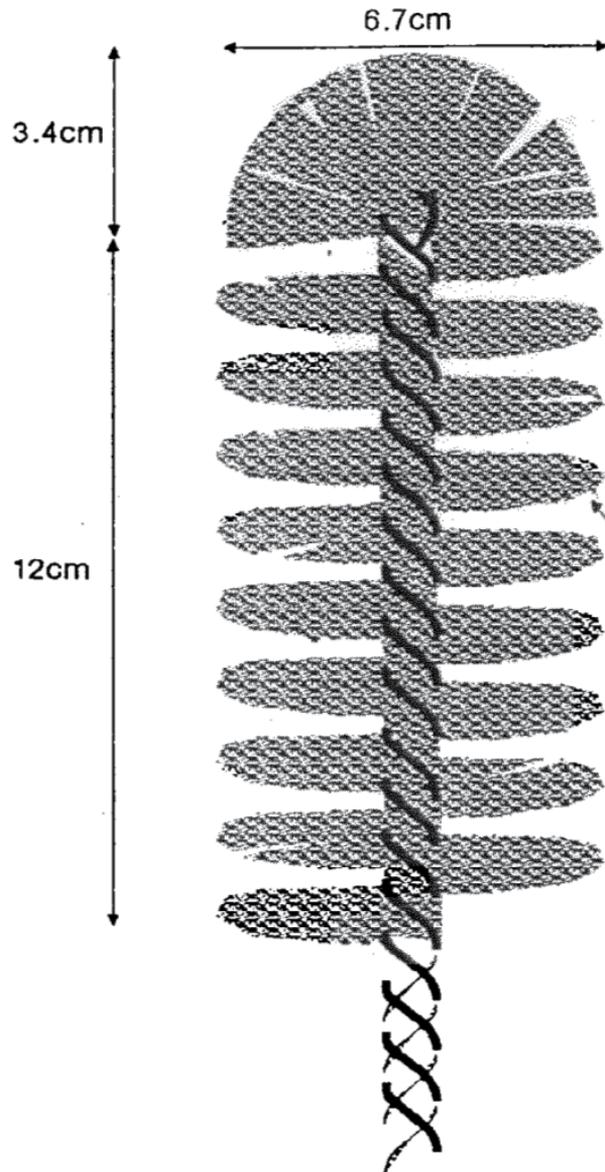


Fig. 7. Some different drift samplers ([http://www.comam.com.br/literatura/Techniques for measuring Spray Drift - Hardi.pdf](http://www.comam.com.br/literatura/Techniques%20for%20measuring%20Spray%20Drift%20-%20Hardi.pdf) )

Reducing the variability in deposit on the drift collectors and increase measured values, several swaths may be sprayed using the same tracks (for comparative studies) or sequential ones (for field drift losses)

## Comperative

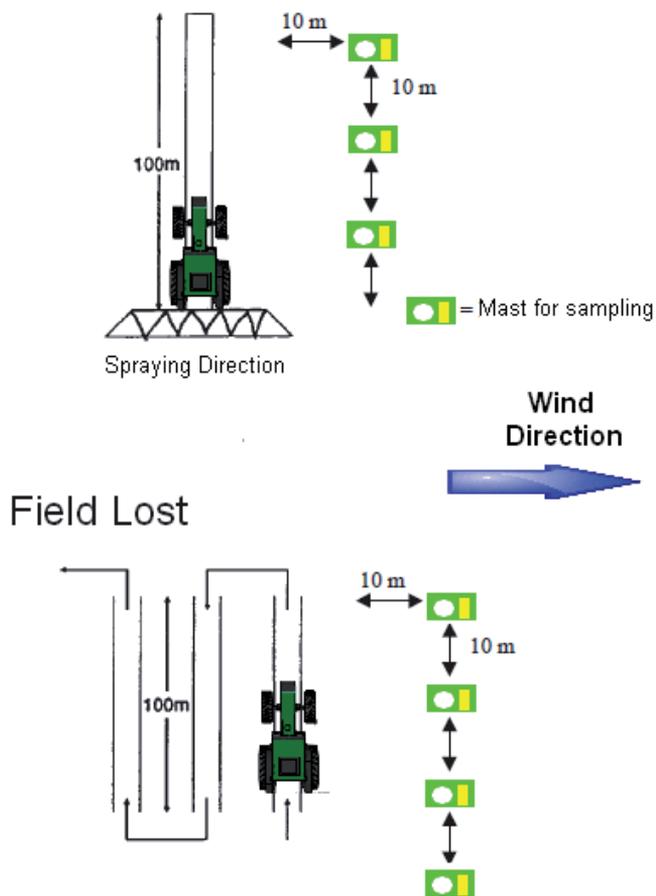


Fig. 8. Typical mast position for spray drift measurement

The distance sprayed up to and beyond the sampling targets needs to ensure the spray cloud is fully developed before, and slight variations in wind direction are considered. Minimum 100 m distance is typically used at most of studies. During and after the spraying wind speed and direction have to be recorded. Also nozzle types, boom width and height must be noted before the application.

Deposits are usually assessed by active ingredients or tracers like colorimetric or fluoremetric dyes. Beside its certain results of using active ingredient is so difficult. Because extracting the results takes so much time and measuring also preparing the process is relatively difficult. These situations are limited using of active ingredients at large number of samples.

Tracers are preferred at most of studies but they have some limitations also. They can interfere from formulation and this will reduce certainty of measurements.

Water sensitive papers can be also used for measuring spray drift. But they must be used very carefully while interpreting the results. Drop numbers, size and cover rate can be extracted from water sensitive papers.

#### 4.1 Estimation of drift potential

An empirical model for predicting spray drift, is developed by Sarker and Parkin at 1995. At this study, they used wind tunnel measurement of spray drift to describe an empirical correlation model of most important parameters of spray drift. They achieved this purpose bu using dimensional analysis. The following equation was developed by them:

$$Dp = 1.612 \times 10^{-3} (C_{dis})^{5.973} \left(\frac{h}{D}\right)^{-0.180} \left(\frac{h}{x}\right)^{1.0451} \theta^{-0.2664} \left(\frac{h}{\sqrt{\frac{u}{Q}}}\right)^{1.618}$$

$D_p$  = drift potential

$C_{dis}$  = coefficient of discharge, which is a measure of the energy loss through an orifice.  $C_{dis}$  is calculated from:

$$C_{dis} = \frac{Q}{A \sqrt{\frac{\Delta P}{\rho}}}$$

where  $Q$  is the discharge ( $m^3 s^{-1}$ ),  $A$  is the orifice area ( $m^2$ ),  $DP$  is the pressure drop across the nozzle and  $r$  is the fluid density

$h$  = nozzle height (m)

$D$  = equivalent diameter of the orifice (m).  $D$  is calculated from

$$D = 2 \sqrt{\frac{A}{\pi}}$$

where  $A$  is the orifice area ( $m^2$ ).

$x$  = downwind distance (m)

$q$  = the angle in the vertical plane that the spray nozzle makes to the airstream. If  $q = 0^\circ$  the nozzle is fully aligned with the airstream. The singularity in the model caused when  $q = 0^\circ$  can be avoided by using  $q = 2^\circ$  for this setting.

$u$  = wind speed ( $m s^{-1}$ ); the wind speed was varied between 1 and 3  $m s^{-1}$  in the experiments.

$Q$  = discharge ( $m^3 s^{-1}$ )

## 5. Common ground spraying equipments

There are different kind of equipments for reducing drift . In general view an equipment which is producing coarse droplets will reduce drift risk. Droplet size can be adjusted easily by changing nozzle type and size. Equipments must be choose for reducing of fine droplets within spraying pattern. Also wiper applicators can be used at sensitive plants and they will prevent the drift risk. There are four basic type of sprayers for ground applications.

**Air assisted sprayers;** are equipments that can be mount on tractor or pull by tractor. Independent and mechanically controlled air flow is being used for spraying. Drift risk is very high because of air flow.

**Boom sprayer;** this sprayers have very wide variety of equipments. Spraying are doing my spray nozzles which are arranged as a row on a boom. Boom height can be adjusted related to nozzle specifications. Spray drift can be minimized by appropriate height adjustment of boom for every different applications and nozzles.

**Boomless sprayers;** have nozzle groups or spinning disc which can be spray target area. As a result of that, there is no need to boom at this kind of sprayers. Groups of nozzles usually are tend to produce bigger droplets and this is reducing drift risk also. Spinning disc will produce small droplets and this will increase drift risk also.

**Wiper systems;** have mechanic contact with target surface. Drift risk is so less at this kind of application because it is depending on rubbing to surface, not spraying.

**Electrostatic sprayers;** are using electrostatic charging technical. Liquid spray droplets are charged negatively. Droplets and plants have opposite charge and they attract each other as a result of physical phenomenon that explain attraction of opposite electrical charged particles. The charged droplets are attracted to the spray target and are able to wrap around objects. There are three different types of electrostatic spraying. These are corona, contact and induction electrostatic spraying methods. (E.Dursun et al, 2005)

## 6. Techniques for controlling drift

There must be considered some issues for reducing drift risk as well as choosing appropriate equipments.

- a. It is very important to choose appropriate nozzle which can have better coverage on surface with optimum size droplets. We also have to consider the place of application while choosing nozzle. For example if we are spraying a place near to ways, using a nozzle which can produce uniform droplets will be more useful.
- b. Reducing the distance between target and nozzle is useful for decreasing drift risk. But coverage will be insufficient if boom is so close to target. .because of these situations distance between target and nozzles must be adjust and monitor continuously. Any change in the height of boom spraying parameters like pressure and flow rate must be calibrated again. Changes must be applied carefully and all of them must be appropriate with manufacturer's specifications.
- c. Operators must be monitor nozzles for physical blockage. Nozzles should be cleaned and replaced if necessary. Calibration of nozzles will be useful for long life usage.
- d. High pressures must not to used at spraying applications. High pressures will caused to produce fine droplets and this will be increased drift risk.

- e. Some additives can be used at spraying chemical for reducing drift. These additives can be increased or decreased viscosity and density of droplets. Also they can effect volatility of spraying chemical. So additives can be used for preventing drift risk.



Fig. 9. Low drift nozzles (Spraying Systems Co.)

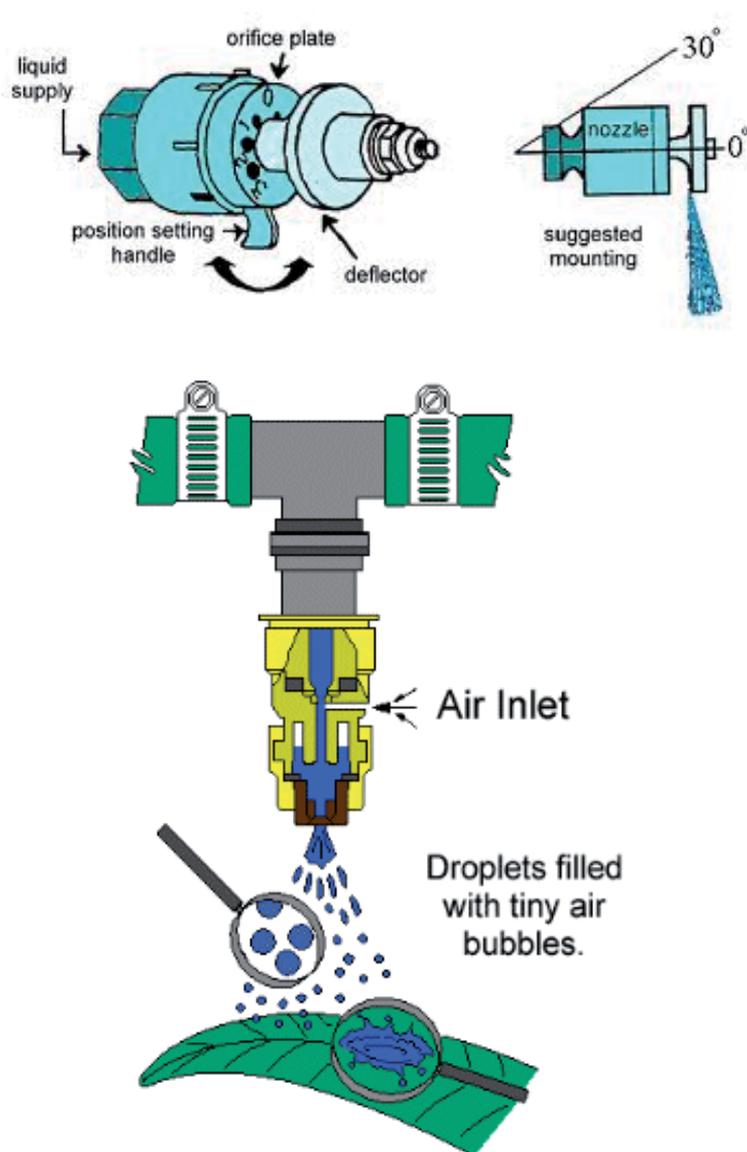


Fig. 10. Low drift nozzles (Greenleaf Tech., Covinton, LA)

## 6.1 New nozzle technologies

### Low-drift nozzles

Production of low-drift nozzles become more important as a result of worldwide attention on preventing drift . When compared with flat nozzles, low-drift nozzles can produce more coarse droplets at same spraying pressure and output. Using of these nozzles decreasing amount of droplets that under  $200\ \mu\text{m}$  at %50-80 rates. As a result of bigger droplet size, drift risk is being decreased. (E.Dursun et al, 2005)

**Pneumatic nozzles**

Air and liquid pressure can be adjusted independently at this kind of spraying nozzles and we can control droplet size as a result of that adjustment.

**Spinning disc nozzles**

We need 10-50  $\mu\text{m}$  droplets at insecticide and fungicide applications. Spinning disc nozzles are good solution for that kind of needs.(Matthews, 1992).

**CP nozzles**

Nozzle orifice can be adjusted by the adjustment mechanism on the nozzle. Required nozzle orifice diameter can be adjust by that feature.

**Twin nozzles**

These nozzles can be used at application which need good coverage. They also have advantage for penetration too. (Çilingir ve Dursun, 2002).

**Multiple nozzles**

This kind of nozzles have group of three-four or five headers which nozzles can be mounted on them. Different kind of nozzles can be mounted on these headers. So choosing a right nozzle for different spraying application will be easier.

**7. Suggestions**

Better understanding of new technologies at spraying will help to reduce drift. Droplet size, understanding of droplet spectrum and weather conditions have effect on these reduction.

Sufficient knowledge about spray drift, factors of spray drift and application tools will make a better control on drift management. Plant protection and drift management must be balanced. For proper and successive spraying application variables below must be considered;

- Spraying pressure
- Nozzle type
- Application rate
- Boom height
- Travel speed
- Wind speed
- Air temperature
- Air stability
- Relative humidity
- Enough buffer space from sensitive areas
- Directives of pesticide producer

Drift is absolutely unwanted situation at anyplace and anywhere;

- Drift cause a decrease at efficiency at both application and economical.
- Drift can cause damage at sensitive crops which are near to application area.
- Chemical residues at food products will reduce product quality.
- Can caused to air and water pollution.
- Drift can make risk for human and animal health.
- Less chemical residue on target surface will decrease efficiency of spraying application. This will increase total costs.

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## **Part 2**

# **Latest Advances in Biopesticides**



# Natural Pesticides and Future Perspectives

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## 1. Introduction

Biopesticide is a term that includes many aspects of pest control such as microbial (viral, bacterial and fungal) organisms, entomophagous nematodes, plant-derived pesticides (botanicals), secondary metabolites from micro-organisms (antibiotics), insect pheromones applied for mating disruption, monitoring or lure and kill strategies and genes used to transform crops to express resistance to insect, fungal and viral attacks or to render them tolerant of herbicide application (Copping & Menn, 2000). Botanicals include crude extracts and isolated or purified compounds from various plants species and commercial products (Liu et al., 2006). Not unlike pyrethrum, rotenone and neem, plant essential oils or the plants from which they are obtained have been used for centuries to protect stored commodities or to repel pests from human habitations and use as fragrances, condiments or spices, as well as medicinal uses (Isman & Machial, 2006). Quantitatively, the most important botanical is pyrethrum, followed by neem, rotenone and essential oils, typical used as insecticides (e.g. pyrethrum, rotenone, rape seed oil, quassia extract, neem oil, nicotine), repellents (e.g. citronella), fungicides (e.g. laminarine, fennel oil, lecithine), herbicides (e.g. pine oil), sprouting inhibitors (e.g. caraway seed oil) and adjuvants such as stickers and spreaders (e.g. pine oil) (Isman, 2006). Plants are capable of synthesizing an overwhelming variety of small organic molecules called secondary metabolites, usually with very complex and unique carbon skeleton structures (Sarker et al., 2005). By definition, secondary metabolites are not essential for the growth and development of a plant but rather are required for the interaction of plants with their environment (Kutchan & Dixon, 2005). The biosynthesis of several secondary metabolites is constitutive, whereas in many plants it can be induced and enhanced by biological stress conditions, such as wounding or infection (Wink, 2006). They represent a large reservoir of chemical structures with biological activity. It has been estimated that 14 - 28% of higher plant species are used medicinally and that 74% of pharmacologically active plant derived components were discovered after following up on the ethnomedicinal uses of the plants (Ncube et al., 2008). Plants and their secondary metabolites are an important source for biopesticides and the development of new pesticides. The recognition of the important role of these compounds has increased, particularly in terms of resistance to pests and diseases. The intensive use of synthetic pesticides and their environmental and toxicological risks have generated increased global interest to develop alternative sources of chemicals to be used in

safe management of plant pests. Recently, in different parts of the world, attention has been paid towards exploitation of higher plant products as novel chemotherapeutics for plant protection because they are mostly non phytotoxic and easily biodegradable (Isman, 2006). Currently, different botanicals have been formulated for large scale application as biopesticides in eco-friendly management of plant pests and are being used as alternatives to synthetic pesticides in crop protection. These products have low mammalian toxicity and are cost effective. Such products of higher plant origin may be exploited as eco-chemical and biorational approach in integrated plant protection programs (Dubey et al., 2009). In order to increase food safety and develop integrated and sustainable strategies for plant protection, which are safe to the consumer, producer and the environment, the use of natural pesticide need to be promoted. According to Ehlers (2009) in order to reach these goals *we need less, rather than more registration requirements*. Recently, Nthalli and Menkissoglu-Spiroudi (2011) reviewed the main chemical classes of plant secondary metabolites that have been used in crop protection focusing on the most recent advances in the chemicals disclosed, their mode of action and their fate in the ecosystem, their current use in pest management underlying registration procedures and commercialization potential. Numerous scientific articles, reviews and chapters book have been written on this subject. The main goal of this chapter is to review this actual topic on natural pesticides in a concise format that is easily understandable. Several topics will be emphasized and in particular: (1) the regulation of plant protection products from natural origin in European Union; (2) their regulation and importance in organic farming; (3) the actual *State of art* of three most widespread botanical pesticides: rotenone, azadirachtin and pyrethrins and (4) the future perspectives of natural pesticides.

## 2. Plant protection products from natural origins

### 2.1 Regulation in European Union

Registration of Plant Protection Products (PPPs) based on botanicals, semiochemicals and micro-organisms follow rules originally developed for the risk assessment of synthetic chemical compounds. Data requirements for authorization and marketing of PPPs under Directive 91/414/EEC according to the categories are present in Table 1. Since PPPs can be harmful to humans and the environment their risks need to be evaluated and their active ingredients must be authorized according to Directive 91/414/EEC prior to commercial use. The authorization for commercial use is only given if unacceptable negative effects to humans and the environment can be excluded.

Biochemicals are the products that are intended for use in plant protection and can contain powdered plants parts, plant extracts and possibly co-formulants. While plant extracts are obtained by treating plants or parts of them, with a solvent, which is further concentrated through evaporation, distillation or some other process. Plant extracts can be obtained also by soft extractions with water and/or ethanol. All relevant available information must be presented in the summary dossier, and must be of sufficient quality to allow an assessment of possible risks of the proposed use. However supplementary data can be requested on a case-by-case basis by the competent authorities in order to allow finalizing the risk assessment. SANCO/10472 document contains a list of plants and plant extracts to which reduced data requirements should apply. Data required according to SANCO/10472 are: 1. reference list established on the basis of available information including literature, evaluation done in OECD countries, European pharmacopoeia, and weight of evidence

Category of PPPs	Description under Directive 91/414/EEC	Data requirements given in
Biochemicals (Plant extracts and plant strengtheners)	Chemical substances	SANCO 10472
Semiochemicals (allelochemicals and pheromones)	Chemical substances	OECD Series on Pesticides 12
Micro-organism and viruses	Viable entities in scope of Directive 91/414/EEC	OECD Series on Pesticides 23 Directives 2001/36/EC
Macro-organisms	Not covered by 91/414/EEC	OECD Series on Pesticides 21 FAO and EPPO guidelines

Table 1. Categories of PPPs and data requirement for authorization and marketing under Directive 91/414/EEC

which indicates that the plant is not harmful to human, animal and environment, edible parts of plants used for animal or human feed herbal drugs in EU pharmacopoeia; 2. Description of the known active substances, providing the concentration range of any toxic substances that are relevant for human, animal health and environment, if the active substance (s) is (are) not identified the definition of a representative marker, providing analysis report of 5 batches of different manufacture, collected over several periods; 3. Physico-chemical properties of all identified "active substances"; 4. Data regarding the application; 5. Validated analytical methods; 6. Efficacy data; 7. Residues in or on treated products food and feed; 8. Fate and behavior in environment; 9. Ecotoxicological studies. Semiochemicals are chemicals emitted by plants, animals, and other organisms that evoke a behavioral or physiological response in individuals of the same or other species. They include pheromones and allelochemicals. Allelochemicals are semiochemicals produced by individuals of one species that modify the behaviour of individuals of a different species (i.e. an interspecific effect). They include allomones (emitting species benefits), kairomones (receptor species benefits) and synomones (both species benefit). Pheromones are semiochemicals produced by individuals of a species that modify the behaviour of other individuals of the same species (i.e. an intraspecific effect). Data required for semiochemicals are given in OECD Series on Pesticides 12 guidelines. Regarding micro-organisms and viruses, data required are given in OECD Series on Pesticides 23 guidelines. In addition nematodes and macro-organisms are not covered by 91/414/EEC in most European countries (Denmark, Finland, France, Greece, Germany, Italy, Portugal and Spain) and usually no registration is required except for exotic species, which have never been used in biological control in an ecosystem. The regulation of plant protection products in the European Union (EU) was firstly harmonized under Directive 91/414/EEC, which came into force on 26 July 1993. This Directive established agreed criteria for considering the safety of active substances, as well as the safety and effectiveness of formulated products. It also provided the establishment of a positive list of active substances (forming 'Annex I' of the Directive) consisting of already existing and reviewed or new ones. A re-registration process was adopted for products already on the market containing a newly listed active substance in Annex I. The review of existing pesticides has led to the removal from the market of pesticides which cannot be used safely. Out of almost 1 000 active substances commercialized on the market in at least one

Member State before 1993, only 26 %, corresponding to about 250 substances, have passed the harmonized EU safety assessment. The majority of substances (67%) has been eliminated because registration dossiers were either not submitted, incomplete or withdrawn by industry (Fig. 1.). About 70 substances failed to be reviewed and have been removed from the market, because the evaluation carried out did not show safe use with respect to human health and the environment. Regulation (EC) 1107/2009 (published on 24 November 2009) replaces Directive 91/414/EC and applies from 14 June 2011. It continues to harmonize plant protection products across the EU as well as introduces some new criteria for registration of plant protection products from plant origin as basic substances and low risk pesticides (Table 2, Fig. 1.). Active substances are considered as basic substances if they fulfill the criteria of the foodstuffs listed in Table 2. Low risk substances should not be considered if at least one of the criteria listed in Table 2 is satisfied. The substances which are currently listed in SANCO/10472 document, «25b list» of the US EPA and all substances with GRAS status reduced data are required for the registration. Some examples are listed in Table 3. In addition it will establish some new

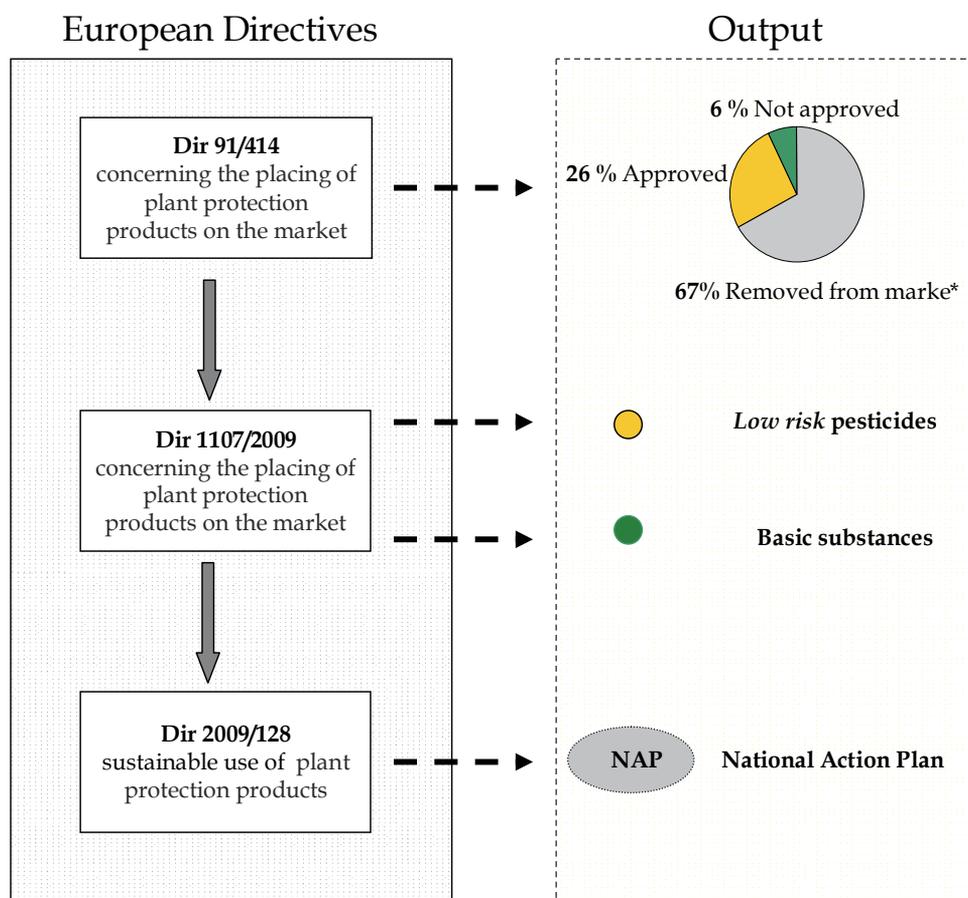


Fig. 1. EU regulations of plant protection products influencing the natural pesticides regulation (\*no dossier submitted, incomplete dossier or dossier withdraw by industry)

<b>Basic substances</b> (fulfill the criteria of a 'foodstuff')	<b>Low risk substances</b> (shall not be considered if at least of one)	
a) is not a substance of concern <i>and</i>	a) carcinogenic,	f) persistent (half-life in soil is more than 60 days),
b) does not have an inherent capacity to cause endocrine disrupting, neurotoxic or immunotoxic effects <i>and</i>	b) mutagenic,	g) bioconcentration factor is higher than 100,
c) is not predominantly used a plant protection product <i>and</i>	c) toxic to reproduction,	h) it is deemed to be an endocrine disrupter, or
d) is not placed on the market as a plant protection product	d) sensitizing chemicals,	i) it has neurotoxic or immunotoxic effects.
	e) very toxic or toxic,	

Table 2. Cutoff criteria for basic and low risk substances

<b>A) Edible parts of plants used for human nutrition or animal feed</b>
artichoke (edible parts), basil (whole plant), black pepper (fruit), carvi (fruit), chives (clove), coriander (fruit), elder (bark, flower, fruit), garden sage (whole plant), garlic (clove), horse tail (leaf), laurel (leaf), mint (whole plant), olive (oil), onion (bulb), oil seed-rape (oil), sesame (seed), soybean (oil), squash (seed), sunflower (oil), tomato (fruit).
<b>B) Parts of plants authorized as herbal drugs</b>
bladder wrack (thallus), feverfew (whole plant), lavender (whole plant), nettle (whole plant), rhubarb (rhizome only), sweet chamomile (whole plant).
<b>C) Plant extracts classified as Minimal risk pesticides</b>
castor oil, cedar oil, cinnamon and cinnamon oil, citric acid, citronella and citronella oil, cloves and clove oil, corn gluten meal, corn oil, cottonseed oil, eugenol, garlic and garlic oil, geraniol, gernanium oil, lauryl sulfate, lemongrass oil, linseed oil, malic acid, mint and mint oil, peppermint and peppermint oil, rosemary and rosemary oil, sesame (includes ground sesame plant) and sesame oil, sodium lauryl sulfate, soybean oil, thyme and thyme oil and white pepper
<b>D) Plant extract classified as GRAS</b>
Lecithin, cinnamon

Table 3. Example of botanicals under the easier procedure for regularization: A) as listed in SANCO/10472; B) as listed in SANCO/10472; C) «25b list» of the US EPA; D) 21 CFR 184.1400 in the REBECA deliverable 14 ([www.rebeca-net.de](http://www.rebeca-net.de))

requirements, such as the introduction of hazard based criteria, assessment of cumulative and synergistic effects, comparative assessment and endocrine disruption. According to the new Dir 2009/128/EC on sustainable use of plant protection products Member states should adopt National Action Plans (NAP), set up their quantitative objectives, targets, measures and time table to reduce risk and impact of pesticides use on human health and the environment by 2012 and encourage the development and introduction of low inputs pesticide production giving whereas possible priority to non chemical methods. Low inputs pest management includes Integrated pest management (IPM) as well as alternatives methods like Organic farming, IPM must be implemented in each Member state by 2014.

Non chemical methods cover also biological pest control according to Dir 2009/128/EC . One example of NAP is a plan called “EcoPhyto2018” in France. The highlighted goals of EcoPhyto 2018 are: “to achieve 50% reduction in the use of pesticides by 2018, if feasible” and “that the total areas certified as organic agriculture go from the present 2% to 6% in 2012, and eventually to 20% by 2020”.

## 2.2 Organic agriculture and EU regulations

Organic agriculture is an ecological production management system that promotes and enhances biodiversity, biological cycles and soil biological activity. It is based on the minimal use of off-farm inputs and on management practices that restore, maintain and enhance ecological harmony (National Organic Standards Board, 1995). Ecological soundness is the key claim, indeed the *raison d'être*, of organic agriculture. Organic farming is a system approach aiming at a sustainable ecosystem, safe food, good nutrition, animal welfare and social justice. Since the beginning of the 1990s till 2009, organic farming has rapidly developed in almost all countries, with more than 37 million hectares managed organically by around 1.8 million producers, constitutes 0.85 % of the agricultural area (FiBL/IFOAM Survey 2011). In 2009 the market value of organic products worldwide reached 60 billion U.S. dollars and consumption of organic vegetables and fruits has increased substantially in the last decade (Sahota, 2009). Quantitatively, organic farming is still of minor importance, but it is one of the most rapidly growing agricultural sectors worldwide. European organic food and drink sales are bouncing back from the economic slowdown in 2009 and while they will return to expand at higher growth rates from 2011 onwards. Growing consumer sophistication is leading to a proliferation in food eco-labels like organic, fair trade, biodiversity, carbon footprint, water footprint, etc. For many consumers, organic foods are the perfect example of quality and/or healthy and organic farming is ‘farming without chemicals’ (Lampkin 1990). Although there is some evidence that consumers are willing to pay more for environmentally ‘added value’ products such as organic produced foods (DEFRA, 2002; Gafsi et al., 2006) research shows that organic consumers are generally more interested in social and environmental aspects of food production than the average consumer (Sylvander & François, 2006). The ethical concerns of organic consumers can be easily categorized according to the three pillars of the concept of sustainability: ecological, social, and economic sustainability. The concerns which exceed the standards set by the EU regulation 834/2008 on organic farming name “*additional ethical*” or “*OrganicPlus*” attributes (Zander et al., 2010). In 2004, the European Commission published an ‘European Action plan for Organic Food and Farming’ (COM, 2004), with the aim to facilitate the expansion of organic farming, to develop the market for organic food and improve standards by increasing efficacy, transparency and consumer confidence. The plan aims to achieve measures such as improving information about organic farming, streamlining public support via rural development, improving production standards or strengthening research. It follows the rapid increase in the number of farmers producing organically and strong demand from consumers during the past few years. The new EU ‘organic regulation’ consists of a framework regulation, complemented by implementation rules and guidelines. Other important regulations/standards are the National Organic Program of the USA, the guidelines of the Codex Alimentarius and the basic standards of the International Federation of Organic Agriculture Movements (IFOAM). Under all these standards, plant protection

management and products are strictly regulated. Organic plant protection management follows a clear hierarchy, above all, plant health is maintained by preventative measures (choice of adapted species and varieties, crop rotation, cultivation techniques, thermal processes and the protection and/or release of natural enemies) and only if these methods are insufficient, plant protection products may be used (Speiser et al., 2006). The substances and their authorized uses are listed in Annex II of Reg. 889/2008 and divided in different categories as substances of crop or animal origin, micro-organisms used for biological pest and disease control, substances produced by micro-organisms, substances to be used in traps and/or dispensers, preparations to be surface-spread between cultivated plants, other substances from traditional use in organic farming, other substances. A very limited range of substances is authorized for use and for some authorized substances and only selected uses are allowed (Speiser et al., 2006). In the EU, new substances can only be authorized if they are consistent with organic farming principles, if they are necessary for sustained production, if they are of plant, animal, microbial or mineral origin in some cases exceptions are possible but non contact criteria has to be respected, if they have no harmful effects on the environment along the life-cycle and lowest negative impact on human or animal health and quality of life, with no negative socio-economic impacts or unfavorable public perception (Article 16 of Reg. 834/2007). In the EU, a legal definition of organic farming practices was first given in 1991 (EC, 1991) and regulations were developed in a lengthy process and represent a broad consensus in Europe (Schmidt & Haccius 2008; Mikkelsen & Schlüter 2009). The new 'organic regulation' consists of a 'framework regulation', complemented by 'implementation rules' and guidelines (EC, 2007). For plant protection, the implementation rule 889/2008 is relevant (EC, 2008). The regulation and authorization of plant protection products in organic farming is recently explained by Speiser and Tamm (2011). The authorization in the EU of new plant protection products has started by requests which are submitted by an EU Member State but not by manufacturers to the Commission. Recently, the Commission has set up an 'expert group for technical advice on organic production' responsible for authorization (EC, 2009). Once an active substance is authorized for use, it is up to the certification authority to determine under which conditions commercial products may be used. Although organic farming is governed by one regulation throughout the EU, the practices of plant protection differ significantly from one country to another. This is due to a complex interaction between organic legislation, national private standards, general pesticide legislation, and commercial activities with respect to plant protection products and regional farming traditions. In the EU organic regulation, plant oils, micro-organisms and pheromones are authorized in a generic way. New substances belonging to one of these three groups can be used in organic farming without any further authorization procedure, if they are allowed for use in general agriculture in the EU. Commercial organic production of any crop is only possible if materials or methods are available to regulate the key pests and diseases. New developments require progress in the range of authorized pesticides due to the farm sizes become larger and farms tend to specialize in a decreasing number of crops, consumers requirements with respect to external quality are continuously important, economical conditions like open market, strong demand for high quality food, seed-borne diseases are likely to gain importance in the future because the not allowed use of synthetic fungicides (Tamm, 2000). Actual statuses of three most widespread botanical insecticides in conventional and organic agriculture are presented

in Table 4. Rotenone is one of controversial substances, its use should be reduced in organic farming and main reasons are explained in the next sections.

Active substances	Category	Annex I (Dir 91/414/EEC)	Regulation (EC)	MRL mg/kg	Organic Farming (889/2009)
Pyrethrins	Insecticide	IN	2008/127	0.05-1	IN
Rotenone	Insecticide	OUT	2008/317	0.01-0.02	IN
Azadiracthin	Insecticide	IN	SCoFCAH Mar 2011	0.01-1	IN

Table 4. Actual status of Pyrethrins, Rotenone and Azaditacthin under Dir.91/414/EEC and Organic farming regulation: IN: Included or authorized; OUT: Not included; MRL maximum residue level (EC No 396/2005)

### 2.3 An actual case of rotenone

A very well known group of natural products occurring in the *Leguminosae* family are rotenoids and their most famous member is Rotenone. Rotenone is extracted from *Leguminosae* species such as *Derris elliptica*, *Tephrosia vogelli* and *Lonchocarpus nicou* (Copping, 1998). A strain of endophytic *Penicillium* sp., isolated from the fresh roots of *Derris elliptica* Benth, might produce rotenone or its analogues and be active against aphids (Hu, 2005). The molecule is named after Roten, who was the first researcher to study this pesticide in Japan at the beginning of the past century. Among 29 rotenoids isolated from cubé resin, obtained from the roots of *Lonchocarpus utilis* and *urucu* from Peru, major components are: rotenone (44.0%, w/w), deguelin (22.0 %, w/w), rotenolone (12a,-hydroxyrotenone) (6.7 %, w/w), and tephrosin (12a,-hydroxydeguelin) (4.3 %, w/w). Additional rotenoids have been isolated and identified but they were likely ascribed to decomposition products generated with the resin processing (Fang & Casida, 1999). A marked insecticide activity is reported for the main rotenoids (Yenesew, 2003), even though rotenone and deguelin show a similar activity much stronger than their derivatives (Fang & Casida, 1999).

#### 2.3.1 Rotenone as biopesticide and mode of action

Rotenone has a long history of use as a toxin for insects and other arthropods, as well as for fish (Ray, 1991). Rotenone shows a pyrethrin-like behaviour but with a stronger action and a higher persistence (Crombie, 1999). It owes part of its efficacy to its rapid neurotoxic action against insects, named "knock down effect"; and it is used to control aphids, suckers, thrips and other insects on fruit and vegetables (Tomlin, 2000). Rotenone increased the insect's mortality and negatively affected its reproduction (Guadan et al., 2000). Despite rotenone being used for many years as a botanical insecticide (Whitehead & Bowers, 1983), there are only a few papers about the feeding deterrent activity of rotenone and its derivatives (Bentley et al., 1987; Nawrot et al., 1989). There appear to be differences in the sensitivity of various Lepidoptera species to this compound (Dowd, 1988; Valles & Capinera, 1993). When ingested, rotenone tended to reduce the amount of food absorbed by the larvae, as well as their ability to convert the absorbed food to biomass (Wheeler et al., 2001). Slow action of rotenone as a stomach or contact poison is known (Fukami & Nakajima, 1971). Rotenone is

highly toxic to fish, with 96-h LC50 values of 23 and 2.6 ng/g to rainbow trout and channel catfish, respectively (Kidd & James, 1991). It has been used by native tribes as a fish poison to obtain food and more recently in fisheries management to achieve the desired balance of species, e.g., the treatment of Lake Davis in California (California Department of Fish and Game, 1997) as well as the treatment of rivers and river systems in the last 5-10 years in Norway to exterminate the parasite *Gyrodactylus salaricus* of North Atlantic salmon by killing the host (Anonymous, 2002). The application of rotenone to fresh waters can also cause significant declines in zooplankton and certain benthic fauna; however, some invertebrates would normally be expected to recover in a few months (Blakely et al., 2005; Melaas et al., 2001), although the recovery rates are largely dependent upon each taxon's recolonisation ability. Rotenone is toxic to bees when used together with pyrethrin (Kidd & James, 1991) but alone is used to control the mite *Varroa jacobsoni* which affects colonies of the honey bees (Jimenes et al., 2000; Martel and Zeggane, 2002). There are several studies that reported the persistence of rotenone on food crops after treatment. The half-life of rotenone on olives has found to be 4 days, while at harvest the residue levels were above the tolerance limit with residues in the oil being higher than those on olives by a factor of 2.4-4.8 (Cabras et al., 2002). Rotenone was detected at concentration of 0.11 mg/kg in honeys (Jimènes et al., 2000). Due to the mentioned evidences and the facts reported in the next paragraphs (2.3.2. and 2.3.3) Rotenone is not included in the Annex I of the European Directive 91/414/EC, as well it is provided with a default maximum residue level (MRL) of 0.01 mg/kg (Table 4). Because of its natural origin, the use of rotenone as an insecticide has been allowed in the last two decades in organic crop production. Rotenone is used in European organic agriculture nowadays, with a strong restriction regarding its environmental hazards. The use of rotenone is partially restricted in Austria, Italy, Spain, Switzerland, and the United Kingdom, but not in Denmark, Netherlands, Portugal, and Slovenia. In the United Kingdom, few private standard-setting organizations allow its use after preliminary permission, while others never permit its use (Speiser & Schid, 2003). In Italy the use of rotenone formulations is allowed until 30 April 2011 only on apples, peaches, pears and cherries.

### **2.3.2 Parkinson Disease (PD) and cancer chemopreventive effect of rotenone**

Rotenone exposure cause neurotoxic effects that may suggest its possible role in the development of a PD-like syndrome in animals. Rotenone treatment causes lesions to the nigrostriatal system that are consistent with PD, via production of Reactive Oxygen Species due to inhibition of mitochondrial Complex I (Sherer et al., 2003 a) and glial activation (Sherer et al., 2003 b). Although the effects of Rotenone on the brain were first tested over 20 years ago, the model received the most attention when reproduced with a chronic mode of intravenous delivery (Betarbet et al., 2000). There are several issues that have disadvantaged acceptance of the chronic Rotenone model, the most important is variability. Different modes of administration have been explored and in specific the intracranial (Saravanan et al., 2005), intravenous (Milusheva et al., 2005) , subcutaneous (Caboni et al., 2004), intraperitoneal (Cannon et al., 2009) and more recently, others such as oral (Inden et al., 2007) and intranasal (Rojo et al., 2007) delivery that are arguably more realistic with regard to potential entry sites for toxin exposure in human PD. Unfortunately, these novel routes have only been reported in single studies with limited pathological analysis. The Rotenone model is evidence demonstrating marked systemic non-specificity is non-specificity within the central nervous system does not reproduce the pathology of PD but

rather induces a pattern of pathological changes. In addition, there remain critical issues regarding the translatability of the model: does the Rotenone model truly recapitulate human PD? These data can be of help for understanding the role of pesticide exposure in human PD development. On the other hand farmers exposed for days or weeks during several years to much lower doses than those used in experimental studies. Therefore, a conclusion on the role of pesticide exposure on the increased risk of developing PD cannot be drawn (Moretto & Colosio, 2011). Future studies should include histopathological analyses of other systems and perhaps attempt to evaluate the model from a different angle, shedding light onto the potential value of these “nonspecific” effects in reproducing other aspects of PD pathology (Cicchetti et al., 2009). Rotenone is known not only as a toxicant but also as candidate anticancer agents (Fang et al., 1997, Rowlands & Casida, 1998). Rotenone induces mitotic catastrophe, mitotic slippage, cell death and cellular senescence in cancer cells (Gonçalves et al., 2011).

### **2.3.3 Chemical and photochemical fate of rotenone in the soils in relation to the soil components**

Both the accumulation of pesticides in the soil and their dispersion in the environment depend chiefly on the characteristics and overall functioning of the ecosystem. Soil represents a major sink for organic xenobiotic contaminants in the environment. It is necessary to establish the fate of the parent compound and its degradation products in the soil in order to completely evaluate the environmental hazard of rotenone. In order to fulfill the lack of information regarding the rotenone fate in the soil several studies have been carried out by the authors. In experiments carried out to study photodegradation of rotenone in soils under environmental conditions, the observed overall degradation of rotenone is not only determined by photolysis itself but also as a function of soil characteristics, and it appears to be reduced and affected by several other physical-chemical mechanisms (Cavoski et al., 2007). Results indicate that the photochemical behavior of rotenone is significantly affected by the soil physicochemical characteristics. The three soils used in the experiment show significantly different net losses due to the sunlight exposure, the photolysis rate ranging from 0.12 to 0.18 min<sup>-1</sup>. However, the contribution of the photochemical processes to the global consumption rate is higher in soils richer in organic matter than that in sandy soil. The photodegradation of rotenone on soil surfaces principally produces an oxidized metabolite, rotenolone (12 $\alpha$ -hydroxyrotenone) (Fig. 2). The photolysis reaction proceeds better fit two compartment or multiple compartment model pathways. A fast initial decrease during the first 5 hours of rotenone irradiation is followed by a much slower decline (lasting more than 10 hours), which clearly indicates the rather complex chemical process of rotenone photodegradation on soil surfaces. In the initial decrease, the degradation in soils of rotenone is mainly effected by direct sunlight irradiation and proceeds at a high rate; then, following rotenone adsorption on soil particle surfaces, it appears to be reduced and be effected by several other physical-chemical mechanisms. On the other hand in the standardized laboratory experiments regarding rotenone degradation in soil we proved that chemical degradation process markedly more complex than its photodegradation on soil surfaces and the contribution of chemical processes to the global degradation rate is higher in sandy soil than in soils richer in organic matter (Fig. 2). The half lives of rotenone and 12 $\alpha$ -hydroxyrotenone (major metabolite), were 8 and 5 days, 23 and 56 days at 20 °C, respectively. However, at 10 °C a tendency for slower degradation of rotenone and 12 $\alpha$ -hydroxyrotenone was observed (25 and 118 days

and 21 and 35 days, respectively). Results show that the degradation rates of both rotenone and 12 $\alpha$ -hydroxyrotenone were greatly affected by temperature changes. Rotenone degradation phenomena are described by a bi-phasic equation, while metabolites degradation kinetics is described by a first order equation. Rotenolone is more persistent than rotenone in the soil. Chemical degradation was strongly affected by soil adsorption properties, soil temperature and rotenone characteristics. Adsorption processes affect its degradation in the soil mainly by modifying its chemical bioavailability and activity. Variability in degradation rate has provided evidence for possible field-to-field variation in the degradation rates of rotenone in the environment. A greater understanding of the factors that influence degradation rates is required to support obtained results.

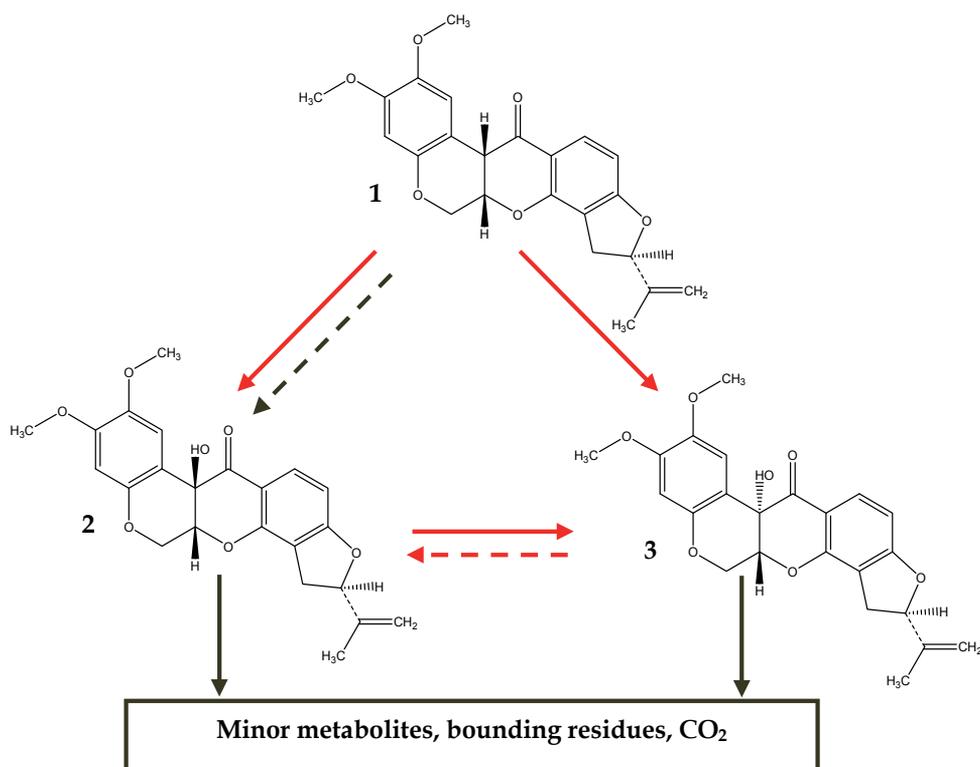


Fig. 2. Chemical (—) and Photochemical (-----) pathway of rotenone in soil. 1- Rotenone, 2- 12 $\alpha$  $\beta$ - hydroxyrotenone and 3- 12 $\alpha$  $\alpha$ - hydroxyrotenone

The principal photochemical reaction of rotenone on soil surfaces is photooxidation (Fig. 2), producing an oxidized metabolite – rotenolone (12 $\alpha$  $\beta$ -hydroxyrotenone). The photodegradation kinetic can be explained by a multiple compartment model. A fast initial decrease during the first few hours of rotenone irradiation is followed by a much slower decline. In the initial decrease, the degradation of rotenone in soils is mainly affected by direct sunlight irradiation and proceeds at high rate (probably initiated by its photosensitizer properties); then, following rotenone adsorption on soil particle surfaces, it is reduced and affected by several other physico-chemical mechanisms. Chemical transformation of rotenone in a soil (Fig. 2), such as hydrolysis and oxidation, are important

phenomena but, however, isomerization has also been observed in standard laboratory studies. Main metabolites were 12 $\beta$ -hydroxyrotenone and its isomer 12 $\alpha$ -hydroxyrotenone, indicating the rather complex chemical process of rotenone degradation in soil with respect to its photodegradation on soil surfaces. In order to understand the role of organic fraction in the soil on rotenone degradation we investigated mechanism by which rotenone binds to the humic acids relative to their origin and properties by means of spectroscopic studies. Results demonstrated that the rotenone adsorption onto humic acids can be achieved by different mechanisms, as a function of the compositional, structural, and functional properties humic material have been obtained (Fig. 3.). The most important

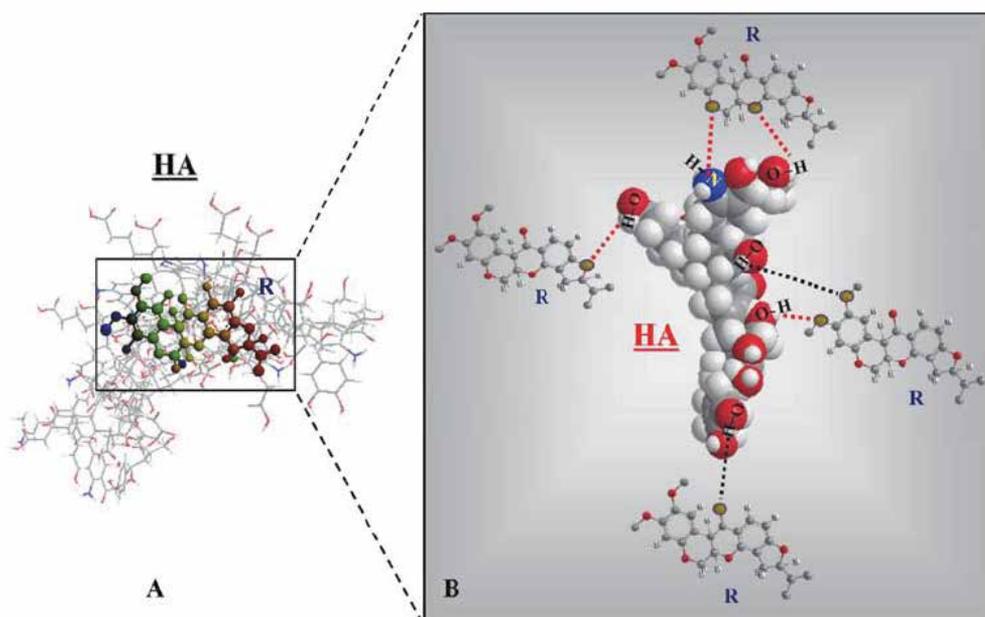


Fig. 3. Schematic representation of binding mechanisms possibly occurring between various structural units of humic acid (HA) macromolecules and rotenone (R): A- hydrophobic and B- hydrogen bonds

parameters determining a prevalence of a specific mechanism with respect to others are the oxygenated group's content and the aromatic degree of humic acids. These characteristics result in a different chemical reactivity and a residual adsorbing capacity of the humic acids toward the non-polar rotenone molecule. The humic acids characterized by a greater aromaticity degree and lower polarity, featured by a mixed aromatic/aliphatic character, rotenone resulted preferentially adsorbed onto these humic acids by hydrophobic interaction, whereas onto mainly aliphatic and acidic humic acids the hydrogen bonds resulted predominant. The most important parameters determining a prevalence of a specific mechanism with respect to others are the oxygenated groups content and the aromatic degree. These characteristics result in a different chemical reactivity and a residual adsorbing capacity of the humic acids toward the non-polar rotenone molecule. The FT-IR spectroscopy data indicated weak interaction between rotenone and humic acids, involving mainly hydrogen bonding, and possibly also hydrophobic forces. Fluorescence

spectroscopies data appear very informative and clear, suggesting that rotenone, preferentially binds to humic acids by hydrophobic interaction (Cavoski et al., 2009). Results provide additional insights about transformation phenomena and metabolite production of rotenone in the environment, describing more clearly the degradation performances as a function of various physico-chemical parameters.

## 2.4 Pyrethrins and Azadirachtin actual status

Neem oil extracted from the seeds of neem tree (*Azadirachta indica*) has been known to contain many bioactive compounds. These compounds are triterpenoids of the class of limonoids. Major limonoids reported in literatures are azadirachtin (azadirachtin A), salanin, nimbin, 3-tigloylazadirachtol (azadirachtin B), and 1-tigloyl-3-acetyl-11-hydroxymeliacarpin (azadirachtin D) (Govindachari et al., 1996, 2000; Isman, 2006, 1990; Kumar, 1996; Mordue & Blackwell, 1993; Morgan, 2009; Mitchell, 1997). These compounds are responsible for diverse activities such as insect antifeedant, insect growth disrupting, insecticidal, nematicidal, fungicidal, bactericidal, etc. (Kavathekar, 2003). Many other compounds are present in smaller quantities in neem seeds (Hallur et al., 2002; Kumar et al. 1996a, 1996b; Ragasa et al, 1997; Siddiqui et al., 1986). Therefore, most products from neem oil are usually represented by their azadirachtin content although they also contain other compounds (Isman, 2006). Azadirachtin content in crude neem oils varies from negligible to more than 4000 mg/kg (Govindachari et al., 1996; Kumar et al., 1997). Limonoids are soluble in polar and mid-polar solvents and slightly soluble in water. Separation of azadirachtin and other limonoids from neem seed or oil can be carried out by using various methods. Still there are studies regarding the recovery of azadirachtin from defatted neem kernels in order to optimize the extraction processes. The hexane induced precipitation is a potential pre-concentration step in the separation of azadirachtin and other limonoids from neem oils where azadirachtin purities in the powders is 14.85% and 7.34%, respectively, which represented more than 180-fold enrichment from initial content in neem oils of 0.1% (Melwita & Ju, 2010). Sunlight photodegradation is the main factor influencing the rate of it decomposition after tomato greenhouse treatment. Under field conditions azadirachtin and other neem constituents, e.g., salannin, nimbin, deacetylnimbin, and deacetylsalannin, are not persistent. Three days post field application at the dose five times higher than recommended by the manufacturer, residues of azadirachtin A and B were 0.03 and 0.01 mg/kg, respectively, while residues of salannin and nimbin were not detectable (Caboni et al., 2006; 2009).

Azadirachtin is one of the 295 substances of the fourth stage of the review program; the peer review process was subsequently terminated following the applicants' decision to withdraw support for the inclusion of azadirachtin in Annex I of Council Directive 91/414/EC by Decision 2008/941. The reasons for this decision were related to incomplete data regarding metabolism in plants, animals and soil by using radio labeled isotope and toxicological-ecotoxicological data for all limonoids present in extract and formulations were required. Approximately 30 additional studies were performed in order to fulfill missing data. IFOM has been sent different letter to EFSA (European Food Safety Association) (letter\_IFOAMEU\_COM\_lime\_sulphur\_azadirachtin\_EFSA\_16.11.2010) for its inclusion, explaining the importance of azadirachtin for the control one of the most dangerous key pests in organic fruit production the rosy apple aphid (*Dysaphis plantaginea*), and its role in resistance management in organic farming for several pests, e.g. the Colorado potato beetle (*Leptinotarsa decemlineata*). At the time when this chapter was written, azadirachtin was

included in Annex I (SCoFCAH Mar 2011 according to Reg. 33/2008) based on EFSA review (EFSA, 2011) (Table 4).

Pyrethrum is a powder obtained by crushing dried flowers of daisies belonging to the family of Asteraceae such as *Chrysanthemum*. spp., *Pyrethrum*. spp., and *Tanacetum*. spp. Pyrethrum is a mixture of six esters, pyrethrins I and II, (the most abundant), cinerin I and II, and jasmoline I and II. Sunlight photodegradation is the main factor influencing the rate of its disappearance after the application at ten times the dose recommended by the manufacturer disappearance time was 2.3 days (Angioni et al., 2005). Pyrethrum was the only botanical included in the Annex I after the fourth stage of re-evaluation. Cultivation is the most suitable option as a large continuous supply of raw material is needed for commercial production. At the moment Kenya produce 70% of pyrethrin world production. In Europe, France (*AgriPlantes*) has been developed the project for pyrethrin production in order to reduction of fluctuation of price and guaranty of formulation quality by controlled harvesting conditions (Isman, 2008). In Mediterranean basin there are various soils and climatic conditions appropriate for pyrethrin growth but high labor cost is limited factor. In the formulation pyrethrum is mixed with piperonyl butoxide, a synergist used for a wide variety of insecticides formulation, in order to increase the effectiveness. At the present time there is strong restriction regarding the use of piperonyl butoxide in the formulations for organic farming. Synthetic piperonyl butoxide is considered a non-allowed "inert ingredient" and pyrethrum products may be synergized only with piperonyl butoxide from a natural source, such as oil of sassafras according to Soil Association. Imported products treated with synthetic piperonyl butoxide can be certified only as "equivalent".

### 3. Conclusion

The researchers suggest that certain organic management practices are not necessarily more environmentally sustainable than conventional systems. An integrated pest management approach might be more suitable, as such a system is flexible enough to include whichever practices have the smallest environmental impact (Bahlail et al., 2010). In order to optimize environmental sustainability, natural pesticides must be evaluated for their environmental impact in the context of an integrated approach, and that policy decisions must be based on empirical data and objective risk-benefit analysis, not arbitrary classifications. Life cycle toxicity assessment of pesticides used in integrated and organic production showed that organic production represents the least toxic pest-control method. The authors concluded that a careful selection of pesticides used in the production can minimize human toxicity impacts by two orders of magnitude while freshwater ecotoxic impacts can be reduced by up to seven orders of magnitude (Jurasko and Sanjuán, 2011). The new categories of low-risk active substances and basic substances under EU regulations (1107/2009) seem to make legal regulation of low-risk active substances more easily and faster but only after submission of a heavy dossier and the complex evaluation process. In addition, basic substance approach appears to be without significant interest for the industrial production of natural pesticides. Directive 2009/128 on sustainable use creates promising opportunities for non-chemical methods and organic farming which must be taken in consideration in National Action Plans. The bottleneck of new plant protection products legal regulation is a time and cost consuming. Possibly it is time to refocus the attention of the research community toward the development and application of known botanicals rather than screen more plants and isolate further novel bioactive substances by developing new technology in botanicals formulation.

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# Neem Seed Oil: Encapsulation and Controlled Release - Search for a Greener Alternative for Pest Control

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## 1. Introduction

### 1.1 Challenge and need of environment-friendly pesticides

Plants constitute the world's primary food source and so there has been a tendency always to maximize agricultural yields which has been achieved through the development of modified high yield crops and the use of synthetic fertilizers. But this productivity is negatively hampered by plant diseases that contribute significantly to the total crop losses. One third of the world's potential food supplies are estimated to be lost to pre and post harvest pests and diseases. According to FAO [Food and Agricultural Organization] estimates, potential losses worldwide are 35% in the field and 14% in storage bringing the total loss to 50%. In Eastern and Southern Africa, these losses are estimated to be even higher. In order to mitigate these losses, pesticides are being used. The discovery of Bordeaux mixture is significant in the history of chemical control of plant diseases. The use of synthetic pesticides has undoubtedly resulted in increased crop production. However, synthetic pesticides that are used to control plant diseases are doing irreparable harm and damage to our fragile environment. Detrimental health effects, environmental issues, insect resistance or marketing opportunities for organically produced food are well-known arguments against the use of pesticides. The increasing awareness and concern about the impact of agricultural practices on the environment and in food and fiber production is promoting the concept of sustainable agriculture, thus, raising the thrust for bio-pesticides over synthetic pesticides. The increasing incidence of pesticide resistance is also fuelling the search for more environmentally and toxicologically safe and more selective and efficacious new bio-pesticides. Thus, in the context of all these challenges, the modern pesticides will be needed to meet, there arises the need of environment-friendly pesticides in order to boost agricultural production with the ever escalating world population the use of pesticide is absolute.

### 1.2 Background history of pesticides

Combating with the diseases of plant, destructive animals and weeds to protect crops started along with the starting of agriculture by human, which is almost a 10,000 year struggle now. Until the early 20th Century, cultural and mechanical methods augmented by

a diverse range of organic and inorganic substances derived from plants, animal and minerals dominated pest control. Effective and affordable synthetic pesticides gained ground by the mid-20<sup>th</sup> Century, due to the maturing chemical industry (Ujváry, 2001). Most of the insecticidal organophosphates were developed during the early 19<sup>th</sup> century, but their effects on insects, which are similar to their effects on humans, were discovered in 1932. Very little progress has been made with regard to treatment of diseases until about 1882 when the value of lime and copper sulphate as a fungicide was accidentally observed in France and very quickly resulted in the development of the Bordeaux mixture by Millardet. The epochal discovery of Bordeaux mixture is believed to be the first important landmark in the history of chemical control of plant diseases (Berger & Kaitisha, 1995). While reckoned back, the discovery of dithiocarbamates in 1934 and introduction of several other pesticides in the late 1960's are considered to be the two most important events in the one hundred year long history of chemical control of plant disease.

Plant extracts were likely the earliest agricultural biopesticides, as history records that nicotine was used to control plum beetles as early as the 17<sup>th</sup> century (biopesticideindustryalliance.org). Biological control related experiments for insect pests in agriculture date back as far as 1835, when Agostine Bassi demonstrated that White-muscadine fungus (*Beauveria bassiana*) could be used to cause an infectious disease in silkworm. Mineral oils as plant protectants were also reported in the 19<sup>th</sup> century. One of the most important discoveries of bio-pesticides was spores of the bacteria *Bacillus thuringiensis* (Bt), in 1901. Japanese biologist Shigetane Ishiwata isolated Bt from a diseased silkworm. It was rediscovered after ten years, by Ernst Berliner in Thuringen, Germany in a diseased caterpillar of flour moth. Bt was started to use as natural insecticide in 1920 while commercially became available in 1938, in France as Bt product, Sporeine. After this, till 1999 several bio-pesticides were discovered, developed commercially, appeared in world market, yet toxic synthetic chemical insecticides were continuously leading the market of pesticides in the 20<sup>th</sup> century as they were cheap.

### 1.3 Pesticides: Synthetic vs natural

Pests refer to the living organisms that occur unwanted or cause damage to the crops. Insects, mice and other animals, unwanted plants (weeds), fungi, microorganisms such as bacteria, viruses, and prions etc. are included in pests. By definition, according to Food and Agricultural Organization [FAO] and the World Health Organization (WHO: UNO, 1963), a pesticide is a substance or mixture of substances intended to prevent, destroy, repel, or to mitigate any pest including unwelcome species of plants or animals; during production and/or storage, transportation, distribution and elaboration of food; agricultural products or food for animals; or that may be administered to animals to fight ectoparasites. The term also includes herbicides and compounds used as growth regulators, insecticides, fungicides, defoliants, desiccants, and inhibitors of fruit thinning and germination. Pesticides include a wide variety of components and display a broad spectrum of chemical properties (biopesticideindustryalliance.org). Pesticides are classified in different ways. According to the source of origin, they may be of synthetic (Chemical) or natural (bio-pesticide). Another way of naming of pesticides is directly by the type of pests they control, e.g.,

- Algicides to control algae,
- Antifouling agents to kill or repel organisms that attach to underwater surfaces,
- Antimicrobials to kill microorganisms (such as bacteria and viruses).

- Attractants to attract pests (for example, to lure an insect or rodent to a trap). (However, food is not considered a pesticide when used as an attractant.)
- Molluscicides to kill snails and slugs,
- Nematicides to kill nematodes (microscopic, worm-like organisms that feed on plant roots).
- Ovicides to kill eggs of insects and mites.
- Pheromones to disrupt the mating behavior of insects.
- Repellents to repel pests, including insects (such as mosquitoes) and birds.
- Rodenticides to control mice and other rodents etc.

Chemical pesticides are usually classified by their common source or production method. There are four basic types of chemical pesticides that are most commonly used- (i) Organophosphate pesticides (ii) Carbamate pesticides (iii) Organochlorine pesticides (iv) Pyrethroid pesticides . Both organophosphate and carbamate pesticides affect the nervous system by disrupting the enzyme that regulates acetylcholine, a neurotransmitter. DDT and chlordane are the example of organochlorines which have been removed from the market due to their health , environmental effects and their persistence. Pyrethrin, a natural pesticide, is obtained from chrysanthemums. Pyrethroid pesticides are developed synthetic products of pyrethrins.

Organochlorides (DDT, dieldrin and aldrin) have high persistence in the environment of up to about 15 years. Organophosphates (parathion, carbaryl and malathion) have an intermediate persistence of several months Carbamates (Tenik, Zectran and Zineb) have a low persistence of around two weeks. Synthetic pyrethroids are non-persistent, contact and residual acting insecticides (cypermethrin, permethrin) and are suitable for a wide range of crops and target insects. Most pesticides are broad-spectrum, that is they kill all insects in a certain area and may kill other animals like birds and small mammals.

A bio-pesticide, according to FAO definition is - *a compound that kills organisms by virtue of specific biological effects rather than as a broader chemical poison. Differ from biocontrol agents in being passive agents, whereas biocontrol agents actively seek the pest. The rationale behind replacing conventional pesticides with bio-pesticides is that the latter are more likely to be selective and biodegradable.* Bio-pesticides are derived from natural materials like animals, plants, bacteria, and certain minerals. For example, garlic, mint, neem, papaya, canola oil ,baking soda etc. all have pesticidal applications and are considered bio-pesticides. Almost all the bio-pesticides are categorized among the three major groups such as (i) microbial pesticides (ii) plant-incorporated-protectants (PIPs) (iii) biochemical pesticides. According to the U. S. Environmental Protection Agency (USEPA), at the end of 1998, there were approximately 175 registered biopesticide active ingredients and 700 products. At the end of 2001, there were approximately 195 registered bio-pesticide active ingredients and 780 products. The most commonly used bio-pesticides are living organisms (bacteria, viruses and fungi) which are pathogenic for the pest of interest. These include biofungicides (Trichoderma), bioherbicides (Phytophthora) and bioinsecticides (Bacillus thuringiensis).

#### **1.4 Advantages and disadvantages of biopesticides and chemical pesticides**

Botanical pesticides also offer various means of combating insects resistant to products currently available. Almost all synthetic pesticides rely on neurotoxic agents, meaning they attack the nervous system of insects. But tropical plants have over time developed literally

thousands of weapons that kill insects in other ways. For example, the makabuhay vine, which grows in the Philippines, burns insects using a chemical that absorbs sunlight.

Synthetic pesticides are rapidly losing their effectiveness. To date, hundreds of insect species have developed resistance to at least one pesticide formula and a dozen or so species are immune to them all. Some scientists fear that pesticide manufacturers will eventually be unable to outwit insects. Chemical pesticides do suffer from several disadvantages due to which the use of bio-pesticides is preferred. Some of the disadvantages associated with the chemical pesticides are-

- i. Environmental pollution,
- ii. Creating health hazards due to the presence of the pesticide residues in food, fiber and fodder
- iii. Development of resistance by the insects.

According to World Health Organization estimates, up to 20,000 people die of pesticide poisoning in the Third World each year. Some synthetic pesticides are accumulating in soil and groundwater where they threaten the health of entire ecosystems.

In contrast, the bio-pesticides offer several advantages over synthetic pesticides which are

- i. Bio-pesticides are less harmful than chemical pesticides because bio-pesticides do not leave harmful residues,
- ii. Bio-pesticides generally target one specific pest or a small number of related pests in contrast to broad spectrum chemical pesticides which affect, apart from the pest, other beneficial insects, birds, mammals or nontarget species.
- iii. Bio-pesticides are effective in smaller quantities, decompose quickly and do not cause environmental problems.
- iv. When used in Integrated Pest Management programs, bio-pesticides can greatly reduce the use of conventional pesticides, while the crop yield remains high.
- v. Bio-pesticides are often cheaper than chemical pesticides.

Few limitations of bio-pesticides -

- i. They are highly specific, due to which exact identification of the pest/pathogen or use of multiple pesticides may be required.
- ii. They often suffer variable efficacy due to the influences of various biotic and abiotic factors (since bio-pesticides are usually living organisms, which bring about pest/pathogen control by multiplying within the target insect pest/pathogen)

### **1.5 Modification of pesticides with polymers-controlled release pesticides**

A significant improvement can be experienced within the agricultural and pharmaceutical sectors by using such environmentally friendly technologies that mainly target the production of healthy, nutritious, quality foodstuff as well as pharmaceutical products by taking environmental characteristics into account, and adapting to them. To achieve such goals mentioned above and to bring sustainable, environmentally human economical systems to the forefront, scientists today are more than ever before being challenged to provide environmentally benign, more economical, and more efficient products for the health and well being of mankind. "Controlled delivery" technologies have emerged as an approach with promise not only to utilize resources in the maximum efficient way but also for the prevention of pollution. Moreover, if the resource is natural or renewable polymer, then it will draw attention as more new, more economical and more eco-friendly source for use of mankind.

Controlled delivery may be defined as a technique or method in which active chemicals are made available to a specified target at a rate and duration designed to accomplish an intended effect (Kyodoneus, 1980). Controlled delivery occurs when a polymer, natural or synthetic, is judiciously combined with an active agent in such a way that the active agent is released from the material in a predesigned manner. The release of the active agent may be constant or cyclic over a long period. It may be triggered by the environment or other external events. In any case, the purpose behind controlling the drug delivery is to achieve more effective therapies while eliminating the potential for both under and overdosing (Peppas, 1997). During the last decade, controlled release technology has received increasing attention in the face of a growing awareness of those substances which are excessively toxic and sometimes ineffective when administered or applied by conventional means. Conventional application of agricultural chemicals provides an initial concentration far in excess to that required for immediate results in order to assure the presence of sufficient chemical for a practical time period. Such overdosing wastes much of the chemical's potential and all too often causes toxicity problems for non target organisms (Kyodoneus, 1980). To improve the efficiency of agricultural pesticides for longer time, the use of controlled release technology is effective. Many of the controlled release formulations are highly efficient in sustaining the release of the biologically active components. It should be recognized that the polymeric material to be used in controlled release must degrade to some fashion before there can be any environmental impact in the chemical, biochemical or biological sense. However, it is encouraging to use naturally occurring polymers or degradable synthetic polymers. To ensure adequate pest control within a suitable period, pesticides are applied in concentrations greatly exceeding those required for control of the target organism, thus increasing the likelihood of runoff or leaching and pollution of surface or groundwater. Controlled release formulations have the potential to reduce the environmental problems associated with the application of pesticides. There are many advantages of controlled release formulations including an increased safety to the user and non-target organisms, a reduction in the amount of pesticides applied, reduced leaching of pesticides and increased persistence of the active ingredient.

The selection of an appropriate coating agent depends on the nature of the agrochemical, environmental aspect of the agent as well as cost effectiveness of its incorporation (Karak, 1999). The different approaches for controlling the release of agrochemicals are-

- i. Controlled release by applying active coating of wax (Herdrie, 1976), sulfur (Miller & Donahue, 1992), polymer (Hansen, 1966a & Hansen, 1966b), neem (Bains et al., 1971), Lac (Kenawy, 1998a). Other coatings include coal tar, mahua oil, metal salts etc.)
- ii. Controlled release by polymeric adduct formation
- iii. Controlled release by incorporation of Nitrification inhibitor
- iv. Controlled release by other techniques.

Controlled release by polymeric adduct formation is one of the best methods so far commercialized. Here, monomer or prepolymer or oligomers are used for adduct formation with active agent by cross linking reactions. Selection of cross linker, time of cross linking and temperature of cross linking reactions are very much important for controlling the rate of release of the active agent.

Over the last few years, there have been plenty of polymeric agrochemical formulations including pesticide, herbicides, growth regulators and fertilizers. However, the major drawback with these techniques is the residual polymers that might accumulate in the soil

and become harmful to the soil and plants. Therefore, an attempt to produce system, which can help to utilize the whole agrochemical and also conserve the water, soil, and economy, is getting considerable interest in the field of modern research.

### **1.6 Advantages of controlled-release pesticides**

The need of controlled delivery device can be understandable by their advantages. Controlled-release technology has many advantages irrespective of its preparation and applications. In this article, the concentration is given mainly on the agrochemicals though other applications are also equally important.

Controlled delivery technology offers several advantages (Kenawy,1998a; Akelah, 1996) over conventional formulations. These can be summarized as follows:

Maintain of constant level of active agent: In conventional formulation, the active agent tends to release first at an overdose then undergoes to the local environment. Controlled releases offer a solution for this problem by maintaining the concentration of active agent between the minimum effect and toxic level.

- Use of smaller dose: This includes more efficient utilization of active agents, resource saving, safety etc.
- Reduces the loss by limiting leaching, volatilization and degradation.
- Minimizes potential negative effect (if there any) associated with overdose.
- Economical because less active material is needed due to reduction of excessive amounts for a given time interval.
- Facilitation of handling and masking of any odor.
- Toxic material becomes chemically nontoxic when attached with polymers.
- Extension of the duration of the activity of less-persistent or non-persistent active agent unstable within the aquatic environment by protecting them from leaching and degradation, hence aiding the practical applications of these materials.
- Reduction of phytotoxicity by lowering the mobility of the active agent in soil, hence reducing its residue in the food chain.
- Convenience: it converts liquids to solids; hence it results in easily transported materials with the reduction of flammability.

### **1.7 Disadvantages of controlled release technology**

Although the advantages of controlled release technology are impressive yet the merits of each application have to be examined individually, and the positive and negative effects weighed carefully before large expenditures for developmental work are committed. In other words, controlled release is not a panacea, and negative effects may, at times, more than offset advantages. Some of the disadvantages of controlled release or the areas that require a thorough appraisal include (McCormick, 1987; Cardarelli, 1980)

- The cost of controlled release preparation and processing is substantially higher than the cost of standard formulations, but this could be compensated by minimizing the repeated applications.
- The fate of using excessive amounts of polymers as matrix and its effect on the environment is very important. This could be eliminated by using biodegradable polymers and improving weight efficiency by using polymers that may be beneficial to the environment when degraded.

- The fate of polymer additives, such as plasticizers, stabilizers, antioxidants, fillers, etc. left behind, once application is over, may cause some impact on environment.
- The polymer degradation products generated by heat, hydrolysis, oxidation, solar radiation and biological degradation may damage the environment.

### 1.8 Mechanisms and types of controlled release systems

Polymer controlled release formulations are divided into two broad categories, physical and chemical combinations. In physical combination, the polymer acts as a rate-controlling device while in chemical combination, it acts as a carrier for the active agent (Akelah, 1996). The choice of the best system to release the active agent in sufficient quantity to achieve the desired effect with minimum biological or ecological side effects depends on many considerations. These include the properties of the active agent, its physicochemical interactions with the polymer; the polymer nature (cross-linking degree, thermal behavior, and compatibility with the active agent); stability of the combination during processing; desired release rate; shape and size of the final product; duration; seasonal conditions; cost and ease of formulation and application.

#### 1.8.1 Physical combinations

Two different approaches have been reported in literature in the case of the physical combination of biologically active agent with polymeric materials. Firstly, the biologically active agent can be encapsulated in a polymeric material in which the release of the active agent is controlled by Fick's law of diffusion through the micro pores in the capsule walls. The equation is given below:

$$R_d = dM_t / dt = A / h D (C_s - KC_e)$$

Where  $M_t$  is the mass of the agent released,  $dM_t/dt$  is the steady state release rate at time  $t$ ,  $A$  and  $h$  are the surface area and thickness through which diffusion occurs,  $D$  is the diffusion coefficient of the active agent in the polymer,  $C_s$  is the saturation solubility of the active agent in the polymeric membrane,  $K$  is the partition coefficient of the active agent and the medium which surrounds the device,  $C_e$  is the concentration of released active agent in the environment.

In the second approach, biologically active agent is heterogeneously dispersed or dissolved in a solid polymeric matrix, which can be either biodegradable or non-biodegradable. The release of the active agent is generally controlled by diffusion and erosion (Kenawy et al., 1992). Release by erosion is a surface area dependent phenomenon, and the general expression which describes the rate of release ( $R_r$ ) by an erosion mechanism is:

$$R_r = dM / dt = K_e C_o A$$

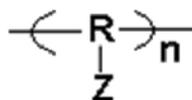
Where  $K_e$  is the erosion rate constant,  $A$  is the surface area exposed to the environment,  $C_o$  is the loading of active agent in the erodible matrix. The design of such physical combinations is generally not influenced by the structure of the active agent molecule.

#### 1.8.2 Chemical combinations

An active agent can be chemically attached to a polymer either as a pendant side groups, or as a part of the main backbone. Obviously only those biologically active agents, that contain

a structural moiety with at least one reactive functional group suitable for use as link to the functionalized polymer, can be used in this technique (Kenawy et al.,1992).

Polymeric chemically bonded active agents can be prepared by two synthetic methods. The first involves chemical modification of a preformed polymer with the desired active agent via a chemical bond, leading to a polymer having the active species linked to the main chain as a pendant group.



Z= active agent, R= monomer unit

Fig. 1(a). Active agent attached to polymer as side chain

The second method requires synthesis and polymerization of a biologically active monomer which leads either to polymer having the active group as repeat units in the main chain backbone.

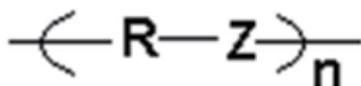


Fig. 1(b). Active agent in the polymer backbone

The chemically attached active agent is released from the polymer by the hydrolytic cleavage of the active agent-polymer linkage or via a slow degradation of the polymer itself induced by the water in the surrounding environment. The kinetic expressions which describe the release rate depend on the extent of branching and crosslinking of the macromolecules, i.e., on whether the cleavage reaction occurs on the surface of an insoluble particle or in the matrix.

Release of the active agent is usually dependent on surrounding environmental conditions that break the linkages via chemical attack (hydrolytic by moisture; thermal / photo by sunlight) or biological degradation (enzymatic by microorganisms), pH of the medium, ionic strength of the dissolution medium, competing ions, electrolyte concentration and temperature etc.

### 1.9 Release mechanisms

The release profile looked for a controlled delivery system is generally the steady state release of active agent or a zero-order release mechanism kinetically. The release rate in such systems is not affected by the amount of active agent released or not released at any moment (Paul, 1976). However, this type of release profile do not exhibit by many controlled delivery systems. The controlled delivery systems can be classified into several classes depending on the mechanism of release rate. According to Fan and Singh (Fan & Singh, 1989), the major release mechanisms involved in controlled delivery formulations are:

i) Diffusion ii) Swelling iii) Osmosis and iv) Erosion or chemical reaction controlled

#### i. Diffusion-controlled systems

Diffusion of active agent through the polymer is the rate-determining step in these systems. The polymer is hardly affected by the environmental factors. There are mainly two types of diffusion-controlled release systems.

*(a) Reservoir systems*

Here, the active agent is released out to the environment by diffusion, through the micropores of the capsule walls. The active agent is surrounded or encapsulated by a thin layer of polymeric membrane. Commonly used techniques in drug delivery systems are microencapsulation, nanoencapsulation, coacervation and spray encapsulation (Nagpal et al., 2001).

*(b) Monolithic systems*

In these systems, the active agent is dispersed or dissolved in the polymer. Release of the active agent may take place either by diffusion or leaching along with diffusion, if there is interaction between polymer and the environment. If a soluble additive is incorporated in the polymer matrix, the environmental fluid can easily penetrate the matrix by dissolving the additive and interconnected channels will be formed, through which the release would be easy. This technique has enormous applicability because, these types of physical combinations need not be influenced by structure of the active agent or polymer.

**ii. Swelling controlled systems**

Here, the dispersed or dissolved active agent in polymer matrix is unable to diffuse to any considerable extent. The active agent is released out slowly when the polymer system gets into contact with a compatible solvent or fluid in the environment and swelling takes place. Examples used in such systems are Poly(hydroxyl methyl methacrylate), polyacrylamide and poly(ethylene glycols) etc. (Omolo et al., 2004).

**iii. Osmosis controlled system**

Osmotic force is the driving force in osmosis-controlled systems. Such systems generally consist of a solid and water-soluble active agent, which is enclosed by a water-permeable, but active agent impermeable polymer membrane with a small opening. Water is transported into the core by permeation and hydrostatic pressure will be built up in the core and subsequently, the dissolved active agent comes out (Fan & Singh, 1989). In the field of controlled delivery, several newer techniques and methods like targeted delivery, viable cell immobilization, microspheres and nanoparticles/nanocomposites are finding great research interest (Hwang et al., 1985; Dua et al., 1996).

**iv. Erosion or Chemical reaction controlled system**

*(a) Erosion-controlled system*

The release of the active agent occurs here by erosion of the polymer. The active agent is physically immobilized in the polymer matrix. Active agent release rate is generally proportional to the erosion rate of the polymer matrix which undergoes surface erosion. A zero order release can be achieved in these systems if the erosion rate is constant and matrix dimension remains unchanged.

*(b) Chemical reaction controlled system*

Here, the active agent is released only when the polymer active agent bond is cleaved or the polymer is degraded. A zero order release profile may be obtained when the active agent is a co-monomeric unit in polymer backbone and release occur by polymer degradation.

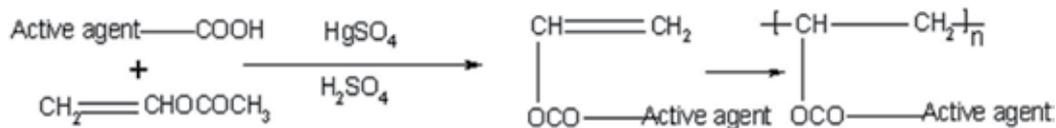
**1.10 Manufacturing techniques of controlled release formulations**

There are several techniques for the preparation of controlled release formulations. Among them, the most widely used techniques are discussed below:

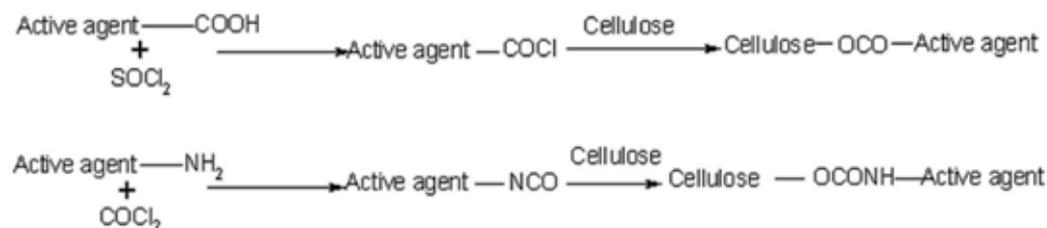
### 1.10.1 Chemically bound

Chemically bound active agents are of two types:

Those which are prepared by attaching a polymerizable site to the active ingredient, followed by polymerization of the new derivative. For example,



Those which are prepared by chemically binding derivatives of active ingredients to a suitable polymer. For example, active agents containing carboxylic functionalities have been reacted to form acid chlorides, which in turn are attached to natural polymers through its hydroxyl group. Similarly, active agent containing primary amino functionality were reacted with phosgene to form isocyanates, which in turn were attached through the hydroxyl group of natural polymers.



Such chemically bound combinations have found application in forestry and agronomic crops. The rate of release can be increased by lowering the molecular weight or increasing the hydrophilicity of the polymer carrier. The rate of release also depends upon the degree of substitution of the herbicide moiety within the polymer, the pH of the hydrolysis medium and the size of the particles.

### 1.10.2 Matrix encapsulation technique

The most common and widely used method for the encapsulation of active agents as controlled release product is the matrix encapsulation technique. Controlled release products obtained by this technique lack a distinctive wall surrounding each particle of the active ingredient. The active ingredient is dispersed within a polymer and becomes entrapped within many small cells of a continuous matrix. The active ingredient may be dissolved or suspended in various polymers to yield ribbons, sheets or granules. Often an excipient such as an inorganic filler is added to such formulations.

### 1.10.3 Microencapsulation

Microencapsulation is the coating of small solid particles, liquid droplets, or gas bubbles with a thin film of coating or shell materials. The product so obtained is termed as microcapsules. Microcapsules are small particles that contain an active agent or core material surrounded by a coating or shell. At present, there is no universally accepted size range that particles must have in order to be classified as microcapsules. However, many

workers classify capsules smaller than 1  $\mu\text{m}$  as nanoparticles and greater than 1000 $\mu\text{m}$  as macrocapsules. Commercial microcapsules typically have a diameter between 3 and 800  $\mu\text{m}$  and contain 10-90 wt% core. A wide range of core materials has been encapsulated, including adhesives, agrochemicals, live cells, active enzymes, flavors, fragrances, pharmaceuticals and inks. Most capsule shell materials are organic polymers, but fats and waxes are also used.

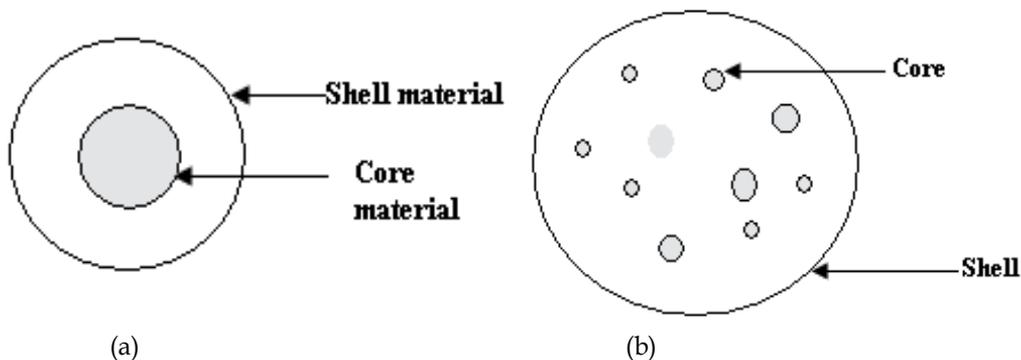


Fig. 2. Schematic diagrams of two representative types of microcapsules: (a) Continuous core/shell microcapsule; (b) Multinuclear microcapsules [Courtesy of C.Thies]

Microcapsules can have a variety of structures. Some have a spherical geometry with a continuous shell as shown in Fig.2(a). Others have an irregular geometry and contain a number of small droplets or particles of core material Fig.2(b).

Within the broad category of microparticles (Fig.3), 'microspheres' specifically refer to spherical microparticles and 'microcapsules' applies to microparticles which have a core surrounded by a material which is distinctly different from that of the core. The core may be solid, liquid or even gas. A microparticle usually refers to a homogeneous mixture of the polymer and active agent, whereas microcapsules have at least one discrete domain of active agent.

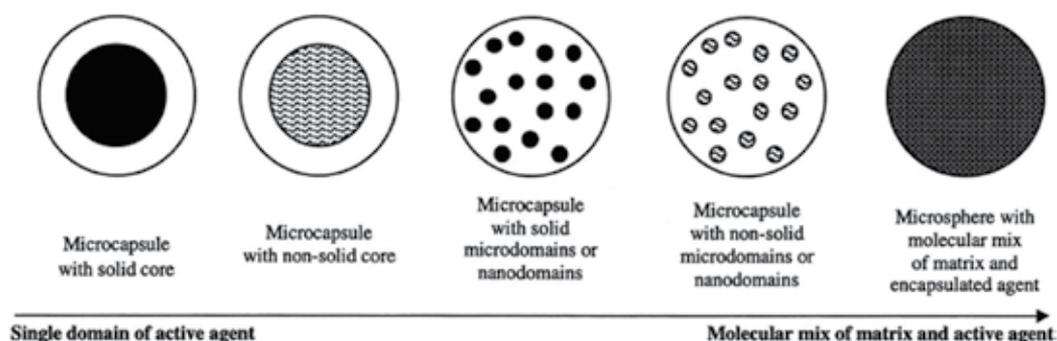


Fig. 3. Different categories of microparticles (Birnaum & Peppas 2004)

A steady stream of encapsulation technologies continues to appear in the patent literature. Some are simply modifications or improvements of the established technologies, where as others new technologies are confined to laboratory scale.

Thus, numerous preparation technologies available for the encapsulation of core material have been reported (Benita, 1996; Arshady, 1999; Sliwka, 1975; Ranny, 1969). Some of the important processes used for microencapsulation are summarized in Table 1.

Chemical processes	Physical processes	
	Physico-chemical	Physico-mechanical
<ul style="list-style-type: none"> <li>Suspension, dispersion and emulsion polymerization</li> </ul>	<ul style="list-style-type: none"> <li>Coacervation</li> <li>Layer-by-layer (L-B-L) assembly</li> <li>Sol-gel encapsulation</li> </ul>	<ul style="list-style-type: none"> <li>Spray-drying</li> <li>Multiple nozzle spraying</li> <li>Fluid-bed coating</li> </ul>
<ul style="list-style-type: none"> <li>Polycondensation</li> </ul>	<ul style="list-style-type: none"> <li>Supercritical CO<sub>2</sub>-assisted microencapsulation</li> </ul>	<ul style="list-style-type: none"> <li>Centrifugal techniques</li> <li>Vacuum encapsulation</li> <li>Electrostatic encapsulation</li> </ul>

Table 1. Different techniques used for microencapsulation.

### 1.10.3.1 Solvent evaporation and extraction based process

#### 1.10.3.1.1 Phase separation or coacervation

IUPAC defined coacervation as: "The separation into two liquid phases in colloidal systems. The phase more concentrated in colloid component is the coacervate, and the other phase is the equilibrium solution." The first systematic approach of phase separation, that is, partial desolvation of a homogeneous polymer solution into a polymer-rich phase (coacervate) and the poor polymer phase (coacervation medium) was realized by Bungenberg and colleagues (Bungenberg de Jong, 1949; Bakkan & Anderson, 1976). These authors termed such a phase separation phenomenon "coacervation".

Currently, two methods for coacervation are available, namely simple and complex processes. The mechanism of microcapsule formation for both processes is identical, except for the way in which the phase separation is carried out. In simple coacervation, a desolvation agent is added for phase separation, whereas complex coacervation involves complexation between two oppositely charged polymers.

#### (a) Simple Coacervation

Aqueous solutions of water-soluble polymers are phase separated in aqueous media when sufficient salt is added to such solutions. This phenomenon is called simple coacervation. As long as phase separation produces a liquid polymer-rich phase, simple coacervation can be used to produce microcapsules (Bakkan & Anderson, 1976). Microcapsules with gelatin, or poly(vinyl alcohol) or different natural polymers shell have been produced in this manner.

#### (b) Complex coacervation

Complex coacervation occurs in aqueous media and is used to encapsulate water-immiscible liquids or water-insoluble solids (Ghosh, 2006). Complex coacervation is carried out by mixing two oppositely charged polymers in a solvent (usually water); the process is shown schematically in Fig. 4.

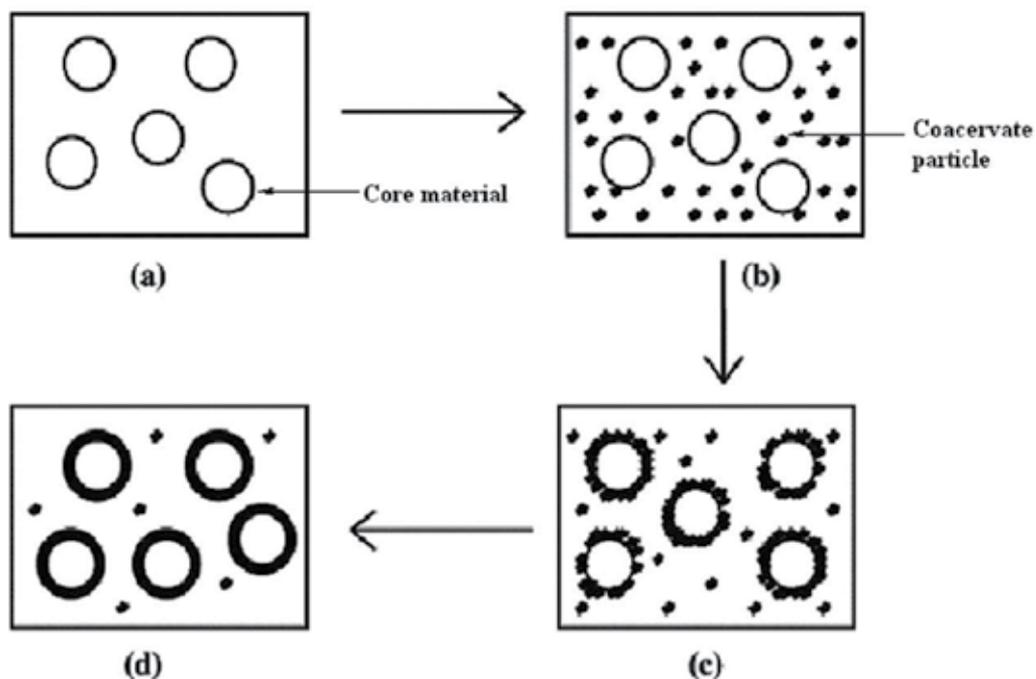


Fig. 4. Schematic representation of complex coacervation process. (a) Core material dispersion in shell polymer solution; (b) separation of coacervate from solution; (c) coating of core material by microdroplet of coacervate; (d) coalescence of coacervate to form continuous shell around core particles.

The three basic steps in complex coacervation are: (i) preparation of the dispersion or emulsion; (ii) encapsulation of the core; and (iii) stabilization of the encapsulated particle. The core material (usually an oil) is first dispersed into a polymer solution (e.g., a cationic aqueous polymer). The second polymer (anionic, water soluble) solution is then added to the prepared dispersion. Deposition of the shell material onto the core particles occurs when the two polymers form a complex. This process is triggered by the addition of salt or by changing the pH, temperature or by dilution of the medium. The shell thickness can be obtained as desired by controlled addition of the second polymer. Finally, the prepared microcapsules are stabilized by crosslinking, desolvation or thermal treatment. Complex coacervation is used to produce microcapsules containing fragrant oils, liquid crystals, flavors, dyes or inks as the core material. Porous microcapsules can also be prepared using this technique. When using this technique, certain conditions must be met to avoid agglomeration of the prepared capsules (Matiowitz, 1999).

### 1.10.3.2 Emulsion based process

#### 1.10.3.2.1 Single emulsion process

This process involves oil-in-water (o/w) emulsification. The o/w emulsion system consists of an organic phase comprising of a volatile solvent with dissolved polymer and the active agent to be encapsulated, emulsified in an aqueous phase containing a dissolved surfactant. A surfactant is included in the aqueous phase to prevent the organic droplets from coalescing once they are formed. The polymer-solvent-active agent solution is emulsified

(with appropriate stirring and temperature conditions) to yield an o/w emulsion. The emulsion is created by using a propeller or magnetic bar for mixing the organic and aqueous phases.

Once the emulsion is formed, it is subjected to solvent removal by either evaporation or extraction process to solidify the polymer droplets. In the case of solvent removal by evaporation, the emulsion is maintained at a reduced pressure or at atmospheric pressure and the stir rate is reduced to enable the volatile solvent to evaporate. The organic solvent leaches out of the droplet into the external aqueous phase before evaporating at the water air interface. In the case of extraction, the emulsion is transferred to a large quantity of water or other quench medium, into which the solvent associated with the oil droplets is diffused out. Several reports are available that discusses on the formation of microspheres, for increase of encapsulation efficiency and release pattern of microspheres formed by this method (Jeyanthi, 1996; Arshady, 1991; Cavalier et al., 1986; Jalil, 1990; Tsai et al., 1986).

#### *1.10.3.2.2 Double emulsion process*

The problem with encapsulating hydrophilic active agent is the loss of the active agent to the external aqueous phase during the formation of the microparticle. Along with the loss of active agent to the external phase, the remaining active agent may migrate to the surface of the droplet before solidifying. To minimize these problems, the organic droplets should be solidified into microparticles as quickly as possible following their formation (Thies, 1992). This is achieved by using a viscous organic solution of polymer and active agent and a large secondary volume of water that attracts the organic solvent into the aqueous phase immediately, thus leaving the microparticle with the encapsulated active agent. The viscous dispersed phase minimizes the volume of organic solvent, facilitating its quick removal from the droplet and also makes it more difficult for the solid active agent particles/crystal to migrate to its surface, resulting in a more homogeneous distribution of the active agent within the particle. Another alternative to encapsulate hydrophilic active agent is to employ the water-in-oil-in water (w/o/w) emulsion process. An aqueous solution of the active agent is added to an organic phase consisting of the polymer and organic solvent with vigorous stirring to form the first w/o emulsion. This emulsion is then dispersed in another aqueous phase containing more surfactant to form the w/o/w emulsion. The problem with this type of emulsion occurs when the inner emulsion is not sufficiently stabilized, resulting in loss of aqueous droplets containing active agent to the external aqueous phase. The choice of surfactants that can be used to stabilize the inner emulsion is limited to materials that will dissolve in the organic solvent. Typically, the fatty acid esters of polyoxyethylene or sorbitan are used due to their high solubility in organic solvents and good biocompatibility.

### **1.11 Neem (*Azadirachta Indica* A. Juss.)**

Neem is a fascinating tree. This plant may usher in a new era in pest control, provide millions with inexpensive medicines, cut down the rate of human population growth, and perhaps even reduce erosion, deforestation, and the excessive temperature of an overheated globe (National Research Council, 1992). Neem played an important role in the life of ancient Indian people. The efficacy of neem as a medicine has been documented in several different ancient treatises. Neem has achieved a relatively wide distribution in the tropical areas of Asia, Africa, South America, and Oceania.

More than 135 compounds have been isolated from different parts of neem and several reviews have also been published on the chemistry and structural diversity of these compounds (Koul,1990; Chatterjee & Pakrashi, 1994; Mitra & Patel, 1963; Taylor, 1984; Champagne et al. 1992; Kraus, 1995; Devakumar &Dev, 1996; Govindachari, 1992). The compounds have been divided into two major classes: isoprenoids and others (Devakumar &Dev,1996). The isoprenoids include diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and csecomeliacins such as nimbin, salanin and azadirachtin. The nonisoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, etc. The details of the chemistry of various compounds falling under these groups have been reviewed (Kraus, 1995; Devakumar &Dev, 1996).

Azadirachtin was first isolated based on its exceptional antifeedant activity in the desert locust, and this substance remains the most potent locust antifeedant discovered to date. Some entomologists concluded that neem had remarkable powers for controlling insects that it would usher in a new era in safe, natural pesticides. Extracts from its extremely bitter seeds and leaves might be the ideal insecticides. They attack many pestiferous species; seem to leave people, animals, and beneficial insects unharmed; biodegradable; and they appear unlikely to quickly lose their potency to a build up of genetic resistance in the pests. Neem seems to provide nontoxic and long-lived replacements for some of today's most suspect synthetic pesticides.

Childs et al.(Childs et al., 2001) reported that there were at least 12 brands of neem pesticides registered in India. Neem-based pesticides refer to those formulated pesticides containing azadirachtin as the major active compound. The most popular product currently seems to be 0.3% azadirachtin EC (emulsifiable concentrate) although some companies are producing products with up to 5% azadirachtin content (Childs et al, 2001).

### **1.11.1 Neem seed oil components**

The neem seed kernel is very rich in fatty acids often up to 50 percent of the kernel's weight. Neem seed oil is very bitter with a garlic/sulfur smell and contains vitamin E and other essential amino acids. Studies of the various components of the oil have been found the following fatty acids (i) oleic acid - 52.8% (ii) stearic acid - 21.4% (iii) palmitic acid - 12.6%

(iv)linoleic acid - 2.1% (v) various lower fatty acids - 2.3%. The percentages vary from sample to sample depending on place and time of collection of the seeds. Neem oil is an excellent moisturizing oil that contains compounds with historical and scientific validity as medicinals. Use of the oil for cosmetics and medicines has been limited by its strongly bitter taste and sulfur/garlic smell.

### **1.11.2 Biologically active compounds from neem seed oil**

Two types of insecticides can be obtained from seeds of the Indian neem tree, *Azadirachta indica* (Maliaceae) (Isman, 2006). Neem oil, obtained by cold-pressing seeds, can be effective against soft-bodied insects and mites but is also useful in management of phytopathogens. Apart from the physical effects of neem oil on pests and fungi, disulfides in the oil may likely to contribute to the bioactivity of the material. Neem seeds actually contain more than

a dozen azadirachtin analogs, but the major form is azadirachtin. Seed extracts include considerable quantities of other triterpenoids, notably salanin, nimbin, and derivatives thereof. Besides azadirachtin, the role of all other biologically active compounds has also been in high esteem.

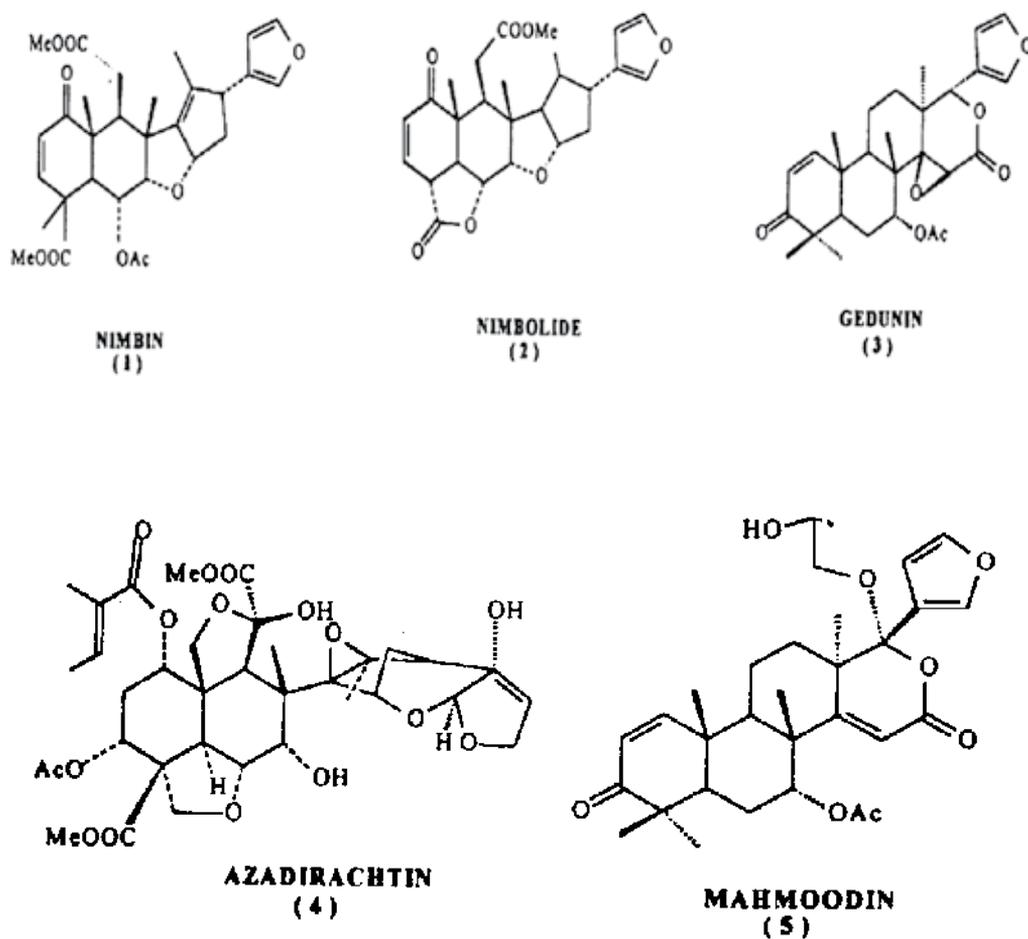


Fig. 5. Major biologically active compounds present in neem seed oil

Neem seeds typically contain 0.2 to 0.6% azadirachtin by weight. From the crude, some tetranortriterpenes, including nimbin, nimbinin, nimbidinin, nimbolide and nimbidic acid have been isolated (Mitra et al., 1971). These entire compounds possess valuable pesticide, repellent as well as medicinal properties. Nimbidin shows antifungal activity by inhibiting the growth of *Tinea rubrum* (Bhargava et al.1970). In vitro, it can completely inhibit the growth of *Mycobacterium tuberculosis* and has been found to be bactericidal (Murthy, & Sirsi, 1958). The spermicidal activity of nimbidin and nimbin (1) is reported in rats. Nimbolide (2) is reported to inhibit the growth of *Plasmodium falciparum* (Rochanakij et al., 1985; Khalid et al., 1989). Nimbolide also shows antibacterial activity against *S. aureus* and *S. coagulase* (Rojanapo et al., 1985). Gedunin (3), isolated from neem seed oil is reported to possess both antifungal (Rao et al. 1977) and antimalarial (Khalid et al., 1989) activities. Azadirachtin (4), highly oxygenated C-secomeliacins isolated from neem seed and have strong antifeedant activity (Butterworth & Morgan, 1968). It can inhibit the development of malarial parasites (Jones et al., 1994). Mahmoodin (5), a deoxygedunin isolated from seed oil, is reported to possess moderate antibacterial action against some strains of human pathogenic bacteria (Devakumar &Dev, 1996).

### 1.11.3 Need of encapsulation of NSO pesticide

Search for environmentally sound pesticides received an impetus following the publication of 'Silent Spring' by Rachel Carson in 1962. It was around this period that Pradhan et al.(Pradhan et al., 1962) reported the feeding deterrent property of neem seed kernel suspension against desert locust, *Schistocerca gregaria*. Subsequently, several bioactive ingredients notably meliantriol and azadirachtin were isolated from various parts of the tree which aroused worldwide interest in the insecticidal bioactivity of the neem tree and thus a substantial work has been carried out globally to establish the insecticidal activity of neem. Isolation of several bioactive ingredients notably meliantriol and azadirachtin from various parts of the tree aroused worldwide interest in the insecticidal bioactivity of the neem. In fact neem has been recommended by most of the authors as a desirable method of pest control and repellent as it does not cause harm to the people and the plant but selectively affects the insects. Neem products affect different physiological processes in insects, e.g. metamorphosis including insect growth regulation, adult fertility, toxicity, and they also affect behaviour, having antifeedant and oviposition deterrent effects. This way the neem extract could influence over 200 species of insects many of which are resistant to modern pesticides. Several review papers have been published regarding the use of neem pesticides in cotton pest management, other vegetable pest management, mosquito repellent activity etc. and almost all the published papers have confirms its strong pesticidal activity.

Though Neem Seed Oil (NSO) has been in high esteem due to its potent pesticide properties, yet, application of it to the soil is limited due to its liquid nature. This limitation can be overcome by the encapsulation of NSO to give rise to a solid formulation. Moreover, azadirachtin, the key component of NSO is rapidly degraded by sunlight. Thus, encapsulation of NSO not only gives it a solid form but also protects it from sunlight. Another reason behind the encapsulation and controlled release of NSO is that, along with potent pesticide activity, it also shows toxicity in some cases. The toxic effect is observed to fish like tilapia and carp and also in human in several isolated cases. NSO intoxication by human produces nausea, vomiting, acidosis, encephalopathy, etc. Microencapsulation and controlled delivery technology seems to be the most useful technique to minimize this toxicity and make an appropriate as well as efficient use of this effective natural resource NSO.

## 2. Literature review

### 2.1 Controlled release agrochemicals

The different classes of polymers viz., elastomers, plastics and fibres were extensively used in agriculture for varied purposes. The major application fields included CR pesticides, herbicides and fertilizers, soil conditioning, plant protection, seed coating and gel planting (McCormick, 1984). Several pesticides like sevin, dimethoate, ethyl trithion, methyl trithion, diazinon, malathion, chloropyrifos and temephos could be incorporated in plasticized poly(vinyl chloride) to obtain CR products (Cardarelli, 1980).

El-Refaie and coworkers (Kenawy, 1998b) prepared controlled release formulations based on crosslinked polyacrylamide derivatives. The release data of the herbicide 2,4-D in vitro from formulations were described. Micro- or macro encapsulation of active agents using polymers is one of the methods widely used for the preparation of CR products. Condensation polymerization reactions yielding polyamide, polyester, polyurea, polyurethane, polycarbonate and polysulphonamide could be well utilized to prepare CR formulations. Crosslinking of the polymer wall provided durable and storage stable capsules (Lowell et al., 1977; Koestler, 1980). Several controlled release pheromone formulations were also synthesized by microencapsulation. The utilization of starch as a polymer matrix for CR agrochemical was reported (Shasha, 1980). Pfister, Bahadir and Korte (Pfister et al., 1986) claimed another system based on calcium alginate with a series of herbicides. Starch was used as an encapsulating material for S-ethyldipropylthiocarbamate (EPTC), atrazine and trifluralin (Shasha et al., 1981a; Trimmell et al. 1984; Shasha, 1981b). Teft and Friend (Teft & Friend, 1993) synthesized controlled-release polymeric microspheres of herbicides Dicamba (DA) based on ethylcellulose, polyarylsulfone or a combination of the two.

Kulkarni, Kumbar, Dave and Aminabhavi (Kumbar et al., 2001a) reported the release kinetics and encapsulation efficiency of urea-formaldehyde (UF) crosslinked matrices of starch, guar gum (GG) and starch+guar gum (St+GG) for controlled release of solid (chloropyrifos) and liquid (neem seed oil) pesticides. In another report, Kulkarni and his group (Kulkarni et al., 2002) claimed the synthesis of novel polymeric sodium alginate interpenetrating network (IPN) beads for the controlled release of chloropyrifos. They also synthesized IPN beads of poly(vinylalcohol)-g-poly(acrylamide) with sodium alginate for the controlled release of cypermethrin pesticide (Kumbar & Aminabhavi, 2002).

Marei et al. (Marei et al., 2000) compared carbofuran encapsulated controlled release formulation with the granular formulation in term of mobility of carbofuran and reported that leaching potential of alginate formulation decreased more than nine times compared with granular formulation. Chitosan gel beads and film were assessed for their ability to control the release of herbicide atrazine and fertilizer urea (Teixeira et al., 1990). Elbahni et al. (Elbahri & Taverder, 2005) developed a technique for encapsulation of herbicide inside ethyl cellulose microsphere and evaluated the shape and size of microspheres by scanning electron microscopy. Polysaccharides like cellulose, chitin, amylose and amylopectin were found to be useful natural polymers for the CR formulations of 2,4-dichlorophenoxyacetic acid and metribuzin (McCormick, 1984). CR formulation of kraft lignin and propachlor had been successfully prepared by Wilkins and Blackmore (Wilkins & Blackmore, 1987). It was reported that rice husk lignin could be combined with 2,4-dichlorophenoxyacetic acid (Kenawy et al., 1992). The application of lignin in CR formulations was reviewed by Wilkins (Wilkins, 1983). Zhu and Zhuo (Zhu & Zhuo, 2001) synthesized a new starch-g-poly(butylacrylate) for encapsulating carboxylic group containing herbicides. Polymerizable

derivatives of pesticides containing acid groups could be prepared by a reaction with alcohols having a vinyl group (Harris & Post, 1974; Harris, 1980). Copolymers of vinyl 2,4-dichlorophenoxyacetate and trimethyl amine methacrylamide were reported to be used for CR application (Kenawy et al., 1992). Increased release of herbicide was obtained as the hydrophilic co-monomer content increased.

Kenawy and his group (Akelah et al., 1993) prepared controlled release systems based on polyureas and poly(Schiff's bases). The effects of structure and temperature of the aqueous environment on the hydrolysis rate of the obtained polymer had been reported. Cheillini and Akelah (Solaro et al. 1991) synthesized polymeric herbicides containing 2,4-D and MCPA by modification of oligoethyleneoxylated styrene/divinylbenzene(DVB) resins. The release features for these systems were greatly affected by the pH.

Akelah et al. (Akelah & Rehab, 1994) reported chemical modifications of a series of polyamides containing hydroxyl groups with 2,4-D in the presence of dicyclohexylcarbodiimide(DCC) as a condensing agent to yield a series of polymer. They reported that the rates of release of 2,4-D from the formulations were mainly dependent on hydrophilicity, the pH and the temperature of the release medium.

Pesticides containing acid groups were converted to more reactive acid chlorides, which could react with polymers containing pendant hydroxyl or amino groups. Acylation of synthetic and natural polymers were possible in this manner (Neogi, 1970; Wilkins, 1976; Allan et al., 1971; Allan et al., 1977). Pentachlorophenol intercalated on mineral clay was reported by Akelah and Rehab (Akelah & Rehab, 1996). The release of pentachlorophenol from the formulations was studied in different media at 30°C and it was concluded that the release of pentachlorophenol from the formulations was dependent on the structure, swelling degree and the medium of release.

A series of preformed polymers modified with pesticides were reported (Kenawy et al., 1992). Chitin (Kemp & Wrightman, 1981; Trenkel, 1997) as a naturally occurring polymer was used as carrier for herbicide metribuzin and the system showed slow release when polymer was directly attached to metribuzin.

## 2.2 Nanocomposite for controlled release of pesticide

Nanocomposites technology with layered silicate as in situ reinforcement has been extensively investigated in recent years. The increasing interest for development of polymer clay nanocomposites is due to the improvement in properties of these composites resulting from the synergic effect of interaction of both the components at nanometer level (Pinnavaia & Beall, 2000). Among the different nanocomposites, the bio-hybrid nano structured materials have evoked considerable interest in recent years. The current trend involves in the development of nanocomposite based on natural polymers and inorganic solid particularly clay minerals. Nano clays and layered double hydroxides are being developed in this regard (Chaoi et al. 2007). Both materials show good biocompatibility, low toxicity, and the potential for controlled release. Chemicals can be loaded between layers of both materials (an arrangement that can be influenced by buffer conditions, in particular pH). In the case of hydrophobic chemicals, this arrangement prevents re-crystallisation, increases solubility, and therefore bioavailability. A number of research activities have been completed with regards to layered double hydroxides as pesticides, growth regulators, plant nutrients, and slow-release fertilizer (Olanrewaju et al., 2000; Lakraimi et al., 2000).

Imazapyr (IMP), a herbicide was bound to polydiallyldimethyl ammonium chloride-montmorillonite composites for reducing the leaching of IMP. IMP release in the soil from

the controlled release formulation was substantially slower than its release from the commercial formulation (Radian & Mishael, 2008). The inorganic Zn-Al layer double hydroxide was used as a matrix to encapsulate the herbicide 2,4-dichlorophenoxyacetate. The release of the herbicide was rapid initially followed by a sustained release. The release behavior was dependent on the type of anions (like chloride, carbonate etc.) and their concentration present in the release medium (Hussain et al., 2005). Mg/Al layered double hydroxide (LDH) was intercalated with herbicides 2,4-D MCPA and picloram. The complexes were assayed for the controlled release of herbicides. It was concluded that LDH had the potentiality for the preparation of slow release formulation of 2,4-D MCPA picloram (Cordoso et al., 2006). The release of alachlor and atrazine into aqueous solution from the controlled formulation prepared from alginate and pectin, with and without the addition of clay minerals, was studied by Gerstl et al. (Gerstl et al., 1998). The addition of clay to the formulations was found to have a profound inhibitory effect on the release of alachlor.

### 2.3 Natural pesticides

Four major types (pyrethrum, rotenone, neem and essential oils) along with three minor types (ryania, nicotine, and sabadilla) of botanical products used for pest control were reported in literature (Isman, 2006). Isman (Isman, 2005) reported that pyrethrum accounted 80% of the global botanical insecticide market. Several plant derived compounds with pesticidal potential were discussed and reviewed in the literatures (Duke, 1990, 1986a, 1986b; Duke & Lydon, 1987; Duke et al., 1988).

Shay et al. (Shay et al., 1998) studied the insecticidal and repellent properties of nine volatile constituents of essential oils against the American cockroach, *Periplaneta americana*. Alali et al. (Alali et al.; 1998) studied the six compounds, representing the mono-tetrahydrofuran (THF) (gigantetrocin A, annomontacin), adjacent bis-THF (asimicin, parviflorin), and nonadjacent bis-THF (sylvaticin, bullatalicin) classes of annonaceous acetogenins, and compared them with technical grades of synthetic amidinohydrazone (hydramethylnon), carbamate (propoxur, bendiocarb), organophosphate (chlorpyrifos), and pyrethroid (cypermethrin) insecticides to determine their dietary toxicities to insecticide-resistant and insecticide-susceptible strains of the German cockroach, *Blattella germanica*.

Mugisha-Kamatenezi et al. (Mugisha-Kamatenezi et al., 2008) demonstrated that usage of botanical pesticides in field pest management was normal around Lake Victoria basin for the subsistence farmers. Mahfuz and Khanam reported (Mahfuz & Khanam, 2007). on the efficacy of seven different plant extracts viz. *Acorus calamus* rhizome, leaves of *Datura fastuosa*, *Datura stramonium* and seeds of *D. stramonium*, *Corchorus capsularis*, *Aphanamixis polystachea* and *Jatropha curcas* on *Tribolium confusum* adult.

Mishra et al. (Mishra et al., 1989) isolated essential oils from leaves of *Chenopodium ambrosioides*, *Cinnamomum zeylanicum*, *Citrus medica*, *Melaleuca lucadendron*, *Ocimum canum* and *O. grattissium*. These oils demonstrated fungitoxicity against *Aspergillus flavus* at 200, 300, 400 and 500 ppm and most of them were shown to be more effective than synthetic fungicides viz; Agrosan G.N., Copperoxychloride, Ceresan, Thiovit and Dithane M45. Asthana et al. (Asthana et al., 1986) found the leaf extract of *Ocimum adscendens* to be fungitoxic against *Aspergillus flavus*. The volatile fungitoxic fraction was identified to be an essential oil, and was observed to be more active than some five synthetic fungicides tested.

Wang et al. (Wang et al., 1990) were able to isolate antifungal and larvicidal polyacetylenes for *Artemisia borealis* (*B. campestris* subsp. *borealis*). Dichloromethane extracts for the whole plant showed antifungal activity against *Cladosporium cucumerinum*.

Upadhyaya and Gupta (Upadhyaya & Gupta, 1990). demonstrated the inhibitory effect of some medicinal plants on the growth of *Curvularia lunata* (*Cochliobolus lunatus*). Ethanol extracts of garlic followed by those of *Ocimum santum*, *Datura alba* and hemp were found to be most inhibitory to growth of the fungus. Aqueous extracts were less effective. Garlic extracts were shown to be inhibitory on the growth of a number of fungi (Tansey & Appleton, 1975). From methanol extracts of twigs of *Oxymitra velutina* - a west african plant, 12 alkaloids; 5 aporphinoids including lysicamine, which is active against *Bacillus subtilis*, *Botrytis cinerea*, *Saprolegnia asterophora* and *Rhizoctonia solani* were isolated (Achenbach & Hemrich, 1991).

Yegen et al. (Yegen et al., 1992) studied the fungitoxic effect of extracts of six selected plants from Turkey. Results indicated that aqueous and essential oils of *Thymbra spicata*, *Satureja thymbra*, *Laura nobilis*, *Mentha spicata*, *Salvia fucicosa* and *Inula viscosa* were fungitoxic to *Fusarium moniliforme*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Phytophthora capsici*. Kumar and Tripathi (Kumar & Tripathi, 1991) screened leaf extracts of 18 plant species belonging to 11 families for their control of *Pythium debaryanum*, *Fusarium oxysporum*, *R. solani* and *Sclerotium rolfsii*.

#### **2.4 Neem seed oil as pesticide**

Pradhan et al. (Pradhan et al., 1962) reported the feeding deterrent property of neem seed kernel suspension against desert locust, *Schistocerca gregaria*. Subsequently, several bioactive ingredients were isolated from various parts of the tree, more notable being the isolation of meliantriol (Lavie & Jain, 1967) and azadirachtin (Butterworth & Morgan, 1968). Both meliantriol and azadirachtin inhibited feeding of locust. In fact neem was recommended by most of the authors as a desirable method of pest control and repellent as it did not cause harm to the people and the plant but selectively affected the insects (Dhawan & Patnaik, 1996). Neem products affected different physiological processes in insects which were reported in literature (National Research Council, 1992). Neem extract could influence over 200 species of insects. Many of which were resistant to modern pesticides. Active constituents like azadirachtin showed larvicidal and anti-feedant (Rao et al., 1988; Morgan & Thornton, 1973; Miller & Chamberlain, 1989) activities.

It was reported that a spray of 1-percent neem oil in water "stopped 95 to 100 percent of the powdery mildew on hydrangeas, lilacs, and phlox." Locke and Larew demonstrated that neem oil could reduce damage caused by various pests, including spider mites. It was also reported that a 2-percent spray of neem seed oil applied directly to spider mite eggs resulted in an 87-percent mortality (Becker, 1994). Neem oil provided effective control of rice plant hoppers like *Nilaparvatha lugens*, *Nephotettix* spp. and *Sogatella furcifera*. A spray of 3% neem oil discouraged settling of hopper, *N. lugens* on treated plants (Jayaraj, 1993).. Similarly, 3% neem oil and 5% neem seed kernel extract were reported to control *Helicoverpa armigera* in bengal gram. Isman (Isman, 1997; Isman, 1996). reported a significantly higher growth inhibition on grubs of rice seedlings while using the crude oils extracted from the seeds of neem, custard apple and china berry. An oil based neem formulation containing 300 ppm of azadirachtin was used effectively against the desert locust, grasshoppers and other lepidopteran pest (Ramarethinam & Marimuthu, 1998). Application of neem oil was also reported to reduce the incidence of plant viral diseases like yellow vein mosaic of okra, yellow mosaic of grain legumes, leaf curl of chillies and ragged-stunt virus of rice. Neem oil was reported to inhibit or reduce the transmission of Tungro virus and Tobacco mosaic virus (Vijayalakshmi et al., 1995).

The use of neem pesticides in cotton pest management was thoroughly reviewed by R.T. Gahukar (Gahukar, 2000). Pesticides derived from neem (*Azadirachta indica* A. Juss.) controlled pests without having the nontarget toxicological effects associated with conventional pesticides (Gahukar, 2000). Both azadirachtin and azadirachtin analogues were studied for antifeedant activity against the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) and azadirachtin was found to be the most active (Simmonds et al., 1995). Neem oil (2%) inhibited normal growth and development of early third instar larvae of *S. litura* under laboratory conditions by affecting growth hormone systems (Koul, 1987). Azadirachtin reduced pupation and adult eclosion in *S. litura* by 47% and 42% respectively (Ayyangar & Rao, 1991; Mohan, 1990). Several reports were found on using neem oil against mosquito and as larvicide (Virendra et al., 1995; Sharma et al., 1993; Fredros et al., 2007; Nagpal et al., 1995; Govindachari et al., 2000).

Antifungal activity of neem oil for mycelia growth inhibition was studied and reported by Mohanty et al. (Mohanty et al., 2008). Wanyika et al. (Wanyika et al., 2009) reported that pyrethrum-botanical oil (neem seed oil, cotton seed oil and yellow oleander oils) blends against maize weevils was effective insecticide compared to pure pyrethrum based insecticide. Senthil-Nathan et al. (Senthil-Nathan et al., 2009) studied on the toxicity and physiological effects of neem pesticides applied to rice and found neem-based pesticides to be more effective to inhibit the growth and survival of *N. lugens*, the brown planthopper. When the synthetic insecticides chlorpyrifos (Termex®), cypermethrin+acetamiprid (Conquest®), and the natural insecticide neem (*Azadirachta indica* A. Juss) seed oil extract were used, the insecticides significantly reduced the populations of diamondback moth, *Plutella xylostella* L., and the cabbage head caterpillar, *Crociodolomia binotalis* Zeller (Umeh et al., 2009). Reports were also available on the advantages of neem oil based pesticides over other chemical pesticides (Gauri, 2007).

## 2.5 Encapsulation and controlled release of NSO

In the field of encapsulation and controlled release of NSO, a number of research works were carried out and reported by Aminabhavi and co-workers. Several controlled release formulations containing NSO were developed by them (Aminabhavi et al., 1998; Kulkarni et al., 1999; Kumbar et al., 2001b; Kulkarni et al., 2000). Preparation and characterization of granules encapsulating the natural liquid pesticide viz., *A. indica* A. Juss. (neem) seed oil (NSO) was reported by Kulkarni et al. (Kulkarni et al., 1999). The polymer matrices used for encapsulation were urea formaldehyde crosslinked starch (UF-St), guar gum (UF-GG) and UF-(St + GG). The release of the active ingredient was depended on the type of matrix and its swelling ability. The entrapment efficiency was more than 95% in majority of the cases indicating the efficient encapsulation. The percentage loading of NSO with different matrices and their density exerted an influence on the release data.

The study regarding the release kinetics and encapsulation efficiency of the urea formaldehyde (UF) crosslinked matrices of St, GG, and St + GG for the controlled release of the solid (chlorpyrifos) and liquid (neem seed oil) pesticides (Kumbar et al., 2001b) was carried out and reported by Kumbar et al. They indicated that variable release rates were related to the polymer type and especially the pesticide type. It was concluded that it was possible to slow the release rates of pesticides using these cheaply available polymers.

Kulkarni et al. studied and reported the encapsulation of NSO as liquid pesticide using sodium alginate (Na-Alg) as a controlled release (CR) polymer after crosslinking with

glutaraldehyde (GA) (Kulkarni et al., 2000). The FTIR spectral study confirmed the absence of chemical interaction between active ingredients and polymer as well as crosslinking agents. An increase in the crosslinking of the polymer resulted in a significant decrease of NSO release from the beads.

Development and invention of an improved granular formulation of neem seed extract containing neem Aza-A having enhanced storage stability (Sreenivasa et al., 2006) and the ability for gradual release of neem Aza-A was reported in a patent. The formulation consists of an inert particulate compound as a carrier, at least one lipophilic substance as a deactivator/binder, optional colorant and neem seed extract containing neem Aza-A (Sreenivasa et al., 2006).

Controlled release microcapsules containing neem (*Azadirachta Indica A. Juss.*) seed oil (NSO) were prepared by encapsulation of natural liquid pesticide NSO in polyelectrolyte complexes of naturally occurring polymers namely carrageenan-chitosan (Devi & Maji, 2009a, 2009b) gelatin-carrageenan (Devi & Maji, 2010) and gelatin-sodium carboxymethyl cellulose (Devi & Maji, 2011). Microencapsulation was carried out at the optimized ratio and pH for complexation between the biopolymers in order to get maximum yield and to form stable polyelectrolyte complex. The microencapsulation method for NSO loading was optimized. The release rates of NSO were studied by varying the percentage of oil loading, concentration of crosslinking agent and polymer concentration. Scanning electron microscopy study demonstrated free flowing to bursting look of the microcapsules for different formulations. Microcapsules formed from different biopolymer polyelectrolyte complex systems were compared. Fourier transform infrared spectroscopy (FTIR) study indicated the absence of any significant interaction between the polyelectrolyte complexes and NSO. The interaction between the complex forming polymers was indicated by the formation of new bond and shifting of peaks in FTIR spectra.

The effect of a natural rubber coating of a capsule obtained from sodium alginate on the release of neem Aza-A was studied by Riyajan et al. (Riyajan & Sakdapipanich, 2009). The effect of physically modifying the hydrophobicity of alginate beads and the optimum conditions for release of neem Aza-A from such capsules were investigated in this study. To enhance the stability of neem Aza-A which is not stable in the environment, a biodegradable novel semi-interpenetrating polymer network based on poly(vinyl alcohol) (PVA) and sodium alginate containing neem (*Azadirachta indica*) in the presence of azadirachtin-A (neem Aza-A) as well as glutaraldehyde as a crosslinking agent was prepared for use in the controlled release of neem Aza-A (Riyajan & Sakdapipanich, 2010).

### 3. Methods of synthesis of encapsulated controlled release NSO pesticides

#### 3.1 Some significant methods used

The most common and frequently used method for preparing granules and beads of NSO is matrix encapsulation technique. This technique was used for preparing crosslinked polymeric granules containing 20, 35 and 50% (w/w) of the natural liquid pesticide viz., *A. indica A. Juss.* (neem) seed oil (NSO) and was reported (Kulkarni et al., 1999). The crosslinked granules were obtained by preparing urea-formaldehyde (UF) prepolymer followed by gelatinizing starch (St), guar gum (GG), or St-GG matrices containing NSO by boiling St, GG, or St-GG combinations in distilled water to form a transparent mucilage. Three concentrations of NSO i.e., 20, 35 and 50% (w/w of the polymer) was added after cooling and then dispersed by mechanical mixing. The pH of the mixture was reduced to 3.0

for completion of crosslinking reaction by using urea-formaldehyde prepolymer as crosslinker. The crosslinked mass was sieved, dried in vacuum oven and further sieved in order to obtain uniform sized granules ranging between 0.5-2mm.

The amount of NSO in the granules, density of the matrices, extent of swelling of the granules and the cumulative release of NSO from the matrix were studied thoroughly and reported. It is concluded that starch matrix is more effective in low moisture containing soil, while in high moisture containing soil, GG matrix is preferred for effective release of NSO (Kulkarni et al.,1999). Urea formaldehyde (UF) crosslinked matrices of St, GG, and St + GG were used to study the release kinetics and encapsulation efficiency of two pesticides-chlorpyrifos, a solid and neem seed oil, a liquid pesticide (Kumbar et al., 2001b).

Sodium alginate beads containing NSO were prepared by mixing NSO in a sodium alginate solution followed by dropwise addition of this solution into methanol containing glutaraldehyde (1%) and HCl(1% of 1N) solution under constant stirring (Kulkarni et al., 2000). The beads were separated from methanol at different time intervals, washed with water and dried.

In our laboratory, microencapsulation of NSO was carried out in three natural polymer systems by the method of polyelectrolyte complexation and then crosslinking. For the carrageenan- gelatin A system (Devi & Maji,2010), the procedure was as follows- known amount of (100ml) 0.5-1.5% (w/v) of carrageenan solution in distilled water was taken in a beaker. This polymer solution was stirred by mechanical stirrer under high agitation at  $70\pm 1^\circ\text{C}$ . This temperature was maintained throughout the experiment. To this, NSO (1-4g) was added under high agitation to form an emulsion. A known amount of (200ml) gelatin-A solution of 1-3% (w/v) was added to the beaker drop wise to attain complete phase separation. However, the weight ratio of carrageenan to gelatin was maintained at 1: 2 during all the experiments. At this ratio, interaction between gelatin and carrageenan was maximum as judged by the measurement of coacervate yield (%) and viscosity. The pH of the mixture was then brought down to 3.5 by adding 2.5% (v/v) glacial acetic acid solution. At this pH, maximum interaction took place as measured by yield (%) and turbidity. The beaker containing the microcapsules was left to rest at the same temperature for approximately 15 minutes. The system was then brought to  $5-10^\circ\text{C}$  to harden the microcapsules. The cross linking of the polymer capsule was achieved by slow addition of certain amount of genipin solution ( a natural crosslinker) (0.5225% w/v). The temperature of the beaker was then raised to  $45^\circ\text{C}$  and stirring was continued for another 3-4 h to complete the crosslinking reaction. The beaker was then cooled to room temperature slowly while stirring. The microcapsules were filtered through 300-mesh nylon cloth, washed with 0.1% (w/v) Tween 80 surfactant solution to remove oil, if any, adhered to the surface of microcapsules. This was further washed with distilled water, freeze dried and stored inside a refrigerator in a glass ampule.

### 3.2 Encapsulation and controlled release studies

UV-visible spectrophotometric methods were used to study the encapsulation efficiencies and controlled release behavior of encapsulated NSO. Encapsulation efficiencies were calculated by estimating the pesticides before (theoretical) and after encapsulation (actual loading). The encapsulation efficiency is dependent on both the nature of the matrix material and the pesticide type. A highly rigid polymer (shell) is obviously a good polymer for encapsulation. A calibration curve of NSO is required for the determination of

encapsulation efficiency, oil content and oil load and release rate of oil from the microcapsules.

### 3.2.1 Calibration curve of NSO

In our study (Devi & Maji, 2009, 2010), it was found that 0.1 gm of NSO could be easily dissolved in 100 ml of water containing 0.1 g Tween 80. A known concentration of NSO in DDI water containing 0.1% Tween 80 was scanned in the range of 200-400 nm by using UV visible spectrophotometer. For NSO having concentration in the range 0.001 to 0.08 g /100ml, a prominent peak at 254 nm was noticed. The absorbance values at 254nm obtained with the respective concentrations were recorded and plotted. From the calibration curve, the unknown concentration of NSO was obtained by knowing the absorbance value.

### 3.2.2 Encapsulation efficiency, oil content and oil load

For the calculation of encapsulation efficiency, oil content and oil load, a known amount of accurately weighed microcapsules are grounded in mortar, transferred with precaution to a volumetric flask containing a known amount of 0.1% (w/v) aqueous Tween 80 solution and kept for overnight with continuous stirring. The encapsulation efficiency (%), oil content (%) and oil loading (%) are generally calculated by using the calibration curve and the following formulae (Maji et al., 2007).

$$\text{Encapsulation efficiency (\%)} = (w_1 / w_2) \times 100$$

$$\text{Oil content (\%)} = (w_1 / w) \times 100$$

$$\text{Oil load (\%)} = (w_2 / w_3) \times 100$$

where

w = weight of microcapsules

w<sub>1</sub> = actual amount of oil encapsulated in a known amount of microcapsules

w<sub>2</sub> = amount of oil introduced in the same amount of microcapsules

w<sub>3</sub> = total amount of polymer used including crosslinker

In all the studied complexes, the oil loading (%), oil content (%), encapsulation efficiency (%) and release rate of oil were dependent on various factors like amount of oil, concentration of polymer, type and concentration of crosslinker. In general, encapsulation efficiency increased as oil load increased. Similarly encapsulation efficiency was found to increase when concentration of polymer ( $\kappa$ -carrageenan-chitosan, gelatin A- $\kappa$ -carrageenan, sodium carboxy methyl cellulose-gelatin A) or cross-linker (glutaraldehyde, genipin, tannic acid) increased. At higher polymer concentration, the availability of the polymer was more to encapsulate oil droplets and thereby efficiency increased. Higher crosslinker concentration (glutaraldehyde /tannic acid/ or genipin) increased the cross-linking which improved the oil retention capacity.

### 3.2.3 Oil release studies

Oil release studies of encapsulated oil can also be carried out by using UV-visible spectrophotometer. A known quantity of microcapsules is placed into a known volume of 0.1% Tween 80 surfactant solution. The microcapsule-Tween 80 mixture is shaken from time to time and the temperature throughout is maintained at 30°C (room temperature). An aliquot sample of known volume (5 ml) is removed at appropriate time intervals, filtered

and assayed spectrophotometrically at 254 nm for the determination of cumulative amount of oil release up to a certain time  $t$ . Each determination is carried out in triplicate. To maintain a constant volume, 5 ml of 0.1% Tween 80 solution is returned to the container. A typical oil release study pattern is given as below.

The faster or slower release rate of oil by the microcapsules prepared by varying different conditions could be explained on the basis of either decrease or increase in the thickness and compactness of microcapsule wall formed due to addition of polymer and crosslinker. Higher oil loading decreased the thickness of microcapsule wall whereas higher concentration of polymer increased the thickness of the wall. This in turn would control the release rate. Microcapsules wall became more compact as the amount of cross-linker increased. This led to decrease in the release rate. Effects of various crosslinking agents (glutaraldehyde, genipin, tannic acid) on NSO encapsulated microcapsules of carrageenan-chitosan complex were compared and glutaraldehyde was found to be the most effective crosslinker followed by genipin and tannic acid.

### 3.3 Characterization

Characterization of encapsulated NSO microcapsules and granules/beads are mainly carried out by using Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) studies. FTIR spectrum of NSO shows the absorption bands at 1745.90, 1463.04 and 1163.85  $\text{cm}^{-1}$  which are due to carbonyl stretching,  $\text{CH}_2$  asymmetric deformation and C-C stretching vibration (Fig.6). The position of these bands in the physical mixture as well as in the NSO loaded microcapsules/beads/matrices remained unaltered (not shown in figure) indicating the absence of any significant interaction between NSO and the polymer complexes or polymer mixtures used for encapsulation.

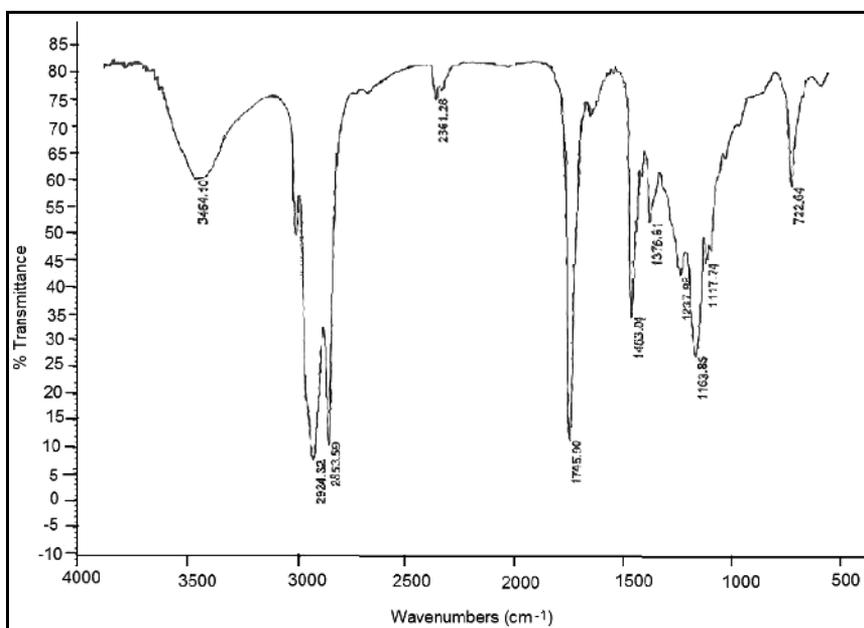


Fig. 6. Pattern of FTIR spectrum of NSO

The DSC thermogram of NSO shows an endothermic peak at around 220°C (Fig.7). Hardly, any remarkable difference in the thermograms of the NSO loaded microcapsules and physical mixture of complex polymer and NSO was observed (not shown in figure). In the thermograms, the endothermic peak due to NSO appeared almost in the similar position. Thus both FTIR and DSC study reveal no interaction between polymers and NSO.

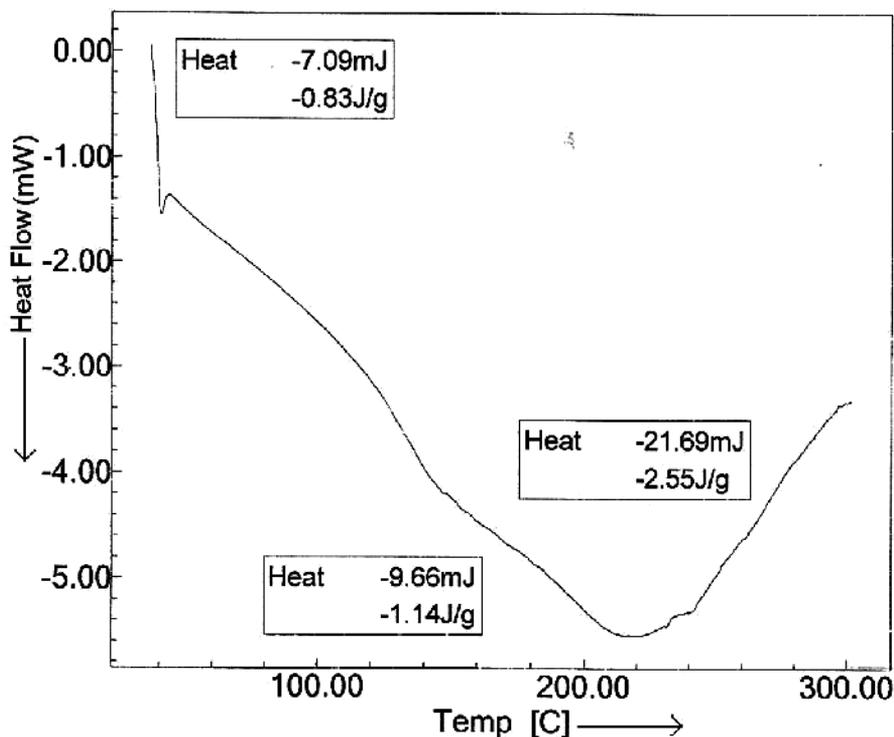


Fig. 7. Endothermic heat absorption pattern of NSO in DSC.

Extent of weight loss due to temperature and temperature of decomposition at different weight loss of NSO as observed in a thermogravimetric analyzer instrument are shown in the Fig.8 and Table 2 below-

Temperature of decomposition ( $T_D$ ) ( $^{\circ}C$ ) at different weight loss (%)							Residue (%) at 500 ( $^{\circ}C$ )
20	30	40	50	60	70	80	
315	340	358	375	395	415	430	7

Table 2. Temperature of decomposition at different weight loss (%) of NSO

Thermal stability of chitosan-carrageenan microcapsules (as suggested by thermogravimetric analysis) was found to improve with the increase in the concentration of crosslinker. In case of various crosslinkers, glutaraldehyde crosslinked samples showed highest thermal stability followed by genipin and tannic acid (Devi & Maji, 2009b).

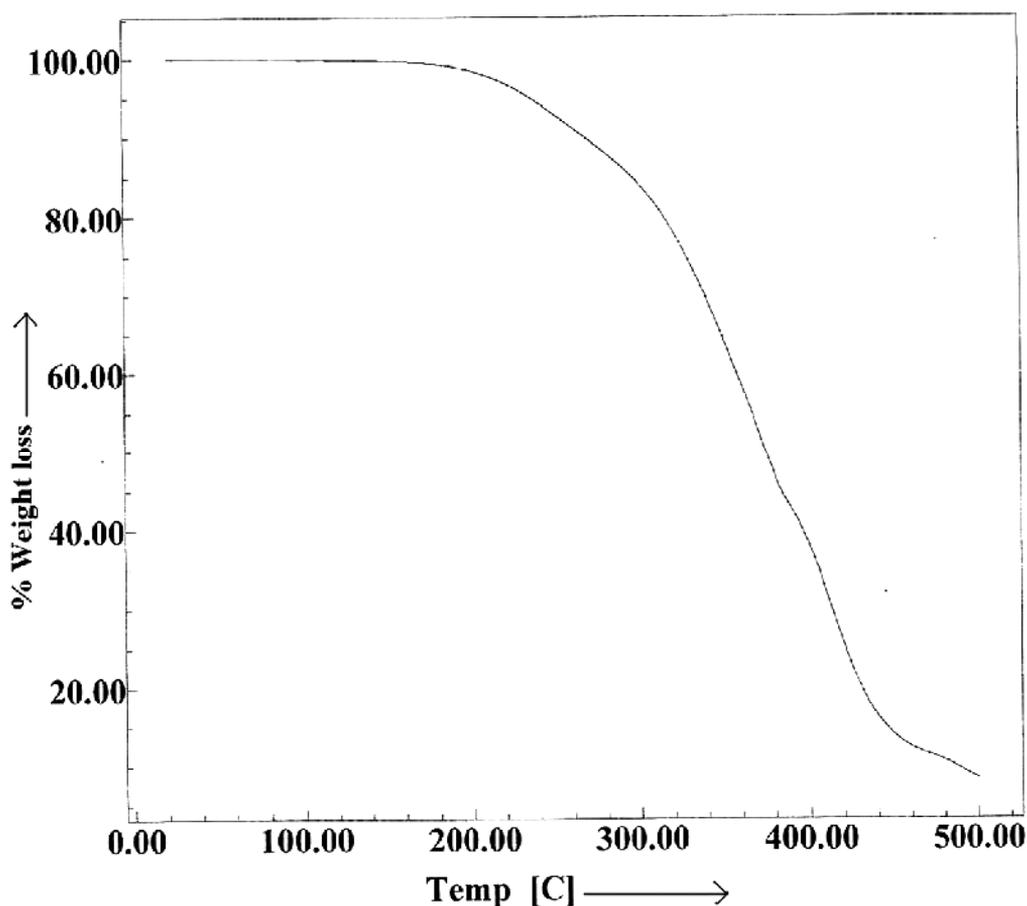


Fig. 8. Weight loss pattern of NSO in TGA

Formation of spherical beads with smooth surface and having particle sizes ranging from 1.21 to 1.40 mm in diameter was reported for sodium alginate beads (Kulkarni et al., 2000). The SEM photographs of capsules of sodium alginate matrix, crosslinked by glutaraldehyde and coated with natural rubber were shaped like an egg (Riyajan & Sakdapipanich, 2009). The mean particle size was 0.14 mm as measured by using both optical microscope and SEM. The diameter of capsules increased drastically from 0.14 mm to be 3 mm and had a smoother surface after coating with natural rubber.

SEM study showed the formation of spherical microcapsules having free flowing to bursting look depending on the extent of oil loading (Devi & Maji, 2009, 2010). SEM micrographs of microcapsules prepared at higher oil loading appeared oily, agglomerated and having a bursting look compared to those of microcapsules prepared at lower oil loading. The sizes of microcapsules increased with the increase of the concentration of the polymer. There was a clear distinction between the polymers with (lump of polymer) or without NSO encapsulation (free flowing spherical microcapsules). Fig.9 below shows the SEM photographs of formed NSO loaded microcapsules of carrageenan-gelatin complex and the neat complex.

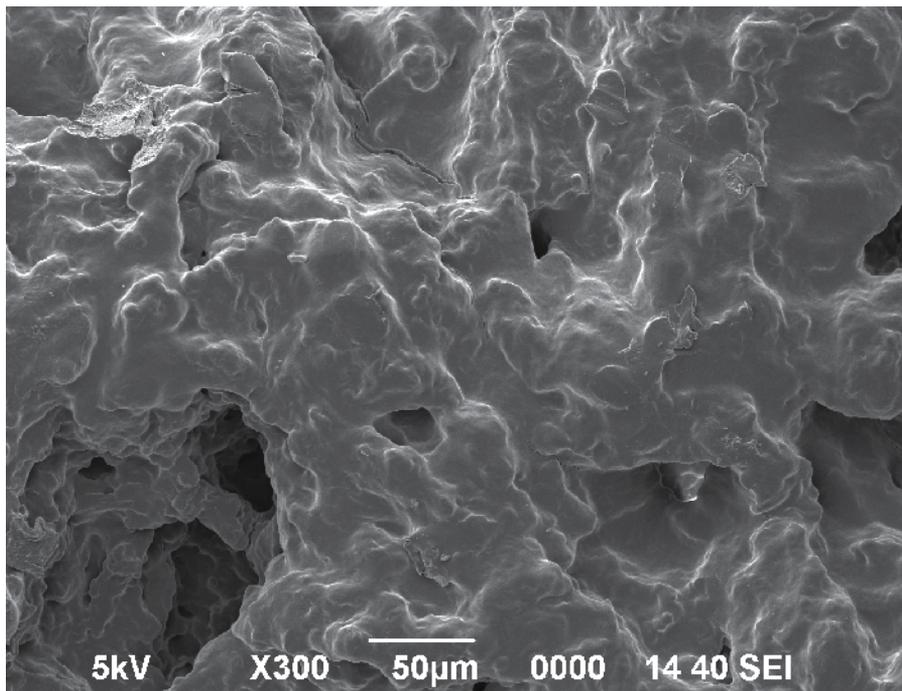


Fig. 9(a). Scanning electron micrograph of polymer complex without NSO encapsulation

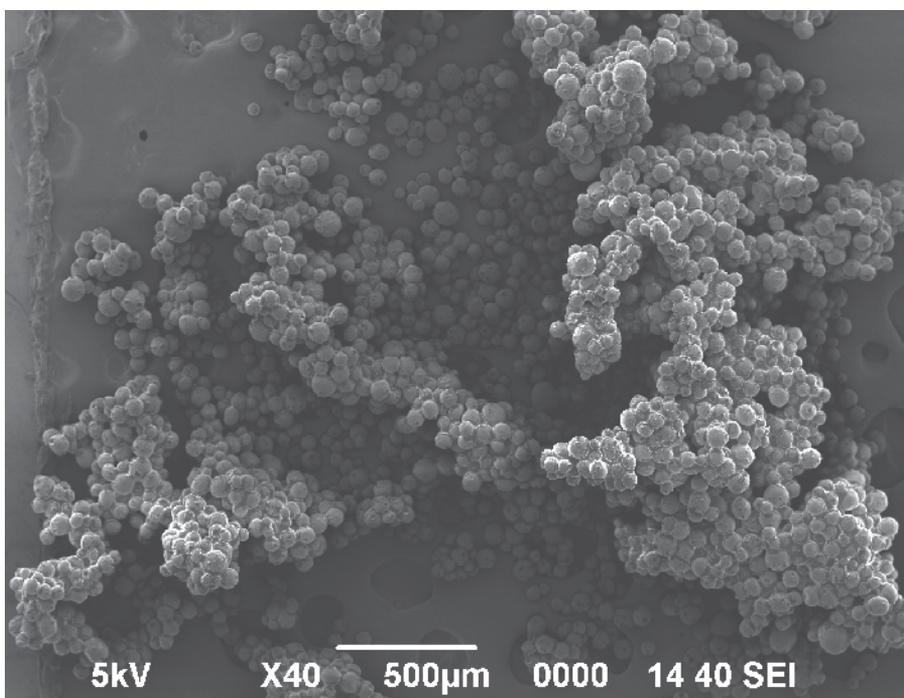


Fig. 9(b). Scanning electron micrograph of polymeric NSO controlled release microcapsules

In a study, the chemical structure of the controlled release neem Aza-A capsule wall (Riyajan & Sakdapipanich, 2010) was evaluated through X-ray diffraction. In addition, the swelling behaviour of the capsules and their thermal stability were investigated. The strength of the capsule wall depended on the polymer (PVA) in the matrix and the crosslinking density. Scanning electron microscopy, electron probe microanalysis and atomic force microscopy data indicated that the structure of the bead walls was rough and nonporous.

#### **4. Commercialization, future direction and conclusion**

##### **4.1 Commercialization of neem and controlled release neem pesticides**

Over 300 species of insects are stated to be controlled by neem all over the world. In India alone 106 species belonging to 10 orders of class Insecta, 12 species of nematodes and a fungus species are reportedly controlled by neem (Singh & Kafaria, 1991). The bio-efficacy results have attracted the attention of the pesticide industry in India and abroad. Nearly five dozen products are either marketed or are awaiting commercialization in India. Most of the products are either oil based or based on various extractives (Table 3) (Parmar & Ketkar, 1996).

##### **4.2 Future direction of research**

While the use of high yield-short duration hybrid varieties of seeds, fertilizers and irrigation increased agricultural productivity, pesticides complemented it by protecting the plants. The focus has now shifted towards sustainable agriculture in the light of harm caused by indiscriminate use of fertilizers and pesticides, laying stress on organic farming and use of biopesticides. Efforts in this direction have led the use of Bt- and plant-derived biopesticides. The later category has provided pyrethrum, nicotinoids, rotenoids for use and rediscovering the applications of neem (*Azadirachta indica*) based formulations. Alternative strategies for plant protection emerging for 21st century comprise of introduction of transgenic varieties of plants resistant to pesticides/pests. They appear to be members of biopesticide consortium, constituting an integral part of integrated pest management (IPM). Efforts are underway to understand the mechanism of action of biopesticides, so that light is thrown at molecular events for designing biopesticides appropriate for different pests. Concerted efforts are hoped to remove limitations in biopesticides raw material availability, potency variations, standardization of extraction methods, quality control, shelf life and improved bioefficacy. Biopesticides appear target specific, do not leave residues on food and feed by virtue of their biodegradability, economical and eco-friendly and hence are hoped to provide cleaner and safer eco-system (Mendki et al., 1994). Thus, the direction for future research of neem seed oil as a greener alternative for future generation technology as well as commercialization of neem products is the most important issue.

Jayaraj et al. (Jayaraj et al. 1996) reported that the future research should aim at the following-

- i. Research on genetic divergence in neem and their phenotypic stability
- ii. Research on maximizing the viability, vigour and storability of neem seed
- iii. Selection of varieties with higher oil content and high yielding capacity
- iv. Research on definitions of quality control and general biological properties
- v. Methods to check the photodegradability of neem formulations
- vi. Enhancement of efficacy of insect repellents and antifeedents of botanical origin by improved botanicals, better timing and improved application methods

Product	Active ingredient(s)	Activity claimed	Manufacturer
Amitul mosquito oil	Oil	Against mosquitoes	M/s Amitul Agrochem Pvt.Ltd., P.O.New Sheopuri Colony, Gorakhpur 273001, U.P.
Azadirachti EC	Oil (300ppm aza)	Pesticidal	M/s Sunida Exports, Mumbai 400 042
Bioneem	Oil (300ppm aza)	Pesticidal	M/s Ajay Bio-tech Ltd., Pune 411 040, Maharashtra State
Godrej Achook	Oil	Antifeedent, repellent, male sterility, molt/chitin inhibitor,ovicidal etc	M/s Bahar Agrochem and Feed Pvt. Ltd. E-24, Lote Parshuram, Firozshah Nagar, Mumbai 400 079
Jaineem	Oil	Pesticidal	M/s Jai Chemicals, Faridabad 121 001, Haryana
Limonool	Oil (300ppm aza)	Pesticidal	M/s Sri Bio-Multi-Tech (P) Ltd., Bangalore, 560 032, Karnataka
Margosom	Oil (300ppm aza)	Pesticidal	M/s Som Phytopharma(India) Ltd., Hyderabad 500 016, A.P.
Moskit	Oil	Mosquito repellent	M/s Investment and commercialization enterprises, Bombay- 400 022.
Neemolin	Oil (300ppm aza)	Pesticidal	M/s Khatan Janker Ltd. Mumbai 400 001
Nimbitor (ZA-199)	Oil (300ppm aza)	Pesticidal	M/s Zandu Pharmaceutical Works Ltd., Mumbai 400 025
Neem oil emulsion	Oil	Pesticidal	M/s Sio Agro Research Laboratories, Dhiraj Apartments, Mulund (West), Mumbai 400 080
Nimbecidine	Oil (300ppm aza)	Antifeedent, repellent, metamorphosis disruptor, synergist	M/s T. Stanes and Co. Ltd., Coimbatore 641 018, Tamilnadu.
Neemrich	Oil (300ppm aza)	Pesticidal	M/s West Coast Herbochem Pvt. Ltd., Bangalore 560 022
TRIC	Oil	Against household pests	M/s Amitul Agrochem Pvt.Ltd., P.O.New Sheopuri Colony, Gorakhpur 273001, U.P.

Table 3. Some of the commercial neem oil based pesticidal products in India including those in the pipelines

- vii. Use of tissue culture and biotechnological approaches to obtain innumerable neem trees with high yield in shorter time.
- viii. Making neem cultivation mandatory in all social forestry and other nutrition gardening programmes, and
- ix. Further research on aspects of manufacturing ready-to-use and effective formulations can be thought of and conducted.

Nanotechnology has the potential to revolutionize the agricultural industry with new tools for the molecular treatment of diseases, rapid detection of deficiency of nutrients etc. Smart sensors and smart delivery systems will help the agricultural industry combat viruses and other crop pathogens. In the near future nanostructured catalysts will be available which will increase the efficiency of pesticides and herbicides, allowing lower doses to be used. Nanotechnology will also protect the environment indirectly through the use of alternative (renewable) energy supplies, and filters or catalysts to reduce pollution and clean-up existing pollutants. Researchers are engaged to develop technologies to make pesticide delivery systems which can respond to environmental changes. Their aim is to tailor these products in such a way that they will release their cargo in a controlled manner in response to different signals e.g. magnetic fields, heat, ultrasound, moisture etc.

## 5. Conclusion

Neem products are finding favour particularly because of their environment friendliness. In the field of pest control, they are revolutionizing the total concept by shifting emphasis from instant pest mortality to gradual, nearly invisible, pest extinction. The use of neem materials for medicinal, toiletries and other purposes has also a large consumer acceptability and almost no report regarding any undesirable effect could be traced (Parmar & Ketkar, 1996). In industrialized countries, it is hard to imagine botanicals playing a greater role in future than at present, except organic food production (Isman, 2006). Organic production is estimated to be growing by 8% to 15% per annum in Europe and in North America (National research council, 2000) and it is in those market places that botanical pesticides face the fewest competitors. In conventional agriculture, botanicals face tremendous competition from the newest generation of 'reduced risk' synthetic insecticides such as the neonicotinoids.

Once a decision is taken to produce and use such products on a large scale, requisite research and development effort will have to be put in to make the neem based technology durable and practically sustainable. Stabilization of neem products against photo, thermal and microbial degradation may be of interest where persistence under such conditions is desired. Easy handling and transportation of the products are also important for industrial scale production and commercialization. In this context the microencapsulation and controlled release technology poses great potential for further research and commercialization of neem oil as a suitable and superior pesticide of the next generation. Extensive and systematic research work is needed to marshal the various facts about it and to speed the realization of its potential for application to plants, soil and water as a greener alternative for future generation technology.

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# Development of Neem Capsule via Biopolymer and Natural Rubber for Its Controlled Release

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## 1. Introduction

At present, population of world is over 6900 million persons, therefore food product is vital. Fig.1 shows the vegetable and fruits such as rice, orange and strawberry.



Fig. 1. Photograph of rice and fruits.

The utilization of synthetic pesticides in the world started in the 1935 and became widespread after World War II. By 1950, pesticide was found to increase farm yield far beyond pre-World War II levels (Nazir Javed et al., 2007).

Farmers depend heavily on synthetic pesticides to control insects in their crops. Today, it is one of the most commonly used approaches in controlling insects but it is relatively high in toxicity and have high environmental impact. At present, natural pesticide is applied thus to solve this problem due to no residue toxic chemical reagent on environment.

Neem is a tree as shown in Fig.2 and is widely distributed in South Asia, South-East Asia, and some other tropical areas, especially in Thailand (Nazir Javed et al., 2007; Kreutzweiser et al., 2004; Wei-Hong & Zhan-Qian, 2006; Kulkami et al., 2001; Kumbarn et al. , 1999; Sundaram & Curry, 1996). The neem seed kernels as presenting in Fig. 3 contains Azadiracthin-A (Aza-A), which is the major insecticidal tetranortriterpinoid. Neem Aza-A is a powerful insect antifeedant and growth-regulating substance, exhibiting considerable promise as an insecticide (Nazir Javed et al., 2007). It can control at least 200 species of agriculture and storage insect pest belonging to different orders, but it has short environmental persistence, and causes negligible hazard to nontarget organisms including humans. Fig. 4 shows insects destroying the vegetable. Its short environmental persistence is due to the presence of sensitive moieties such as *p*-electrons, ester linkages, and epoxide

ring. Thus, neem Aza-A is highly photolabile, either breaking down or isomerizing under sunlight. However, the photodegradation of neem Aza-A in sunlight is the major problem limiting its use in agriculture because the insecticide should persist long enough to cause the death of the insect. Many works solve this problem. The controlled stability of neem containing Aza-A can be done the two major approaches such as addition of antioxidant in neem solution and encapsulation of neem by polymer metric. For example, the addition of UV light absorbers can enhance the photostability of neem Aza-A (Wei-Hong & Zhan-Qian, 2006). The addition of ferulic acid, gallic acid, and rutin provided a moderate degree of photostabilization of neem Aza-A (Wei-Hong & Zhan-Qian, 2006). The present patent invention relates to an improved granular formulation of neem seed extract containing neem Aza-A having enhanced storage stability (Sreenivasa et al., 2006), and the ability for gradual release of neem Aza-A for application to plant rhizosphere. The formulation consist of inert particulate as a carrier at least one lipophilic substance as a deactivator/binder, optional colorant and neem seed extract containing neem Aza-A.



Fig. 2. Images of neem tree and it fruits

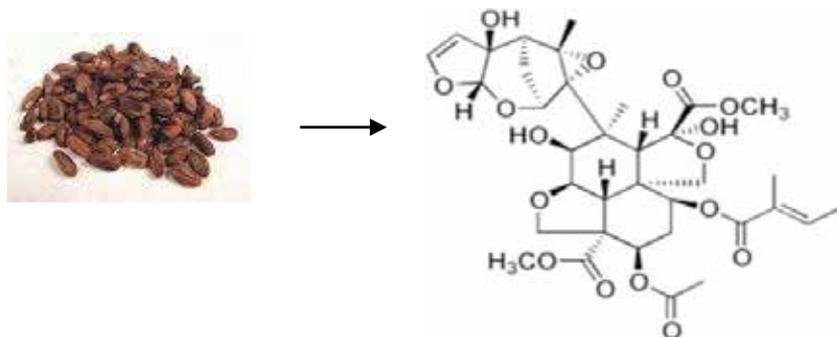


Fig. 3. Neem seed and chemical structure of Azadirachtin

By the way, detail of preparing neem extracted is reported as following (Riyajan & Sakdapipanich, 2009) neem seed kernels (5 g) had their cortex removed then crushed into small pieces, deoiled by grinding in light petroleum (200 mL) and filtered. The grinding and filtering were repeated twice more. The deoiled neem seed powder was stirred in 200 mL of methanol for 2 h and filtered at room temperature. The meal was reextracted with two further portions of methanol. The combined methanol filtrates were concentrated to approximately 50 mL, the aqueous methanol solution was extracted thrice with an equal volume of n-hexane (each was 50 mL) followed by 3×50 ml of dichloromethane (Fluka Company). The methanol-water layer was discarded and the dichlormethane layers were combined and dried over MgSO<sub>4</sub> (Fluka Company) and then evaporated to dryness. Two grams of the product were dissolved in eight mL of hexane during stirring. The liquid was separated into two layers using a separating funnel. The process was repeated by addition of a further 8 mL of ether. The methanol layer was evaporated and the residue was dissolved in 2 mL dichloromethane and then treated with 10 mL n-hexane and 10 mL ether, according to the above-mentioned process. The final yield of 65.0% neem Aza-A was 0.8 g from 1 kg of neem seeds.



Fig. 4. Photographs of insects destroying the vegetables

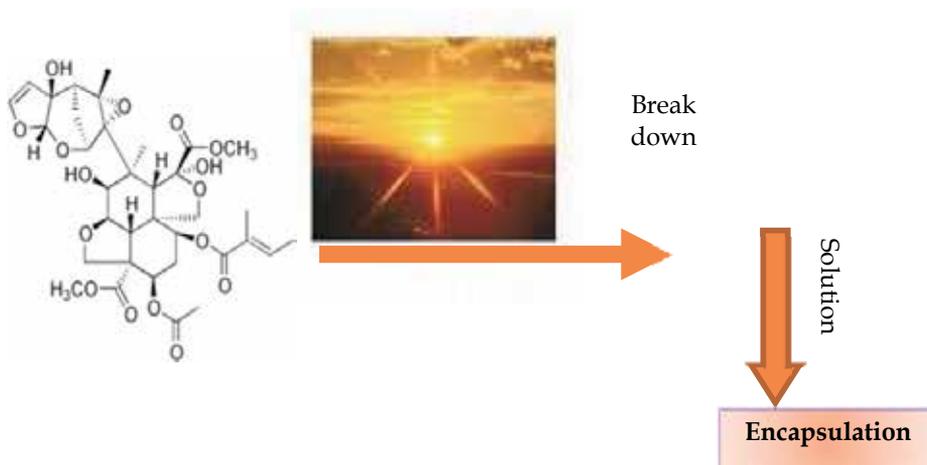


Fig. 5. Formula structure of Azadirachtin break down or isomerize under sunlight

## 2. Encapsulation

The encapsulation is packages the sensitive ingredients within a coating or wall material (Burt et al., 1995; Kim & Jung, 2007; Kulkarni et al., 2000; Fundueanu et al., Ruan et al., 2002; Sun et al., 2008; Vanderaer et al., 1974; Zhu & Zhuo, 2001). Definition of encapsulation is process in which particles or droplets/gases are surrounded by a coating of polymers to give capsules many useful properties. The capsules consisting of a core and a permeable or non-permeable wall have been widely used in release and transfer control. The wall material protects the sensitive ingredient (or core) including catalysts, drugs, anti-fouling compounds or toners, against adverse reactions, prevents the loss of volatile ingredients, and can control the rate of release of the ingredient. In addition, microencapsulation can convert liquids into free-flowing powders, so that they can be more easily handled. The controlled release of the capsule contents strongly depends on capsule wall thickness and porosity (Ji et al., 2001). The model of encapsulation is presented in Fig. 6. Rate of reactive agent release from capsule two patterns is presented in Fig.7. It is clear that the reactive agent from capsule obtained from matrix model start to release faster than that of capsule derived from encapsulate model.

### Polymer type

Polymers using the encapsulated neem made from synthetic polymer and natural polymer. For example, the synthetic polymers are given poly (vinyl alcohol) (PVA), polyethylene glycol and polyacrylamide. In case of natural polymer, they are obtained from chitosan, sodium alginate, cellulose, natural rubber (NR) and starch. NR is used a membrane of capsule for this work due to its hydrophobic behaviors. It consists of 1,4-*cis* polyisoprene and non-rubber component such as protein, carbohydrate and fatty acid (Riyajan & Santipanusopon, 2010)

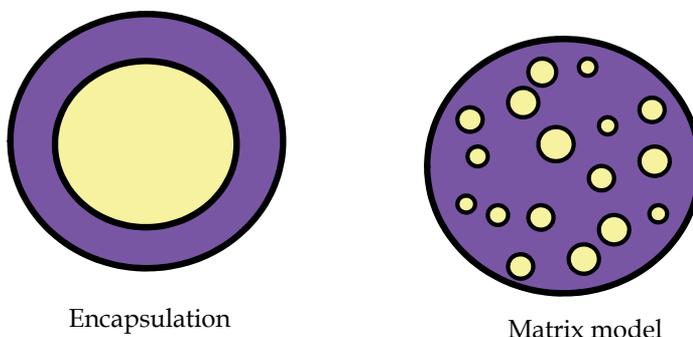


Fig. 6. Model of encapsulation consisting of encapsulation and matrix

### Crosslinking agent

The chemicals of the crosslinking agent are glutaraldehyde, calcium chloride and maleic anhydride.

### The methods for manufacture microcapsules

The methods for manufacture microcapsules are physical method, physical-chemical method and chemical method as following.

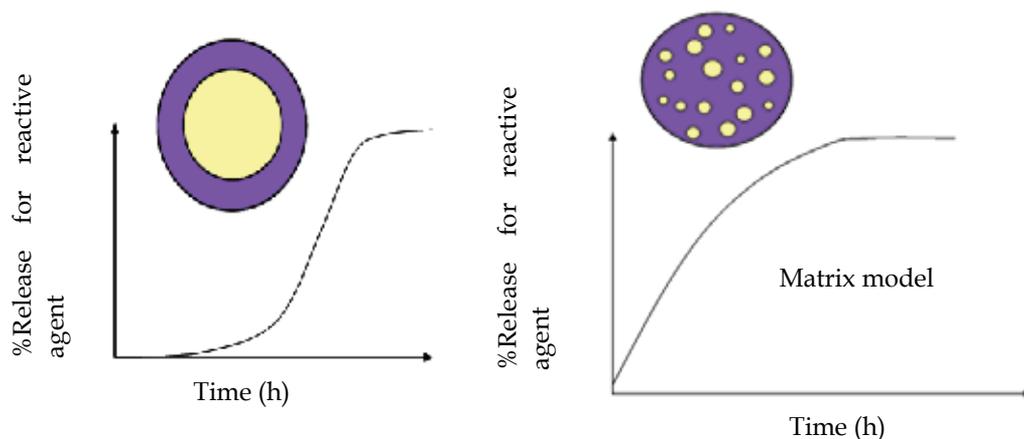


Fig. 7. Rate of reactive agent release of different capsules from encapsulation model and matrix model

### Physical methods

#### Pan coating

The pan coating process is widely used in the preparing capsule application and it is presented in Fig.8. The reactive particles are tumbled in a pan or other device while the coating material is applied slowly to get the capsule. For example, (the nitrate solution is the coating of fertilizers by degradable polymers. The water vapour and liquid diffuse through polymer films detached from their support. Therefore, we may classify polymers as a function of their properties like water vapour and liquid barrier. The capsule of fertilizer was done by pan coating. The electron microscopy used to see the quality of the wall has showed the presence of pores due to the rapid evaporation of solvent. A drying in air current and an annealing could be done to avoid this problem (Devassine et al., 2002).



Fig. 8. Photographs of pan coating equipment from digital camera

#### Fluidized bed process

Fluidized bed process or air-suspension coating of reactive particles by solutions or melts gives better control and flexibility. Fig. 9 presents the photographs of the fluidized bed

process. The reactive agent particles are coated while suspended in an upward-moving air stream. They are supported by a perforated plate having different patterns of holes inside and outside a cylindrical insert. Most of the rising air (usually heated) flows inside the cylinder, causing the particles to rise rapidly. Micro encapsulation by air suspension technique consist of the dispersing of solid, particulate core materials in a supporting air stream and the spray coating on the air suspended particles. The advantage of the fluidized bed technology is well known for its good solid mixing properties leading to lower agglomeration tendency, as well as its good heat and mass transfer rates and an uniform temperature distribution leading to the petrochemical industry (since decades) as well as in the pharmaceutical and food industry (Rosenkranza et al., 2008).

### Spray-drying

Spray drying is used in preparing capsule and it demonstrates in Fig. 10 when an active material is dissolved or suspended in a melt or polymer solution and becomes trapped in the dried particle. The main advantage is the ability to handle labile materials because of the short contact time in the dryer, in addition, the operation is economical. In modern spray dryers the viscosity of the solutions to be sprayed. The main factors that affect encapsulation efficiency of capsule obtained form spraying techniques the type of wall material, the properties of the core materials (concentration, volatility), the characteristics of the infeed emulsion (total solids, viscosity, droplets size) and the conditions of the spray drying process including atomization type, inlet air temperature, air flow and humidity.



Fig. 9. Photographs of the fluidized bed process from digital camera

The main advantage of spray dryer is the ability to handle labile materials because of the short contact time in the dryer, in addition, the operation is economical. In modern spray dryers the viscosity of the solutions to be sprayed. However, disadvantage of this method is degradation of reactive agent.



Fig. 10. Photographs of spray drying from digital camera

#### Physical-chemical method

Coacervate is a technique which consists of a tiny spherical droplet of assorted organic polymer molecules holding together by hydrophobic forces from a surrounding liquid. The particle size of droplet from coacervates method is range of 1 to 100 micrometers across which there is osmotic properties and form spontaneously from certain dilute organic solutions.

#### Chemical methods

##### Interfacial polycondensation

The two reactants in a condensation interface and react rapidly. Under the right conditions, thin flexible walls form rapidly at the interface. Interfacial polycondensation is a method of wide applicability for encapsulation of active ingredients. It offers the possibility of rapid production of polymers, under normal conditions of temperature and pressure, in an almost ready-to-use form. However, the mechanistic aspects of the process are not well understood because of the difficulties in following the fast kinetics and the need to account for the interplay of several equilibrium and rate processes in any comprehensive modeling effort.

The factors on properties of capsule obtained from interfacial polymerization are monomer concentration, diffusion and interfacial reaction. A solution of the pesticide and a diacid chloride are emulsified in water and an aqueous solution containing an amine and a polyfunctional isocyanate is added. Base is present to neutralize the acid formed during the reaction. Condensed polymer walls form instantaneously at the interface of the emulsion droplets. Considering, the different physicochemical rate and equilibrium processes of this capsule (Dhumala et al., 2008) are (i) ionic equilibria for the aqueous phase monomer, (ii) transport of the aqueous phase monomer and/or the organic phase monomer from bulk phases to the site of reaction, (iii) the reaction between the two monomers, and finally and (iv) the phase separation of the formed oligomeric species.

##### Interfacial cross-linking

Interfacial cross-linking is derived from interfacial polycondensation, and was developed to avoid the use of toxic diamines, for health applications. In this method, the small bifunctional monomer containing active hydrogen atoms is replaced by a biosourced polymer, like a chitosan. When the reaction is performed at the interface of an emulsion, the acid chloride reacts with the various functional groups of the protein, leading to the formation of a membrane. The cross-linked protein microcapsules are biocompatible and

biodegradable, and the presence of the protein backbone renders the membrane more resistant and elastic than those obtained by interfacial polycondensation. The method is very versatile, and the properties of the microcapsules (size, porosity, degradability, mechanical and resistance) can be easily tuned by varying the preparation parameters. A carbohydrate can be added to the protein, for the modulation of particle biodegradability. For example, the thermoresponsive properties of the macromers are imparted by *N*-isopropylacrylamide, with additional co-monomers including pentaerythritol diacrylate monostearate, acrylamide and hydroxyethyl acrylate (Klouda et al., 2011). The latter monomer contains hydroxyl groups that can be modified to acrylate or methacrylate moieties. These moieties can be covalently cross-linked with the addition of a water soluble, thermal free radical initiator system. Increased stability and higher viscosity values could be achieved when the hydrogels were physically and chemically gelled, as opposed to only thermally gelled controls.

### **In-situ polymerization**

In a few microencapsulation processes, the direct polymerization of a single monomer carried out on the particle surface. Usual deposition rates are about 0.5 $\mu\text{m}/\text{min}$ . Coating thickness ranges 0.2–75  $\mu\text{m}$  (0.0079–3.0 mils). The coating is uniform, even over sharp projections. For, example, aluminum pigment was encapsulated with styrene–maleic acid copolymer by in situ polymerization (Liu et al., 2008a). In addition, poly(trimethylolpropane triacrylate)/flaky aluminum composite particle (PTMPTA/Al) was prepared by in situ polymerization in order to improve the corrosion resistance and adhesive performance of aluminum pigments (Liu et al., 2008a).

### **3. Mechanism of release**

The methods of reactive agent release from capsule are erosion of polymer, diffusion and stress driven. Fig.11 represents the release pattern of reactive agent from capsule in medium. Even when the aim of a microencapsulation application is the isolation of the core from its surrounding, the wall must be ruptured at the time of use. Many walls are ruptured easily by pressure or shear stress, as in the case of breaking dye particles during writing to form a copy. Capsule contents may be released by melting the wall, or dissolving it under particular conditions, as in the case of an enteric drug coating. In other systems, the wall is broken by solvent action, enzyme attack, chemical reaction, hydrolysis, or slow disintegration. The capsule made from polylactic acid, polyglycolic acid and their polymer slowly biodegrade in nature. Encapsulation can be used to slow the release of a drug into the environment. This may permit one controlled release dose to substitute for several doses of non-encapsulated drug and also may decrease toxic side effects for some drugs by preventing high initial concentrations in the medium. There is usually a certain desired release pattern. In some cases, it is zero-order, i.e. the release rate is constant. In this case, the microcapsules deliver a fixed amount of drug per minute or hour during the period of their effectiveness. This can occur as long as a solid reservoir or dissolving drug is maintained in the microcapsule. A more typical release pattern is first-order in which the rate decreases exponentially with time until the drug source is exhausted. In this situation, a fixed amount of drug is in solution inside the microcapsule. The concentration difference between the inside and the outside of the capsule decreases continually as the drug diffuses.

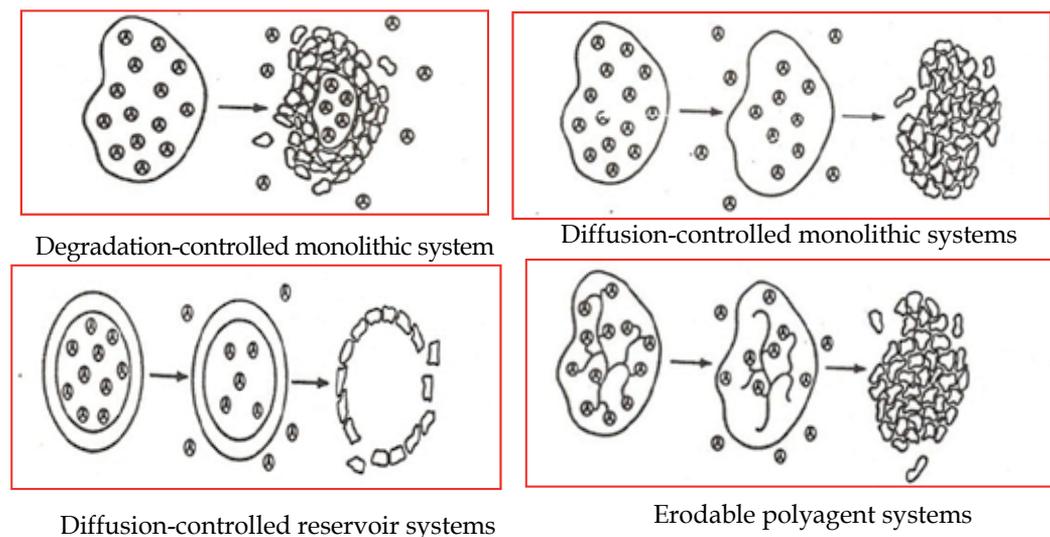


Fig. 11. Possible mechanism of release pattern of reactive agent from capsule (Baker 2000)

#### Mechanism of reactive lease from capsule

A coacervate is a technique, which consists of a tiny spherical droplet of assorted organic polymer molecules holding together by hydrophobic forces from a surrounding liquid. The particle size of droplet from coacervates method is range of 1 to 100 micrometers across which there is osmotic properties and form spontaneously from certain dilute organic solutions.

### 4. Preparation of capsule

The two preparing capsules were coaservation and spray drying as following.

#### i. Coaseveration

A 4% sodium alginate solution in distilled water was prepared by heating mental. After complete cooling the amounts of neem Aza-A (7000 ppm) was added and mixed thoroughly using a magnetic stirrer. The polymer solution containing neem Aza-A was added dropwise into methanol containing 1% glutaraldehyde (GA) and 1% of 1 N HCl, using a 25-ml hypodermic syringe (0.8 mm diameter) with constant stirring. The beads formed were removed from methanol at a selected time interval say 10, 20 and 30 min. The beads were washed with water and then dried. Fig.12 shows procedure of preparing capsule neem obtained from coaservation. The efficiency of entrapment was calculated as the ratio between the initial mass of neem Aza-A to be encapsulated and its mass in the final product. About 20 mg of exactly weighed microcapsule sample was extracted in distilled water to form a homogeneous solution. The total neem Aza-A in the solution was extracted for 48 h with a 50/50 MeOH/H<sub>2</sub>O mixture and its mass was determined by HPLC (PerkinElmer LC).

#### Drying rate study of the beads

A 3 samples of the beads formed after crosslinking with GA were selected for the drying study and were allowed to dry in an oven (VELP) maintained at  $31 \pm 2^\circ\text{C}$  (the initial mass of the beads should be nearly equal). The masses of the beads were taken at definite intervals of time until the constant mass was achieved. All the mass measurements were done on a

Mettler single pan balance (Model AB 204, Mettler). In order to obtain reproducible results, experiments were conducted in triplicate, and the average values were used for the calculation and plotting of the data vs. time. Results of drying are displayed in Fig. 13 indicate that the beads with longer time of exposure to the crosslinking agent exhibit higher drying rates than the beads exposed to shorter time to glutaraldehyde. The beads exposed for 10 min dried quickly (i.e., within 40 h) when compared to beads exposed to the crosslinking agent for 30 min (i.e., 72 h), while an intermediary drying time (i.e. 60 h) was required by the beads exposed to crosslinking for 20 min. This may be due to an increased rigidity of the polymer formed after a longer exposure time to the crosslinking agent thereby showing a decreased desorption rate of liquid from the beads.

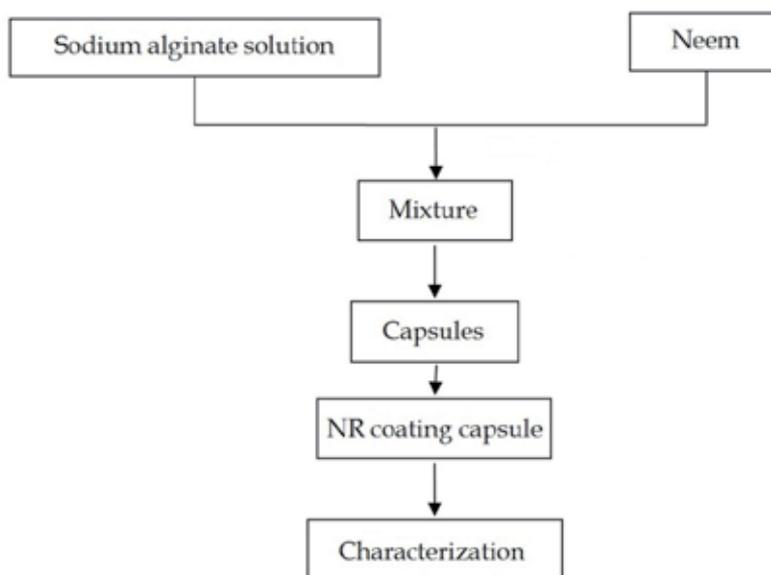


Fig. 12. Procedure of preparing capsule neem Aza-A obtained from coaservation

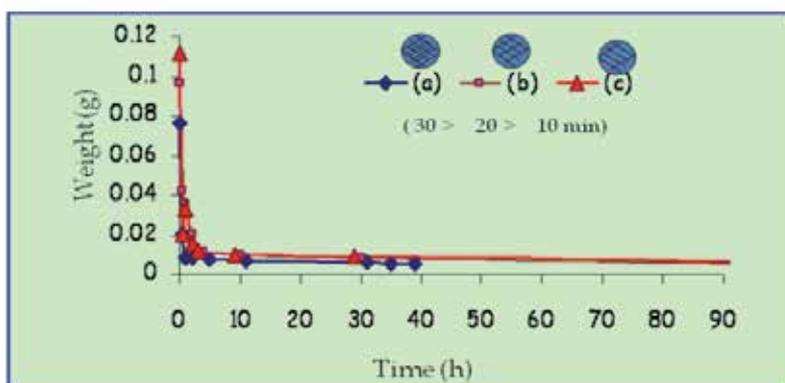


Fig. 13. Effect of crosslinking on drying of beads at different exposed times to GA solution (Riyajan & Sakdapipanich, 2009a)

### Coating of capsule with NR

Concentrated NR latex used in this study is high ammonia latex received from Jana company, Co., Ltd. (Songkhla Thailand).

% TSC of latex is calculated from the percentage by weight of the concentrated latex which is non-volatile at a definite temperature in an open atmosphere. The %TSC of concentrated NR latex in this study was determined by using method described in ASTM D107688 as shown in equation (1).

$$\%TSC = (W / W_t) \times 100 \quad (1)$$

Where,  $W$  = weight of dry NR sample (g)

$W_t$  = weight of NR latex sample (g)

%DRC of latex is defined as the percentage by weight of the concentrated latex which is precipitated by acetic acid. The %DRC of concentrated NR latex was determined (equation 2) by using method described in ASTM D1076-88.

$$\%DRC = (W_x / W_t) \times 100 \quad (2)$$

Where,  $W_x$  = weight of dry NR coagulum (g)

$W_t$  = weight of NR latex sample (g)

The 5 g of dried NR were dissolved in toluene (50 ml) in beaker (250 ml). The capsules (5 g) were dipped to the toluene solution and dried at room temperature.

So, the dry capsules of crosslinked sodium alginate mixed with neem Aza-A (7000 ppm) were dipped into a toluene solution of NR (5% w/w). Then, the coating capsules were dried at 30°C for 24 h. Multiple coatings were prepared by the immersion of the single-coated neem capsules into a NR with 30%DRC. Thereafter, the procedure was the same as during the preparation of single-coated neem capsules. The third-coated neem capsules were derived by the dipping of double-coated neem capsules into a NR with 30%DRC and then the same methodology as that given above mention. Fourth-coatings were prepared by the immersion of the third-coated neem capsules into a NR with 60 DRC and dried at 60°C until its weight was constant.

#### ii. Spray dryer method

In a number of processes, a core material is imbedded in a polymeric matrix during formation of the particles. A simple method of this type is spray-drying, in which the particle is formed by evaporation of the solvent from the matrix material. However, the solidification of the matrix also can be caused by a chemical change. The ratios of polymer with 0, 40% and 87% hydrolyzed polyvinyl acetate (PVAc) and distilled water containing glutaraldehyde 5% w/v and 0.1 % hydrochloric acid, given in Table 1, were prepared for the encapsulation of the neem Aza-A product. Suitable amounts of the neem Aza-A product in solution were added to the polymer solutions in water to obtain mixtures of the neem Aza-A solution: polymer in the proportion of 10:5 (w/w). Microcapsules were obtained by spraying the solutions through a mini spray dryer Buchi-191 equipped with a 0.7 mm nozzle at 206 kPa. The microparticles were collected and stored under vacuum at room temperature for 48 h. Fig. 14 shows the procedure of preparing capsule neem obtained from spray dryer.

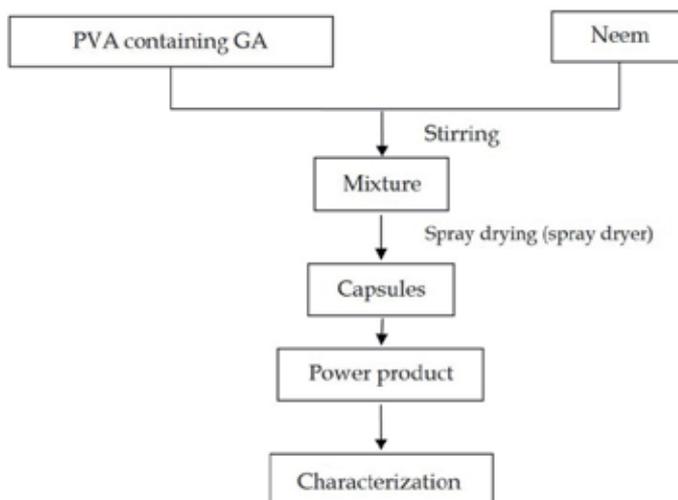


Fig. 14. Procedure of preparing neem Aza-A capsule obtained from spray dryer

## 5. Characterization of capsule

### Particle size and morphology of capsule

Five samples of the completely dried beads from different formulations were selected and their sizes were measured by using a micrometer screw gauge (Sargent, USA) with an accuracy of  $\pm 0.01$  mm for capsule obtained from coaservation. Capsules were prepared using sodium alginate and PVA solutions at 5% (w/v) concentration. The average diameter of the capsules ranged from 1.2 to 1.4 mm. The particles produced in this work were analyzed for their sizes using the light scattering method. The SEM photographs of capsule were shown in Fig. 15 and it is clear that the particles are egg in shape. The mean particle size was 0.14 mm observed by both OM and SEM. After the capsules were coated with NR, their diameter was drastically increased from 0.14 mm to be 3 mm and more smooth on surface of capsule was also observed. In case of spray dryer, the average of particle size of capsule was roughly 50 micron (Riyajan & Sakdapipanich, 2009b). In addition, the shape of this capsule depend on the blend ratio between PVA and sodium alginate, in which it change from spherical to egg-like due to the different viscosity of the mixture between PVA and sodium alginate (Riyajan & Sakdapipanich, 2010). The surface of the capsule was investigated by many techniques such as electron probe microanalysis, atomic force microscopy and SEM and detail was displayed in (Riyajan & Sakdapipanich, 2010).

### Swelling study of the individual beads

Swelling property of the beads was subjected to a measurement of swelling ratio in aqueous medium as a function of time. The bead samples exposed to GA at different time were selected and incubated with distilled water in a watch glass. The mass of all bead samples was taken at different interval period times and the average value was calculated. During this process, care should be exercised while it was handed of the swollen beads so as to avoid any weight loss due to breaking or erosion of the beads. All the mass measurements of the swollen beads were taken on a Mettler single pan balance and having accuracy up to fifth decimal. The percentage swelling ratio of bead was calculated as in equation 3.

$$\% \text{Swelling ratio} = \frac{(W_1 - W_2)}{W_1} \times 100 \tag{3}$$

Where,  $W_1$  and  $W_2$  are wet weight and dried weight, respectively.

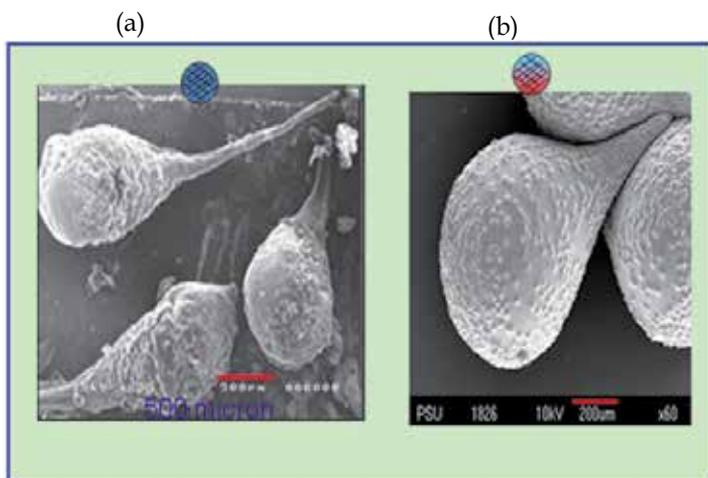


Fig. 15. Scanning electron microscopic photographs of capsule beads (a) sodium alginate and (b) sodium alginate: PVA (75:25) (Riyajan & Sakdapipanich, 2009a).

Ratio between polymer and distilled water	% Efficiency of yield (%S.D)	% Efficiency of encapsulation (%S.D)
0% Hydrolyzed PVAc: distilled water		
1:20	85 (2)	75 (4)
1:35	95(2)	78(4)
1:40	96(2)	79(2)
40% Hydrolyzed PVAc distilled water		
1:20	86(3)	76(2)
1:35	94(3)	78(2)
1:40	96(3)	80(3)
87% Hydrolyzed PVAc: distilled water		
1:20	84(4)	79(3)
1:35	96(2)	80(5)
1:40	97(2)	81(2)

Table 1. Efficiency of yield and efficiency of encapsulation obtained at different ratios between polymer and distilled water (Riyajan & Sakdapipanich ; 2009)

The swelling of any capsule in a aqueous medium depends on crosslinking density (Riyajan & Sakdapipanich, 2009a), polymer blend ratio (Riyajan & Sakdapipanich 2010), NR coating layer (Riyajan & Sakdapipanich, 2009), and % hydrolysis of PVAc (Riyajan & Sakdapipanich, 2009b) as well as crystalline region (Riyajan & Sakdapipanich, 2010).

Fig. 16 reveals the effect of crosslinking on percentage of swelling ratio by beads at various exposure times to GA. It was found that all the beads show a maximum amount of water absorption during the first hour, but beads formed by exposing for only 10 min to the crosslinking agent absorb more water than the beads formed by exposing for 20 and 30 min. The particles produced in this work were analyzed for their sizes using the light scattering method. NeemAza-A release from the beads were subjected to a number of physical and chemical parameters including those related directly to the release medium, the release conditions (temperature) and those resulting from change in the characteristics of the controlling release device (beads).

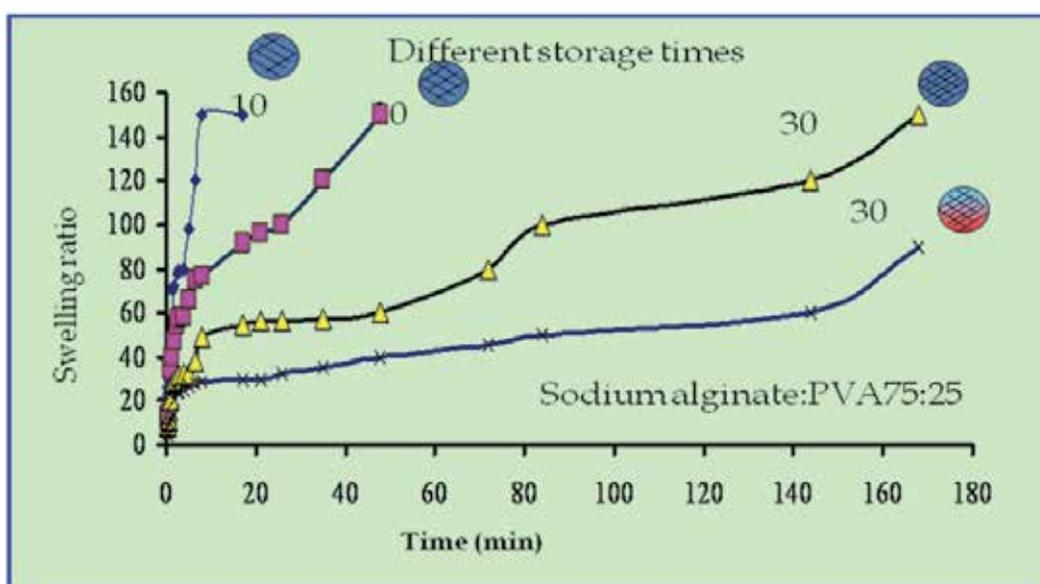


Fig. 16. Effect of crosslinking on percentage of water uptake by beads obtained from sodium alginate and sodium alginate/ PVA (75:25) (Riyajan & Sakdapipanich, 2010)

Fig. 17 exhibit the effect of NR layer on percentage of swelling by beads prepared at 30 min storage time of GA solution. From the results reported in section, it can be said that the structure of capsules with influence the release rate of neem Aza-A should be improved by coating the capsule with NR. Diffusion in polymers is an important mechanism in pharmacy for the controlled release of drugs (Vanderaer, 1974). Diffusion in polymeric systems is passive, if the driving force is purely a brownian molecular motion, but diffusion can also be activated by external effects, either by the influence of the release medium by swelling or biodegradation, or by the effects of physical forces as electrical, osmotic or convective forces. The fundamental of diffusion is based on Fick's laws which describe the macroscopic transport of molecules by a concentration gradient (Vanderaer, 1974).

The suitable coating material must be nonreactive, essentially immiscible with the material being encapsulated and capable of being rapidly hardened to form a film. NR was selected to

be a coating agent of neem Aza A-sodium alginate capsules to provide an adequate barrier wall. So, the dry capsules of crosslinked sodium alginate mixed with neem Aza-A (7000 ppm) were dipped into a toluene solution of NR (5% w/w). Then, the coating capsules were dried at 30°C for 24 h. Multiple coatings were prepared by the immersion of the single-coated neem Aza-A capsules into a NR with 30 DRC. Thereafter, the procedure was the same as during the preparation of single-coated neem Aza-A capsules. The third-coated neem Aza-A capsules were derived by the dipping of double-coated neem Aza-A capsules into a NR with 30%DRC and then the same methodology as that given above mention. Fourth-coatings were prepared by the immersion of the third-coated neem Aza-A capsules into a NR with 30% DRC and dried at 60°C until its weight was constant. It is clear that the rate of swelling decreased dramatically after coating neem Aza-A capsule with NR compared with that of the bead without coating. When NR layer on capsules increased, the swelling ratio of these resulting capsules dramatically decreased, especially capsule bead obtained from three-coated NR. The swelling ratio of neem Aza-A obtained from first-coated neem Aza-A in aqueous medium at 2, 24, 72 and 240 h of storage neem Aza-A was 10, 29, 38 and 60%, respectively. When the NR-coated on capsules increased from 1 to be 3 layers, the swelling ratio of neem Aza-A obtained from first-coated neem Aza-A in aqueous medium at 2, 24, 72 and 240 h of storage neem Aza-A was 2, 5, 5 and 30%, respectively.

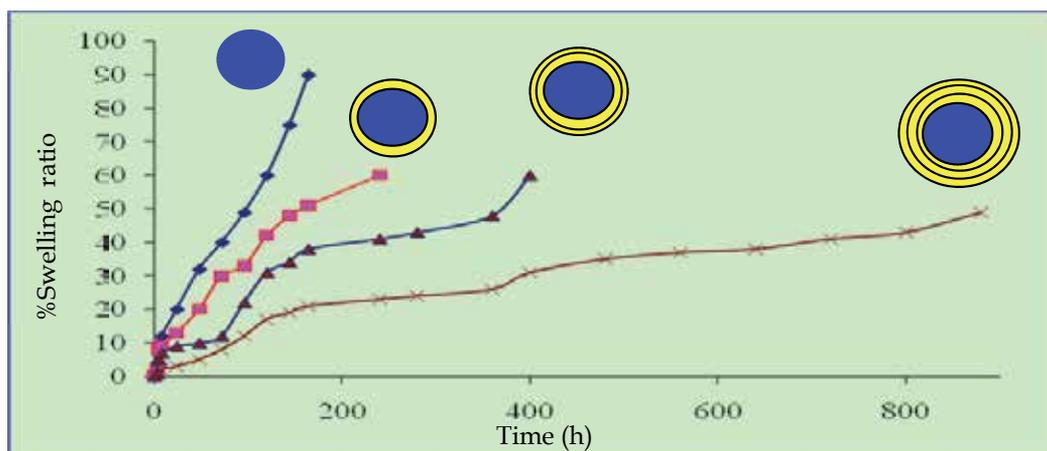


Fig. 17. Effect of NR on percentage of water uptake by beads obtained from sodium alginate and sodium alginate/ PVA (75:25) (Riyajan & Sakdapipanich, 2009a)

### Interaction of capsule and neem

Fourier transforms infrared; FTIR and XRD data were obtained to detect any chemical interactions between neem and sodium alginate and sodium alginate alone. The possible reaction between sodium alginate and GA in presence of hydrochloric acid is presented in Fig. 18. The presence of a large number of hydroxyl groups in PVA resulting from semi-interpenetration between sodium alginate and PVA and strong hydrogen bonding will affect the solubility of PVA in aqueous medium. The changes of the characteristic spectra peaks reflect the chemical interactions when two or more substances are blended (Riyajan & Sakdapipanich, 2010). After adding GA to blend samples, the intensity of the diffraction peak at 19° for PVA becomes flatter and broader (Riyajan & Sakdapipanich, 2010).

### Photodegradation and thermal studied

Thermal studies were conducted with Mettler-Toledo instrument (TGA/SDTA 851) analysis the heating rate for the thermogravimetric analysis of sodium alginate alone and capsule beads was 30°C/min. A small amount (1-3 mg) of sample was taken for the analysis and the samples heated from 30 to 800°C at in nitrogen. The TGA and DTG curves are drawn for each sample. The increase in NR layer on capsule was further confirmed by TGA analysis. The weight loss before 400°C in the TGA curves was attributed to the thermal degradation of polymer coating from capsules, from which the polymer content in the capsules particle. The TGA and derivative thermogravimetry (DTG) curves for the pyrolysis of capsules are shown in Fig.19. It was found that pure sodium alginate began to degage at 100°C and the residue left was about 0% at 450°C and reaches to maximum at 243°C. The NR shows better thermal stability than that of sodium alginate alone. For neem Aza-A capsules, the degradation of neem Aza-A experienced a comparatively long time and wide temperature range until 450°C. In the case of capsule NR coating, below 250°C there is no degradation. In the temperature range of 250–400°C about 85% of the material is degraded. During this stage weight loss and volatilization of degradation products take place rapidly. Beyond 420°C the weight loss is about 6–7%. The weight loss contents of capsule with NR coating at from 320 to 450°C increased with NR coating layers on capsules.

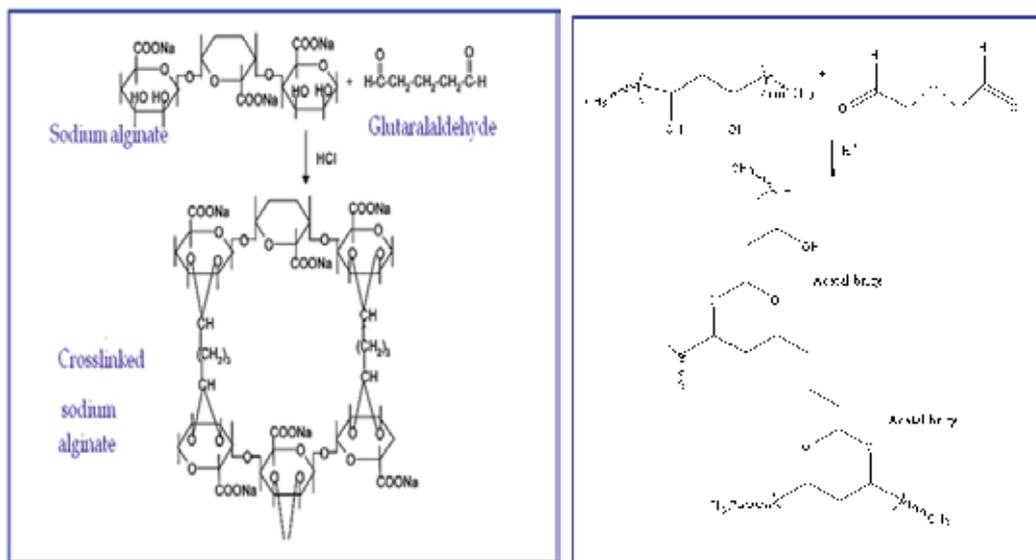


Fig. 18. The crosslinking product (a) between sodium alginate and GA containing acid and (b) between sodium alginate and GA containing acid (Riyajan & Sakdapipanich, 2010)

The rate of neem Aza-A degradation was reduced rapidly from the time of initiation and became constant after 30 h of UV irradiation as shown in Fig.20. When the neem oil was irradiated for 10 and 30 h, the residual neem Aza-A was 50 and 19%, respectively. The results responds to (Sundaram, & Curry, 1996). Based on the UV spectral data, UV protectants can be selected and matched to stabilize UV-labile pesticides. The employed material matrix for encapsulation of neem Aza-A was 0, 40, and 87% hydrolyzed PVAc. These results show that the efficiency of thermal stability for encapsulated neem Aza-A

obtained from the 87% hydrolyzed PVAc was higher than that of other samples. The residual of neem Aza-A for encapsulated neem Aza-A obtained from the non hydrolyzed PVAc was 85 and 78% after 10 and 30 h of UV irradiation time.

### 6. Release content uniformity, dissolution and releasing studies

Beads were evaluated for the neem content and this was done by refluxing a known mass of the beads with 100 ml of methanol at 65°C. Refluxing was continued for 1 h to ensure complete extraction of neem Aza-A from the beads. Then the absorbance of methanol containing the extracted amount of neem Aza-A was taken at a wavelength of 211nm in a HPLC (PerkinElmer LC) using pure methanol as a blank.

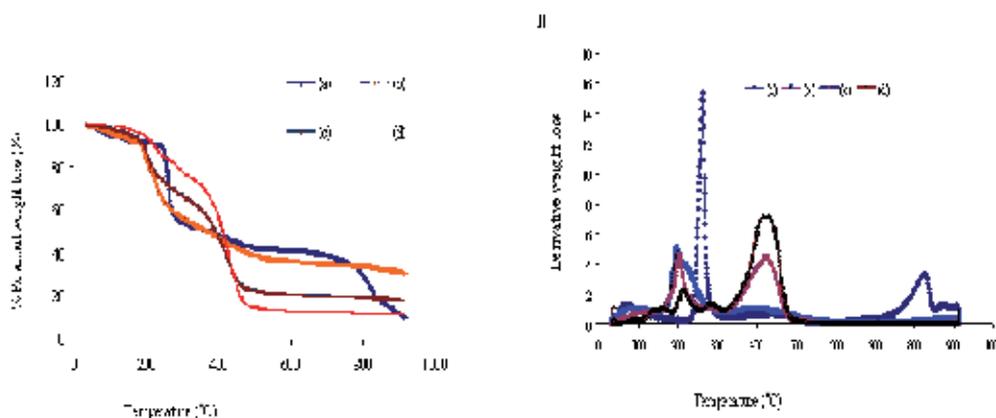


Fig. 19. Thermal behaviour of the capsule analyzed by I) TGA and II) DTG of sodium alginate alone (a) and capsules with (b) 0, (c) 1, (d) 2, and (e) 3 layers of NR coating

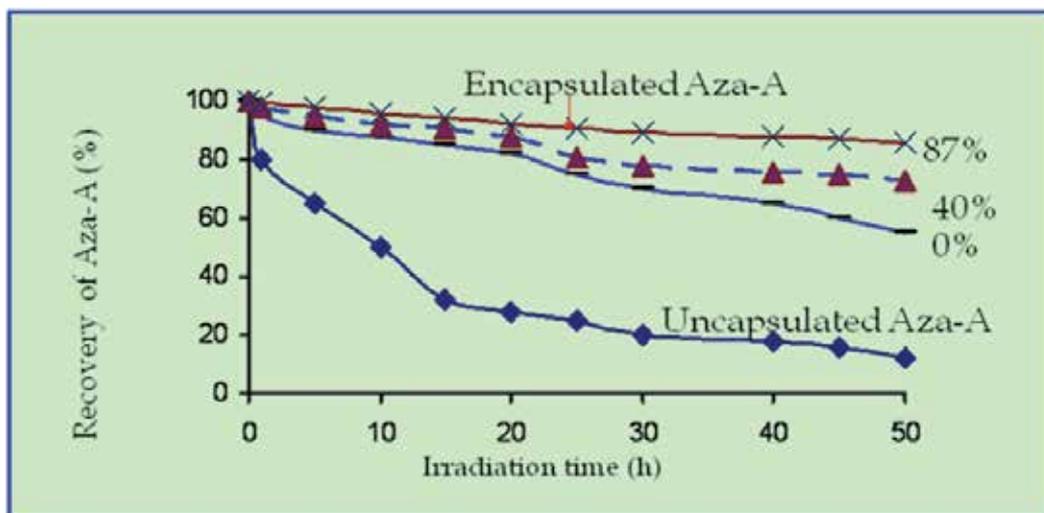


Fig. 20. Photodegradation behaviour of the capsule under UV irradiation (Riyajan & Sakdapipanich, 2009b)

The dissolution study was done in 250-ml conical flasks containing the dissolution media (0.1% Tween-80 solution in distilled water) with the closer caps which were kept in an incubator (WTB Binder, Germany) maintained at 35°C as shown in Fig.21. Two to three beads weighing about 10 mg were taken in the dissolution media. At definite intervals of time, the conical flasks were shaken well and a 10-ml aliquot was taken for the analysis of neem Aza-A using HPLC (PerkinElmer LC) at 211 nm. Experiments were performed in triplicate in order to minimize the variation error. The cumulatively release of neem Aza-A from capsule beads was estimated from HPLC experimental.



Fig. 21. Photographs of dissolution equipment from digital camera

#### Coaservation (Riyajan & Sakdapipanich, 2009a)

The encapsulation of neem (as called capsule) was prepared by sodium alginate as a controlled release polymer after crosslinking with GA, and then the capsule was coated with NR solution. The optimum condition for encapsulation of neem such as storage time in GA solution was investigated. In order to optimize the drying conditions, some samples of the beads with different extent of crosslinking were selected such that the initial weight is nearly equal. The percentage entrapment efficiency was varied by varying exposure time periods to the crosslinking agent. It was found that the percentage entrapment efficiency decreased drastically with a decrease in aqueous medium. Beads produced in 5.0% HCl in methanol exposed for 10 min showed the highest entrapment efficiency i.e., 91.2% and the lowest entrapment efficiency i.e., 73.6% was observed for 0.5% HCl content in methanol exposed for 30 min. Neem Aza-A is soluble in aqueous media and hence, an increase in the percentage entrapment efficiency was observed with an decreasing storage time period in GA containing HCl as a catalyst due to the increased release of the neem Aza-A from the matrix at longer time of exposure. Neem Aza-A release from the beads were subjected to a number of physical and chemical parameters including those related directly to the release medium, the release conditions (temperature) and those resulting from change in the characteristics of the controlling release device (beads). The effect of degree of crosslinking of sodium alginate beads on the kinetics of neem Aza-A release is depicted in Fig. 22. It is found that the higher the exposure time to GA the higher the release rate. The release rate of neem Aza-A beads at 10 min with exposure to GA have shown 100% release in the 5 h, whereas the neem Aza-A-loaded beads with exposure to GA have shown 100% at 10 h, but neem Aza-A-loaded beads with exposure to GAe have shown 100% release at 25 h. To observe the effect of the extent of crosslinking on the release kinetics of the beads exposed to the crosslinking agent, exposure to GA at 30 min were selected for NR coating. The effect of release rate of neem Aza-A from the beads coated with different layers of NR and exposed for 30 min to GA are presented in Fig. 23. The release profile from neem Aza-A without NR coating is also shown for comparison. It is obvious that the neem Aza-A release rate is reduced significantly by NR coating, which is consistent with the results of the swelling study. The NR film is very strong, rigid and hard to

swell, so the diffusion through this coating is the rate limiting step for swelling and neem Aza-A release. The release was prolonged by additional natural rubber layers on the capsule surface. The neem Aza-A cumulatively release of capsule derived from 2, 24, 72 and 240 in aqueous medium was 31, 69, 81 and 100%, respectively and when NR coated on capsule increase from 1 to be 3 layers, the neem Aza-A cumulatively release of capsule stored in at the same condition was 8, 29, 36 and 60%, respectively.

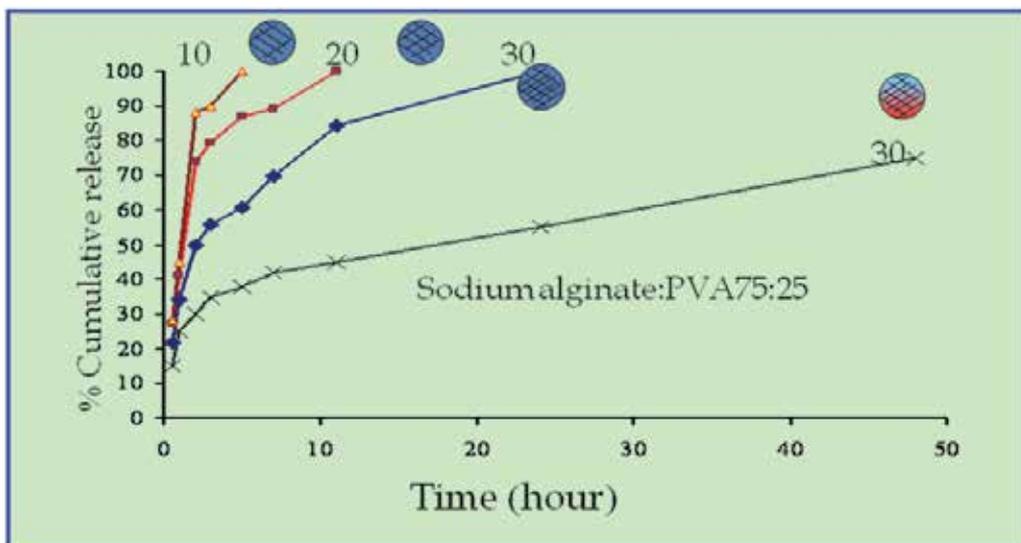


Fig. 22. Effect of crosslinking on releasing neem Aza-A for beads obtained from sodium alginate with different times and sodium alginate/PVA (75:25)

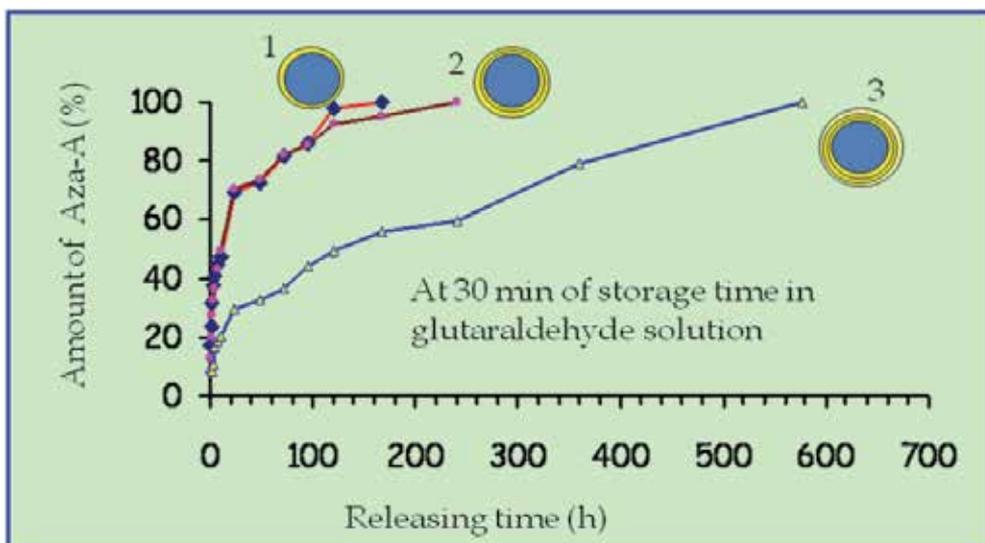


Fig. 23. Effect of crosslinking on the release neem for capsules coated NR (a) 1 layer (b) 2 layers and (c) 3 layers of NR (Riyajan & Sakdapipanich, 2009a)

It is to be noted that with an increase in NR coating, the capsule matrix becomes more dense resulting in a decrease in the rate of diffusion of neem Aza-A through the swollen beads, especially beads with three- NR.

### **Spray dryer** (Riyajan & Sakdapipanich, 2009b)

The neem Aza-A was dispersed evenly throughout the matrix of the capsule and was unable to diffuse to any significant extent within the matrix. However when the polymer matrix was placed in a thermodynamically compatible medium, the hydrolyzed PVAc swelled owing to absorption of the medium, then the neem Aza-A in the swollen part diffused out of the polymer matrix. The release of the neem Aza-A from the polymer matrix has been schematically described in Fig. 24.

Results of a study of the effect of polymer types on its rate of release in distilled water from capsules with 0, 40 and 87% hydrolyzed PVAc is shown in Fig. 24 and Table 1. It is clear that the release rate of neem Aza-A from the microcapsules was proportional to the release time. The release rate of neem Aza-A from microcapsules obtained from non hydrolyzed PVAc was high during the first 10 h followed by a slow release. Release of neem Aza-A from the non hydrolyzed PVAc microcapsules was found to be almost complete within about 15 h. This result indicates that high amount of neem Aza-A was presented on its surface of capsules. In the case of capsules obtained from the 40 and 87% hydrolyzed PVAc, the release rate of neem Aza-A from the microcapsules was high during the first 15 h. followed by a slow release. Finally, release of neem Aza-A from the microcapsules obtained from 40% and 87% hydrolyzed PVAc was found to be almost complete within about 25 and 30 h, respectively. This result indicates that neem Aza-A was entrapped in the polymer matrix. This could be explained by the amount of neem Aza-A released being dependent on its hydrophilicity in the polymer matrix as well as its solubility in water.

## **7. Mechanism**

The release results were investigated by using an empirical equation to estimate the value of  $n$  as follows (equation 4).

$$M_t / M_\infty = Kt^n \text{ or } \log (M_t / M_\infty) = \log (K) + n \log(t) \quad (4)$$

Where  $M_t/M_\infty$  is the released fraction at time  $t$ ,  $n$  is the release exponent, and  $K$  is the release factor. From the slope and intercept of the plot of  $\log (M_t/M_\infty)$  against  $\log (t)$ , kinetic parameters  $n$  was calculated as shown in Fig 25.

The  $n$  value of neem Aza-A coated with NR is represented, estimated from Fig.25. It was found that the  $n$  value of this sample obtained from 0, 1, 2 and 3 layers was 0.3515, 0.3766, 0.4476 and 0.3497, respectively at regression of 0.9988, 0.9875, 0.9870 and 0.9991, respectively. Thus, the neem Aza-A release mechanism of beaded coated with NR was Fickian diffusion. From previous works, on the basis of the diffusion exponent, an  $n$  value of 0.5 indicates that the release mechanism approaches a Fickian diffusion controlled release, whereas when  $n$  is equal to 1.0 this indicates the release mechanism approaches a zero-order release. In addition, an  $n$  value from 0.5 to 1.0 indicates a reactive agent release mechanism for non-Fickian diffusion (Riyajan & Sakdapipanich, 2010). Authours found that the value of  $n$  of 0.33 for the sample obtained from 75/25 PVA/sodium alginate after 30 min storage time in GA solution indicates that the release in this system deviates from a Fickian diffusion controlled release (Riyajan & Sakdapipanich, 2010).

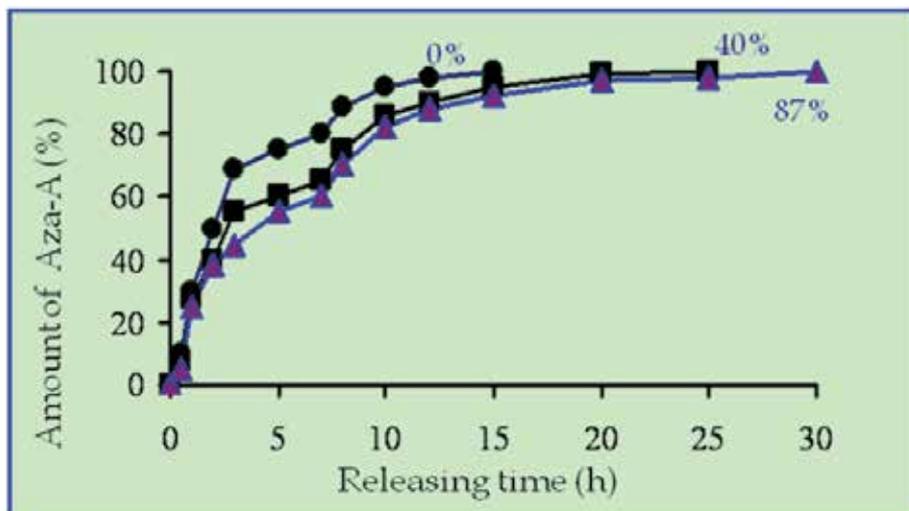


Fig. 24. Relationship between release rate of neem Aza-A and release time from microcapsules derived from encapsulated neem Aza-A in different hydrolyzed PVAc containing GA (Riyajan & Saktapipanic, 2009b)

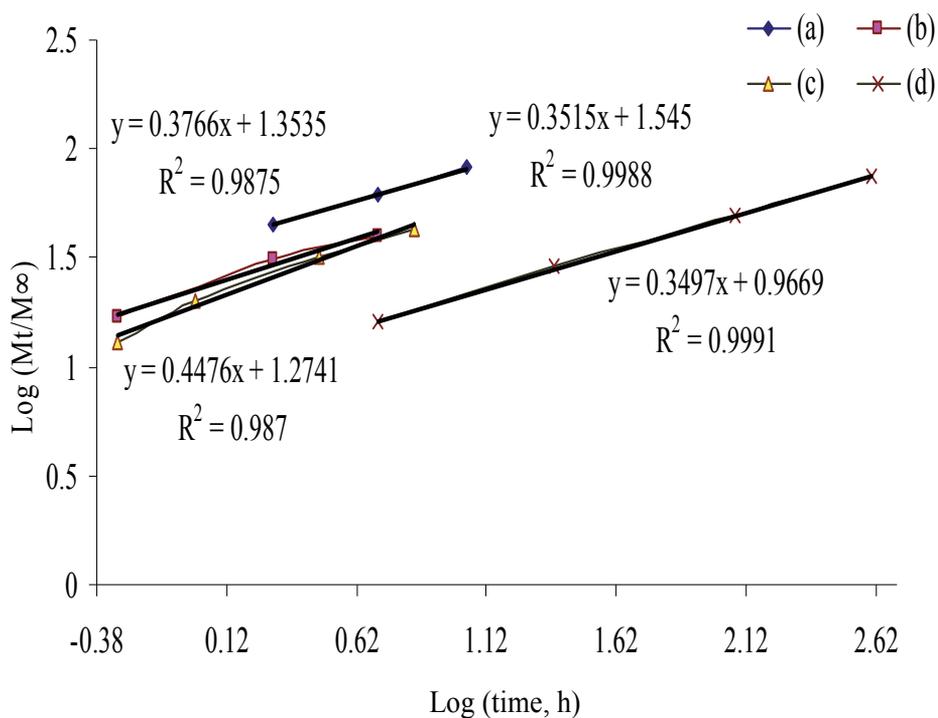


Fig. 25. Kinetic behaviour of the capsule with uncoated NR (a) and coated with (b) 1, (c) 2 and (d) 3 layer (s) of NR (Riyajan & Saktapipanic, 2009a)

The representation of swelling type controlled release system of neem Aza-A in aqueous medium is presented in Fig.26. The neem Aza-A from capsule coated with NR may be released by swelling process under particular conditions. In other systems, the wall is swollen by water action (Riyajan & Sakdapipanich, 2009a). In parallel, there are cases where the polymer matrix shows swelling with no significant limitations. The many factors affecting on the rate of the diffusion transfer of a solvent, including (a) the polymer transition from glassy to rubberlike state; (b) relaxation transitions on the surface and in the bulk of a sample; (c) dependence of the diffusion mobility of water on its concentration in the polymer; (d) expansion of the sample, reaching several tens or even a few hundred percentages with respect to the initial dimensions, requires development of a complicated multiparametric model of the water transport in polymers. In addition, the rate of neem Aza-A from capsule depend on NR coating. Thus, the time required to neem Aza-A from capsule increased with increasing NR coating layers.

In further work, all countries concentrate to the increasing concern globally regarding food production due to the exponentially increased human population by no changing environment. Therefore, the agriculture researchers find new pesticide herb and develop the method of encapsulation of herb with biopolymer. From previous work, the rapid increase in the number of publications was observed by the ISI Web of Knowledge concerning about "herbicide(s) or fungicide(s) or insectides(s)" and "metabolomics" or "metabonomics" or "metabolic fingerprinting" or "metabolic profiling" over the last decade (Aliferis, 2011). In addition, the particle size of capsule will be reduced to nano size. In parallel, the equipment of preparing capsule would be also developed.

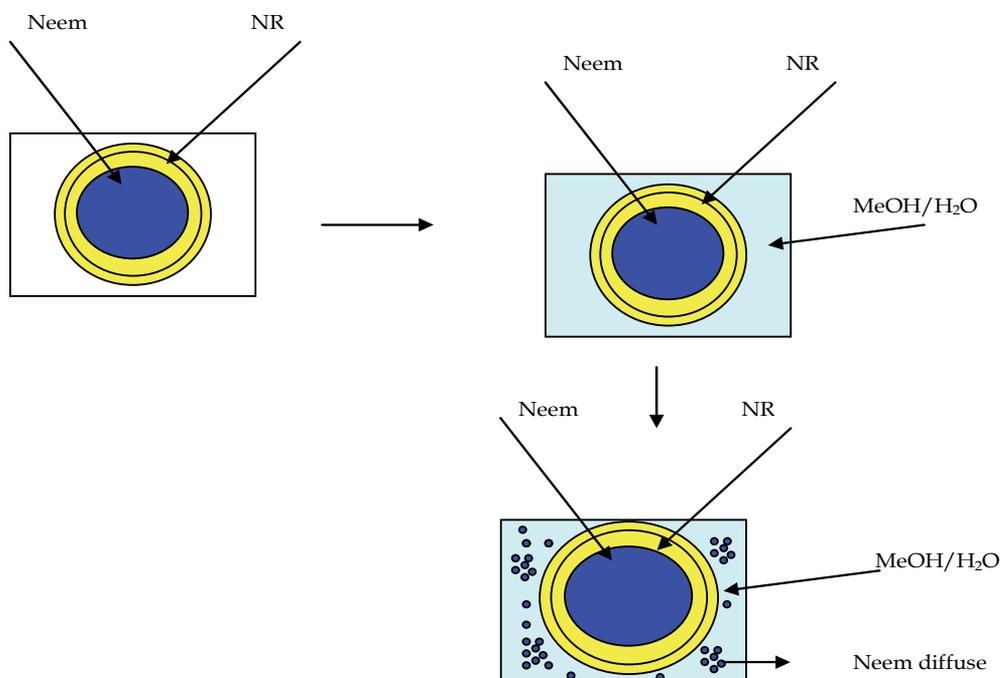


Fig. 26. Schematic representation of swelling type controlled release system of neem containing capsule dispersed in matrix Aza-A in aqueous medium

## 8. Conclusion

The neem Aza-A can be successfully encapsulated into biopolymer like sodium-alginate, PVA and PVA/sodium alginate containing GA and then it was coated with NR matrix. The two approaches of the encapsulated neem Aza A were investigated. In the case of the first method, the microcapsules of neem Aza-A with partially hydrolyzed vinyl acetate with 0, 40 and 87% hydrolysis as a matrix has also been prepared by spray techniques. Optimum condition of encapsulation for neem Aza-A by using spray drying was investigated. The SEM data indicated that the structure of the walls the beads are smooth and nonporous. The swelling results indicated that swelling of the polymeric beads decreases with increasing exposure time to the crosslinking agent. At particular intervals, the remaining concentration of neem Aza-A was analyzed by HPLC. The release data have been fitted to an empirical equation to estimate the kinetic parameter. It was found from the experiment that the ratio of 87% hydrolyzed poly (vinyl acetate) to water being 1:40 gave the highest concentration of neem Aza-A in the device. Finally, the successful encapsulation of neem Aza-A, was used by coaseversion technique and then coating with NR form. The swelling results indicated that swelling of the polymeric beads decreased with increasing exposure time to GA and reduced the rate of release of the pesticide. The degree of release of neem Aza-A from capsules was controlled by their condition of formation. In addition, we studied the effect of PVA/sodium alginate ratio on release of neem Aza-A from capsule. The degree of neem Aza-A release from capsule dramatically decreased when amount of PVA in composite blend increased. Inclusion, the obtained capsules have possible potential used in agriculture field.

## 9. Acknowledgment

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# Use of Botanical Pesticides in Modern Plant Protection

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## 1. Introduction

The European Union has made very clear political decisions to increase environmental awareness. A Thematic Strategy on the Sustainable Use of Pesticides was launched by the Commission of the European Communities in 2006. It was decided to minimize the hazards and risks to health and the environment caused by the use of plant protection products. In 2009, the European Parliament accepted a new framework directive on the sustainable use of pesticides. Directive 2009/128/EC fosters the development of plant protection and integrated pest management (IPM) in the EU. The directive states that *“when pesticides are used, appropriate risk management measures should be established and low-risk pesticides as well as biological control measures should be considered in the first place”*. Biological control comprises various technologies of which one option is the use of botanical products. Many kinds of plant species and technologies have been used in the production of botanical pesticides. Some but not many of the plant-based pesticides have already become established plant protection products (Isman 2006).

There are many methods to extract essential oils and liquids from plant material, the most popular being steam distillation. Other methods are expression, enfleurage, maceration, solvent extraction and pyrolysis. Pyrolysis has been thoroughly described by Tiilikkala et al. (2010), proving that slow pyrolysis has been known for thousands of years. It has been used in the production of charcoal (biochar), tar, pitch and wood vinegar. The liquids which are most useful as biocides and pesticides are tar and wood vinegar. Tar has mainly been used as a wood preservative. The production and use of wood vinegar has increased rapidly in Asian countries, including Japan, China, India and Thailand. As a result of active development numerous botanical pesticides have been put on the market during the last 10 years (Tiilikkala et al. 2010). Simultaneously, pyrolysis has also become a frequently used technology in waste treatment which may lead to a rapid increase in the production of liquids based on the use of organic matter.

The raw materials used for the production of botanicals will differ in different parts of the world because of divergent natural resources. In Finland we have a huge reserve of wood

material and thus it was decided at MTT Agrifood Research Finland to search for modern pesticides from birch. The defence mechanisms of birch (*Betula* sp.) and the chemical compounds involved have been well studied (Novriyanti et al. 2010). The defence mechanisms are activated when a plant is under stress. Pests (including mammals) and plant diseases often attack birches and disturb their normal growth. Strong defence mechanisms are needed to survive the attack by herbivores and birches react to these in various ways. Plant defence can be divided into constitutive defences and induced defences. The defence methods used by birches within these categories are chemical, physical and phenological, depending on the plant organ. All these methods are found in leaves, but in the bark and woody parts the main defence mode is chemical. Different models, such as the optimal defence, carbon-nutrient balance, growth rate and growth differentiation hypothesis have been developed to explain variations in plant defence against herbivores. However, in birch the defence mechanisms are very complex and variations may depend on the type of stress or consumer and the genetical regulation (Novriyanti et al. 2010).

Pyrolysis was the “leading technology” three hundred years ago in Finland when the country’s “bioeconomy” was based on pine tar trade in Europe. Consequently, pyrolysis was a self-evident technology for extracting plant-based pesticides from wood. However, much of our experience of the use of pyrolysis liquids as pesticides can be applied in the commercialization of all kinds of botanical pesticides. The route to bring an effective botanical to the market from the stage of an innovation is not predicated on the extraction technology but on many other factors of the commercialization procedure. REGULATION (EC) No 1107/2009 (EU commission 2009) is the most important guideline for SMEs planning registration of biological plant protection products in Europe.

The aim of this article is to demonstrate the potential of slow pyrolysis liquids as pesticides and give examples of the use and role of botanical pesticides in modern IPM programmes. The information requirements and the bottlenecks in the commercialization of botanicals are discussed.

## **2. Efficacy of pyrolysis liquids as plant protection products**

### **2.1 Usability of pyrolysis liquids as repellents and insecticides**

Efficacy studies on birch tar oil (tar and wood vinegar) have been in focus for several years at MTT and at the University of Helsinki. After screening possible organisms suitable for further investigation by treating pests, diseases and weeds with two birch tar oils either alone, as a mixture or together with Vaseline®, the most promising target organisms were found. It was proved that birch tar oil, when painted on fences and pots, most efficiently prevented the molluscs *Arianta arbustorum* and *Arion lusitanicus* (Lindqvist et al. 2010) from crossing the barriers to reach the food behind the fence or in the pots. In addition, repeated treatments with birch tar oil in combination with short intervals or mixed with Vaseline were needed to achieve a long-term effect against slugs and snails (Figures 1 and 2).

The repellence of birch tar oil was also seen in a choice bioassay experiment with egg laying psyllids (*Trioza apicalis*) but did not affect flies (*Delia floralis*) (Tiilikkala & Segerstedt 2009). In a laboratory experiment in Greece, aphids (*Myzus persicae*) on eggplants were effectively killed (95 %) when sprayed once with birch tar oil (1% v/v aq solution) (Figure 3). However, the application seemed to be phytotoxic.

The repellence was observed in mammals as well. Field voles (*Microtus agrestis*) housed in a terrarium, avoided treated apple branches (Figures 4 and 5) if untreated branches or other food was available (Tiilikkala & Segerstedt 2009). Similarly, voles in the apple orchard injured birch tar oil-treated apple stems only when untreated trees were not available or when the vole population density was peaking. Orihashi et al. (2001) reported similar results in evaluating the deterrent effect of rosin and three wood tars on gray sided voles (*Clethrionomys rufocanus bedfordiae*) – high population density seemed to correlate with slight effect of rosin. Moreover, they proved that all tars were effective in repelling voles, but wood vinegar without tar did not inhibit voles from barking the tree material tested.



Fig. 1. Pots moved out from an experimental field where a high number of slugs (*Arion lusitanicus*) attacked the pots every night. Chinese cabbage was eaten by the slugs within one week when grown in pots without the birch tar oil treatment.



Fig. 2. Pots painted with a repellent made of birch tar oil (mixture of tar and wood vinegar). No slugs or snails were found from the pots and the test plants were untouched at the end of the experiment.

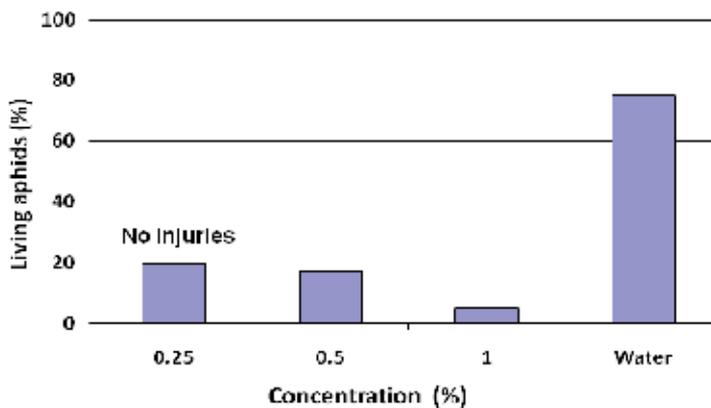


Fig. 3. Number of aphids on eggplant four days after spraying with birch tar oil. Three different concentrations were used: 1%, 0.5% and 0.25%. The last bar on the right refers to a birch tar oil free control.



Fig. 4. Branches of an apple tree were cut and treated with birch tar oil. Field voles did not touch the branches if untreated branches were available.



Fig. 5. Untreated branches of an apple tree were totally eaten within one day by field voles in a test terrarium.



Fig. 6. Moose repellents must be covered with a material to prevent the impact of rainwater. If not covered, wood vinegar will leach to the soil within a few days.

It has been observed that excessive moisture or rainwater will easily dilute active components of pyrolysis liquids (Figure 6). Efficacy tests may not show any impact if the repellents have not been protected against rainwater (Härkönen & Heikkilä 2009). Further evaluation of fractions made from wood tar revealed that all fractions contained repelling components (Orihashi et al. 2001). Beyond these results a literature review on the efficacy of wood vinegar used as a pesticide indicated that only a limited number of scientific publications are available (Tiilikkala et al. 2010). In spite of this wood vinegar has been widely used as a pesticide based on old traditions and knowledge of users and local producers.

## 2.2 Weed control with pyrolysis liquids

In a weed control experiment Tiilikkala & Segerstedt (2009) showed that birch tar oil could control broad-leaved weeds such as *Chenopodium album* and *Stellaria media*. Preliminary field experiments already showed the potential of wood vinegar as a herbicide (Figure 7). In weed control acetic acid is probably the most important component (Tiilikkala unpublished data). Experimental work with the pyrolysis liquids has shown clearly that the efficacy of botanicals depends very much on the application technology. Sprayers developed for use with synthetic chemicals are not well suited for the application of wood vinegar. It is obvious that the development of plant-based pesticides should be linked to the development of novel application technologies. With suitable application technology wood vinegar can be used for control of broad-leaved weeds including invasive alien species such as *Heracleum sp.* (Figure 8).



Fig. 7. One spray with wood vinegar controlled weeds in a carrot plot effectively (left). On the right, dense weed vegetation completely smothered the carrots.



Fig. 8. Birch tar oil proved to be an effective herbicide for control of giant hogweed (*Heracleum* sp). The oil was poured inside the gut vessel of the weed which did not recover after the treatment.

### 2.3 Use of pyrolysis liquids as a fungicide

Preliminary results of a laboratory experiment indicated that birch tar oil (10 and 30 % v/v aq solution) inhibited growth of wood rotting fungi (*Cylindrobasidium evolvens*, *Libertella* sp. *Stereum hirsutum* and *Chondrostereum*) on Petri dishes (Tiilikkala & Segerstedt, 2009). The same effect was seen on the cut surfaces of birch branches treated with birch tar oil (Figure 9). In laboratory conditions, volatile components of birch tar oil effectively inhibited growth of potato late blight (*Phytophthora infestans*) fungi (Figure 10). A similar control effect was difficult to achieve outdoors when the same product was sprayed with conventional equipment (Tiilikkala & Segerstedt 2009).

Many control technologies have been developed to inhibit fungi that cause discolouration on timber. It has been shown (Velmurugan et al. 2009) that wood vinegar made from bamboo and broad-leaved trees is effective against sapstaining fungi. The results revealed that compounds of Chikusaku-eki and Mokusaku-eki markedly inhibit fungal growth and the product possesses both antifungal and antioxidant properties as well as potential for use as a natural preservative in woodworking industries. The antifungal efficiency of wood vinegars was reported to be strongly dependent on their phenolic compound content (Baimark et al. 2009).



Fig. 9. One cut surface of a birch log (left) was dipped in birch tar oil and photographed after storage for one year outdoors. Many kinds of wood rotting fungi were growing on the untreated cut surface (right) but not on the treated surface.



Fig. 10. Untreated potato leaves covered with potato late blight mycelium (left) and leaves sprayed with birch tar oil before inoculation with the fungi.

### 3. Toxicology of birch tar oil

Hagner et al. (2010a) reported that the risk to the soil environment caused by birch tar oil (concentration 500–1360 L/ha) is insignificant and short-term compared to many synthetic plant protection products. A proper dose of the pyrolysis liquid when sprayed on the soil surface activated soil organisms shortly after the application (Figure 11). The authors concluded that pyrolysis liquids could be listed as “Minimal Risk Pesticides” like many essential oils in the USA.

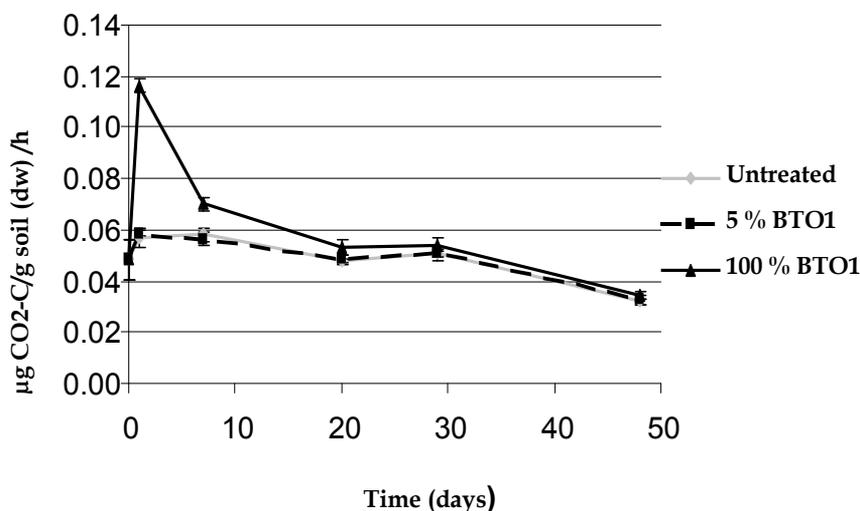


Fig. 11. Impact of a birch tar oil treatment (BTO1) on the activity of soil microbes (mean values + S.D., n=5). Time is given in days from the start of the pot experiment. The bioactivity of soil microbes is presented as CO<sub>2</sub> emission.

In their toxicity tests with a wide spectrum of aquatic organisms Hagner et al. (2010b) reported that these biota are invariably sensitive to birch oil distillates. However, the authors suggested that birch tar oil does not pose a severe hazard to aquatic biota (Figure 12).

Due to variations in manufacturing techniques, pyrolysis liquids differ in chemical composition and, consequently, the toxicological studies are divergent (Schmid et al. 1996). Very little is known about the toxicity of birch tar and wood vinegar, although the liquids have been used for hundreds of years in East European countries like Russia (Nozdrin et al. 2004). The lack of knowledge about the toxicology of pyrolysis liquids has been noted in many publications (Orihashi et al. 2001).



Fig. 12. Test fish swimming in a box treated with birch tar oil (left) and in a control box without birch tar oil (right). No negative impact was recorded on fish although the fish were hardly visible because of the birch tar oil treatment.

#### 4. Chemical composition of pyrolysis liquids

The characterization of fast pyrolysis liquids has been continued for a long time. The products contain many organic components and the composition is very complicated. According to literature, the main organic components of liquids from fast pyrolysis are methanol and acetic acid (Tiilikkala et al. 2010). Other components are acetone, methyl acetone, acetaldehyde, allyl alcohol, furan and furfural, as well as formic, propionic and butyric acids. The settled tars can be fractionated into light and heavy oil fractions. The former consists of aldehydes, ketones, acids and esters. Various phenols, including a high proportion of cresols and pitch, are present in the heavy oil fraction.

The chemical composition, physical properties and fuel oil quality of fast pyrolysis liquids has been extensively developed and described by Oasmaa & Meier (2005). Similar information on slow pyrolysis products is needed. The production of a standard wood vinegar from divergent plant material is a challenging task for the SMEs which produce biochar, charcoal and wood vinegar. Practical quality criteria for pyrolysis products are needed and technology for quality assurance should be developed (Tiilikkala et al. 2010). The variability of botanical products is a well known phenomenon and carefully considered e.g. in the production of botanical medicine (Shane-McWhorter 2001).

## 5. Registration of biological plant protection products

Isman (2006) wrote that pyrethrum has been approved as a pesticide worldwide, but the commercialization of other botanical insecticides is variable. The use and marketing of wood vinegar has grown rapidly in many Asian countries but not in Europe (Tiilikkala et al. 2010). Isman (2006) listed several barriers to commercialization of botanical insecticides and, respectively, Tiilikkala et al. (2006) discussed the regulatory problems of pyrolysis liquids in Europe. Regulatory barriers to all biological control agents were dealt in the report of the REBECA Project (Ehlers 2006). The authors of the REBECA report concluded that SMEs applying for registration of new botanicals should be financially supported by specific programmes and should be given detailed guidance by the regulatory authority. The REBECA group suggested that funding could come from various sources, such as rural development agencies, IPM and organic action plans, promotion of SMEs or taxes on pesticides. A follow-up of the commercialization of wood vinegar in Finland has proved that innovative SMEs rarely have sufficient resources to obtain e.g. the toxicological data which is required for the registration of all kinds of pesticides.

## 6. Conclusion

In the future the use of pesticides will be tightly regulated because of well-documented environmental risks in the use of synthetic chemicals (Ongley 1996). This may lead to a growing demand for biological plant protection agents including use of botanicals. Based on hard data and scientific publications it seems evident that plant extracts are biodegradable and thus will not cause similar environmental risks as many of the widely used synthetic chemicals. The option of replacing fossil oil-based chemicals with plant extracts fits well with food and agriculture policies directed to the future (Lee & Neves 2009). Sustainable food security cannot rely on the use of fossil oil as has been the case for a long time in the highly developed countries. Local resources must be utilized in agriculture and thus also recentralized production of biopesticides should become a common practise. In fact, this is already the case in many parts of the world where farmers have never had money to buy synthetic chemicals. The development of organic farming as predicted by FAO (2009) may boost the use of botanical pesticides and biological pest control globally.

Various extraction technologies will be used for production of plant-based liquids. Many kinds of raw material can be used as the source of the bioactive molecules. Slow pyrolysis is a powerful technology, because different types of materials can be processed. The price of the by-products, such as tar and wood vinegar, will be reasonably low which will make it possible for all farmers to use pyrolysis liquids as pesticides. In the future, production of biochar or agrichar will increase the volume of pyrolysis liquids substantially if claims

(Verheijen et al. 2009) concerning the positive impacts of biochar in agriculture can be proved. One of the main challenges will be the production of liquids with standard quality. This problem applies to all extraction methods and producers who are using plants or waste as raw material for the production of green chemicals.

The efficacy of wood tar as a biocide has been known for thousands of years, but evidence to prove all the claims about the efficacy of wood vinegar as a plant protection product needs to be verified. However, it is obvious that pyrolysis liquids can be used as raw material for making repellents, insecticides, molluscicides, herbicides and fungicides. In most of the products the efficacy is based on a mixture of many components. This is one of the main difficulties in the registration of botanicals as pesticides. The registration procedure has been developed for registration of one single active (synthetic) ingredient but not for mixtures of green chemicals. In practice, the efficacy of botanicals depends very much on the formulation of the product and on the application technology. Water can wash wood vinegar from plants and repellents, which means that rain or soil moisture can eliminate the efficacy of the vinegar in a short time. Standard efficacy studies must be adjusted to the functions of botanicals. Very often more frequent use of botanicals is needed compared to use of synthetic chemicals and the push and pull theory must be known e.g. in control of insects and other mobile pests. Formulation of slow-release products will increase the efficacy of botanicals such as wood vinegar (Lindqvist et al. 2010).

Pyrolysis liquids have been used for thousands of years without any records of the risks on the environment. However, this kind of historical information is not usable in the registration process of pesticides. Official data proving environmental impacts must be obtained for every product from laboratories which have an official quality system. Scientific evidence has proved that birch tar oil is an environmentally friendly product (Hagner et al. 2010a, 2010b). Similarly, it has been shown that essential oils do not pose any threat to the environment (Misra & Pavlostathis 1997). However, it is still uncertain if scientific publications are valid documents in the registration process for biological plant protection products.

Biodegradability of botanicals may be an important factor which will increase demand for plant-based products. On the other hand, we have found that the rapid biodegradability of botanicals may hinder the registration process. It is very difficult to obtain data indicating the spread of botanicals in water, because all measurable components break down in soil within a few days. There is no single active ingredient or decomposition product which could be used as an indicator of the leaching risk. No data - no progress in registration.

Risks to human health are a very important factor which must be analysed and documented before the registration of all kinds of pesticides. Many of the tar and wood vinegar products have been used as skin ointments and thus spread intentionally on the skin of patients. However, this kind of historical knowledge does not alleviate the need to obtain scientifically sound data proving the low risks of every single botanical pesticide on human health. It is generally well known that plant-based components may cause e.g. allergic reactions in sensitive people. Botanicals consist of hundreds of components as a mixture and the impacts of the mixtures must be studied in depth.

Commercialization of synthetic chemicals is very expensive because of the need for hundreds of documents which are required for the registration process of an active ingredient and a plant protection product. Only economically strong companies have been able to fulfil the demands for information. The SMEs which produce botanical pesticides do

not have the resources to cover all the costs of registration. Public funding is needed from many sources as was suggested in the reports of the REBECA Project (Ehlers 2006).

Botanicals may have an important role as pesticides in the modern world. However, it will take a long time before the potential of new innovations can be realized. The whole agribusiness is tightly committed to the use of fossil oil-based agrochemicals and the structure of the business must be radically changed before full-scale commercialization of botanicals is possible. While waiting for the business to change, much research work needs to be done to justify the claims of the already known and used plant-based products. Studies on ecotoxicology and toxicology need a lot of time and funding. Simultaneously the application technology used for spraying synthetic chemicals needs to be rebuilt so that modern technologies will adapt to the needs of the modern plant protection products – botanicals.

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# **Bacillus-Based Biological Control of Plant Diseases**

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## **1. Introduction**

Plant diseases cause considerable losses in crop production and storage. Nowadays, growers still rely heavily on chemical pesticides to prevent, or control these diseases. However, the high effectiveness and ease of utilization of these chemicals can result in environmental contamination and the presence of pesticide residues on food, in addition to social and economic problems. Consequently, there is an increasing demand from consumers and officials to reduce the use of chemical pesticides. In this context, biological control through the use of natural antagonistic microorganisms has emerged as a promising alternative. Indeed, these biopesticides present many advantages in term of sustainability, mode of action and toxicity compared to chemical pesticides. Here, we focus in details on the versatile utilization of *Bacillus* based products as biopesticides. More precisely, a special emphasis is given to the three main specific mechanisms involved in biocontrol of plant diseases by this bacterial genus: competition for ecological niche/substrate in the rhizosphere, production of inhibitory chemicals and induction of so-called systemic resistance in host plants. Beside this, strategies for enhancing the efficacy of *Bacillus*-based biopesticides are also discussed.

## **2. Potential for microbial biocontrol agents in agriculture**

### **2.1 Interest in the development of biopesticides**

As all living organisms, plants must face infections and diseases following the attacks of a mass of plant pathogens and pests from animal, microbial or viral origin. These diseases can be minor causing solely a reduction of plant-growth capacities or can be at the origin of much more severe damage leading to plant death in the worst case. Plant diseases are responsible for the loss of at least 10% of global food production, representing a threat to food security (Strange & Scott, 2005). Agrios (2004) estimated that annual losses caused by disease cost US\$ 220 billion. Worldwide, plant diseases were responsible for severe famines in the past (Agrios, 2004). For example, potato blight caused by the plant pathogenic oomycete *Phytophthora infestans* on potato cultures caused more than one million deaths in Ireland during the “the great famine” between 1845 and 1849 (O'Neill, 2009).

To prevent or control these diseases, producers have become increasingly dependent on agrochemicals, especially over the past few decades, as agricultural production has intensified. However, despite the great effectiveness and ease of utilization of these products, their use or misuse has caused many problems including significant pollution of soils and ground water reservoirs, accumulation of undesirable chemical residues in the food chain, emergence of fungicide-resistant strains of pathogens, not to mention health concerns for growers (Fig. 1). According to the Stockholm convention on persistent organic pollutants, 10 of the 12 most dangerous and persistent organic chemicals are pesticides (Gilden et al., 2010). An example is the synthetic pesticide dichlorodiphenyltrichloroethane, well known as DDT, which was extensively used in agriculture between 1950 and 1980 and was found genotoxic in human and responsible for endocrine disorders (Cohn et al., 2007). Consequently, there is nowadays an increasing demand from consumers and authorities for more safe, rational, sustainable and eco-friendly strategies. This has resulted not only in stricter regulations concerning pesticide use, commercialization and production but also in the development of alternative strategies including genetic adaptation of crops, modification of cultural practices and use of biopesticides.

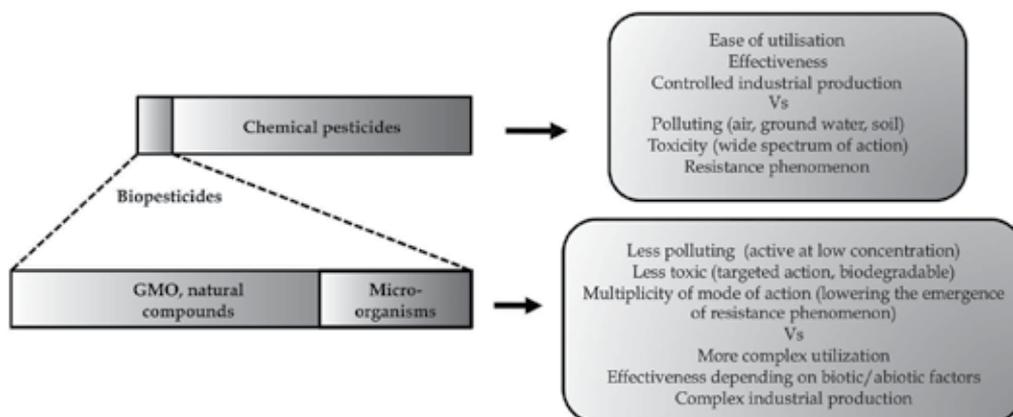


Fig. 1. Market share and (dis)advantages of microbial biopesticides versus chemical phytosanitary products.

## 2.2 Advantages and marketshare of biopesticides

Biopesticides, which are used to suppress pathogen populations, are living organisms or natural products derived from these organisms. They can be divided into four main groups: microorganisms (microbial pesticides), other organisms (nematodes, insects...) used to control pests, natural substances that are derived from living organisms (biochemical pesticides) and plant-incorporated protectants (genetically modified plants) (EPA, 2011; Thakore, 2006). Biopesticides show several advantages when compared to chemical products. They decompose more quickly in the environment and are generally less toxic towards non-target species (Thakore, 2006). Additionally, their modes of actions are usually distinct from those of conventional pesticides. This implies that they can often help suppress resistant pathogens and that they can be applied in alternation with other pesticides to avoid resistance development (see below and Fig. 1).

Among biopesticides, microorganism-based products represent about 30% of total sales and have a variety of applications. They are used in field crops and greenhouses to reduce

diseases on various cereals, legumes, fruits, flowers and ornamental plants caused either by soilborne, foliar or post-harvest pathogens. These plant protective microorganisms, mainly fungi and bacteria, are often isolated from suppressive environments. In other words, these beneficial microorganisms are generally obtained from aerial or underground parts of plants that are naturally less or not at all affected by a pathogen that devastates a neighboring group of the same plant species (Cook & Baker, 1983; Ryan et al., 2009). One of the advantages of microbial biopesticides compared to most other phytosanitary products is the multiplicity of their ways of actions globally based on competition for nutrients and space, direct antagonism of plant pathogen growth and host plant immunization (see below). Compared to GMOs, microbial pesticides benefit from a better consumer acceptance. In Europe, there are also several legal barriers against GMOs. In comparison with natural extracts, microbial pesticides often retain the advantage of having a persistent activity through time. Indeed, microbial agents can establish themselves in the phytosphere and produce continuously bioactive compounds *in situ*. Moreover, as these active molecules are produced in direct contact or very close to the target organisms, only limited quantities are needed for efficacy.

In addition to their potential to directly reduce the incidence of diseases, some microbial products also have other positive effects on crops such as promoting plant growth and nutrition (biofertilizers and phytostimulators) and/or facilitating interaction between the host plant and other beneficial organisms (Antoun & Prevost, 2006). A large amount of nutrients present in the soil are in an insoluble form that is unavailable for the crops (Francis et al., 2010). Biofertilizers act through the direct improvement of plant nutrition either by solubilizing these nutrients or by fixing atmospheric N<sub>2</sub>. In the case of solubilization, several mechanisms may be involved depending on the nature of the nutrient. For example, phosphate can be released from insoluble organic forms by several microbial enzymes like phytases or non-specific phosphatases, while inorganic phosphorus stocks are solubilized through the production of organic acids by the beneficial bacteria. Phytostimulation is the direct promotion of plant growth through the modulation of the plant's hormonal balance. Several microorganisms are capable to produce and excrete a variety of plant hormone-like compounds including auxin, gibberellins, cytokinins etc. Some microbial agents produce enzymes that degrade a precursor of ethylene thus limiting the levels of this hormone in the plant thereby increasing plant growth especially under stress conditions (Francis et al., 2010; Lugtenberg & Kamilova, 2009). Both biofertilization and phytostimulation are important phenomena in the context of the constant need to produce more food on fewer surfaces with the simultaneous wish to reduce reliance on chemical fertilizers. Moreover, a microorganism that possesses a combination of these growth-promoting activities and biocontrol potential offers the advantage to supply the crop in one application with a biopesticide and a biofertilizer. In addition, better nutrition of the plant often enhances its overall resistance against pathogens and other stress factors (Bent, 2006).

Despite providing such advantages, biopesticides take up only a small share of the pesticide market, 2.5% in 2005, which still represents an important business as global pesticide sales in 2005 reached 26.7 billion dollars. Biopesticides have gained more and more interest over the years with a market share in 2000 of only 0.2% and an expected 15% annual market growth. Moreover, conventional pesticides have been slowly losing ground since 2000, with an expected decline rate of 1.5% per year (Thakore, 2006).

### 2.3 Biopesticides and integrated pest management

Biopesticides thus play an important role and are legally accepted for use in integrated pest management and organic agriculture. According to the US Environmental Protection Agency (EPA), Integrated Pest Management (IPM) is an effective and environmentally sensitive approach that relies on a combination of common-sense practices (EPA, 2011). IPM programs use current, comprehensive information on the life cycles of pests and on their interaction with the environment. This information, in combination with available pest control methods, is used to manage pest damage by the most economical means, and with the least possible hazard to people, property, and the environment. IPM may involve a judicious use of pesticides by contrast with organic food production that applies many of the same concepts as IPM but limits the use of pesticides to those that are produced from natural sources, as opposed to chemicals.

An example of integration of alternative/biological methods in IPM is given here for the control of lily diseases and pests. This program was developed in a company specialized in the cultivation of lily, located in Holambra, SP, Brazil, with a history of intensive use of fungicides, insecticides and miticides. Phytosanitary problems in lily culture of high value, limit its cultivation. Diseases may originate from several agents such as the fungi/oomycetes *Botrytis elliptica*, *Phytophthora*, *Fusarium*, *Sclerotinia*, *Penicillium*, *Rhizoctonia* and *Pythium* or pests such as aphids, fungus gnats, leaf miners, thrips and caterpillars. To solve these problems, in 2000, over 30 different chemical pesticides had to be used routinely at a cost of US\$ 10.00/m<sup>2</sup>/year in a cultivated area of 13,500 m<sup>2</sup>. For these products to keep working properly, growers needed to use increasingly higher doses and more toxic products, but losses due to pests and diseases kept increasing. Facing such a situation, the decision was made to change the production system. To achieve integrated control of cultural problems, the use of chemical pesticides was gradually replaced by the integration of biocompatible methods to control pests and diseases like introducing a diversity of microorganisms for biocontrol. Along with this substitution of chemical pesticides, an adaptation of fertilization procedures was needed to improve the survival of the biocontrol agents. The first step was to stop using the most toxic pesticides which took about two years. One additional year was required to successfully replace the use of chemical pesticides of less toxic levels. In general, the current production is based on the treatment of a steam-disinfested substrate with aerobic compost tea and beneficial microorganisms such as *Trichoderma*, *Metarhizium*, *Beauveria* and *Bacillus*. *Clonostachys rosea* and *Trichoderma* sp. are sprayed weekly to control *Botrytis* and other pathogens. When necessary, neem oil, propolis, phosphite and others alternative products are used. Associated with these products and with balanced fertilization, a sanitation program is maintained in all the greenhouses with the elimination of diseased plants or plant's parts. Also, traps and monitors for controlling the relative humidity in greenhouses are used. Currently, no chemical pesticides are used, except for bulbs, which are treated with imidaclopride before planting to control aphids, in order to comply with phytosanitary standards for exportation. The success is due, not only to the substitution of chemical pesticides by biopesticides and biocompatible products, but also by reconsidering the entire production system. The acreage today is 27,500 m<sup>2</sup> with an approximate cost to control disease and pest problems of US\$ 3.00/m<sup>2</sup>/year (Wit et al., 2009). The same strategy is used for the control of disease on *Spathiphyllum*, avoiding any chemical pesticide input and involving *Bacillus subtilis* for the control of *Cylindrocladium spathiphylli* (Wit et al., 2009).

### 3. Interest in *Bacilli* as biopesticides

Bacterial products represent the majority of the microorganism-based biopesticides but fungal biocontrol agents were also developed as efficient products (recently reviewed by Shores et al., 2010). Among the bacterial biocontrol agents, *Bacillus thuringiensis* accounts for more than 70% of total sales. This bacterium is essentially used for insect pest control and is the origin of the gene used in insect resistant "Bt GMO crops". Other isolates from several bacterial genera have been used successfully for crop protection and numerous products listed in Table 1 are currently commercialized in the world for the control of important plant diseases (Table 1). As illustrated in this table, about half of the commercially available bacterial biocontrol agents are *Bacillus*-based products but strains of the other genera, including *Streptomyces* and *Pseudomonas*, were also marketed for biocontrol in the recent years.

The *Bacillus* genus encompasses a large genetic biodiversity. *Bacilli* are present in an extremely large palette of environments ranging from sea water to soil, and are even found in extreme environments like hot springs (Hoch et al., 1993). This bacterium could be one of the major sources of potential microbial biopesticides because it retains several valuable traits (Ongena & Jacques, 2008). Firstly, *Bacilli*, such as *B. subtilis*, are well-studied organisms which facilitates their rational use. Secondly, the US Food and Drug Administration (USFDA) has granted the "generally regarded as safe" (GRAS) status to *Bacillus subtilis* which is thus recognized non-pathogenic (Harwood & Wipat, 1996). This is of course essential regarding its application as a biopesticide. Thirdly, *Bacilli* have the capacity to produce spores (Piggot & Hilbert, 2004) which are extremely resistant dormancy forms capable to withstand high temperatures, unfavorable pH, lack of nutrients or water, etc. They are produced by the bacteria when environmental conditions are unfavorable which probably helps these microorganisms to survive in the phytosphere. The phenomenon can also be exploited in industrial production as sporulation can be induced at the end of cultures (Monteiro et al., 2005). This greatly facilitates post-culture conditioning as bacterial suspensions can be converted to easy to handle powder formulations without the impressive bacterial mortality observed with non-sporulating bacteria (Lolloo et al., 2010). Shelf life of biopesticides based on sporulated bacteria is generally longer and require less storage precaution compared to other products containing living organisms. *Bacilli* are also relatively easy to produce industrially as they are not particularly exigent regarding nutritional sources. Beside its spore forming ability, *B. subtilis* possess several characteristics that enhance its survival in the rhizosphere and thus its effectiveness as a biopesticide (Losick & Kolter, 2008; Rosas-Garcia, 2009). This bacterium known to live in aerobic environments can also behave as facultative anaerobe surviving and evolving under low oxygen concentration (Nakano & Hulett, 1997). This is a real advantage in the rhizosphere as oxygen availability may fluctuate during time and is generally low. Additionally, *B. subtilis* is a motile bacterium that readily moves towards and on the root surface which facilitates colonization of new ecological niches. Another reason for the high interest in *Bacilli* is the diversity of their modes of action. They can display almost all the mechanisms of biocontrol and bio-stimulation/fertilization mentioned here below and above. Moreover, one strain may often acts through several mechanisms. This enables these bacteria to be effective in many conditions (variety of pathogens, plants, environmental conditions) as one mechanism may act instead of another.

Product	Bioagent / mode of action	Diseases / target pathogens	Crop	Company	Registered and commercialized
<i>Bacillus</i> spp.					
Avogreen®	<i>B. subtilis</i> / antibiosis	<i>Colletotrichum gloeosporioides</i> and <i>Cercospora</i> spot	Avocado	Ocean Agriculture	South Africa
Bacillus SPP®	<i>Bacillus</i> spp./ antibiosis	<i>Pseudomonas syringae</i> pv. <i>syringae</i> , <i>Xanthomonas campestris</i> pv. <i>vesicatoria</i> and <i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	Several crops	Bio Insumos Nativa Ltda., Chile	Chile
Ballad®	<i>B. pumilus</i> / antibiosis, competition, growth promotion and resistance induction	Root rot ( <i>Rhizoctonia oryzae</i> ), rust ( <i>Puccinia</i> spp., <i>Uromyces betae</i> , <i>Puccinia sorghi</i> ), rice blast ( <i>Pyricularia oryzae</i> ), powdery mildew ( <i>Peronospora manshurica</i> , <i>Erysiphe graminis</i> , <i>Erysiphe betae</i> , <i>Erysiphe polygoni</i> ), leaf spot ( <i>Cercospora</i> , <i>Cercospora beticola</i> <i>Entyloma</i> , <i>Dreschlera</i> , <i>Exserohilum turcicum</i> , <i>Helminthosporium</i> , <i>Bipolaris maydis</i> , <i>Cochliobolus heterostrophus</i> , <i>Cochliobolus</i> , <i>Ceratobasidium</i> , <i>Ramularia</i> ), bacterial spot ( <i>Xanthomonas</i> spp.), Asian soybean rust ( <i>Phakopsora pachyrhizi</i> ), brown spot ( <i>Septoria glycines</i> ), white mold ( <i>Sclerotinia sclerotiorum</i> )	Cereals, oil plants, sugar beet	AgraQuest Inc., USA	USA
Bio safe®	<i>B. subtilis</i> / antibiosis	Foliar blight	Soybean, bean, cotton	Lab. Biocontrol Farroupilha, Brazil	Brazil (not sold, only for use in the company)
Biosubtilin	<i>B. subtilis</i> / antibiosis, competition	<i>Fusarium</i> , <i>Verticillium</i> , <i>Pythium</i> , <i>Cercospora</i> , <i>Colletotrichum</i> , <i>Alternaria</i> , <i>Ascochyta</i> , <i>Macrophomina</i> , <i>Myrothecium</i> , <i>Ramularia</i> , <i>Xanthomonas</i> and <i>Erysiphe polygoni</i>	Cotton, cereals, ornamental plants and vegetable crops	Biotech International Ltd.	India
Botrybel	<i>B. velezensis</i>	<i>Botrytis cinerea</i>	Tomato, lettuce, pepper, grape, strawberry and vegetables	Agricaldes, Spain	Spain
Cease®	<i>B. subtilis</i>	Soilborne pathogens ( <i>Rhizoctonia</i> , <i>Pythium</i> , <i>Fusarium</i> , <i>Phytophthora</i> ) and foliar pathogens ( <i>Botrytis</i> , <i>Erwinia</i> , <i>Xanthomonas</i> )	Several crops	BioWorks Inc., USA	USA, Mexico

Product	Bioagent / mode of action	Diseases / target pathogens	Crop	Company	Registered and commercialized
Companion®	<i>B. subtilis</i> / antibiosis, competition, growth promotion, resistance induction	Root rots ( <i>Aspergillus</i> , <i>Golovinomyces cichoracearum</i> , <i>Fusarium oxysporum</i> , <i>Fusarium nivale</i> , <i>Magnaporthe poae</i> , <i>Phytophthora</i> , <i>Pythium</i> , <i>Rhizoctonia solani</i> , <i>Sclerospora graminicola</i> , <i>Sclerotinia minor</i> ), leaf spot ( <i>Alternaria</i> , <i>Botrytis cinerea</i> , <i>Colletotrichum orbicular</i> , <i>Colletotrichum</i> , <i>Didymella bryoniae</i> , <i>Erwinia carotovora</i> , <i>Erwinia tracheiphila</i> , <i>Plasmodiophora brassicae</i> , <i>Podosphaera xanthi</i> , <i>Pseudomonas syringae</i> , <i>Xanthomonas campestris</i> )	Cotton, bean, pea, soybean, peanut, corn, and others	Growth Products Ltd., USA	USA
EcoGuard™ Biofungicide	<i>B. licheniformis</i> / antibiosis and enzymes	Antracnose ( <i>Colletotrichum graminicola</i> ) and dollar spot ( <i>Sclerotinia homeocarpa</i> )	Golf courses, sports turf, lawns, turf farms and arboretums	Novozymes A/S, Denmark. Novozymes Biologicals, USA	USA
Ecoshot	<i>B. subtilis</i>	Gray mold ( <i>B. cinerea</i> )	Grape, citrus, vegetables, legumes and others	Kumiai Chemical Industry Japan	Japan
FZB24®WG, li and TB	<i>B. subtilis</i>	Root rot and wilts ( <i>Alternaria</i> , <i>B. cinerea</i> , <i>Curvularia radicola</i> , <i>Curvularia inequalis</i> , <i>Corynebacterium michiganense</i> , <i>E. carotovora</i> , <i>Fusarium avenaceum</i> , <i>Fusarium culmorum</i> , <i>F.oxysporum</i> f. sp. <i>cucumerinum</i> , <i>F. oxysporum</i> f. sp. <i>dianthi</i> , <i>F. oxysporum</i> f. sp. <i>gerberae</i> , <i>F. oxysporum</i> f. sp. <i>gladioli</i> , <i>F.oxysporum</i> f. sp. <i>lycopersici</i> , <i>F. oxysporum</i> f. sp. <i>narcissi</i> , <i>Gaeumannomyces graminis</i> , <i>Gerlachia niveale</i> , <i>Phoma chrysanthemi</i> , <i>Phomopsis sclerotioidea</i> , <i>Pyrenochaeta lycopersici</i> , <i>P.ultimum</i> , <i>R. solani</i> , <i>S. sclerotiorum</i> , <i>Stromatinia freesia</i> , <i>Verticillium</i> spp.)	Several crops	ABiTEP GmbH, Germany	Germany
HiStick N/T® / Subtiltex® / Pro-Mix®	<i>B. subtilis</i>	Root rot and seed treatments ( <i>Fusarium</i> , <i>Rhizoctonia solani</i> , <i>Aspergillus</i> , <i>Pythium</i> and <i>Alternaria</i> )	Soybean, ornamental plants and other crops	Becker Underwood, USA Premier Horticulture Inc., Canada	USA, Canada
Kodialk®	<i>B. subtilis</i> / antibiosis, competition, growth promotion, resistance induction	Soilborne diseases ( <i>Rhizoctonia</i> and <i>Fusarium</i> )	Cotton	Gustafson Inc., USA	USA

Product	Bioagent / mode of action	Diseases / target pathogens	Crop	Company	Registered and commercialized
Rhapsody®	<i>B. subtilis</i>	Anthraco-nose ( <i>Colletotrichum</i> spp.), bacterial leaf spot ( <i>Erwinia</i> , <i>Pseudomonas</i> , <i>Xanthomonas</i> ), leaf spot ( <i>Cercospora</i> , <i>Entomosporium</i> , <i>Helminthosporium</i> , <i>Myrothecium</i> , <i>Septoria</i> , <i>Diplocarpon rosea</i> ), gray mold ( <i>B. cinerea</i> ), downy mildew ( <i>Peronospora</i> spp.), early blight ( <i>Alternaria</i> ), powdery mildew ( <i>Erysiphe</i> , <i>Oidium</i> , <i>Podosphaera</i> , <i>Sphaerotheca</i> ), rust ( <i>Puccinia</i> ), scab ( <i>V. inaequalis</i> ), root rot ( <i>R. solani</i> , <i>Pythium</i> , <i>Fusarium</i> , <i>Phytophthora</i> ), dollar spot ( <i>Sclerotinia homeocarpa</i> ), rice blast ( <i>Pyricularia grisea</i> ), soilborne diseases ( <i>Rhizoctonia</i> , <i>Pythium</i> , <i>Fusarium</i> and <i>Phytophthora</i> )	Turf, forest, ornamental plants	AgraQuest Inc., USA	USA, Canada
Rhizo Plus®	<i>B. subtilis</i> FZB24	Soilborne pathogens	Gardening (Several crops)	ABiTEP GmbH, Germany	Germany
RhizoVital®42 li and RhizoVital 42TB	<i>B. amylolique-faciens</i>	Soilborne pathogens	Potato, corn, strawberry, tomato, cucumber, ornamental plants	ABiTEP GmbH, Germany	Germany
Serenade®	<i>B. subtilis</i> / antibiosis	Gray mold ( <i>B. cinerea</i> ), Botrytis ( <i>B. cinerea</i> ), black Sigatoka ( <i>Mycosphaeraella fijiensis</i> ), early blight ( <i>Alternaria solani</i> ), late blight ( <i>Phytophthora infestans</i> ), powdery mildew ( <i>Leveillula taurica</i> , <i>Oidiopsis taurica</i> , <i>Erysiphe chichoracearum</i> , <i>Erysiphe</i> spp., <i>Sphaerotheca macularis</i> , <i>Sphaerotheca</i> spp., <i>Podosphaera clandestina</i> , <i>Podosphaera leucotricha</i> , <i>Uncinula necator</i> ), downy mildew ( <i>Bremia lactucae</i> , <i>Peronospora</i> spp.), early leaf spot ( <i>Cercospora</i> spp.), Botrytis neck rot ( <i>Botrytis</i> spp.), scab ( <i>Venturia</i> spp.), leaf-drop ( <i>Sclerotinia</i> spp.), bacterial spot ( <i>Xanthomonas</i> spp.), walnut blight ( <i>Xanthomonas campestris</i> ), fire blight ( <i>Erwinia amylovora</i> ), anthracnose ( <i>Colletotrichum</i> ), white mold ( <i>Sclerotinia sclerotiorum</i> )	Grape, apple, pear, banana, cherry, walnut, peanut, hop, leafy vegetables, tomato, peppers, cucurbits, mango, bean, onion, garlic, potato, broccoli, carrot	AgraQuest Inc., USA	Chile, USA, New Zealand, Mexico, Japan, Israel, Costa Rica, Philippines, Guatemala, Honduras, Argentina, Italy, France, Turkey, Switzerland, Korea, Ecuador, Peru, and others

Product	Bioagent / mode of action	Diseases / target pathogens	Crop	Company	Registered and commercialized
Sonata®	<i>B. pumilus</i>	Powdery mildew( <i>Oidiopsis taurica</i> , <i>Erisiphe</i> spp., <i>Erysiphe cichoracearum</i> , <i>Uncinula necator</i> , <i>Sphaerotheca</i> spp., <i>Sphaerotheca macularis</i> , <i>Podosphaera leucotrica</i> ), early blight ( <i>Alternaria solani</i> ), late blight ( <i>Phytophthora infestans</i> ), downy mildew ( <i>Peronosporaspa</i> , <i>Pseudoperonospora</i> spp., <i>Bremia lactucae</i> )	Tomato, potato, grape, strawberry, cucurbits, peppers, apple, pear	AgraQuest Inc., USA	USA, Mexico, Peru, Switzerland, Germany
Sublic®	<i>Bacillus</i> sp.	Damping-off, root rot, and wilt ( <i>Botrytis</i> , <i>Rhizoctonia</i> , <i>Colletotrichum</i> , <i>Sclerotinia</i> , <i>Macrophomina</i> , <i>Phomopsis</i> and <i>Pythium</i> )	Several crops	ELEP BiotechnologiesItaly	Italy
Yield Shield®	<i>B. pumilus</i>	Root rot ( <i>R. solani</i> and <i>Fusarium</i> )	Soybean	Bayer CropScience, USA	USA
<b><i>Streptomyces</i> spp.</b>					
Actinovate® SP	<i>S. lydicus</i> /antibiosis, enzymes, competition, growth promotion	Damping-off and root rot ( <i>Pythium</i> , <i>Rhizoctonia</i> , <i>Fusarium</i> , <i>Phytophthora</i> , <i>Verticillium</i> ), powdery mildew ( <i>Erysiphe</i> , <i>Oidium</i> , <i>Podosphaera</i> , <i>Sphaerotheca</i> ), downy mildew ( <i>Pseudoperonospora</i> , <i>Peronospora</i> ), gray mold ( <i>B. cinerea</i> ) and alternaria blight ( <i>Alternaria</i> spp.)	Ornamental plants, vegetables, turf, forest species	Natural Industries Inc., USA	USA
Mycostop®	<i>S. griseoviridis</i> /antibiosis, competition, parasitism, growth promotion	Root rot, damping-off, and wilt caused by <i>Fusarium</i> , <i>Alternaria brassicola</i> , <i>Phomopsis</i> , <i>Botrytis</i> , <i>Pythium</i> , <i>Phytophthora</i> and <i>Rhizoctonia</i>	Several crops	Verdera Oy, Finland	USA, Germany, Canada, Finland
<b><i>Rhizobium radiobacter</i></b>					
Agrogal I 30®	<i>R. radiobacter</i>	Crown gall disease ( <i>Agrobacterium tumefaciens</i> )	Ornamental, fruit and nut plants	Probial Bioestimulantes Foliare Profer Chile	Chile
Dygall®	<i>R. radiobacter</i>	Crown gall ( <i>A. tumefaciens</i> )	Ornamental and nut plants, pear, blueberry, grape, and other plants	AgBioResearch Ltd., Canada	Canada, New Zealand
Galltrol-A®	<i>R. radiobacter</i>	Crown gall ( <i>A. tumefaciens</i> )	Ornamental, fruit and nut plants	AgBioChem, USA	USA
Nogall™	<i>R. radiobacter</i>	Crown gall ( <i>A. tumefaciens</i> )	Ornamental, fruit and nut plants	Becker Underwood Pty Ltd., Australia	Australia, USA

Product	Bioagent / mode of action	Diseases / target pathogens	Crop	Company	Registered and commercialized
<i>Pseudomonas</i> spp.					
Bio-save® 10/11	<i>P. syringae</i>	Blue mold ( <i>Penicillium expansum</i> ), green mold ( <i>Penicillium digitatum</i> ), gray mold ( <i>B. cinerea</i> ), mucor rot ( <i>Mucor piriformis</i> ), Fusarium dry rot ( <i>Fusarium sambucinum</i> ), helminthosporiosis ( <i>Helminthosporium</i> spp.)	Apple, pear, citrus, berries, sweet potato, potato	Jet Harvest Solutions, USA	USA
Cedomon®	<i>P. chlororaphis</i>	Seedborne disease	Barley and oats	Lantmännen BioAgri AB, Sweden	Italy, Finland, Sweden, Denmark, Poland
Cerall®	<i>P. chlororaphis</i>	<i>Tilletia caries</i> , <i>Septoria nodorum</i> and <i>Fusarium</i> spp.	Wheat, rye and triticale	Lantmännen BioAgri AB, Sweden	Sweden, Finland, Swiss, Austria, Lithuania
Spot-Less Biofungicide®	<i>P. aureofaciens</i>	Anthraxnose ( <i>Colletotrichum gramminicola</i> ), root rot ( <i>Pythium aphanidermatum</i> ) and dollar spot ( <i>Sclerotinia homeocarpa</i> )	Turf	Turf Science Laboratories, USA	USA
<i>Burkholderia cepacia</i>					
Botrycid®	<i>B. cepacia</i>	Soilborne pathogens ( <i>Rhizoctonia</i> , <i>Thielaviopsis</i> , <i>Verticillium</i> , <i>Fusarium</i> and <i>Pythium</i> ), disease caused by <i>Botrytis</i> , <i>Mycosphaerella</i> , <i>Erwinia</i> , <i>Xanthomonas</i> , <i>Agrobacterium</i> and <i>Ralstonia solanacearum</i>	Several crops	Safer Agrobiologicos Colombia	Colombia

Table 1. Bacterial biopesticides commercialized for the control of plant pathogens.

### 3.1 *Bacillus thuringiensis* as insecticide

The best-known *Bacillus* species used as a biopesticide is *B. thuringiensis* (Bt). This bacterium produces the proteins Cry and Cyt which are highly toxic to insects but not to mammals or for the environment. This contributes to explain the early use of this biopesticide which was first applied in 1938 (Sanahuja et al., 2011). Cry toxins are part of the structure of the *B. thuringiensis*' spores. When these bacterial spores are ingested by an insect, the Cry proteins act through pore formation in the gut wall of the animal allowing the bacteria that emerge from the spores to feed on the contents of the insect's body cavity. This generates a new bacterial population and thus a new source of spores after the death of the insect (Sanahuja et al., 2011).

Bt toxins are highly specific regarding their mode of action. The proteins are present in the spore in an inactive form but cleavage in the insect gut renders them toxic. Key factors for this event are the presence of specific proteases and an alkaline environment. This and the fact that toxicity also requires the presence of specific receptors in the insect's gut explains why the toxins are only effective on a small host range and thus often have limited effect on non target populations. Major insect families which can be controlled with Cry/Cyt toxins are Coleoptera, Lepidoptera and Diptera. Much of the research done on this biopesticide focuses on discovering new Cry toxins or combination of toxins to cover new ranges of insect pests. A diversity of products based on Bt toxins are being used on many crops and Bt toxins are even used to limit mosquito population in the context of malaria (Sanahuja et al., 2011). The mode of action of Cry also implies that the toxins must be present on the plant parts eaten by the target insect. Use in field is actually limited by the fact that the active

agent may be rapidly degraded or washed off the leaves surface. Pulverization has thus to be repeated more or less frequently depending on environmental conditions. Many efforts have been made with variable success to limit this drawback but the ultimate solution to this problem came in the form of GMO crops capable to produce the Bt toxins by themselves. These plants represent approximately 36% of all biotech crops cultured in 2009 corresponding to more than 50 million hectares. The advantage of the GMO crops compared to pulverization is the reduced loss of active component into the environment limiting need for repeated pulverization and potentially reducing pollution as, after plant death, crop residue does not seem to impact soil health indicators like earthworm populations. By contrast, risks are the emergence of new pests and the development of resistant pest populations. The risk of new pest emergence is mainly driven by the specificity of the toxin. The target pest being suppressed this opens an ecological space for a new pest. Efforts are made to limit resistant population development mainly by using combinations of Cry toxins and/or by planting non-resistant plants near GMO parcels thereby limiting selective pressure. Meanwhile, GMO crops continue to be a subject of intense political debate (Sanahuja et al., 2011).

### 3.2 Other *Bacillus* spp. for the control of multiple diseases

*B. thuringiensis* is an important microbial pesticide that has been the topic of recent reviews. In this chapter, we will better illustrate the agronomic interest of other *Bacillus* species. A variety of strains of *Bacillus* and particularly *B. subtilis* are currently commercialized as biopesticides (Table 1) and numerous studies are in support of the great potential of such strains at controlling multiple diseases occurring on a wide range of host plant species (Table 2). This potential is further illustrated in the following concrete examples.

Crops	Pathogens	References
<b>Fungi</b>		
Abricot	<i>Moniliana laxa</i>	(Altindag et al., 2006)
Alfalfa	<i>Fusarium graminearum</i>	(Chan et al., 2003)
Amaranthus	<i>Choanephora cucurbitarum</i>	(Emoghene & Okigbo, 2001)
Apple	<i>Botrytis cinerea</i>	(Touré et al., 2004)
Avocado	<i>Rosellinia necatrix</i>	(Cazorla et al., 2007)
	<i>Colletotrichum gloeosporioides</i>	(Demos & Korsten, 2006)
	<i>Lasiodiplodia theobromae</i> , <i>Dothiorella aromatica</i>	
	<i>Thyronectria pseudotrichia</i>	
	<i>Phomopsis perseae</i> <i>Pseudocercospora purpura</i>	(Korsten et al., 1997)
Banana	<i>Pseudocercospora musae</i> <i>Colletotrichum musae</i>	(Fu et al., 2010)

Crops	Pathogens	References
Bean	<i>Uromyces phaseoli</i>	(Baker et al., 1985) (Bettiol & Varzea, 1992)
Beet	<i>Cercospora beticola</i> <i>Pythium spp.</i>	(Collins & Jacobsen, 2003)
Blueberry	<i>Monilinia vaccinii-corymbosi</i>	(Dedej et al., 2004) (Scherm et al., 2004)
Carrot	<i>Alternaria dauci</i>	(Hernandez-Castillo et al., 2006)
Cauliflower	<i>Pythium ultimum</i>	(Abdelzاهر, 2003)
Citrus	<i>Colletotrichum acutatum</i> <i>Guignardia citricarpa</i> <i>Phytophthora citrophthora</i> <i>Phytophthora parasitica</i> <i>Penicillium digitatum</i>	(Kupper, 2009) (Amorim & Melo, 2002)  (Leelasuphakul et al., 2008)
Chir-pine	<i>Macrophomina phaseolina</i>	(Singh et al., 2008)
Coffee	<i>Hemileia vastatrix</i>	(Bettiol & Varzea, 1992) (Haddad et al., 2009)
Corn	<i>Fusarium moniliforme</i> <i>Fusarium verticillioides</i> <i>Aspergillus flavus</i> <i>Fusarium solani</i> <i>Pythium spp.</i> <i>Rhizoctonia solani</i>	(Bacon et al., 2001) (Cavaglieri et al., 2005)  (Nesci et al., 2005) (Cavaglieri et al., 2005)
Cotton	<i>F. oxysporum</i>	(Gajbhiye et al., 2010)
Cucumber	<i>Pythium aphanidermatum</i> <i>Phytophthora nicotianae</i>  <i>R. solani</i> <i>Phomopsis spp.</i> <i>Colletotrichum lagenarium</i> <i>Sphaerotheca fuliginea</i>	(Grosch et al., 1999)  (Kita et al., 2005)  (Ongena et al., 2005) (Bettiol et al., 1997)
Grape	<i>Eutypa lata</i>  <i>B. cinerea</i>  <i>F. oxysporum</i> <i>Botryodiplodia theobromae</i>	(Ferreira et al., 1991)  (Rodgers, 1989)  (Swain et al., 2008)
Lentil	<i>Fusarium oxysporum f.sp. lentis</i>	(El-Hassan & Gowen, 2006)
Lettuce	<i>P. aphanidermatum</i>	(Corrêa et al., 2010)
Litchi	<i>Peronophythora litchi</i>	(Jiang et al., 2001)

Crops	Pathogens	References
	<i>Alternaria alternata</i> <i>Cladosporium spp.</i>	(Sivakumar et al., 2007)
Mango	<i>Oidium mangiferae</i>	(Nofal & Haggag, 2006)
Mellon	<i>Podosphaera fusca</i>	(Romero et al., 2007b)
Mustard	<i>Alternaria brassicae</i>	(Sharma & Sharma, 2008)
Nectarine	<i>Monilinia laxa</i>	(Casals et al., 2010)
Oilseed rape	<i>Sclerotinia sclerotiorum</i>	(Hu et al., 2005) (Yang et al., 2009)
Potato	<i>R. solani</i>	(Brewer & Larkin, 2005) (Schmiedeknecht et al., 1998)
Pear	<i>Monilinia fructicola</i>	(Pusey & Wilson, 1984)
Peach	<i>M. laxa</i> <i>M. fructicola</i>	(Casals et al., 2010) (McKeen et al., 1986) (Fan et al., 2000)
Pepper	<i>Phytophthora capsici</i> <i>R. solani</i> <i>P. capsici</i> <i>P. aphanidermatum</i>	(Ahmed et al., 2003)  (Lee et al., 2008) (Nakkeeran et al., 2006)
Pinus	<i>Ophiostoma picea</i> <i>M. phaseolina</i>	(Silo-Suh et al., 1998) (Singh et al., 2008)
Rice	<i>Aspergillus flavus</i> <i>Pyricularia oryzae</i> <i>R. solani</i>	(Reddy et al., 2009) (Bettiol & Kimati, 1990) (Yang et al., 2009)
Rose	<i>B. cinerea</i>	(Tatagiba et al., 1998)
Strawberry	<i>B. cinerea</i> <i>Podosphaera aphanis</i>	(Helbig & Bochow, 2001) (Pertot et al., 2008)
Soybean	<i>Septoria glycines</i> <i>F. oxysporum</i> <i>F. graminearum</i> <i>Sclerotinia sclerotiorum</i>	(Mantecon, 2008) (Zhang et al., 2009)
Sorghum	<i>P. ultimum</i>	(Idris et al., 2008)
Tobacco	<i>P. aphanidermatum</i> <i>Cercospora nicotiana</i>	(Maketon et al., 2008)
Tomato	<i>F. oxysporum</i> <i>Fusarium semitectum</i> <i>F. oxysporum f.sp. lycopersici</i>	(Chebotar et al., 2009) (Nihorimbere et al., 2010) (Abd-Allah et al., 2007) (Baysal et al., 2008)

Crops	Pathogens	References
	<i>P. aphanidermatum</i>  <i>R. solani</i>	(Jayaraj et al., 2005) (Ongena et al., 2005)  (Kondoh et al., 2000) (Kondoh et al., 2001)  (Montealegre et al., 2003)
Wheat	<i>Gaeumannomyces graminis var. tritici</i>	(Liu et al., 2009)
Yam	<i>F. oxysporum</i> <i>Botryodiplodia theobromae</i>  <i>B. theobromae</i> <i>Fusarium moniliforme</i> <i>Penicillium sclerotigenum</i> <i>Rhizoctonia spp.</i>	(Swain et al., 2008)    (Okigbo, 2003)
<b>Bacteria</b>		
Arabidopsis	<i>Pseudomonas syringae</i>	(Bais et al., 2004)
Brassica	<i>Xanthomonas campestris pv. campestris</i>	(Wulff et al., 2002)
Mulberry	<i>Ralstonia solanacearum</i>	(Ji et al., 2008)
Soybean	<i>X. campestris pv. glycines</i>	(Salerno & Sagardoy, 2003)
Tobacco	<i>R. solanacearum</i>	(Maketon et al., 2008)
Tomato	<i>Xanthomonas euvesicatoria</i> <i>Xanthomonas perforans</i>	(Roberts et al., 2008)
<b>Nematodes</b>		
Tomato	<i>Meloidogyne</i> <i>Meloidogyne incognita</i>	(Araújo & Marchesi, 2009) (Siddiqui & Futai, 2009)
Soybean	<i>Heterodera glycines</i>	(Araújo et al., 2002)

Table 2. Potential of *Bacillus subtilis* for the control of plant pathogens.

Bean rust caused by *Uromyces appendiculatus* can cause severe damages when it occurs early in culture. Relying on genetic resistance is not really successful due to the large variability of the causal agent and thus the control of the disease was traditionally achieved by using chemical fungicides. In greenhouse assays, Baker and collaborators tested the soil originating strain APPL-1 of *Bacillus subtilis*. Treatment with this isolate decreased the number of pustules of bean rust by 95%, when applied on plants 2 to 120 h before the inoculation of uredospores of *Uromyces appendiculatus* (Baker et al., 1983). In field conditions, reduction of at least 75%, in the occurrence of rust, was observed upon three weekly applications of the strains APPL-1 and PPL-3 (Baker et al., 1985). Centurion (1991) obtained a reduction of 80 to 100% in the number of pustules of rust by applying *Bacillus subtilis* strain

W401 in greenhouse assays (Centurion, 1991). Mizubuti (1992) observed significant reduction in the germination of uredospores of *Uromyces appendiculatus* by five strains of *Bacillus subtilis*, and a reduction in the number of pustules per leaf when the strains were applied 48 h before inoculation with the pathogen in the greenhouse (Mizubuti, 1992). Bettiol and collaborators observed that extracts of *B. subtilis*, obtained by the precipitation of metabolites with ammonium sulphate or by acidification at pH 2.0, at a concentration of 1000 ppm totally inhibited the germination of urediniospores of *U. appendiculatus*, and controlled the rust (84%) when sprayed on bean leaves (Bettiol et al., 1992). Nowadays, the product Bio safe® (Table 1) with cells of *B. subtilis* is used to control anthracnose in bean and Asian soybean rust. In recent years, the product has been used in integrated pest management for these crops.

Coffee leaf rust (*Hemileia vastatrix*) is the most important coffee disease in Brazil. Bettiol and Varzea observed that cell suspensions of *Bacillus subtilis* strains AP-3 and AP-150 totally inhibited urediniospore germination of various races of *Hemileia vastatrix* (Bettiol et al., 1992). Spraying of sterilized and unsterilized *Bacillus* cell suspensions on detached leaves of coffee cv Caturra allowed reduction of the number of lesions by 72% to 87% depending on the pathogen race. The same disease control trend was observed when these strains were sprayed on whole coffee plants. Under commercial conditions, Haddad and collaborators (2009) also showed that the strain B157 of *Bacillus* sp. can be considered a potential biocontrol agent for coffee leaf rust in organic crop systems in Brazil (Haddad et al., 2009).

As third example, a product formulated with *Bacillus subtilis* and *Bacillus licheniformis*, has been used for the control of diseases caused by the nematode pathogens *Meloidogyne incognita*, *Meloidogyne javanica*, *Pratylenchus brachyurus* and *Pratylenchus coffeae* on potatoes and carrots in Brazil. In 2008, more than 12,000 kg of this product were commercialized only for the treatment of potato and carrot. 5 - 10 kg/ha of the product, with  $2 \times 10^{10}$  CFU/g, were applied by irrigation and other ways. The cost was approximately US\$ 160-300/ha. This product has replaced nematicides.

#### **4. Deciphering the mechanisms involved in biocontrol of plant diseases by *Bacillus***

By taking benefits from the nutrients constantly released from roots or leaves of growing plants, beneficial bacterial strains efficiently colonize leaf surfaces and root systems and their surrounding soil layer. In turn, they beneficially influence the plant by protecting it from infection by plant pathogens via three main mechanisms: competition for ecological niche/substrate, production of inhibitory allelochemicals, and induction of systemic resistance in host plants. It should be noted that none of these mechanisms described below are necessarily mutually exclusive, and frequently several modes of action are exhibited by a single biocontrol agent. In the next sections, we mainly consider beneficial microbes introduced in soil but the same principles and mechanisms apply for isolates used to combat foliar diseases.

##### **4.1 Competition for niche and nutrients**

Competition for resources such as nutrients and oxygen occurs generally in soil among soil-inhabiting organisms. For biocontrol purpose, it occurs when the antagonist directly competes against pathogens for these resources. Root inhabiting microorganisms compete

for suitable sites at the root surfaces. Competition for nutrients, especially for carbon, is assumed to be responsible for the well-known phenomenon of fungistasis, characterizing the inhibition of fungal spore germination in soil (Alabouvette et al., 2006). Given the relatively low abundance of substrates in the rhizosphere, the efficiency of nutrient uptake and catabolism by bacteria is a key factor in competitiveness. Competition for trace elements, such as iron, copper, zinc, manganese etc., also occurs in soils. For example, iron is an essential growth element for all living organisms and the scarcity of its bio-available form in soil habitats results in a furious competition (Loper & Henkels, 1997). Siderophores, low molecular weight compounds with high iron affinity, are produced by some microorganisms (and also by most biocontrol agents) to solubilize and competitively acquire ferric ion under iron-limiting conditions, thereby making iron unavailable to other soil microorganisms which cannot grow for lack of it (Haas & Défago, 2005; Loper & Henkels, 1997). Suppression of soilborne plant pathogens through competition for niche and nutrients has been demonstrated in some instances for some beneficial bacteria such as *Pseudomonas* (Haas & Défago, 2005). Experimental proof concerning *Bacillus* is scarce but these competitive phenomena should also occur with this bacterium given its natural rhizosphere competence.

## 4.2 Direct inhibition of phytopathogens

### 4.2.1 Antibiosis

Members of multiple *Bacillus* species such as *B. amyloliquefaciens*, *B. subtilis*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, and *B. pumilus* are known as very efficient producers of antibiotic molecules. *Bacillus subtilis* has an average of 4-5% of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial compounds (Stein, 2005). In strain FZB42, which is proposed as a paradigm for plant-associated *Bacillus amyloliquefaciens* as well as in other isolates, an even larger part of the genome (~8%) is seemingly involved in antibiotic synthesis (Arguelles-Arias et al., 2009; Chen et al., 2009; Ruckert et al., 2011). Among the vast array of biologically active molecules synthesized by *Bacillus*, some have been reported for their inhibitory activity against plant pathogens and this antagonistic activity or antibiosis is probably the best-known and the most important mechanism used to limit pathogen invasion in host plant tissues.

*B. cereus* UW85 produces two fungistatic antibiotics, zwittermicin A and kanosamine, which are suggested to contribute to the suppression of damping-off disease of alfalfa caused by *Phytophthora medicaginis* (Silo-Suh et al., 1994). Zwittermicin A may also control the fruit rot of cucumber (Smith et al., 1993) and suppress other plant diseases (Silo-Suh et al., 1998). Bacillaene, difficidin and macrolactin are polyketides displaying a broad spectrum of antibacterial activities that may be involved in the biocontrol activity of the producing strain such as in the case of fire blight, a serious disease of orchard trees caused by *Erwinia amylovora* (Chen et al., 2009). The phosphono-oligopeptide rhizoctin produced by *B. subtilis* also displays antifungal and nematocidal activities, but does not retain any bactericidal properties (Borisova et al., 2010). Peptide compounds represent the predominant class of *Bacillus* antibiotics. They are of various sizes, may be composed entirely of amino acids but some contain other residues. Cyclic or linear oligopeptides, basic peptides and aminoglycoside antibiotics usually occur (Stein, 2005). Low molecular weight and hydrophobic or cyclic structures, with unusual constituents like D-amino acids, are also

common characteristics of peptide antibiotics normally synthesized by *Bacillus*. Moreover, they are generally resistant to hydrolysis by peptidases and proteases of animal and plant origin. *Bacillus brevis* (*BreviBacillus brevis*) and *Bacillus polymyxa* (*PaeniBacillus polymyxa*) produce gramicidin S and polymyxin B peptide antibiotics that strongly inhibited *Botrytis cinerea* germination *in vitro* but also exhibited high activity under natural field conditions against the *Botrytis* grey mould disease caused by this fungus on strawberry (Haggag, 2008). Another group of peptide antibiotics usually produced by *Bacillus subtilis* are lantibiotics (Stein, 2005). These compounds display strong antibacterial properties against gram-positive bacteria but their involvement in the biocontrol activity of plant-associated *Bacillus* isolates has not been clearly demonstrated so far. More simple molecules, such as the dipeptide bacilysin (L-Ala linked to the non-proteinogenic amino acid L-anticapsin), also retain strong bactericidal effect and are seemingly involved in the control of some plant pathogens (Chen et al., 2009).

A major class of *Bacillus* peptide antibiotics are cyclic lipopeptides (cLPs) which may vary in the type and sequence of amino acid residues, the nature of the peptide cyclization and in the nature, length and branching of the fatty acid chain (Ongena & Jacques, 2008). In various species of *Bacillus*, the three main families are surfactins, iturins and fengycins. They encompass structural variants depending on the genetic background of the considered strain. Surfactins are heptapeptides interlinked with a  $\beta$ -hydroxy fatty acid to form a cyclic lactone ring structure. Iturins, with 7 variants including bacillomycins and mycosubtilin, are also heptapeptides but are linked to a  $\beta$ -amino fatty acid chain with a length from C<sub>14</sub> to C<sub>17</sub>. Fengycins A and B, also called plipastatins, are lipodecapeptides with an internal lactone ring in the peptidic moiety and with a  $\beta$ -hydroxy fatty acid chain (C<sub>14</sub> to C<sub>18</sub>) that can be saturated or not. Beside these three main families, other classes of bioactive lipopeptides synthesized by *Bacillus* species have been identified (Hathout et al., 2000; Lee et al., 2007).

Each family of *Bacillus* cLPs displays specific antibiotic activities and may thus be differentially involved in the antagonism of the various plant pathogens. In the case of soilborne diseases, iturin A produced by *B. subtilis* RB14 was involved in the control of damping-off of tomato (a seedling disease) caused by *Rhizoctonia solani* (Asaka & Shoda, 1996). Overexpression of mycosubtilin in *B. subtilis* ATCC 6633 also led to a significant reduction of seedling infection by *Pythium aphanidermatum* (Leclère et al., 2005). As examples in the control of phyllosphere diseases, a contribution of both iturins and fengycins was shown in the antagonism of *B. subtilis* toward *Podosphaera fusca* infecting melon leaves (Romero et al., 2007a). This was notably demonstrated by showing the strong inhibitory effect of these cLPs on *P. fusca* conidia germination, and by recovering cLPs from bacterial-treated leaves and using cLP-deficient mutants. In the protection against post harvest diseases, *Bacillus subtilis* strain GA1, which efficiently produces cLPs from the three families and notably a wide variety of fengycins, protected wounded apple fruits against gray mold disease caused by *Botrytis cinerea*. The role of fengycins was demonstrated by the very effective disease control provided by treatment of fruits with cLPs-enriched extracts and by *in situ* detection of fengycins in inhibitory amounts (Touré et al., 2004). To further illustrate the broad range of fungal targets, fengycins were also reported for their antagonistic activity against *Fusarium graminearum* (Wang et al., 2007), and iturins for their inhibitory effect towards the anthracnose-causing agent *Colletotrichum dematium* (Hiradate et al., 2002), *Penicillium roqueforti* (Chitarra et al., 2003), *Aspergillus flavus* (Moyné et al., 2001), *Rhizoctonia solani* (Yu et al., 2002), wood-staining fungi (Velmurugan et al., 2009) and nematophagous fungi (Li et al., 2007). In some instances, the fungitoxic activity was clearly related to the

permeabilization of spore/conidia therefore inhibiting germination or alternatively to hyphal cell perturbation. As revealed by transmission electron microscopy techniques, both phenomena most probably result from membrane damaging by the cLPs (Chitarra et al., 2003; Etchegaray et al., 2008; Romero et al., 2007a).

A few studies have revealed some insecticide activity of cLPs from *B. subtilis*. Surfactin and iturin were described for their antagonistic effect against fruit fly *Drosophila melanogaster* (Assie et al., 2002) and cLPs contained in a crude extract were efficient at inhibiting the development of larvae of the mosquito *Culex quinquefasciatus* (Das & Mukherjee, 2006). Although active doses are quite high (approx. 200  $\mu\text{M}$ ) and mechanisms underpinning such biocidal effect have not yet been investigated, treatments with cLPs are presented as possible alternatives for the use of the endotoxin producer *B. thuringensis* in the biocontrol of insects for which this bacterium is not efficient.

#### 4.2.2 Other inhibitory mechanisms

Distinct from antibiosis that involves low-molecular weight compounds and does not require physical contact, predation/parasitism is also an important mechanism used by some biocontrol microorganisms, mainly fungi such as *Trichoderma*. It is based on enzymatic destruction of the fungal pathogen cell wall. The ability of bacteria to parasitize and degrade spores or hyphae of pathogens through the production of various cell-wall degrading enzymes has also been suggested (Whipps, 2001). As examples, isolates related to *Bacillus ehimensis* (Hoster et al., 2005) produce chitin-degrading enzymes while *Bacillus subtilis* AF1 displays some fungitoxicity through the secretion of *N*-acetyl glucosaminidase and glucanase (Manjula & Podile, 2005). Some more specific pathogen–biocontrol strain interactions leading to pathogen restriction were reported such as interference with biofilm formation, inactivation of pathogen germination factors and degradation of pathogenicity factors such as toxins but will not be detailed here.

#### 4.3 Plant resistance triggering

The isolation of some PGPR strains efficient in biocontrol but lacking the ability to exert any antagonistic activity toward pathogens shed new light on the diversity of their modes of action and suggested that such strains may activate defense systems in the host plant. This stimulation of the plant immune system represents one of the most newly discovered aspects of plant-microbe interactions (Bakker et al., 2007). Some isolates are indeed able to reduce disease through the stimulation of a primed state in the host plant which allows an accelerated activation of defense responses upon pathogen attack, leading to an enhanced resistance to the attacker encountered (Conrath et al., 2006). Conclusive evidence for the role of induced systemic resistance (ISR) in disease reduction by a given bacterium tested on a particular pathosystem is obtained by verifying the spatial separation of the pathogen and the resistance-inducing agent in order to exclude any direct antagonistic interaction. ISR can be globally viewed as a three-step process involving sequentially i) the perception by plant cells of elicitors produced by the inducing agents that initiates the phenomenon, ii) signal transduction that is needed to propagate the induced state systemically through the plant and iii) expression of defense mechanisms *stricto sensu* that limit or inhibit pathogen penetration into the host tissues (Van Loon, 2007). Defense molecules include phytoalexins, pathogenesis-related (PR) proteins (such as chitinases,  $\beta$ -1,3-glucanases, proteinase inhibitors, etc.) and lignin for reinforcement of cell walls (Van Loon, 2007). Cell wall thickenings, wall appositions or rapid death of the injured plant cells resulting in necrosis of

the immediate adjacent tissues are barriers which cut the pathogen off its nutrients and contribute to slowing down the fungal invasion (Lugtenberg et al., 2002).

The list of bacteria identified as ISR inducers has grown rapidly over the last two decades and includes Gram-negative bacteria such as members of the *Pseudomonas* and *Serratia* genera but also Gram-positive bacteria and more particularly *Bacillus* spp. (Bent, 2006; Kloepper et al., 2004). Rhizobacteria-mediated ISR can occur in many dicotyledonous and monocotyledonous plant species. By analogy with the pathogen-induced SAR, protection afforded through ISR is quite non-specific regarding the nature of the infectious agent. Because of its systemicity, the enhanced defensive capacity is expressed in roots as well as in leaves. Control of diseases caused by fungi, bacteria, and viruses has been demonstrated thoroughly but ISR may also be a successful strategy in management of nematode and insect pests in several crops. Rhizobacteria-mediated ISR does not confer a total protection against pathogen infection but as the phenomenon is long-lasting and not conducive for development of pathogen resistance (multiplicity and variety of induced defense pathways), ISR-based biocontrol strategies are promising and some trials were successfully performed under field conditions.

Volatile compounds such as 2,3-butanediol (Ryu et al., 2004) and lipopeptides are the sole compounds formed by *Bacillus* spp. that were identified as elicitors of ISR. The potential of *Bacillus* cLPs as plant resistance inducers was demonstrated by testing pure surfactins and fengycins that provided a significant induced protective effect similar to the one induced by living cells of the producing strain (*B. amyloliquefaciens* S499). In a complementary approach, experiments conducted on bean and tomato showed that overexpression of both surfactin and fengycin biosynthetic genes in the naturally poor producer *B. subtilis* strain 168 was associated with a significant increase in the potential of the derivatives to induce resistance. Moreover, the macroscopic disease reduction induced by the surfactin overproducer was associated with defense-related metabolic changes in the host plant tissues (Ongena et al., 2007).

## 5. Improving biopesticides and conclusive statements

Microbial biopesticides and *Bacillus* based products in particular improve plant health through many mechanisms and gain increasing interest for commercial application as exemplified above. Unfortunately, these products often offer only partial protection against pathogen and pest attacks. Another weakness of microbial biopesticides is their inconsistent effect. As the active ingredient is a living organism, its efficacy is more strongly dependant on application conditions compared to conventional pesticides. The activity of the beneficial organism depends on its global ecology, in other words the interactions between the beneficial organism, the plant host, the pathogen and the biotic and abiotic environmental parameters (Butt et al., 1999; Fuentes-Ramirez & Caballero-Mellado, 2006).

Unfortunately, our knowledge about the ecology of most beneficial microorganisms used today is still poor, limiting rational field applications. In a more "critical" point of view, we will point out that, at least in the case of *Bacillus*, one of the main causes of this lack of information is the often-observed deficiency in connections between field trials and more controlled laboratory experiments. For instance, it is often speculated that the frequent occurrence of *B. subtilis* in its natural environment might be due to the selective advantage conferred by the panoply of bioactive metabolites that it may produce. However, even if some *B. subtilis* strains are well equipped genetically to produce a vast array of antibiotics,

only a limited part of this antibiotic-devoted genetic background may be readily expressed in soil and thus, only a part of this arsenal may be actually produced under natural conditions. The recent developments in biotechnology and analytical chemistry open the doors to new approaches for investigation and new tools to study *in situ* antibiotic production by valuable strains in their ecological niche. This will surely contribute to enhance our knowledge about *Bacillus* fitness in natural living conditions which is a crucial point for optimizing biocontrol strategies using this organism.

There are many other possible approaches for the improvement of biopesticide efficacy and consistency of protection. Strain selection also probably deserves further improvement. The methods through which this selection is conducted are subject of debate. *In vitro* screening, though easy to implement, often includes the drawback of not taking into account environment conditions for field application. In order to optimize selection process, a middle path has to be found between more realistic and easier selection methods. A potential long-term solution could be to invest more in fundamental research linking field observations to readily testable parameters.

In the context of biopesticide improvement, one must consider when possible the whole agricultural system. A key to improve plant health and growth is to find adequate combinations between biopesticides, chemical pesticides, plant fertilization, agricultural practices like different types of tillage etc. as embodied by integrated pest management (Chakraborty et al., 2010; Dukare et al., 2011; Kumar et al., 2010). Moreover, when implementing biopesticides in integrated pest management, the biological product must be compatible with conventional pesticides. This parameter is of particular importance when the applications of the biological and chemical ingredients occur simultaneously for example in seed treatment or in combined foliar sprays.

The simultaneous implementation of several active ingredients in one commercial product is certainly a way to guarantee the global efficacy under varying conditions. A microbial strain can be used together with other strains, with natural extracts or other none chemically transformed products or with chemical pesticides (Shanmugam & Kanoujia, 2011; Liu et al., 2011; Akila et al., 2011; Kondoh et al., 2001). These combinations are generally more effective and reliable. For example, combinations of strains can be selected to broaden pathogen spectrum by blending strains with distinct action mechanisms, or to enhance reliability by mixing isolates with different ecological competences (Jijakli, 2003; Ramamoorthy et al., 2001). Moreover, the combination of strains can induce synergic effects improving biocontrol. An advantage of combining products is also that several treatments are applied at once reducing labor for the farmer. The main setbacks of combination products are: potential antagonisms between the active ingredients (even between two strains) (Whipps, 2001) and more fastidious homologation procedures as the ingredients contained in the mix must pass legal tests individually. This is mainly a problem in Europe where pesticide legislation is very strict. In the U.S., biopesticide homologation is simpler as the one used for classic pesticides as these first products are considered less dangerous (Jijakli, 2003).

Formulation and application methods are also key issues influencing the efficacy of commercial products and research on these topics should be focused on specific environmental applications. For example, in the case of formulation, a possibility is the addition of molecules that favor the adhesion of the bacteria to fruit or leaves when used as a spray (Rabindran & Vidhyasekaran, 1996). Another option could be to combine the strain with a substrate like chitin which may stimulate biocontrol activity (Ahmed et al., 2003).

In conclusion, we can say that microbial biopesticides have great potential that is and should be even more used to make the future agriculture more sustainable. Much work has been done but a lot is still to do for scientists and industrials, to improve reliability and efficacy of these products and keep gaining an increasing market share.

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# The Effect of Some Botanical Pesticides Against Citrus Leafminer (CLM) and Two Spotted Mite (TSM)

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## 1. Introduction

The novel natural extracts of the higher plants have a variety characteristics including insecticidal, antifungal, antiviral and antibacterial activity, repellence to pests, antifeedant effects, insect growth regulation, toxicity to nematodes, mites, agricultural pests, (Prakash and Rao, 1997). Insecticidal activity of many plants has been demonstrated (Carlini and Grossi-de-Sá, 2002). The deleterious effects of plant extracts on insects can be manifested in several manners including toxicity, mortality, and reduction of reproduction, fecundity and fertility. The plants that used for pest insect control have strongly correlated to medicinal and pesticidal plants (Yang and Tang, 1988). The plant substances contain components that are toxic to insects through a novel mode of action. In addition to the obvious implication of discovering a new target site against which to design insecticides, existing mechanisms of resistance may not confer cross-resistance to these plant extracts.

The citrus leafminer (CLM), *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae), is an important pest of citrus and related rutaceae and ornamental plants almost worldwide (Achor *et al.*, 1996). The CLM mines leaves, surface tissue of young shoots and stems, and less frequently the fruit (Sponagel and Diaz, 1994). Although citrus leafminer causes indirect damage to young leaves, which predisposes them to infection by canker so, controlling citrus leafminer is a vital component of canker management (Pena *et al.*, 1996; Belasque *et al.*, 2005). The first record of CLM from southern and northern Iran, with a dramatic increase and widespread dispersal, was noted in 1961 and 1994, respectively. The pest has about 5-9 generations over year, with peak periods in early summer and early autumn. Preliminary field trials with selected insecticides indicate the superiority of Dimilin (diflubenzuron) over Diazinon, Zolone (Phosalone) and Ekamet (Etrimfos) in controlling CLM in the northern Iran, but it is not totally effective (Jafari, 1996). There is no biological control plan of CLM, but some studies are underway for planned extension activities using biorational insecticides. Several different insecticides, such as Avant, Buprofezin and Pyriproxifen was used (Amiri, 2006 (a)), but these may involve interference in control of the pest by natural enemies (Guerra *et al.*, 1997). However, biological control is the best option for control (Pena, 1997), but the effective control of CLM is very complicated because of its high migration ability from outside of orchards, high fertility, present epidermis of citrus leaf as substantial protection and the difficulty of direct contact of chemical to the larval body. However, CLM

has a long history of resistance to many insecticides and development of resistance against the chemicals sometimes makes it difficult to obtain enough control effect (Tan and Huang, 1996, Mafi and Ohbayashi, 2006).

Recently, the use of biorational insecticides (any type of natural or synthetic material active against pest populations) has been extremely increased. *Bacillus thuringiensis* sub sp *Krustaki* is the soil bacterial insecticide most widely used for controlling Lepidoptera larvae population (Broderick et al., 2000; Lacey et al., 2001) that is safe for many non-target insects with a minimal environmental impact (Jyoti, J and Brewer 1999). Because CLM is protected inside the mine it is suggested that the mineral oils would be as a surfactant and reduce the surface tension and increase the penetration of the *Bacillus thuringiensis* suspension through the epidermis of the citrus leaf (Dias et al., 2005). *Bacillus thuringiensis* and *Bt* plus MO are active against the leafminer, demonstrating that these biopesticides penetrate into leaf mines, thereby killing the larvae (Amiri, 2007).

Chlorpyrifos-methyl (Reldan) is a wide-spectrum insecticide which belongs to the organophosphate group. It is effective against rice stem borer, aphids, cutworms, plant and leaf hoppers, mole crickets, some moths and stored grain pests. Currently, its main use in Iran is in stored grain. The mode of action of Reldan is cholinesterase inhibition. Methoxyfenozide (Runner 240 SC), a moult accelerating compound and an ecdysone agonist which is currently submitted for registration in Iran, is a compound compatible with IPM (Integrated Pest Management) that has strong, activity against lepidopterous pests.

Spinosad is a mixture of two macrolide lactones, Spino- syn A and D, produced by fermentation of the soil actinomycete *Saccharopolyspora spinosa* (Mertz and Yao, 1993). Spinosad is classified by the U.S. Environmental Protection Agency as a reduced-risk material due to its low environmental persistence and very low toxicity to most vertebrates (Thompson et al., 2000).

There is no biological control plan for CLM, but studies are underway using bio-rational insecticides. Several different insecticides, such as indoxacarb (Avaunt) and pyriproxifen (Esteem), have been used<sup>4, 5</sup>, but these may interfere in the control by natural enemies<sup>13</sup>. Biological control is the best option for long-term control, however the effective control of CLM is complicated by its high migration ability from orchards, high fertility, and protection of the larvae inside the citrus leaf making contact with the chemical difficult. As well, CLM has a history of developing resistance to insecticides making it difficult to achieve sustainable control<sup>27, 18</sup>.

Biopesticides, including botanicals, can offer a safe and effective alternative to conventional insecticides for controlling major insect pests within an integrated pest management program. The use of biopesticides such as Tondexir which is extracts from hot pepper, discourage insect pests from laying eggs on leaves and pose lower risk to humans and the environment than other pesticides. Pepper worked better when insects are soft-bodied during the larval stage because the chemical is able to penetrate. It worked fast.

The citrus leaf-miner (CLM), *Phyllocnistis citrella* Stain-ton (Lepidoptera: Gracillariidae), is an important pest of citrus and related Rutaceae and ornamental plants world-wide (Achor et al., 1996). The CLM mines leaves, the surface tissue of young shoots and stems, and less frequently the fruit (Sponagel and Diaz, 1994). Although CLM causes indirect damage to young leaves, which pre-disposes them to infection by canker, controlling CLM is a vital component in (1996; Belasque et al., 2005). The first record of CLM from southern and northern Iran, with a dramatic increase and widespread dispersal, was noted in 1961 and 1994, respectively. There is no biological control plan for CLM, but some studies are

underway for planned extension activities using biorational insecticides. Several different insecticides, such as Avant, Buprofezin and Pyriproxifen have been used (Amiri, 2006a, b, 2007), but these may cause interference in the control of the pest by natural enemies (Guerra et al., 1997). CLM has a long history of resistance to many insecticides and the development of such resistance makes it difficult to achieve control (Mafi and Ohbayashi, 2006).

*Bacillus thuringiensis* (*Bt*) subsp *Krustaki* is the bacterial insecticide most widely used for controlling Lepidoptera larvae population (Broderick et al., 2000). It is safe for many non-target insects and has a minimal impact on the environment (Jyoti & Brewer, 1999). Since CLM is protected inside the mine it is suggested that the mineral oils used as a surfactant would reduce the surface tension and increase the penetration of the *Bt* suspension through the leaf epidermis. Petroleum oil reduced infestation by preventing oviposition and there is a negative effect between the number of mines/leaf and concentration of oil (Dias et al., 2005).

Botanical insecticides are important products for pest management in industrialized countries (Isman, 1997). The pesticide resistance and negative effects on non-target organisms, for example, man and environment, are the main problems (Franzen, 1993). The natural insecticides are relatively inactive against the nontarget organism. The pests mainly controlled by synthetic pesticides in the last fifty years, but today the main strategy in aim of the plant protection is reduction of synthetic pesticides and use of botanically insecticides.

The two-spotted spider mite (TSSM), *Tetranychus urticae* Koch is widely distributed in the world and a common pest of many plant species in greenhouses, nurseries and field crops. A population of TSSM can increase rapidly especially during hot and dry periods. It infests many crops including tomatoes, beans, peepers, eggplants and ornamental plants (Cagle, 1949).

Many new acaricides are now available in the market but they have a high cost associated with their use and application restrictions listed on the label to prevent the development of resistance.

The aim of this study was to evaluate the toxicity of the commercial formulation of *Bacillus thuringiensis* as a biorational insecticide, mineral oils as a selective pesticide, Methoxyfenozide (Runner), Spinosyn A and D (Tracer), Insecticidal Gel (Palizin, IG), Insecticidal Emulsion (Sirinol, IE), Tonexir with/without Mineral Oils (MO), chlorpyrifos-methyl (Reldan), methoxyfenozide (Runner), spinosad (Tracer), an insecticidal soap (Palizin, IS), an insecticidal emulsion (Sireinol, IE), plant secondary metabolites on second and third instars larvae of the CLM in laboratory and field conditions and also toxicity evaluation of Nissorun, Palizin, Sirinol and Tondexir on *Tetranychus urtica* Koch a pest of *Brassica napus* L.

## 2. Materials and methods

### 2.1 Laboratory bioassay and experimental design

The toxicity of commercial and biorational insecticides (table.1) to the citrus leafminer was tested in the laboratory of toxicology at Sari Agricultural and Natural Resources University in 2005, 2006, 2007, 2008, 2009, 2010. Newly growth leaves were sampled from citrus Thomson tree of different orchards at Sari district in North of Iran. The bioassay method (Leaf-dip method) was devised to test toxicities of above biorational insecticides. Leaves were examined in a laboratory with the aid of a stereo-microscope. The numbers of larvae, pre pupae, pupae and adult leaf miners on leaves  $\geq 10$  mm in length were recorded.

Common name	Trade name	Chemical group	Formulation company	LD50 for rats (mg/kg)	Dose (ml/l)
Tondexir chlorpyrifos-methyl	Tondexir Reldan	Pepper extract organophosphate	EC Kimia Sabzaver 50 EC	> 5000 > 3000	2 1
Methoxyfenozide	Runner	Diacylhydrazin(IGR)	Dow AgroSciences 24% SC	> 5000	1
Spinosad	Tracer	Spinosyn A & D	240sc	>5000	0.75
<i>Bacillus thuringiensis</i> sub sp morrison (BT)	Bitiran	Biopesticides	LD 3.6% (liquid dispersal)	NT	4
MO	PO	MO	O 80%	4300	0.05
Insecticidal emulsion (IE)	SIRENOL	Garlic extract insecticide	EC Kimia Sabzaver	>5000	2 & 4
Insecticidal Gel (IG)	PALIZIN	Insecticidal and miticidal soap	Kimia Sabzaver WS	>5000	2 &4
Avant					
Buprofezin	Indoxicarp	Oxadiazon	SC150	2198	2
Pyriproxyfen	Aplaud	IGR (Chitin Synthesis Inhibitors)	EC40%	>5000	0.5
	Admiral	IGR (Juvenile Hormone Mimic)	EpC 10%	>5000	0.5

Table 1. Pesticide used in experiments.

In assay, only leaves with actively feeding second or third instars leaf miner larvae were completely excised with petioles from citrus Thomson trees and used for bioassays. To keep the leaves turgid during the bioassay, each petiole was covered by wet cotton. Leaves were dipped separately for approximately 10 seconds into each of three different pesticides (Table 1). After dipping, the leaves were air-dried for approximately 2 hours and placed at the bottom of the plastic Petri dishes (9 cm diameter × 2.5 cm high) which was lined with a wet filter paper and covered with a plastic lid. The experiment for each treatment was replicated four times along with distilled water treated as a control group. After 24, 48, 72

and 96 hours of post-treatments the numbers of live and dead larvae for each replicate were counted in the laboratory under a stereomicroscope.

## 2.2 Semi field assay

The toxicity of the six different pesticides (Table 1) to CLM was examined in the nursery and Laboratory at Sari Agricultural Science and Natural Resources University in 2007. 72 young trees (4 years) of the citrus variety of Thomson navel (*Citrus sinensis*) nursery within Sari agricultural University and spray method bioassay were used<sup>7</sup>.

Experiments for each treatment were replicated four times, and distilled water was used as a control. At 24, 48, 72 and 96 hours post-treatment, the numbers of live and dead larvae in each replicate were counted in the laboratory under a stereomicroscope.

## 2.3 Topical spray toxicity assay

In topical spray assay, leaves containing actively feeding second and third instars larvae of citrus leafminer were placed into plastic Petri dishes (9cm diameter × 2.5 cm high). The leaves were sprayed with one ml aliquots of each chemical pesticide (Table 1) in a Potter Precision Spray Tower (Burkard Manufacturing Co. Ltd., Rickmansworth Herts, UK). Each treatment was replicated four times along with distilled water treated as a control group. The leaves in the control group were sprayed with 1 ml of distilled water. Following these treatments, the leaves were individually transferred to clean plastic Petri dishes. After 24 and 48 hours of post-treatment the numbers of live and dead larvae for each replicate were counted in the laboratory (as above).

## 2.4 Measurements and statistical analysis

This experiment was conducted in a completely randomised design using factorial arrangement of treatments. Variables measured per replicate of each treatment were the average number of mines per leaf, larval mortality (the proportion of larvae that were dead). Normality was assessed using probability plots. The normal distributed was approximated for the number of dead larvae per leaf when these data were reciprocally transformed using  $\text{ArcSin}\sqrt{\frac{y}{100}}$ . Mortality data were corrected using Abbott's formula (Abbott, 1925).

The analysis of data was performed on each dependent variable using the ANOVA procedure (SPSS, 1993). If a significance effect of variables was calculated, means were contrasted by Duncan's multiple range test and Tukey's test.

The insecticides and respectively concentrations used were *Bt* (0.5, 1, 3, and 6 g L<sup>-1</sup> of water) in experiment 1, different percentage (0.1, 0.2, 0.3, 0.5) of mineral oil (MO) in experiment 2 and *BT* (0.5, 1, 3, and 6 g L<sup>-1</sup>) + 0.5 % MO in experiment 3. In each experiment a control group was run using sterile water. The leaf-dip bioassays were devised to test the toxicities of *Bt* pesticide. In assay, only leaves with actively feeding second or third instar leafminer larvae were completely excised with petioles from citrus Thomson trees and used for bioassays. To keep the leaves turgescient during the bioassay, each petiole was covered by wet cotton. Leaves were dipped separately for approximately 10 seconds into each treatment. Air-dried for approximately 2 hours and placed at the bottom of the plastic petridishes (9 cm diameter × 2.5 cm high). These dishes were lined with a wet filter paper and covered with a plastic lid. The experiment for each treatment was replicated four times along with distilled water treated as a control group. After 24,

48, 72 and 96 hours of post-treatment the numbers of live and dead larvae for each replicate were counted under a stereo-microscope. Variable measured per replicate of each treatment were the average number of mines per leaf larval mortality (the proportion of larvae that were dead).

Tondexir, Sirinol and Palizin with 3, 1, 0.5, 0.1 mL<sup>-1</sup>dose from Kimia Sabz co. were used in this experiment. One bioassay methods (Leaf-dip method) were devised to test toxicities of these pesticides at five concentration (3, 1, 0.5, 0.1 mL<sup>-1</sup>) in distilled water. Leaves with actively feeding adult TSSM excised with petioles from *Brassica napus* L and used for bioassays. This experiment was conducted in a completely randomized design using factorial arrangements of treatments with two factors (TONDEXIR, SIRINOL and PALIZIN with 4 dose plus control and post spraying period with 4 times) in four replicates. The average number of mortality (the proportion of larvae that were dead) was detected. Normality was assessed using probability plots. The normal distributed was approximated for the number of dead larvae per leaf when these data were reciprocally transformed using

$ArcSin\sqrt{\frac{y}{100}}$  (Sato et al., 2005). The analysis of data was performed on each dependent variable using the ANOVA procedure (SPSS, 1993). If a significance effect of variables was calculated, means were contrasted by Duncan's multiple range and LSD tests. The lethal concentrations (LC<sub>50</sub> and LC<sub>90</sub>) were calculated using probit analysis (Finney, 1971). The percentage mortality was calculated and corrections for mortality when necessary were done by using Abbot's (1925) formula.

### 3. Results

#### 3.1 Effect of pyriproxifen, avant and buprfezin on CLM

Analysis of variance indicated that there were significant differences (P<%1) among methods of bioassays and type of chemical insecticides used at the present study (Table 2). The interaction effect of methods of bioassays and type of chemical insecticides was significant (P<%5), but no significant differences were found between post spraying methods.

Sources of variance	df	Sum of squire	Mean of squire	Significance
Type of bioassays (A)	1	0.905	0.905	**
Type of pesticides (B)	3	1.67	0.557	**
Post spraying period (C)	1	0.144	0.144	ns
A×B	3	0.550	0.183	*
A×C	1	0.008	0.008	ns
B×C	3	0.0470	.015	ns
A×B×C	3	0.0088	0.0029	ns

\*\* Significant at the 1% level, \* Significant at the 5% level, ns - nonsignificant.

Table 2. The ANOVA of effects of different factors on citrus leafminer larval mortality.

Interaction effect of post spraying methods on treatment was also not significant (Table 2).

Among the used chemical insecticides at the present study, Avant pesticide was more effective (35.58%) than Buprofezin (21.25%) and Pyriproxyfen (19.31%) on citrus leafminer larval mortality (Table 3).

Treatment	Subset for Alpha =0.01		
	c	b	a
Control	7.96±3.05		
Pyriproxyfen		19.31±3.5	
Avant			35.58±5.7
Buprofezin		21.25±4.8	

<sup>a, b, c</sup> Means followed by different letters are significantly different.

Table 3. Effects of fixed factors (three different commercial chemical insecticides) used in the model on citrus leafminer larval mortality (%).

Multiple slope of Duncan’s test showed that between two types of spray methods, leaf-dipping method was more effective (29.5%) than topical spray method (12.20%) on pest mortality (Table 4).

Spray methods	Pesticides			
	Control	Pyriproxyfen	Avant	Buprofezin
Leaf-dipping	10.94±3.5 <sup>dc</sup>	22.07±5.3 <sup>bcd</sup>	49.75±8.09 <sup>a</sup>	36.67±5.3 <sup>ab</sup>
Topical Spray	5.0±2.67 <sup>e</sup>	19.55±2.97 <sup>cde</sup>	21.42±4.08 <sup>bc</sup>	5.0±2.3 <sup>de</sup>

<sup>a</sup> Means followed by the same letter are not significantly different

Table 4. Comparison between two bioassay methods on citrus leafminer larval mortality (%).

No significantly difference in citrus leafminer larval mortality was detected between periods of post spraying in pest mortality (Table 5).

Post spraying	Pesticides			
	Control	Pyriproxyfen	Avant	Buprofezin
24 h post treatment	6.25±2.25	19.57±7.07	42.96±7.30	35.11±9.15
48 h post treatment	15.62±9.37	24.57±8.74	56.53±14.88	38.23±6.81

Table 5. Comparison between two post spraying treatments on citrus leafminer larval mortality (%).

### 3.2 Effect of Bt and Mineral Oils (MO) on CLM

A significant reduction in the number of larval mortality per leaf in all chemical treated groups (19.31-35.58%) compared to untreated leaves as a control group (7.96%) was achieved (Table 7).

Analyses of variance indicated that there were significant differences among *Bt* treatments ( $P<0.01$ ). The results clearly demonstrated that the efficacy of *Bt* against CLM increased with increasing *Bt* concentration (Table 6).

Treatment	Mean comparison		
	c	b	a
Control	8.49±1.5		
0.5	35.40±7.5	35.40±7.5	
1		40.07±5.3	40.07±5.3
3		46.96±5.7	46.96±5.7
6			61.16±7.5

a, b, c, Means did not followed by the same letters in rows are significantly different ( $P<0.01$ ).

Table 6. The effect of different concentrations of *Bt* (0.5, 1, 3 and 6 gram per liter of water) on percentage of CLM larvae mortality (Mean±sd).

The comparison between different post treatment times showed significant differences ( $P<0.05$ ) among the periods of the post treatments (Table 7).

Post treatment time (h)	Mean comparison	
	b	a
24	16.81±3.8	
48	35.37±4.64	35.37±4.64
72		47.45±6.70
96		54.10±7.15

a, b, Means did not followed by the same letters in rows are significantly different ( $P<0.01$ ).

Table 7. The effect of different post treatment time of *Bt* on percentage of CLM larvae mortality (Mean±sd).

Post-treatment for 96, 72 and 48 h were more effective than 24 hour on pest mortality. There was no significant difference between interaction of *Bt* and post treatment time on CLM larvae mortality. Results for different concentrations of *Bt* plus MO indicated significant differences among treatments. The results showed that the treatment of *Bt* plus MO increased the mortality of CLM larvae at higher concentration of *Bt* (Table 8).

Treatments	Mean comparison		
	c	b	a
control	8.48±1.5		
0.5		53.38±3.4	
1		54.72±5.6	54.72±5.6
3		56.25±4.8	56.25±4.8
6			63.13±5.5

a, b, c, Means did not followed by the same letters in rows are significantly different ( $P<0.01$ ).

Table 8. The effect of different concentrations of *Bt* (0.5, 1, 3 and 6 gram per liter of water) plus MO (0.5%) on percentage of CLM larvae mortality (Mean±sd).

The comparison between different post treatment times showed significant differences ( $P < 0.05$ ) among the periods of the post treatments on CLM larvae mortality (Table 9).

Post treatment time (h)	Mean comparison	
	b	a
24	42.6±10	
48	59.27±9.6	59.27±9.6
72	64.82±8.3	64.82±8.3
96		85.82±5.9

a, b, Means did not followed by the same letters in rows are significantly different ( $P < 0.05$ ).

Table 9. The effect of different post treatment time of *Bt* plus MO (0.5%) on percentage of CLM larvae mortality (Mean±sd).

No statistically differences were observed in CLM larvae mortality between *Bt* treated groups in comparison with their counterparts *Bt* plus MO groups (Table 10).

Treatments(dose) mg/l	BT	BT+MO
0	8.49±1.51	8.49±1.51
0.5	35.40±7.60	56.04±3.46
1	38.22±5.62	53.73±5.65
3	46.96±5.72	54.24±4.91
6	61.16±7.56	63.21±5.61

Table 10. Mean comparison between the effect of *Bt* and *Bt* plus MO (0.5%) on percentage of CLM larvae mortality (Mean±sd).

### 3.3 Effect of *Bt*, MO, IG (Palizin) IE (Sirinol) on CLM

There were significant differences ( $p < 0.001$ ) among different biorational insecticides and post spraying methods that used at the present study (Table 11 & 12), but the interaction effect of insecticides and time was not significant. These results showed that each insecticides and time have independent and separate effect on percentage of larval mortality.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment(A)	.017	4	.004	10.706	**
Day(B)	.016	3	.005	13.872	**
treat * day	.002	12	.000	.524	ns
Error	.016	40	.000		

\*\*showed significantly different ( $P < 0.01$ )

ns not significantly different

Table 11. The ANOVA of different biorational insecticides on citrus leaf miner larval mortality

The percentage of larvae mortality in IE, IG, BT and MO was  $67.83 \pm 9.10$ ,  $62.45 \pm 8.10$ ,  $49.08 \pm 6.70$  and  $37.70 \pm 8.50$ , respectively (Table 12). There were significant differences in larvae mortality between control and treatment groups ( $p < 0.0001$ ).

treatment	Subset for alpha = .05		
	1a	2	3
control	$19.16 \pm 5.25$		
MO	$37.75 \pm 8.47$	$37.75 \pm 8.47$	
BT		$49.08 \pm 6.68$	$49.08 \pm 6.68$
IG			$62.50 \pm 8.10$
IE			$67.83 \pm 9.09$

<sup>a</sup> Means followed by the same number are not significantly different

Table 12. The comparison of the mean of different Biorational insecticides on percentage of larval mortality of CLM consist of Tukay 's test.

A significantly differences in CLM mortality was found among post spraying period (Table 4). The percentage of larvae mortality among 96 and 72 hours of post treatments ( $71.9 \pm 5.9$  and  $54.4 \pm 6.3$ ) were more effective than 24 and 48 hours post treatments ( $25.2 \pm 6.8$  and  $37.4 \pm 7.8$ ). The results have shown that 96 and 72 hours post spraying period were more effective than 48 and 24 hours post spraying period on mortality of the CLM (Table 13).

Time Hours(h)	Subset for alpha = .05		
	1	2	3
24	$25.2 \pm 6.8$		
48	$37.4 \pm 7.8$	$37.4 \pm 7.8$	
72		$54.4 \pm 6.3$	$54.4 \pm 6.3$
96			$71.9 \pm 5.9$

<sup>a</sup> Means followed by the same letter are not significantly different

Table 13. The comparison of the mean of different post spraying period on percentage of larval mortality of CLM consist of Tukay 's test ( $P < 0.01$ ).

Among different biorational insecticides at the present study, IE with  $67.83 \pm 9.10$  and IG with  $62.45 \pm 8.10$  were more effective than Bt with  $49.08 \pm 6.70$  and MO with  $37.70 \pm 8.50$  percentage on citrus leafminer larval mortality (Table 14).

Treatment	Mean
Bacteria	49.08 ± 6.68*
Mineral oil	37.75 ± 8.47**
.Insecticide gel	62.50 ± 8.10**
Insecticide emulsion	67.83 ± 9.09**
Control	19.16 ± 5.25
Tukay(0.1)	17
Tukay(0.5)	22

\*\*showed significantly different (P<0.01)

\*showed significantly different (P<0.05)

Table 14. Mean comparison between the effects of biorational insecticide on percentage of CLM larval morality (Mean ± sd).

### 3.4 Effect of Reldan, Runner, Tracer, Sirinol, Palizin and MO on CLM

The toxicity of Reldan, Runner, Tracer, Sireinol, Palizin and oil (Table 1) on CLM was investigated using a leaf-dip bioassay. There were significant differences (p<0.001) among the different treatments and also among the intervals before assessment (Table 15).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	0.072	6	0.012	104.192	**
Time	0.010	3	0.003	28.043	**
Treatment * time	0.006	18	0.000	2.855	**
Error	0.006	56	0.000		

\*\* Significantly different (p<0.01)

Table 15. ANOVA details for different insecticides and times to assessment.

The percent of larvae dead with Reldan, Runner, Tracer, oil, Palizin and Sireinol was 89.33 %, 86.66 %, 100 %, 84.66 %, 83.66 % and 50 %, respectively, at 96 hours after treatment (Table 16). The difference between the control and all other treatments was significant at p<0.0001.

Treatment	Time after treatment			
	24 h	48 h	72 h	96 h
Control	8.33 ± 0.8 a <sup>1</sup>	8.33 ± 1.55 a	8.33 ± 1.66 a	8.33 ± 1.66 a
Tracer	41 ± 4.9 d	93.33 ± 6.66 d	93.33 ± 6.66 d	100 d
Reldan	79 ± 10.59 d	89.33 ± 5.36 d	89.33 ± 5.36 d	89.33 ± 5.36 d
Runner	54.33 ± 1.73 cd	61 ± 20.1 cd	73.66 ± 1.54 cd	86.66 ± 6.88 cd
Palizin(IS)	41 ± 4.93 c	52 ± 15.63 c	66 ± 8.62 c	83.66 ± 2.33 c
Sireinol(IE)	24.33 ± 4.33 b	31 ± 5.85 b	43.33 ± 3.33 b	50 ± 5.77 b
Oil	14.66 ± 8.11 c	60 ± 3 c	60 ± 3 c	84.66 ± 8.41 c

Means followed by the same letter are not significantly different

Table 16. Comparison of the mean time on Tracer, Reldan, Runner, IS, IE and oil on the percentage mortality of CLM larvae using Tukey's test.

Reldan, Runner and Tracer, with 96-h mortalities of 89.33 %, 86.66 % and 100 %, respectively, were more effective than IS, oil and IE with mortalities of 84.66 %, 83.66 % and 50 % at the rates used (Table 3). CLM mortality also varied significantly with the post-spraying interval tested, increasing with time (Table 5).

The total percentage of larvae mortality achieved by Reldan, Tracer, Runner, MO, IG and IE were  $86.75 \pm 3.2$ ,  $81.91 \pm 7.5$ ,  $68.91 \pm 7.16$ ,  $54.83 \pm 8.07$ ,  $60.66 \pm 6.24$  and  $37.16 \pm 3.69$ , respectively (Table 17). Significant differences in larvae mortality between the control and the various treatments were observed ( $P < 0.0001$ ).

treatment	Subset for alpha = .05 <sup>a</sup>			
	a	b	c	D
Control	8.33+_0.0			
IE		37.16 ± 3.69		
IG			60.66 ± 6.24	
MO			54.83 ± 8.07	
Runner			68.91 ± 7.16	68.91 ± 7.16
Tracer				81.91 ± 7.5
Reldan				86.75 ± 3.2

Means followed by the same letter are not significantly different

Table 17. Comparison of the different insecticides using the overall mean percentage mortality of CLM larvae and Tukey's test.

Significant differences in the mortality of CLM larvae were also found between the lengths of time after treatment (Table 18).

Time Hours (h)	Subset for alpha = .05 <sup>a</sup>		
	a	b	c
24	37.52 ± 7.8		
48		56.42 ± 6.8	
72		62 ± 6.3	62 ± 6.3
96			71.80 ± 5.9

<sup>a</sup> Means followed by the same letter are not significantly different

Table 18. Comparing the mean effect of different post-spray assessment periods on percentage mortality of CLM larvae using Tukey's test ( $P < 0.01$ ).

The percentage mortality at 96 and 72 h post-treatment ( $71.80 \pm 5.9$  and  $62.1 \pm 6.3$ , respectively) was considerably higher than the mortality at 48 and 24 h post-treatment ( $56.42 \pm 6.8$  and  $37.4 \pm 7.8$ , respectively). Thus, the 96 and 72 h post-treatment periods were more effective than the 48 and 24 h treatments on CLM mortality. Among the different pesticides tested, Reldan ( $86.75 \pm 3.2$ ) and Tracer ( $81.91 \pm 7.5$ ) were more effective than the others on percentage CLM larvae mortality.

Future studies should also investigate the toxicity of insecticide residues after exposure in the field for various intervals.

### 3.5 Toxicity evaluation of Nissorun, Palizin, Sirinol and Tondexir on *Tetranychus urtica* Koch a pest of *Brassica napus* L

There were significant differences among different treatments ( $P < 0.01$ ). The comparison between different post treatment times has shown that treatments significant differences ( $P < 0.01$ ); Table 19). These results showed that each factor has independent and separate effect on percentage of mortality.

The results of current experiment showed that among different treatments of the Nissorun with 3/1000 and tondexir with 3/1000 (95 %) were more effective than the others on citrus TSSM mortality (Table 20 Fig. 1).

In addition, among periods of the post spraying methods, 76 h was significantly different with 48 h and they were more effective than 24h on pest mortality of the TSSM (Table 3).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
treat	.023	4	.006	99.047	**
day	.001	2	.001	9.163	**
treat * day	.000	8	3.29E-005	.558	ns

\*\* showed significantly different ( $P < 0.01$ )

ns not significantly different

Table 19. The ANOVA of the different treatments

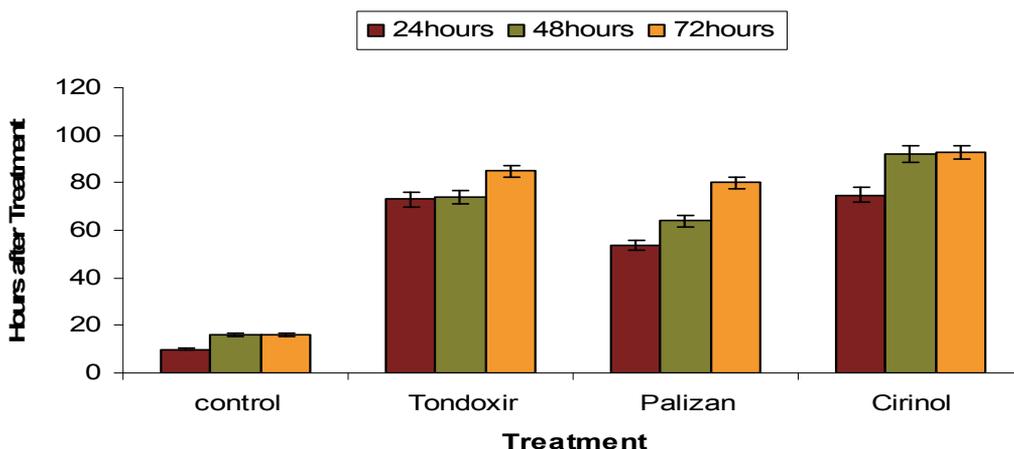


Fig. 1. Comparison of the effect of different toxins on TSSM

Treatment Time	Time Hours(h)		
	24	48	72
Control	11.67±4.41a	13.33±4.41a	13.33±4.41a
Cirinol(3/1000)	74.33 ± 3.18c	91.67± 3.71c	95.67 ± 3.33c
Palizan(3/1000)	47.33±5.48b	60.33 ± 2.60b	75.33 ± 8.19b
Nisiron(3/1000)	71.67±7.68d	97.33± 1.66d	97.33 ± 1.66d
Tondoxir(3/1000)	75.33±14.17c	87.33±6.64c	95±4c

Means followed by the same letter are not significantly different

Table 20. The comparison of the mean of different treatments on percentage of morality of TSSM consist of Tukay ´s test.

#### 4. Discussions

The goal of control program is to protect the main growth flushes particularly the summer shoots grown in greenhouses which are important as fruit-bearing branches. The CLM larvae are protected by a cuticle layer of the leaves in the serpentine mine and the pupal stage is also protected by the rolled leaf margins (Raga *et al.*, 2001). The use of chemical and synthetic insecticides in developing countries and its concomitant problems is necessary to test the alternative traditionally oriented methods for pest control. The present work revealed the effect of the biorational insecticides including Insecticidal Emulsion (IE), Insecticidal Gel (IG), *Bacillus thuringiensis* (Bt) and the mineral oils (MO) on CLM. These insecticides had significant insecticidal activity against CLM larvae, but the IE and IG were more effective than the Bt and MO. The IE and IG are used against a wide rang of pest. However, they have very low toxicity against vertebrate and mammals (With LD<sub>50</sub> more than 10000 mg/kg). These results suggest that there may be different compounds in IE and IG possessing different bioactivities.

Pesticides may be applied to protect new flushes of growth when the leaves are most vulnerable to CLM damage. However, the best foliar insecticides confer only 2 weeks of leaf miner infestations (Michaud and Grant. 2003). Recently, Mafi and Ohbayashi (2006) found that the percentage corrected mortality of eggs of the citrus leafminer exposed to insecticides (dipping method bioassay) ranged from 3 to 44%, but all the insecticides tested showed almost over 90% mortality to the first instar larvae of citrus leafminer. It is important to select less toxic chemicals against the natural enemies in order to expect both the activity of natural enemies and control effect of insecticides for suppressing the infestation of CLM.

The results of this study has shown that the IE, IG, BT, Reldan, Runner, Tracer, SirenoI, Palizin and MO are active against the leafminer demonstrating that these biopesticides penetrate into leaf mines, killing the larvae as observed by Shapiro et al (1998) and the oil of the IE, IG and MO might be reduced the infestation by acting as an oviposition deterrent in the field (Liu et al 1999). The IE, IG, Reldan, Runner, Tracer, SirenoI, Palizin and MO solution that tested in current experiment had two different effects on CLM mortality. Firstly, they had insecticidal activity when applied these biopesticides against the CLM at the recommended dose. Secondly, they increased the efficacy of the commercial formulation

of IE, IG, Bt and MO by helping them to penetrate the plant cuticle and enhanced the activity of the active ingredient of these biorational insecticides when applied to the pest, probably due to increased penetration through the mine stomata into the mines. Since CLM preferentially mine the abaxial surface of leaves, enhanced stomatal infiltration is especially useful against CLM.

The petroleum oil spray residues reduced infestations of CLM by preventing oviposition and its effects depended on concentration of oil and time of spraying (Beattie *et al.*, 1995, Smith *et al.* 1997). Raga *et al.* (2001) reported that both of Abamectin and Lufenuron pesticides along with petroleum oil provided a significant increase in CLM larval activity. However, the efficacy of petroleum-derived spray oils used as oviposition deterrents to control citrus leafminer is related to time of spraying, the amount of oil dose and the persistence of oil molecules on sprayed surfaces or efficacy is also related to increasing molecular weight of oil molecules as reflected by *nCy* values and, therefore, persistence of oil molecules on sprayed surfaces (Liu *et al.*, 2001). Therefore, the petroleum oils alone or combine with microbial agent as emulsifier which has synergist and less harmful effect for the environment recommended for using in IPM program (Khyami and Ateyyat, 2002).

Plant allelochemical may be quite useful in increasing the efficacy of biological control agents because plants produce a large variety of compounds that increase their resistance to insect attack (Murugan *et al.*, 1996; Senthil Nathan *et al.*, 2005a).

It has been shown that neonicotinoid, pyrethroid and growth regulator insecticides have a significant, negative impact on some predators which are appearing to be the most important biological control agents of leafminers. Thus, it is necessary to be aware about the effect of these pesticides on beneficial insects, therefore, the usage of biorational insecticides, such as BT, are recommended (Grafton and Gu, 2003; Villanueva-Jiménez *et al.*, 2000). In addition, the toxicity of pesticides such as Avant was higher than Pyriproxyfen and Buprofezin (Amiri (b), 2006). IE, IG, BT and MO are relatively much safer compounds than the conventional organophosphorus insecticides, Buprofezin, Avant and Pyriproxifen (Maha and Abdalla, 1999). Chemistry of natural compounds is a very complex subject and screening for activity will have to face, among other factors, isolation and identification of the products variability due to the plants or the environment, a synergism due to the mixtures of compounds in crude extracts (Chiue, 1989). The high toxicity of the IE and IG may be due to penetration through mine stomata into the mines. Since CLM preferentially mine the abaxial surface of leaves, enhanced stomatal infiltration is especially useful against these pests. The low toxicity of the mineral oils in this study may be due to different factors including cuticle properties, ambient temperature, and the molecular size and the volume of oil molecules. According to Cole (1994), the choice of insect and bioassay can greatly influence the outcome of a screening. However, to develop a useful commercial product, testing against agricultural pests is important. Amonkar and Reeves (1970) found that garlic killed an insecticide resistance strain of *Aedes nigromaculis* as well as susceptible *Aedes* species. However, the neem formulations can be used as follow-up sprays under heavy infestation and as prophylactic sprays during new flush emergence (Verghese and Jayanthi, 2004). Howard (1993) has shown that Azadiractin and abamectin both as a biorational insecticide were potentially useful for controlling CLM.

The results of this study indicated that the plant-based compounds such as IE and IG may be effective alternative to conventional synthetic insecticides for the control of CLM. For further understanding it is necessary to investigate the third generation pesticides such as growth regulators (IGRs) and Biorational insecticides in combination with mineral oil, to get

much more suitable results in the field conditions. Since the spring population density of CLM is very low, it is not necessary to control CLM before late June in most parts of the citrus growing regions in north of Iran. On the other hand, it is so important to protect the new shoots of the young or top grafting citrus trees from the infestation of summer generations of CLM.

The effect of insecticides in citrus orchards against the CLM is difficult to achieve the maximum CLM larval mortality and it is not very sufficient because several generations of CLM are usually overlapping and the CLM larvae are protected by a cuticular layer of the leaves in the serpentine mine and the pupal stage is also protected by the rolled leaf margins (Raga *et al.*, 2001). The results of present study clearly demonstrated that the efficacy of *Bt* and *Bt* plus MO against CLM increased with the increasing *Bt* concentration. It has been shown that the larval mortality vary with spray volume suggesting that the oil reduced the infestation by acting as an oviposition deterrent (Liu *et al.*, 2001).

In our experiment, by comparing the activity of the commercial formulation between *Bt* and *Bt* plus MO against the CLM, we observed that the CLM larval mortality was higher (not statistically) in *Bt* plus MO treated groups than the *Bt* alone. Several research groups have shown that, the application of Abamectin in combination with petroleum oil provides the most synergistic effect to control of the *Helicoverpa armigera* and CLM (Wang *et al.*, 2005).

Sometimes the indirect damage of CLM is very important. Mining of immature foliage by the larvae can lead to reduced growth rates, yield and mined surfaces serve as foci for the establishment of diseases such as citrus canker, *Xanthomonas citri*. In the absence of citrus canker, citrus leafminer is a serious pest of rapidly growing immature or pruned trees. But in presence of citrus canker, it is a major pest of both immature and mature trees (Liu *et al.*, 2001). Therefore, it is important to select less toxic chemicals against the natural enemies in order to expect both the activity of natural enemies and control effect of insecticides for suppressing the infestation of CLM. The higher activity of *Bt* in *Bt* plus MO treated groups at the present study may be due to increased penetration of *Bt* through the mine by helping of MO.

For better understanding it is necessary to investigate the third generation pesticides such as growth regulators in combination with mineral oil, microbial and fungi insecticides to get much more suitable results in the field conditions. However, more field studies will need to be performed to understand the effect of *Bt* and *Bt* plus MO against *P. citrella* and to determine the optimum timing of the multiple application.

The CLM is one of the key pests in citrus growing, especially in nurseries, top-grafted trees and newly planted trees in north of Iran. Therefore, it is so important to protect new shoots of young or top-grafted trees from the damage caused by summer and autumn generations of CLM. The goal of cultural, chemical and other control programs is to protect the main growth flushes. The results obtained at the present study indicated that insecticidal control is difficult to achieve the maximum CLM larval mortality. Because the larvae of the CLM are shielded within the mines by the leaf epidermis and the pupal stage is also protected by the rolled leaf margins (Raga *et al.*, 2001). Foliar sprays may be applied to protected new flushes of growth when the leaves are most vulnerable to CLM damage. However, the best foliar insecticides confer only 2 weeks of leaf miner infestations (Michaud and Grant. 2003).

Recently, the toxicity of different insecticides to the citrus leafminer and its parasitoids was evaluated under laboratory conditions in Japan (Mafi and Ohbayashi, 2006). They found that the percentage corrected mortality of eggs of the citrus leafminer exposed to insecticides (dipping method bioassay) ranged from 3 to 44%, but all the insecticides tested showed almost over 90% mortality to the first instar larvae of citrus leafminer. Comparison between two spray methods at the present study leaf-dipping method was more effective than topical spray method on pest mortality. According to several authors, the application of Abamectin in combination with petroleum oil provides the most effective control of the CLM. It has been reported that in the absence of citrus canker, citrus leafminer is a serious pest of rapidly growing immature or pruned trees. But in presence of citrus canker, it is a major pest of both immature and mature trees (Liu *et al.*, 1999). In our study, it was shown that the toxicity effect of Avant was higher than Pyriproxyfen and Buprofezin pesticides. Previously, it has been shown that pesticides such as Buprofezin and Pyriproxifen decreased the number of laying eggs, hatched eggs and also short life cycle in *Bemisia tabaci* (Yasui *et al.*, 1987; Ishaaya *et al.*, 1994). They concluded that Buprofezin and Pyriproxifen affects on reproductive system of *Bemisia tabaci* at immature stage of life cycle (Ishaaya *et al.*, 1988). It has been shown that the two pest control agents, Buprofezin and Super Royal are relatively much safer compounds than the conventional organophosphorus insecticides (Maha and Abdalla, 1990). Exposure of adult beetles species (*Circellium bacchus*) to Pyriproxyfen did not affect egg production or the viability of eggs, nor did the compound have adverse effects on immature development, indicating that Pyriproxyfen is unlikely to be the cause of the observed population depression of *Circellium bacchus* (Kruger and Scholtz, 1997). In our study, no significant difference in effectiveness was found between periods of post spraying in citrus leafminer larval mortality. The results obtained at the present study suggest that the Avant chemical pesticide can be account as an effective tools in controlling the spreading of citrus leafminer in citrus growing regions in north of Iran. Since the spring population density of CLM is very low, it is not necessary to control CLM before late June in most parts of the citrus growing regions in north of Iran. For further understanding it is necessary to investigate the third generation pesticides such as growth regulators in combination with mineral oil, microbial and fungi insecticides to get much more suitable results.

These data represent some of the first published information on the effects of Reldan, Runner, Tracer, Sirenol, Palizin and oil on CLM. It is very difficult to protect the new shoots of young trees from CLM damage, especially in nurseries and newly planted orchards in north Iran. The goal of any CLM control program is to protect the main growth flushes, particularly the summer shoots of young trees. The insecticides studied here had significant activity against CLM larvae, but Reldan, Runner and Tracer were more effective than Sirenol (IE), Palizin (IS) and oil. The above pesticides are used against a wide range of pests. However, they have very low toxicity to vertebrates and mammals. The results from this study suggest that there may be different compounds in IE and IG which have different bioactivities.

This study has shown that Reldan, Runner, Tracer, Sirenol, Palizin and oil are active against CLM, demonstrating that dips of these pesticides penetrate into leaf mines. , and the adjuvant ingredient of Reldan, Runner, Tracer, Sirenol, Palizin and MO might reduce the infestation by acting as an oviposition deterrent in the field (Liu *et al.*, 1999).

The results of this study will contribute to a significant reduction in the application of synthetic insecticides, which in turn will increase the opportunity for natural control of

various important horticultural pests by botanical pesticides. Since these are often active against a limited number of species including specific target insects, are easily biodegradable, non-toxic products, and potentially suitable for use in CLM control programs (Alkofahi *et al.*, 1989), they could lead to the development of new safer classes of insect control agents. Plant allelochemicals may be quite useful in increasing the efficacy of biological control agents because plants produce a large variety of compounds which increase their resistance to insect attack (Senthil Nathan *et al.*, 2005a). In addition, the translocation and translaminar properties of the above insecticides make them available in the host plant tissues to control leaf feeders, however, surface residues disappear quickly, thus making them safe for parasitoids and most natural enemies (Brunner *et al.*, 2001).

Reldan has a lower mammalian toxicity, LD<sub>50</sub> (oral, rat) is 3000 mg/kg, than Dursban with an LD<sub>50</sub> (oral, rat) of 135 mg/kg (Kalyanasundaram *et al.*, 2003). Reldan is mainly effective against rice stem borer, aphids, cutworms, plant and leaf hoppers, mole rickets, some moths and stored grain pests. Reldan also has high toxicity to CLM, as high as Runner and Tracer in these experiments.

Methoxyfenozide is a dibenzoylhydrazine insect growth regulator, similar to tebufenozide in its mode of action, its ability to induce a lethal molt and its specificity for Lepidoptera (Carlson *et al.*, 2001). Methoxyfenozide has a much lower ability to bind with receptors in non-lepidopteran species, making it a highly selective insecticide and useful in a number of crops. Low levels of resistance to methoxyfenozide in codling moth, beet armyworm and oblique banded leaf roller have been found, necessitating precautions similar to those for tebufenozide. In our study, methoxyfenozide (Runner), although considerably less toxic than the other insecticides based on LC<sub>50</sub> levels, still resulted in substantial (83%) mortality of CLM after 96 h.

A significant advantage of spinosad is that it is effective against strains of *R. dominica* which are resistant to pyrethroids and methoprene (Nayak *et al.*, 2005). Some of the newer insecticides, such as spinosad, indoxacarb, and emamectin benzoate, have been shown to be relatively safe on predacious hemipterans, mites, coccinellids, lacewings and some parasitoids. Relatively rapid degradation of surface residues in the field would definitely improve the compatibility potential with natural enemies. This would likely be the case with spinosad (Williams *et al.*, 2003).

This study indicated that plant-based compounds such as IE and IS may be effective alternatives to conventional insecticides for the control of CLM. For further understanding it is necessary to investigate the third generation pesticides such as growth regulators (IGRs) and bio-rational insecticides in combination with mineral oil, to ensure that they work in field conditions.

A number of novel insecticides with unique modes of action were registered during the late 1990s and early 2000s for insect control in agriculture. These new insecticides have several advantages over older insecticides. Firstly, low mammalian toxicity allows for short re-entry and pre-harvest intervals, allowing the insecticides to be easily incorporated into pest control programs. Many also have greater selectivity and so are less likely to harm natural enemies than the broad-spectrum organophosphate, carbamate, neonicotinoid and pyrethroid insecticides. As such, they are less likely to cause outbreaks of secondary pests, and may be used as “clean-up” sprays to manage outbreaks of such pests.

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## **Part 3**

# **Pesticides Management and Sustainable Development**



## Ecological Effects of Pesticides

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### 1. Introduction

Pesticides which are used for preventing or destroying pest is having more negative impact on our ecological system when compared to its desired action. Pesticides are carried by wind to other areas and make them contaminate. Pesticides are also causing water pollution and some pesticides are persistent organic pollutants which contribute to soil contamination. (Rockets & Rusty, 2007).

The amount of pesticide that migrates from the intended application area is influenced by the particular chemical's properties: its propensity for binding to soil, its vapor pressure, its water solubility, and its resistance to being broken down over time (Tashkent, 1998) Some pesticides contribute to global warming and the depletion of the ozone layer.

### 2. Water

Pesticides were found to pollute every source of water including wells. (Gilliom et al., 2007). Pesticide residues have also been found in rain and groundwater. (Kellogg et al., 2000). Pesticide impacts on aquatic systems are often studied using a hydrology transport model to study movement and fate of chemicals in rivers and streams. 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).

The main routes through which pesticides reach the water are:

1. It may drift outside of the intended area when it is sprayed.
2. It may percolate, or leach, through the soil.
3. It may be carried to the water as runoff.
4. It may be spilled accidentally or through neglect. (States of Jersey, 2007).

They may also be carried to water by eroding soil. (Papendick et al., 1986) Factors that affect a pesticide's ability to contaminate water include its water solubility, the distance from an application site to a body of water, weather, soil type, presence of a growing crop, and the method used to apply the chemical. Maximum limits of allowable concentrations for individual pesticides in public bodies of water are set by the Environmental Protection Agency in the US (Pedersen, 1997).

### 3. Soil

Many of the chemicals used in pesticides are persistent soil contaminants, whose impact may endure for decades and adversely affect soil conservation (U.S. Environmental

Protection Agency,2007).The use of pesticides decreases the general biodiversity in the soil. Not using the chemicals results in higher soil quality verified needed, (Johnston,1986) with the additional effect that more organic matter in the soil allows for higher water retention.(Kellogg et al.,2000).This helps increase yields for farms in drought years, when organic farms have had yields 20-40% higher than their conventional counterparts.(Lotter et al.,2003) 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.(Kellogg et al.,2000).

#### **4. Air**

Pesticides can contribute to air pollution . Pesticide drift occurs when pesticides suspended in the air as particles are carried by wind to other areas, potentially contaminating them. (Kellogg et al.,2000). Volatile pesticides applied to crops will volatilize and are blown by winds to nearby areas posing a threat to wildlife.(Reynolds,1997). Sprayed pesticides or particles from pesticides applied as dusts may travel on the wind to other areas, or pesticides may adhere to particles that blow in the wind, such as dust particles.(National Park Service,2006). Compared to aerial spraying ground spraying produces less pesticide drift. (U.S. Environmental Protection Agency,PR,2007) Farmers can employ a buffer zone around their crop, consisting of empty land or non-crop plants such as evergreen trees to serve as windbreaks and absorb the pesticides, preventing drift into other areas.

#### **5. Effects on biota**

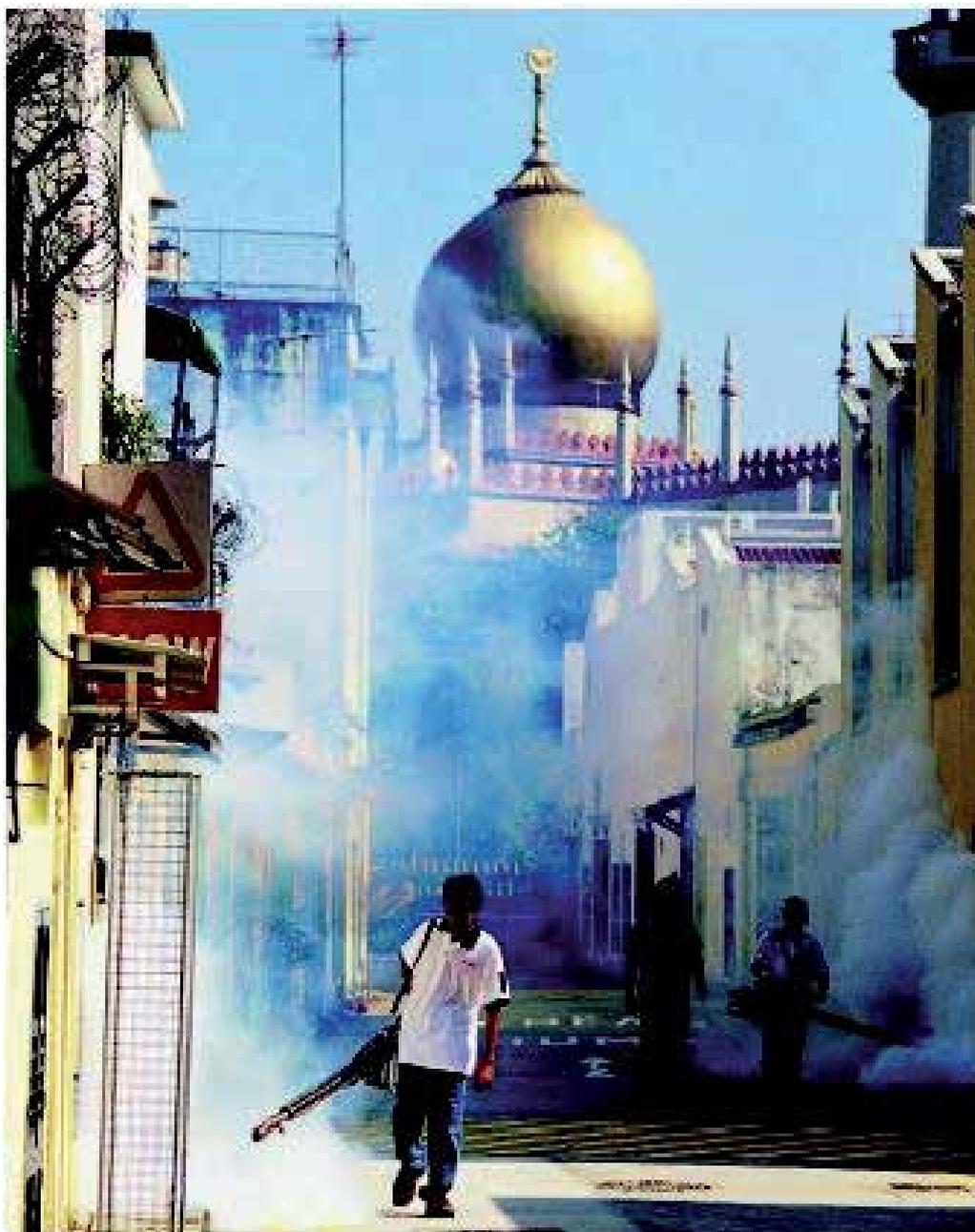
##### **5.1 Plants**

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 reduces crop yields(Rockets&Rusty,2007). Root nodule formation in these plants saves the world economy \$10 billion in synthetic nitrogen fertilizer every year.(Fox et al.,2007).

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, (Wells, 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,(Cornell University,2007) 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%..(Rockets & Rusty,2007),

##### **5.2 Animals**

Pesticides inflict extremely widespread damage to biota, and many countries have acted to discourage pesticide usage through their Biodiversity Action Plans.Animals may be poisoned by pesticide residues that remain on food after spraying, for example when wild animals enter sprayed fields or nearby areas shortly after spraying. (Palmer et al.,2007).



Fumigators walking down a street in the Sultan Mosque area of Singapore and spraying a pesticide to rid the area of mosquitoes

Widespread application of pesticides can eliminate food sources that certain types of animals need, causing the animals to relocate, change their diet, or starve. Poisoning from pesticides can travel up the food chain; for example, birds can be harmed when they eat insects and worms that have consumed pesticides. Some pesticides can cause bioaccumulation, or build up to toxic levels in the bodies of organisms that consume them over time, a phenomenon that impacts species high on the food chain especially hard. (Cornell University, 2007).

Pesticides can affect animal reproduction directly, as evidenced by the deleterious effect of the persistent organochlorine insecticides on reproduction in raptors and other birds. Eggshell thinning due to the uptake of organochlorine insecticides that affect calcium (Ca) metabolism has been observed in predacious birds (Keith *et al.*, 1970; Newton *et al.*, 1986; Wiemeyer *et al.*, 1986; Opdam *et al.*, 1987). Fish-eating birds are more severely affected than terrestrial predatory birds, because the fish-eating birds acquire more pesticides via their food chain than the other predators (Pimentel, 1971; Littrell, 1986).

Pesticides can also affect reproduction in the invertebrates; for example, sublethal doses of DDT, dieldrin, and parathion increased egg production by the Colorado potato beetle by 50, 33 and 65 per cent, respectively, after two weeks (Abdallah, 1968). The herbicide 2,4,5-T was found to reduce the reproduction of soil-inhabiting Collembola (Eijsackers, 1978). Populations of invertebrates with high rates of increase can recover stable populations much more rapidly than those of bird and mammal populations (Pimentel and Edwards, 1982).

### 5.2.1 Human

Pesticides can enter the human body through inhalation of aerosols, dust and vapor that contain pesticides; through oral exposure by consuming food and water; and through dermal exposure by direct contact of pesticides with skin. (Department of Pesticide Regulation, 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.

There is increasing anxiety about the importance of small residues of pesticides, often suspected of being carcinogens or disrupting endocrine activities, in drinking water and food. In spite of stringent regulations by international and national regulatory agencies, reports of pesticide residues in human foods, both imported and home-produced, are numerous.

Over the last fifty years many human illnesses and deaths have occurred as a result of exposure to pesticides, with up to 20,000 deaths reported annually. Some of these are suicides, but most involve some form of accidental exposure to pesticides, particularly among farmers and spray operators in developing countries, who are careless in handling pesticides or wear insufficient protective clothing and equipment. Moreover, there have been major accidents involving pesticides that have led to the death or illness of many thousands. One instance occurred in Bhopal, India, where more than 5,000 deaths resulted from exposure to accidental emissions of methyl isocyanate from a pesticide factory.

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 & Eric, 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. (Department of Pesticide Regulation,2008). The chemicals can bioaccumulation 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. (Lorenz & Eric,2009)

### **5.2.2 Aquatic life**

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 became very low due to pesticide contamination

Fish and other aquatic biota may be harmed by pesticide-contaminated water. Pesticide surface runoff into rivers and streams can be highly lethal to aquatic life, sometimes killing all the fish in a particular stream.(Toughill,1999).

Application of herbicides to bodies of water can cause fish kills when the dead plants rot and use up the water's oxygen, suffocating the fish. Some herbicides, such as copper sulfite, that are applied to water to kill plants are toxic to fish and other water animals at concentrations similar to those used to kill the plants, Repeated exposure to sub lethal doses of some pesticides can cause physiological and behavioral changes in fish that reduce populations, such as abandonment of nests and broods, decreased immunity to disease, and increased failure to avoid predators,(Helfrich et al.,1996).

Application of herbicides to bodies of water can kill off plants on which fish depend for their habitat.(Helfrich et al.,1996).Pesticides can accumulate in bodies of water to levels that kill off zooplankton, the main source of food for young fish.(Pesticide Action Network North America,1999).Pesticides can kill off the insects on which some fish feed, causing the fish to travel farther in search of food and exposing them to greater risk from predators.The faster a given pesticide breaks down in the environment, the less threat it poses to aquatic life. Insecticides are more toxic to aquatic life than herbicides and fungicides. (Helfrich et al.,1996).

### **5.2.3 Birds**

Pesticides had created striking effects on birds, 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 will also 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.

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.

Bald eagles are common examples of nontarget organisms that are impacted by pesticide use. Rachel Carson's landmark book *Silent Spring* dealt with the loss of bird species due to bioaccumulation of pesticides in their tissues. There is evidence that birds are continuing to be harmed by pesticide use. In the farmland of Britain, populations of ten different species of birds have declined by 10 million breeding individuals between 1979 and 1999, a phenomenon thought to have resulted from loss of plant and invertebrate species on which the birds feed. Throughout Europe, 116 species of birds are now threatened. Reductions in bird populations have been found to be associated with times and areas in which pesticides are used. (Kerbs et al.,1999) In another example, some types of fungicides used in peanut farming are only slightly toxic to birds and mammals, but may kill off earthworms, which can in turn reduce populations of the birds and mammals that feed on them. (Palmer et al.,2007).

Some pesticides come in granular form, and birds and other wildlife may eat the granules, mistaking them for grains of food. A few granules of a pesticide is enough to kill a small bird. (Palmer et al.,2007).

The herbicide parquat, when sprayed onto bird eggs, causes growth abnormalities in embryos and reduces the number of chicks that hatch successfully, but most herbicides do not directly cause much harm to birds. Herbicides may endanger bird populations by reducing their habitat.(U.S. Environmental Protection Agency,2007).



A bird that died as a result of pesticide use. ( U.S. EPA. Reproduced by permission.)

## 6. Threatening reports on hazardous effects of pesticides

Endosulfan is a harmful insecticide , it causes several health hazards in human beings.endosulfan was aerial sprayed on cashew plantations in india especially in northern parts of kerala for more than 20 years.the terrain was unsuitable for aerial spraying considering the relatively high rainfall and its geological structure. unusual diseases and even deaths were observed in and around the region.

Endosulfan is a chlorinated hydrocarbon insecticide of the cyclodiene subgroup which act as a contact poison in wide variety of insects and mice is primarily used on food crops like tea,fruits,vegetable and grains. exposure to endosulfan will result from ingestion of contaminated food.it does not easily dissolve in water and transport is likely occur if it attached to soil particles in surface runoff.endosulfan residues have been found in numerous food products at very low concentration.

Endosulfan is rapidly degraded and eliminated in mammals with very little absorption in gastrointestinal tract. in these areas, where aerial spraying was done lot of children who have exposed are considered to be living martyr.

Studies consistently show that endosulfan is highly poisonous and easily causes death and severe acute and chronic toxicity to various organ systems including mental impairment, neurologic disturbances, immunotoxicity and reproductive toxicity and most of the newborn were physically handicapped and showing epilepsy.

Classified by the US Environmental Protection Agency as highly hazardous, endosulfan was at the centre of controversy in the Philippines in the 1990's. (Nishand .P,2006)

## 7. Alternative methods for eliminating pesticides

### 7.1 Diversified planting

A common practice among home gardeners is to plant a single crop in a straight row. This encourages pest infestation because it facilitates easy travel of an insect or disease from one host plant to another. By intermingling different types of plants and by not planting in straight rows, an insect is forced to search for a new host plant thus exposing itself to predators. Also, this approach corresponds well with companion planting. (Ann R. Waters, 2011)

### 7.2 Low toxicity pesticides

Formulated, biodegradable pest-control substances are commercially available. Although these products are pesticides, they have low toxicity to mammals and do not last long in the environment. The local County Extension Service can provide information on these and other pesticide products. (Ann R. Waters, 2011)

Many alternatives are available to reduce the effects pesticides have on the environment. There are a variety of alternative pesticides such as manually removing weeds and pests from plants, applying heat, covering weeds with plastic, and placing traps and lures to catch or move pests. Pests can be prevented by removing pest breeding sites, maintaining healthy soils which breed healthy plants that are resistant to pests, planting native species that are naturally more resistant to native pests, and use biocontrol agents such as birds and other pest-eating organisms. (National Audubon Society, 2003).





Photos of some victims of endosulphan tragedy

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# Changing to Minimal Reliance on Pesticides

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## 1. Introduction

The most commonly accepted method of controlling pests in Australian crops and throughout the world is the use of pesticides (eg. Pimental 1997, Pimental *et al.* 1997, Page and Horne 2007). There is a reliance on pesticides and the majority of farmers look to chemical companies to continue to provide the familiar means with which they can deal with the complete range of pests that are of concern. In 1997 Pimental *et al.* estimated that US farmers used 400 million kg of pesticides annually and worldwide 25 million tonnes of pesticides were applied. An example of the dependence on pesticides can be seen by the close association between the primary producer organizations and pesticide companies and resellers. For example, in Australia, there is an acknowledged strong association between the vegetable industry, as represented by AusVeg (see <http://ausveg.com.au>) where chemical industry sponsorship of the organization is seen as desirable by both groups. This is alongside an acknowledged effort to implement IPM in vegetables crops in Australia by both AusVeg and several chemical companies. Similarly the Grains Research and Development Corporation (GRDC) in Australia would like to be seen as promoting IPM but the reality is that pesticide applications are the main controls used.

However, there are many examples worldwide of a desire to reduce reliance on pesticides and one particular example of this is by the adoption of integrated pest management (IPM) strategies. It is an approach that is widely seen as desirable and is promoted by many agencies worldwide, including the United Nations, the World Bank and the Food and Agriculture Organisation (FAO) (Maredia 2003, Olsen *et al.* 2003) and also government agencies in Australia (Williams and Il'ichev 2003). Despite this support the change to using this approach is often slow, even though there are examples of success and proven methods of implementing IPM strategies. So, can we successfully reduce our reliance on pesticides if farmers and their advisors do not adopt the strategies that scientists have developed? Also, have scientists developed strategies that are too narrow in design, by dealing with only a narrow pest spectrum?

In this paper we look at what is required to implement change, specifically regarding pest management practices so that there is less reliance on applications of pesticides. The reasons for wanting to reduce reliance on pesticides include insecticide resistance, destruction of natural enemies and other non-target species, residues in produce, environmental concerns and effects on human health (Perkins and Patterson 1997).

### 1.1 IPM

Integrated Pest Management (IPM) is the use of all available control measures used in a compatible way, but in our opinion should be based on biological and cultural controls with

pesticides used only as support tools. The use of these support tools is decided on the results of monitoring of both pest and beneficial species. The basis of this paper is that adoption of IPM is the best method to reduce reliance on pesticides (Perkins and Patterson 1997). This is supported by many publications over many years, (see Table 1 and Horne et al. 2008). So the change to minimal reliance on pesticides depends largely on the adoption of IPM strategies which are based on biological and cultural controls.

Advantages of IPM	Disadvantages of IPM
Reduced dependence on pesticides	More complex than control by pesticide alone and requires a shift in understanding
Increased safety to farm workers, spray operators and the community	Requires a greater understanding of the interactions between pests and beneficials
A slower development of resistance to pesticides	Requires a greater understanding of the effects of chemicals
Reduced contamination of food and the environment	Increased time and resources
Improved crop biodiversity	Level of damage to the crop may initially increase during transition to an IPM programme, in some horticultural crops

Table 1. Advantages and disadvantages of adopting IPM

There are also very many publications that report the fact that levels of adoption of IPM are very often low and rates of adoption are slow (Bajwa and Kogan 2003, Herbert 1995, McNamara *et al.* 1991, Olsen *et al.* 2003, Sivapragasam 2001, Wearing 1988). Even in horticultural crops where the theory of IPM is well developed, achieving widespread adoption on farms remains a challenge (Page and Horne 2007; Boucher and Durgu 2004). There is a very simple reason for the low rates of adoption of IPM – and it is that the current methods (pesticide applications) are still effective, are legal, and are familiar. There is only a general desire to do something different when this set of reasons is not true. That is, there is a desire to find a new method of pest control when either the pesticides fail (insecticide resistance), or the pesticides are no longer available (withdrawn or banned). Then the pressure to try something unfamiliar becomes more compelling.

The way in which information about IPM is presented, and indeed what is presented, is something that is contentious amongst IPM workers around the world, and we of course have our own opinion. There is a large amount of information available, including websites, CD-ROMs, videos, posters and information sheets that are used to provide farmers with the necessary information. Direct contact with farmers is also an option but even this is not an assured method of adoption.

We have a different view on the presentation of IPM information to most (but certainly not all). We believe that there is a scientific basis to the method of presenting IPM that we use, and also that there is a great distinction between information that increases *awareness* of IPM and that which increases *adoption* of IPM.

## 1.2 What information is presented? Entomologists presenting advice on individual species, farmers dealing with whole crop

An extremely important aspect of advice given about pest management, and in particular pesticide applications, is how wide the consideration is given to the impact of any application. That is, if any action, including pesticide application, was made with the aim of controlling any particular pest, then what consideration is made as to the impact on the control of other pest species? It is not only the degree of control of the target pest achieved by a pesticide application that may have been applied, but also the effect of that pesticide on the biological control agents of other pests.

For example, a key target pest in brassica crops is *Plutella xylostella* (diamondback moth). It is certainly not the only pest in brassica crops but this has been sometimes forgotten by entomologists focusing on this pest. (Given the importance of this species of pest it is understandable that there would be an over-emphasis on this pest alone). The focus was created because of the insecticide resistance that developed in *P. xylostella* in strategies of pest control that were based on insecticide applications alone. An outcome of the singular focus on diamondback moth has been the presentation to brassica growers of strategies labelled in terms such as an: "IPM Strategy for Diamondback Moth". This obviously does not consider the full range of pests, or actions that brassica growers may need to make to deal with a range of pests including other caterpillars and aphids, or the beneficial species that contribute to the control of all of these pests. What is required is an IPM Strategy for Brassica Crops.

The development of strategies based on pesticides to control *P. xylostella* include insecticide resistant management strategies (IRM) and this has been the focus of some researchers in Australia (eg: [[http://www.sardi.sa.gov.au/pestsdiseases/horticulture/horticultural\\_pests/diamondback\\_moth/insecticide\\_resistance\\_management](http://www.sardi.sa.gov.au/pestsdiseases/horticulture/horticultural_pests/diamondback_moth/insecticide_resistance_management)]). Whether or not these strategies work on control of *P. xylostella* is not the only consideration for a brassica grower. To have a brassica such as broccoli or cauliflower accepted by the market there must be adequate control of a range of pests, not just *P. xylostella*. A broccoli or Brussels sprouts crop free of *P. xylostella* but infested with aphids is still not acceptable in the market. What this means of course is that a brassica grower needs to have a strategy to control all pests, not just some. Research entomologists can mistake the target pest on which they are working as the only pest to be considered.

## 1.3 Disruption of biological control

The IRM strategies that have been developed in various places are examples of how advice on how to control one pest (for example the major pest) can disrupt control of other pests. This is because pesticides that may kill the major pest will also kill the beneficial species that control other pests. This is the most serious problem with pesticide applications in most cases. However, there are other effects that can and do influence the control of pests other than the major pest.

One example is when some of the newer pesticides such as emamectin ("Proclaim") or indoxacarb ("Avatar") are sprayed for the control of caterpillar pests. These are much more selective than organophosphate and synthetic pyrethroid insecticides and can be used within IPM strategies but their impact on the beneficial species that control other pests can still be significant. For example, emamectin will kill wasp parasitoids and also predatory bugs such as *Nabis conformis* and indoxacarb will kill a range of beneficial species but is not particularly residual ([www.ipmtechnologies.com.au](http://www.ipmtechnologies.com.au)). So the application of these pesticides

may achieve the immediate aim of killing target caterpillar pests, but in the process there will be a loss of the biological control agents that are required for on-going and sustainable control. In addition, the control of other pests such as aphids or mites could easily be disrupted because of the loss of a different set of beneficial species. This does not mean these products must never be used but the impact of using them needs to be understood before they are applied. In particular, what species of beneficials are present and is there the possibility of re-invasion of these species?

A common desire amongst many farmers and agronomists, and even some entomologists, is to produce a list rating pesticides as “safe” or “not safe” in absolute terms, not taking into consideration the effect on different species of beneficials. This simplistic approach can be a major setback for IPM adoption as growers tend to simply shift to using less-broad-spectrum insecticides and think they are using IPM. What is required is detailed information on the effects (acute and sub-lethal) of each pesticide on each particular species.

#### **1.4 Awareness versus adoption**

There is a tendency amongst some entomologists to concentrate more on awareness of IPM than adoption of IPM. While there needs to be awareness before there can be adoption, the adoption step requires a different set of skills and is something that some organizations promoting IPM would rather avoid. Awareness of IPM may involve talks, workshops, videos, leaflets, manuals, DVD's etc and the production of these tools is a more attractive option to many public organizations as they would seem to be able to reach a large number of people and involve little risk. However, adoption, or implementation, of those practices requires a further step to be taken by the farmer even when there may be awareness of what is possible with IPM. There are different steps needed to implement the change to a totally different method of pest management to that which has been used by the farmer and is familiar.

#### **1.5 What is required to implement change?**

In many cases the catalyst for making the change to something unknown (ie IPM) occurs when there is a crisis in pest control, because either the pesticides that have been relied on stop working (insecticide resistance) or the pesticide is no longer available (eg registration withdrawn or the product is withdrawn from sale). While there are other factors that influence the decisions on whether or not to use a pesticide-based strategy (referred to in chapter 2) these two are the most common reasons for a sudden increase in adoption of IPM. Obviously if the methods that are being used no longer work then something different must be done to control pests. In such circumstances farmers are more naturally responsive to embrace a different approach, and examples of different reasons for making changes are given later in this chapter.

It is entirely possible to have farmers understand and implement IPM without any particular crisis. The key factors to success are well documented (eg Herbert 1995) and involves the collaborative and participatory approach to working with individual or small groups of farmers and providing expert, site-specific advice when required.

#### **1.6 Familiar versus different**

There is a common experience from a farmer's (or advisor's) point of view when deciding between whether to use a pesticide-based strategy or an IPM strategy. When the starting

point for any individual farmer or farm is that a pesticide-based strategy is known, legal and it works then the decision to adopt something unfamiliar and unproven (on the farmer's own crop) is extremely difficult and is seen as unnecessary and risky. One way to reduce the perceived risk is to have regular monitoring of both pests and beneficial species conducted, in order to check that the desired biological control component of IPM is working as expected.

### **1.7 The role of monitoring**

Monitoring does not control any pests! The reason for saying this is that very often, in our experience, farmers expect that because their crop is being monitored by professional crop monitors then there will automatically be a decrease in pest problems. The only reason for conducting monitoring is to allow timely and informed decisions to be made. Monitoring will allow an assessment to be made about the risk of economic damage by any particular pest. This will include an assessment as to the degree of likely biological control.

### **1.8 Information on pesticides**

It may seem strange at first, but the most important information required to help implement IPM and especially the biological control component, is knowledge about pesticides. This includes information about the impact of pesticides on beneficial species but also involves information about the correct selection of pesticides (including fungicides) for any given problem and in particular to ensure that the pesticide application achieves the result that is desired. Again, it may seem surprising but the practical limitations such as weather and temperature, water volume, adjuvants that should be used with different products are not always (or often) known by farmer or their advisors (including chemical re-sellers).

In an IPM strategy insecticides are used as a support tool when biological and cultural controls are assessed by monitoring as not being sufficient to give adequate levels of control. It is therefore absolutely essential that any application of pesticide is well-timed and correctly applied or else it will not achieve the intended aim of supporting the other control elements. Too often this aspect of IPM is not implemented well. Although research may be done by entomologists (including us) to evaluate the impact of pesticides on beneficial species, it needs to be applied. That is, farmers need to be able to use that information, not just hear about it.

This brings into question the role of entomologists as researchers as opposed to advisors. Worldwide there has been a huge investment in IPM research, and it is the same in Australia. The problem for investors in this research is that there has been a poor level of adoption of IPM as a result of research and the question is why? The answer, we believe, is that researchers do not usually sell or market IPM the way a chemical company or reseller can sell a chemical. What is required is for IPM (and the research that has developed IPM strategies) to be brought into the commercial world rather than stay in the (usually) public funded research environment.

Given that there are only three control options for pest management (biological, cultural or chemical) then it is essential that all three methods work in collaboration, not in opposition. The biological controls in most cases are naturally occurring and cannot be manipulated (apart from not being killed) and cultural controls are usually underestimated. This means that for the farmer, the association between pesticide application and pest control is still paramount. The advisor or crop monitor may know more, but the farmer will be tempted to measure pest control with sprays applied. This means that the decision-making that leads

to a pesticide application needs to be absolutely site-specific and take into account the many factors that may be influencing any decision (eg. pest pressure, levels of beneficials, types of beneficials, the observed trend in success of biocontrol, time until harvest, age of crop, time of year, stages of pests, stages of beneficials, intended market etc). These many factors are too many for a general advisory note to include and so site-specific advice is what is needed.

### **1.9 Collaboration (participatory trials)**

We believe that the best way to implement a change to the adoption of IPM is by farmers and advisors working together to obtain site-specific solutions. However, if this is not possible because a lack of such advisors then there are still great changes and advances that farmers can make. With the current technology of the internet, e-mail and digital photos available (and it is certain to improve still more) it is possible to find information and advice relating to the above points. There may not be absolute information regarding particular pest/crop/pesticide/location, but there is usually some information regarding the relative toxicity of pesticides to a range of species (eg. side-effects guides on web-sites such as [www.koppert.com](http://www.koppert.com)). Using the available information a farmer can begin to make changes to what has been the major decision involved in pest management – namely, “what should be sprayed”. An option in this decision that involves relying on biological and cultural controls is the option to spray nothing. Information on what pesticides do to a range of beneficial species and not just the pests is the most important tool that most will need to begin to make changes to pest management.

Once the change in pesticide application has been trialled then the farmer may also be more interested in looking at what cultural controls can be utilized along with the different spray regime. Again, information relating to cultural controls can be found or potential (historical/ colloquial) methods trialled on-farm without risking the entire crop.

## **2. Examples of implementing change: Case studies**

### **2.1 Colin Hurst, Arable cropping farmer, Canterbury, NZ**

Colin Hurst runs a 700 ha family farm near Waimate in the southern Canterbury region of New Zealand. The main crops grown are wheat, grass seed, brassica seed and he also grazes sheep. Cropping accounts for 60% of the farm area each year.

Prior to 2006 Colin was operating a conventional system using synthetic pyrethroid sprays (“Karate”) as the main defence against aphids and the barley yellow dwarf virus that they can vector. However, in 2006 Colin had an introduction to the concept of IPM via a project initiated by the Foundation for Arable Research (FAR). The initial discussion that aroused Colin’s interest was that the sprays applied for aphid control could be causing disruption of the control of other pests, and in particular slugs. In wet years slugs were a significant problem and were expensive to deal with (using baits).

The idea of an IPM approach sounded interesting, especially as it offered an alternative to over-reliance on a single insecticide and the development of resistance, but it was also very different to the mainstream approach being used at the time in New Zealand. The idea of seeing pests increase in number but not applying an insecticide spray was one of the biggest changes to be made and one of the biggest concerns in the early stages. So Colin trialled the approach on half a paddock. This allowed him to see the results of each method and compare the results not only in terms of cost of pesticides (including baits) but also in terms of yield.

The first year's trial was very encouraging, with no pest issues, reduced pesticide use and an increased awareness of beneficial species. Colin had back-up with insect monitoring and decision-making during this time from Plant and Food Research (a government agency in New Zealand) and IPM Technologies P/L visiting from Australia as a part of the project. The realisation that there were many beneficial species present in his crops was something that Colin had not utilised before and he decided that he should investigate further.

Therefore, in the next 2 years of the 3 year project, Colin progressively adopted an IPM approach as he felt more comfortable with the new IPM strategy and decision-making based on monitoring. The monitoring that he uses consists of direct searching for a range of predators and parasitoids, sticky traps to assess what is flying at any time, aphid flight information provided by the Foundation for Arable Research (with Plant and Food Research). Using IPM has meant a much greater use of monitoring than in the past and so any action is based largely on observations on the farm rather than pre-determined sprays or district - wide information. Colin now uses seed dressings of synthetic insecticides rather than sprays as a part of the strategy to use minimal insecticides and instead relies on cultural and biological controls.

The change has been dramatic and in 2010 (4 years after implementing change) the only slug problems requiring treatment was on a border with a neighbours field where there was invasion from outside the farm. The only control measure required in this case was a border application of slug bait. Colin now believes that carabid beetles provide a significant level of control of slugs and is keen not to disrupt this control with sprays targeting aphids.

After 3 years of trials Colin now implements an IPM strategy over the entire farm, and only uses selective insecticides to support the biological and cultural controls as necessary. Although initially daunting, the change in practice has proved worthwhile.

## **2.2 IPM at Henderson hydroponics, Tasmania**

*(This is the basis of an article published by Good Fruit and Vegetables magazine in 2009 that describes the change to using IPM with the assistance of the authors of this chapter).*

Rob Henderson (and his family) grow hydroponic capsicums near Devonport in Tasmania and experienced major problems during the 2007 - 8 season with tomato spotted wilt virus (TSWV). This virus affects the plant and the fruit, causing affected fruit to be unsalable. The only treatment of infected plants is their removal and disposal which resulted in approximately 75 % of TSWV susceptible cultivars being removed prior to the end of the season, which resulted in considerable financial pain. The problem virus is spread by several species of thrips, including western flower thrips (WFT) which had not previously been present at Henderson Hydroponics. WFT are resistant to many insecticides and that was the problem in this case. The thrips were surviving the insecticides that were used and so were literally out of control. Rob needed to do something different to manage these pests and insecticides did not look like the answer.

In 2008 before his latest crop was planted he met with Dr Paul Horne and Jessica Page of IPM Technologies to discuss implementing an IPM approach. Paul and Jessica were in Tasmania to help develop IPM in a range of vegetable crops and were introduced to Rob by an agronomist (Peta Davies from Roberts Ltd) who saw that this may be the answer to their problem.

A range of predators were introduced throughout the season to control fungus gnats, WFT, aphids and two-spotted mite. It also meant that the broad-spectrum insecticides that he

had used in the past could no longer be used, and extreme care had to be taken to ensure that these beneficial species were not disrupted by attempts to control other pests .

Rob admits “We were sceptical at first about IPM, but now we are converts ”. He said that “The western flower thrips were present in the latest crop but the predators, in time, controlled them and total damage was reduced to below 2% infection, down from 75% the previous year”. Two spotted mite were becoming an increasing problem in previous seasons. However excellent control was achieved with the release of *Persimilis*, which displayed a ravenous appetite for two spotted mite.

There were some very nervous moments early on in the season when WFT were obviously present and before the predatory mites (known as *cucumeris*) had taken control of the pests. However, the results later in the season speak for themselves and the next seasons expanded crop will again be grown using IPM, but with less nervousness now that Rob knows what to expect.

Rob will be trialling a new thrips predator called Orius that is a new possibility for WFT control. It is being produced in WA.

The project that allowed this to happen is funded by Horticulture Australia and the AusVeg levy.

Rob estimates in his first year of IPM he would have spent approximately treble that which he would have normally spent on insecticide. However he is hopeful that next season, having had a season of IPM experience behind him, this cost may reduce. However as a qualified agricultural economist Rob considers the expenditure on IPM in his greenhouses to be an extremely sound and profitable investment both financially and environmentally.

Henderson Hydroponics staff enjoy working in an environment free of insecticides and have noted the dramatic increase in natural predators, particularly frogs and lady birds seen this season in the greenhouses.

Henderson Hydroponics customers have also been pleased to purchase quality fruit grown without the use of insecticides.

### 2.3 What has Henderson hydroponics learnt from one season of IPM?

- IPM does work.
- Constant crop monitoring is very important.
- Don't be afraid of seeing small numbers of pests, leave the insecticide locked in the chemical store and only think about using it as a last resort.
- Predators take time to multiply. If pest numbers are increasing, purchase and release more predators rather than waiting for predators to breed up.
- Good advice is readily available. Make use of it. Henderson Hydroponics are extremely grateful for the advice provided by Paul and Jessica during their visits and by them being available to answer questions on the telephone and email at short notice.

### 3. Conclusion

Reduction of the current reliance on pesticides for the control of pests in agriculture will be best achieved by the adoption of IPM strategies. The poor results (overall) with regard to adoption of IPM is explained in large part by the poor use of known strategies (participatory research) and the fact that IPM is still largely associated with the publicly funded (Government) organisations rather than with commercial aims. It is essential that IPM adoption shifts to the commercial sector to compete with pesticide focussed strategies.

#### 4. Acknowledgements

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# Increasing IPM Knowledge Through FFS in Benin

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## 1. Introduction

Over the next two generations 4 billion more people will live in cities, increasing the proportion of the urban population from 50 to 80 per cent of the total world population (NRC, 1999). Thus a sustainable development needs to focus on meeting the needs of an increasing human population, reducing poverty and hunger while at the same time sustaining the life support systems of the planet (NRC, 1999). While the Green Revolution technologies enabled extensive monocultures and higher yields through improved seeds, chemical fertilizers and synthetic pesticides, biodiversity in and around the agro-ecosystems have been reduced, causing the loss of natural pest and disease control (Gallagher et al., 2005). This has increased the need for synthetic pesticides in the agricultural sector to the current global use of 2.56 billion  $\text{kg yr}^{-1}$  (Pretty, 2008), with associated negative effects for humans and the ecosystem becoming evident. While the externalities of pesticides in rice systems in China cost \$1.4 billion per year through adverse effects on biodiversity and people's health (Norse et al., 2001), the annual mortality rate due to pesticides in the remote Ecuadorian highlands is among the world's highest, 21 per 100 000 people (Sherwood et al., 2005). On the other hand, in the Philippines, agricultural systems that do not use any synthetic pesticides experience higher net social benefits due to reduced illnesses among farmers and their families, and associated lower medical costs (Rola & Pingali, 1993, Pingali & Roger, 1995). According to FAO and ILO estimates, 2 to 5 million agricultural workers yearly experience severe pesticide poisoning and related illnesses of which 40 000 are lethal (FAO & ILO, 2009). However, pesticide poisoning incidents are often underreported, as indicated by a study among farmers in Senegal, Mali and Benin, where over 80% of the respondents faced adverse effects after spraying pesticides, to the extent of blurred vision, unconsciousness or severe dizziness, but only 2% sought medical attention for these symptoms (Thiam & Touni, 2009). Thus recent studies, where 4% to 9% of the surveyed farmers reported poisoning incidents the last year, estimate a yearly 25 - 45 million poisoning cases (Kishi, 2005).

Africa only accounts for 4% of pesticides used globally, an estimated 75-100 metric tons of pesticide active ingredient (compared to 350,000 tons in Europe), and average pesticide use per hectare cultivated land in Africa (1.23kg/ha) is very low compared to Latin America and Asia (7.17kg/ha and 3.12kg/ha respectively) (Thiam & Touni, 2009). Still, the risks and impacts associated with synthetic pesticides are not necessarily lower in Africa as many of the pesticides used in the continent are adulterated, poor quality and unlabelled and application and handling practices are often highly unsafe (Thiam & Touni, 2009, Lund et

al., 2010). In a study among farm families in Senegal and Benin, the number of pesticide poisoning incidents were 619 and 84 respectively of which 16% and 23% were fatal (Thiam & Touni, 2009). Only 2% of the farmers in the studied villages used a full set of protective gear (gloves, boots, and masks or glasses), showing how unavailability and impracticality of protective gear has an enormous impact on poisoning levels and farmers' health (ibid.). The farmers' families and communities also experienced negative effects of pesticides, like accidental poisonings, because pesticides are often stored freely available in kitchen or bedrooms, empty pesticide containers are reused for food and drinks and pesticides are purchased in non-original containers (ibid.). Governments tend to focus on the ones handling pesticides directly assuming that those face the highest poisoning risk, but data from Benin, Senegal, Ethiopia (ibid.), Ecuador (Cole et al., 2002) and India (Mancini et al., 2005) shows high frequencies of pesticide poisonings among women and children even though they are generally not applying pesticides.

The World Health Organization (WHO) has classified pesticides in Ia (extremely hazardous), Ib (highly hazardous), II (moderately hazardous), III (slightly hazardous) and unlikely hazardous. There is increasing pressure from and work done by government regulators and civil society to prohibit the use of the Class Ia and Ib pesticides (Thiam & Touni, 2009). The frequent incidents of acute and fatal poisonings from Class II pesticides in Benin and Senegal, illustrates the dangerous effects of even "moderately hazardous" pesticides in conditions of poverty and poor education, showing that also Class II and Class III compounds (e.g. malathion) should be considered for restrictions (ibid.). The Persistent Organic Pollutant Endosulfan (Class II), which has been widely used in West African cotton growing areas, was banned by governments in the region in 2008, as it had been associated with acute and fatal poisonings (ibid.). Pesticides cause long-term health problems such as birth defects and cancers (Lichtenberg, 1992) and several studies link pesticide exposure to respiratory problems, memory disorders (Arcury et al., 2003), dermatologic conditions (O'Malley, 1997), anxiety, depression (Beseler et al., 2008), and neurological disorders (Ascherio et al., 2006). WHO estimated that long-term exposure to pesticides may result in a yearly 735,000 people globally suffering specific chronic defects and 37,000 cases of cancer (WHO, 1990). Thus also health Ministries in six Central American countries have proposed a regional ban on the Class II pesticides endosulfan, paraquat and chlorpyrifos, in addition to pesticides in class 1a and 1b, based on results from an eight year poisoning surveillance program (Rosenthal, 2005).

Pesticide residues may interfere with the legume-rhizobium chemical signalling reducing nitrogen fixation and crop yields (Fox et al., 2007), and over 95% of applied herbicides and 98% of insecticides reach other destinations than their target, including non-target species, water, air, bottom sediments, and food (Miller & Spoolman, 2009). The use of synthetic pesticides among vegetable producers in urban and peri-urban areas of West-Africa has increased to the extent that certain insect pests have developed resistance to the pesticides (Atcha-Ahowé et al., 2009). Additional negative effects are increasing insecticide resistance in insect vectors due to the leakage of insecticides to mosquito breeding sites (Akogbeto et al., 2008), to the extent that insecticide resistance in *Anopheles* mosquitoes is threatening the success of malaria control programs (Djouaka et al., 2005), and pesticide resistance in target pests has made pest resurgence a common phenomenon in cotton, vegetables, rice and fruit crops production systems (Lim, 1992). Recent research also indicates that toxic compounds

may be dispersed to even remote areas via atmospheric deposition (Rosendahl et al., 2009). In West-Africa, agricultural land is being degraded by poor agricultural practices and the use of chemical products (Pimbert et al., 2010). Still, African farmers often use credit to buy inputs such as seeds, fertilizers and synthetic pesticides at high costs, making them dependent on good yields to break even and manage their debts (Williamson, 2003). In this situation, many may get caught in the pesticide treadmill where they do not dare to reduce the use of synthetic pesticides for fear of yield loss. While the externally funded West-African agricultural research system increasingly focus on the use of imported fertilizers and pesticides, the use of traditional seeds and organic manure is declining and small-scale producers have felt lack of citizen control over knowledge production (Pimbert et al., 2010). January 2006, the local government of Sikasso in Mali hosted the Citizen Space for Democratic Deliberation on GMOs and the Future of Farming in Mali where local farmers made policy recommendations based on expert evidence from various sources (ibid.). The farmers requested a re-orientation of public research from the current focus on input-intensive farming and GM seeds, towards ecological farming not requiring chemical inputs, improved local seeds and landraces, regeneration of local markets and food systems, supporting small-scale producers. They also suggested that farmers set the research objectives and called for more exchange between farmers and researchers as well as the development of new Integrated Pest Management (IPM) strategies and training in these strategies taking local knowledge into account (ibid.). Also the recent International Assessment of Agricultural Knowledge, Science and Technology for Development (IAASTD) panel supported by over 400 experts under the co-sponsorship of the FAO, GEF, UNDP, UNEP, UNESCO, the World Bank and WHO, called for new farmer-scientist partnerships, to improve understanding of agro-ecology, i.e. by IPM, and develop an integrated agro-ecosystem and human health approach to enhance food security and safety, stating that: "The way the world grows its food will have to change radically to better serve the poor and hungry if the world is to cope with growing population and climate change while avoiding social breakdown and environmental collapse" (McIntyre et al., 2009). A recent UNEP and UNCTAD report based on 24 African countries states a 100% yield increase with organic or near-organic practices, concluding that organic practices in Africa outperformed industrial, chemical-intensive conventional farming and in addition improved soil fertility, water retention and draught resistance, making it a promising approach for food security in the continent (UNEP & UNCTAD, 2008).

As the 'top-down' recommendations for pest control, have often failed to reduce pest damage, pesticide use or enable farmers to learn about IPM (Williamson, 1998), there is a need for new ways of learning (Orr, 1992, Bentley et al., 2003, Liebelin et al., 2004, Bawden, 2005, Chambers, 2005). One learning method focusing on the farmers' own development is the farmer field school (FFS), which is increasingly being used to promote IPM. Since IPM-FFS was introduced by the Global IPM Facility in West Africa (Ghana) in 1996, it has spread to over a dozen countries, from Senegal to South Africa (WB, 2004). IPM-FFS has been adopted in the national policies in Mali, Burkina Faso and Senegal, and IPM curricula, initially made for rice, have been developed for other crops including vegetables (ibid.). Community IPM, which has been used to increase the community involvement and adaptation of IPM in Asia for 15 years, is now being tested in Africa, including Burkina Faso, Ghana, Mali and Senegal in West Africa (ibid.). In 2003, the International Institute of

Tropical Agriculture (IITA) initiated the project 'Healthy Vegetables through Participatory Integrated Pest Management (IPM) in Peri-urban Gardens of Benin' (hereafter referred to as the project) to enhance farmers' efforts to produce quality vegetables through informed decisions on the choice and use of IPM options (James et al., 2006). The project was unique as it was the first time that a vegetable FFS was conducted by IITA in West Africa. This chapter will discuss the effects of IPM-FFS in pest management, including the IITA vegetable IPM-FFS as a case study.

## 2. The use of IPM and FFS in pest management

### 2.1 IPM and FFS

Mature ecosystems' state of dynamic equilibrium buffers against large shocks and stress, but modern agro-ecosystems have weak resilience (ability to resist stress and shocks) (Holling et al., 1998, Folke, 2006, Shennan, 2008). Thus developing sustainability requires a focus on structures and functions to improve the resilience, such as increasing the biodiversity to recreate natural pest and disease control, rather than seeking to eliminate those populations (ibid). In ecosystems, multi-trophic interactions are vital (Shennan, 2008). For example foliar herbivory in grasslands impact the functions of soil food webs (Wardle, 2006), which, together with changed nutrient dynamics, in turn affect the plant attractiveness to herbivores (Awmack & Leather, 2002, Beanland et al., 2003). Also due to the crops' systemic defense mechanisms above-ground attack may trigger responses to below-ground attack and vice versa (Bruce & Pickett, 2007). These complex crop-weed-disease-pest interactions imply that farm practices such as crop rotation, tillage, pesticides and fertilizers affect disease incidence, weed and pest populations (Bruce & Pickett, 2007), while practices like utilizing nitrogen fixing legumes, natural enemies for pest management and applying zero-tillage may enhance the sustainability (Pretty, 2008). As the importance of the complex interrelationships between the crop, weed, disease and pests is increasingly documented, the reductionist view of applying a synthetic pesticide to fix a specific pest problem is being questioned (NRC, 2010). Thus the pressure for pesticide reductions has influenced research to shift its focus towards non-chemical alternatives (ibid.). Increased emphasis is being paid to the approach Integrated Pest Management (IPM), which appreciates the complexity of the agro-ecosystem and "utilizes all suitable techniques in the socioeconomic, environment and population dynamics context of farming systems in a compatible manner to maintain pest population levels below those causing economic injury" (Dent, 1995: 1). IPM has proved able to reduce or eliminate the use of synthetic pesticides while improving the natural capital in and around agro-ecosystems (Lewis et al., 1997). However, the understanding of IPM varies, so while FAO recently changed its definition of IPM towards greater emphasis on ecologically based management, with pesticides as a last option (W. Settle, presentation to the committee on January 14, 2009 in NRC, 2010), other actors continue to use a narrower definition focusing on improved pesticide use (Shennan et al., 2001).

In pest management, proper soil maintenance to support the microbial, fungal, and nematode community suppressing pathogenic fungi and nematodes, inducing crop resistance responses, and reducing viable weed seed populations is important. Biological control has proven successful for arthropod pest management (NRC, 2010), like the introduction of the parasitoid *Epidinocarsis lopezi* controlling the mealy bug *Phenacoccus manihoti* attacking cassava in Africa (Neuenschwander et al., 2003). Bio-pesticides require that users understand they are working with biological processes or living organisms (Waage, 1996). Thus poor understanding of the

function of microbial agents, influenced by the marketing of bio-pesticides as biological versions of conventional pesticides, has reduced the usefulness of many microbial agents (*ibid*). However, to convince farmers about the value of bio-pesticides and make them choose it over chemical products, the farmers need to be able to assess the impact of the control agent (Williamson, 1997). Conservation biological control enhances indigenous populations of natural enemies such as insects, spiders, and other arthropods by providing habitat in the field (Shennan, 2008), or by planting hedgerows (Letourneau, 1998, Letourneau & Bothwell, 2008, Nicholls et al., 2001). Also "neutral" arthropods, like plankton feeders and detritivores, are important in controlling the pests as they stabilize the natural enemy populations by providing alternative food sources for the latter (Settle et al., 1996). Thus structurally complex landscapes lead to increased parasitism levels and decreased crop damage (Thies & Tschardt, 1999, Pullaro et al., 2006), as concluded in a review landscape diversity effects on biological control (Tschardt et al., 2008): "Complex landscapes characterized by highly connected crop-noncrop mosaics may be best for long-term conservation biological control and sustainable crop production".

IPM requires that the farmers, through observation and experimentation, learn about their agro-ecosystem so as to develop site specific technologies and practices (Pretty, 2008). The Farmer Field School (FFS) learning method, which focuses on the farmer as the key decision-maker in pest management and on the facilitation of a discovery-learning process using non-formal education methods (Williamson, 1998), has proven successful in situations of severe pest infestations and excessive synthetic pesticide use (see discussion below). FFS encompasses the following four principles: production of a healthy crop, conservation of natural enemies, performance of regular field observations and belief on the expertise of farmers in their own fields (Pontius et al., 2000). FFS is based on the idea that 'Learning is the process whereby knowledge is created through the transformation of experience' (Kolb, 1984: 38). Experiential learning is a process whereby we, based on the experience of a phenomenon, reflect and allocate meaning to that experience and develop knowledge from it. By experiencing the interactions of the agro-ecosystems and developing their analytical skills, farmers are empowered to realize which factors are within their own control (Fleischer et al., 1999).

According to Long (2001), knowledge is a cognitive and social construction constantly made by the experiences and discontinuities that emerge in the intersection between different actors' 'life worlds', defined as a person's life (or lived) experience, background and values which influence how the person sees the world (Schutz, 1962). Knowledge is a product of dialogue and negotiation, and includes transformation rather than transfer of meaning (Long, 2001). Although farmers may classify observable and culturally important insects better than many entomologists, they may not be aware of the parasitoids and insect pathogens in the agro-ecosystem. This was the case for Honduran smallholders, who distinguished a range of bees and wasps by their flight patterns, but were unaware of the existence of parasitic wasps and the carnivorous nature of wasp larvae (Bentley, 1992). Thus, for the learning and development of knowledge about pest management and the agro-ecosystem complexity, it is important to value both farmers' and scientists' knowledge and experiences. Participatory research approaches combining farmer knowledge and experience with research information could increase the ability to predict when synergies or negative interactions are likely to occur in the field and adjust management accordingly (NRC, 2010). As active farmer participation and public education are often a prerequisite for successful biological control (Williamson, 1998), the lack thereof may reduce the impact of introduced control agents, as was the case during the introduction of the parasitoid

*Diadegma semiclausum* Hellen (Hym., Ichneumonidae) in Southeast Asia. The parasitoid largely failed to control diamondback moth (*Plutella xylostella* L.) in brassicas in areas where farmers sprayed heavily with broad spectrum insecticides eliminating both native and introduced natural enemies (Waage, 1996, Gyawali, 1997).

IPM-FFS has been used for decades in Asia, where positive social changes (Pontius et al., 2000), have been documented along with reduced pesticide use, increased yield (Pretty & Waibel, 2005) and associated positive human and environmental health effects (e.g. Rola et al., 2001, Erbaugh et al., 2002, Godtland et al., 2004, Praneetvatakul & Waibel, 2006, WB, 2006) such as reduced pest resurgence (Matteson et al., 1994). FFS may provide an arena for shared learning between farmers, scientists and decision makers, with the emphasis on the farmers' experiential learning process, as in the Philippines, Pakistan and Honduras. In a vegetable IPM-FFS in the Philippines, farmers participated in releases of the diamondback moth parasitoid *Diadegma* sp. in their cabbage terraces and built wooden emergence boxes for the parasitized cocoons provided by the local university (ADB, 1996). From their observation of parasitized diamondback moth larvae and exercises demonstrating the effects of commonly used insecticides on the parasitoids, the farmers reduced their pesticide application by 80% and started asking the visiting agrochemical salesmen whether the products they sold were "Diadegma-friendly". Even the mayor of Atok town in the Cordillera region in the Philippines, got convinced about biological control to the extent that he banned all advertising of synthetic insecticides in his municipality (Cimatu, 1997). In Pakistan, FFS was undertaken to tackle the resurgence of the whitefly *Bemisia tabaci* (Gennadius Hom., Aleyrodidae), vectoring cotton leaf curl virus, and the increasing insecticide use in cotton (Poswal & Williamson, 1998). Whitefly may be kept in control by natural enemies, thus the whitefly outbreaks in Pakistan seemed to be a result of the elimination of the key natural enemies in cotton fields due to early and increased insecticide applications. The FFS participants learned about natural enemies and crop compensation by observing whitefly parasitization by *Encarsia* and *Eretmocerus* spp. (Hym., Aphelinidae), predation of jassids by mites, ants and spiders, and of whitefly by anthocorid and reduviid bugs, staphylinid beetles and spiders. Due to increased IPM knowledge the participants did not apply any insecticides on the IPM decision making plots the first 8-10 weeks after planting, thus allowing natural enemy populations to build up, and thereby reducing the total number of pesticide applications while yields increased. Having experimented with whitefly resurgence caused by the application of organophosphate, one FFS group demonstrated the impact of unnecessary pesticide applications to the Department of Agriculture officials, neighbouring farmers and local agrochemical salesmen.

In Honduras, the farmers learning about natural enemies and insect reproduction in the Natural Pest Control Course run by Zamorano (the Pan-American School of Agriculture), were able to enhance predation of pests in maize, potatoes and vegetables (Bentley et al., 1994, Rodríguez, 1993). As the farmers had learned the underlying principles of the agroecosystem, not merely specific techniques, they could also apply what they had learned to new situations (ibid.). These techniques adapted or invented by the farmers were tailored to their pest problems and resources in a way that standard 'recipes' could never be (ibid.). These results indicate why agricultural systems with high levels of social and human assets are more able to adapt to change and innovate in the face of uncertainty (Uphoff, 1998, Chambers et al., 1989, Bunch & Lopez, 1999, Olsson & Folke, 2001, Pretty & Ward, 2001). However, collective adoption of IPM techniques is vital, because the effect of IPM will be reduced if neighbouring farmers continue relying on chemicals for pest control killing beneficial parasites and predators, and exposing IPM farmers and local ecosystems to chemical spill overs (WB, 2006).

### 3. Effects of a vegetable IPM-FFS in Cotonou, Benin on pest management

In this section, the vegetable IPM-FFS by IITA-Cotonou will be discussed with emphasis on the extent to which the IPM-FFS training influenced the participants regarding their knowledge and use of IPM options including pesticides, their awareness of health hazards of synthetic pesticides and corresponding handling practices. Also the knowledge created through the interactions between the vegetable producers' and the scientists' 'life worlds' will be explored. The IPM-FFS project conducted two FFS sessions for farmers selected to participate in the Trainer Of Trainers (ToT) in 2003/04, covering the crops *Solanum macrocarpon* L. (Solanaceae) (a variety of the African eggplant, locally known as gboma), *Daucus carota* L. sativus Hayek (Apiaceae) (carrot), *Lactuca sativa* L. (Asteraceae) (lettuce) and *Brassica oleracea* L. capitata L. (Brassicaceae) (cabbage). To scale out the knowledge created in the ToT sessions, each ToT participant was to arrange horizontal sharing of knowledge and skills gained during the ToT sessions. The major factors limiting vegetable production in the urban and peri-urban areas of Benin are soil infertility, pests, weak irrigation infrastructure and poorly developed vegetable enterprises (James et al., 2006). Thus towards improved vegetable pest management, the IITA vegetable project found a strain of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina: Hyphomycetes) (isolate Bba5653) to be effective against the diamondback moth *Plutella xylostella* L., the most devastating pest of cabbage (Godonou et al., 2009a, Godonou et al., 2009b). As the microbial control agent is environmentally friendly and harmless to humans, it was part of the IPM options promoted through FFS training by the project along with botanical nematicides (Loumedjinon et al., 2009), as alternatives to synthetic pesticides.

The FFS training by the IITA vegetable IPM project created interfaces where the knowledge of the vegetable producers was challenged by the knowledge of other producers and scientists. Most of the vegetable producers believed that all arthropods in their fields would damage their crops, and therefore the project focused on distinguishing harmful insects from harmless and beneficial insects (e.g. *Cotesia plutellae* (Kurdjumov) (Hymenoptera: Braconidae), a parasitoid of *P. xylostella*, and the predator *Exochomus troberti* Mulsant (Coleoptera: Coccinellidae); (James et al., 2006). During the training, the participants were therefore exposed to IPM, beneficial organisms, plant health, agro-ecosystems and the concept of quality vegetables. Agro-ecosystem analysis (AESAs) was used to assist participants to base their decisions on whether to apply synthetic pesticides on several criteria connected to observing the development of the plants, pests and diseases in vegetable plots. Vegetable producers were expected to change inappropriate IPM practices as a result of the training offered by the project.

#### 3.1 Methods of data collection

The research was conducted in urban and peri-urban areas of Cotonou, department of Littoral, in 2006/07. In Benin, the law 1991/004 regulates packaging, labelling, transport, storage, usage and disposal of synthetic pesticides on the market, and the national plant protection service (Service de la Protection des Végétaux, SPV) is in charge of the quality control of synthetic pesticides and authorization of salesmen. The Centre d'Action Régional pour le Développement Rural (CADER) was in charge of the control of the use of synthetic pesticides, but it had closed down at the time of the survey. In this study, three vegetable gardens (Houeyiho, Office National d'Édition de Presse et d'Imprimerie (ONEPI) and Gbgamey), where vegetable IPM-FFS had been conducted, were compared with three

vegetable gardens in Godomey where no FFS had been held (control group). The gardens where FFS had been held were selected on the basis of accessibility (short travel distance from Cotonou), that they were still in use as vegetable gardens and some of the crops covered in the project (carrot, lettuce, cabbage and gboma) were cultivated there. The control sites were the only vegetable gardens in Cotonou, which had not participated in the project. All the respondents produced vegetables for the market, and nearly all the land area in the gardens studied was used for vegetable production, while the respondents lived in other parts of or outside Cotonou. Each vegetable producer had his/her defined cropping area, consisting of several beds, with an average size of 7.2 m<sup>2</sup> each (James et al., 2006), but none of them owned the land they cultivated or had formal contracts with the landowner, thus their situation was very insecure (Zossou, 2004).

During the vegetable IPM-FFS, IPM options within the categories 'chemical, biological, mechanical and cultural' were taught. To distinguish how the different vegetable producers understood the concept of IPM, the number of IPM techniques (chemical, biological, mechanical and cultural) in which they mentioned IPM tools when answering the open-ended question, 'What does IPM mean?' was counted. It was also noticed whether the respondents only listed IPM options or explained a holistic approach using AESA. When IPM tools were mentioned within only one IPM approach, the respondents were considered as having a narrow understanding of the concept of IPM. When IPM tools were mentioned within all the four IPM approaches, their understanding of the concept of IPM was considered broad. While the 'concept of IPM' was based on how the respondents described IPM, the respondents' 'knowledge of IPM' was evaluated by the number of IPM techniques, in the four mentioned categories, in which they mentioned IPM tools as response to various questions during the interview. AESA was used in the IPM-FFS training to assist the farmers to take management decisions based on the conditions of their fields. The farmers observe the biotic and abiotic factors, analyse how these impact their crops and thereafter take proper management decisions based on the analysis (Pontius et al., 2000). To perform a sound AESA requires the farmers to have a good understanding of ecosystem interactions such as pest-predator relationships and the existence of beneficial insects. Insect zoos (enclosed pot with a plant, a pest and/or a beneficial insect) are often used to visualize the pest-predator relationships (Pontius et al., 2000).

A transect walk was done in all the vegetable gardens to get preliminary information about the area. Convenience sampling of the snowball type was used (Bryman, 2004), and the most available vegetable producers in the gardens were identified. Among these, the sample of producers was selected on the basis of gender, age, education, and economic and social status. The list of respondents was checked by the leadership of the community-based farmers union in Cotonou (Union Communale des Producteurs, UCP) to ensure that all socioeconomic categories were represented. From the IPM-FFS project area, 15 ToT participants, 9 FFS participants and 19 non-participants were selected. Twelve control respondents were selected from the area where no IPM-FFS had been conducted. Fifty-four semi-structured interviews with open-ended questions were carried out with the vegetable producers. The interviews focused on knowledge and use of IPM options, awareness of health hazards of synthetic pesticides, handling practices of synthetic pesticides, and knowledge and use of protection gear. Focus group interviews were held with the producers in the ToT, FFS and non-participant groups to collect data on synthetic and botanical pesticides, the IPM-FFS training and the production environment in the vegetable gardens. Female and male producers were interviewed separately for open discussions. Key informant interviews were held with an

ambulant salesman of agro-chemical inputs; two elderly, experienced vegetable producers; and various NGO staff and government employees, as well as with project stakeholders from an NGO specialized in biological agriculture (Organisation Béninoise pour la Promotion de l'Agriculture Biologique), SPV, the national institute of agricultural research in Benin (Institut National de Recherche Agricole du Bénin), IITA in Benin and the UCP. Data was collected on pesticides (rules, regulations and sales) and on the IPM-FFS training (structure and curriculum). Triangulation and follow-up questions were used within the interviews to capture the respondents' real view. An interpreter was used for all the interviews with the vegetable producers so that the interviews were held in the common local language 'Fon' (spoken by most of the interviewed vegetable producers) or French.

As far as possible, the double difference model, comparing the differences in change over time between two populations, was used. The change in behaviour within the ToT and FFS groups was compared with the change in behaviour of the non-participants and control groups. A modified version of Mangan and Mangan's (Mangan & Mangan, 1998) model was used to assess producers' understanding of beneficial insects. The questions 'If you had the possibility would you like to kill all the insects in your field?' and 'Are there any insects that might be beneficial to have in your field (if yes give an example)?' were asked at different points during the interview. If the respondent did not want to kill all insects and could give examples of beneficial insects, she/he would be grouped as having a 'good concept' of beneficial insects.

### 3.2 Results and discussion

#### 3.2.1 Increased IPM knowledge and plant health

All the ToT respondents, 56% of the FFS respondents, but only 16% of the non-participants were familiar with the term IPM. The ToT respondents had a broader understanding of the concept of IPM, but in general, most of the vegetable producers who were familiar with IPM had a narrow understanding of the concept of IPM (Fig. 1).

All the producers understood IPM as a separate management tool, but while most of them associated IPM with chemical control, the ToT and FFS respondents were more concerned about reducing the use of synthetic pesticides and using botanicals (such as neem, papaya, pepper, orange and cassava epidermis). 'Knowledge of IPM' was based on the IPM tools reported by the respondents during the interview. All the respondents had a broader 'knowledge of IPM' than 'concept of IPM', indicating that even though many vegetable producers were not familiar with the scientific term 'IPM', they knew about various pest management methods. A larger proportion of the ToT respondents (60%) had a good concept of beneficial insects than the FFS (22%), control (18%) and non-participant respondents (none). As the ToT participants were only shown pictures of the beneficial and harmful insects, but did not have any insect zoo, they lacked the experience of visually understanding pest-natural enemy relationships. As a consequence, many did not transform the information into reliable knowledge, as illustrated by a ToT participant: *"In the ToT I learned that beneficial insects eat the pest, but it is too risky to rely on it so I rather kill all the insects. I have to see how the beneficial insects behave in practice before I can trust that they won't damage my vegetables, but I don't have enough space to experiment with this"*. In terms of AESA activities, the largest improvements in observing pests and crop interactions were among the ToT respondents (Fig. 2), who shifted from using preventive application to applications based on frequent observations of changes in crop, pest and natural enemy developments.

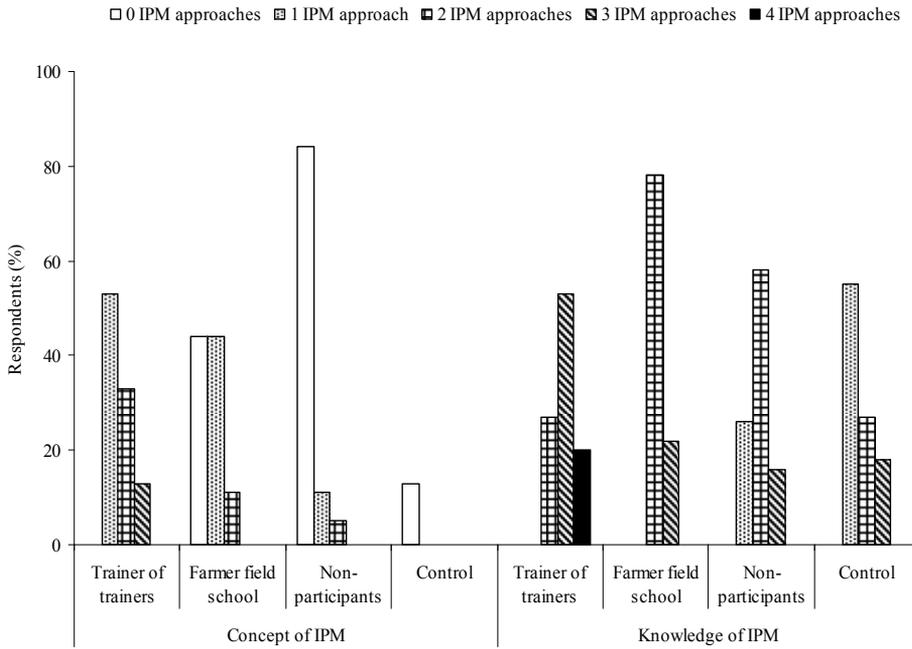


Fig. 1. Broadness of "Concept of IPM" and "Knowledge of IPM"

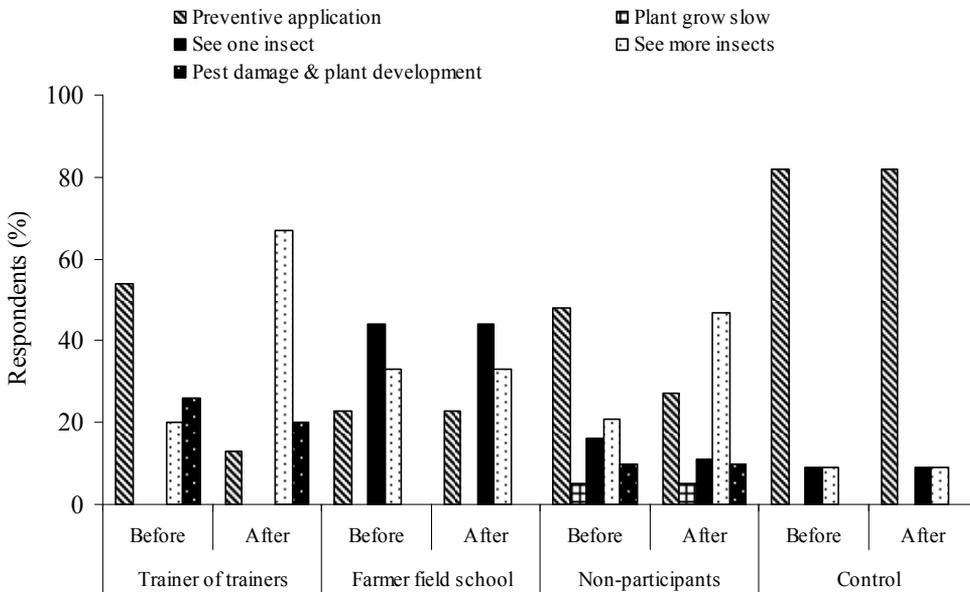


Fig. 2. Decision criteria used by vegetable producers to apply synthetic pesticides before and after the training

Sixty-seven percent of the FFS respondents said that the project had reinforced their knowledge to observe pests and crops, as told by a ToT participant: *"Now (after the Project) I greet my plants in morning and ask how they spent the night. If bad, I have to consider what to do"*. Nevertheless, only one ToT respondent used agro-ecosystem analysis and based the management decisions on a holistic analysis of the field; the rest based their decisions on subjective assessment of increasing pest densities. The rate of calendar application was higher when the producers did not have a good understanding of beneficial insects, indicating that having a good understanding of beneficial insects may reduce the calendar application or vice versa. The largest change was among the ToT respondents (33%), shifting from consulting family and neighbours and not measuring at all, to reading the label and using information from the ToT/FFS training.

The term 'plant health', seemed to be a well-known term in the area, but how the respondents understood plant health differed. While the majority of the FFS (89%), non-participant (68%) and control respondents (63%) emphasized the use of synthetic pesticides to get healthy plants, the ToT respondents placed more importance on botanically and biologically based pesticides. Using organic matter to build the soil is important for plant health, and the ToT and FFS respondents were more aware of the importance of applying organic matter before sowing. However, the use of compost seemed to be dependent on the availability and price, as 82% of the respondents in Houeyiho used it, but nearly none of the respondents from the other areas used it. Also seed quality is important for plant health, and only ToT respondents (67%) knew how to use germination testing to check the seed quality, but even though all but one of them claimed to use it, no beds where germination testing was performed were observed during this study. The experiments in the project consisted of giving two beds different treatments and comparing the results. None of the respondents in the project experimented in this way as a result of the training. Forty-seven per cent of the ToT respondents and 22% of the FFS respondents, however, experimented in other ways after the ToT/FFS training, meaning that they tried out methods taught in the project such as the dose of synthetic pesticides, botanical pesticides, observing their fields, and organic and chemical fertilizers. While one respondent said: *"Cultivating vegetables is about experimenting"*, others were more risk averse, and lack of time and land constraints were the most common reasons for not experimenting.

The respondents used 32 different types of synthetic pesticides (Fig. 3) and of these pesticides all the class 1b, but also some class II (Endosulfan and Fenprothrin) and even class III pesticides (Orthene and Malathion) contained substances that are banned or severely restricted in the European Union (PANEurope, 2009), posing serious health concerns for the producers, consumers and the environment. Two respondents used endosulfan, which is prohibited in Benin and proposed by the POPs Review Committee to be eliminated from the global market (StockholmConvention, 2010).

ToT respondents were more often applying the correct pesticides against targeted pests than the other respondents. On the other hand, a large proportion of the control (82%), ToT (80%), FFS (44%) and non-participant respondents (42%) used 'cocktails', mixing up to four different pesticides. Two respondents illustrated the producers' difficult situation: *"I use Talstar against leaf miners and field crickets although I know it is not effective against those pests"* and *"I have no solution for the nematodes on my gboma. I think Kinikini would be effective, but I do not have money to buy it"*. The use of cocktails of different pesticides made it difficult to evaluate the project's impact on frequency and quantity of pesticides used (Table 1).

The project recommended to use correct pesticides and to follow the prescriptions from the manufacturer, but as one respondent said: *“The pesticides they (the Project) recommended us to use are not available, so we have to use what is available”*. Thus, due to unavailability and expensiveness, the majority of the producers sometimes or always bought synthetic pesticides in non-original packages not knowing what they were using. Even if the vegetable producers bought synthetic pesticides in the original packages, some respondents might be illiterate and the labels were often in foreign languages. Most of the respondents were very much aware of and respected the recommended pre-harvest interval. As indicated by one respondent, economic constraints may reduce the safety in pesticide applications: *“There is shortage of land and people need money so it is difficult to wait the recommended days before harvest”*. On the other hand, the safety is also influenced by the individual and collective perception of ethics, as one respondent said: *“It has to be made socially unacceptable to not respect the pre-harvest interval”* and another respondent describing the changes due to increased awareness: *“Earlier some people sold the cucumber five days after spraying, but now everybody know they have to respect the harvest interval, so people will inform the buyer if the vegetable producer has sprayed too close to the harvest”*.

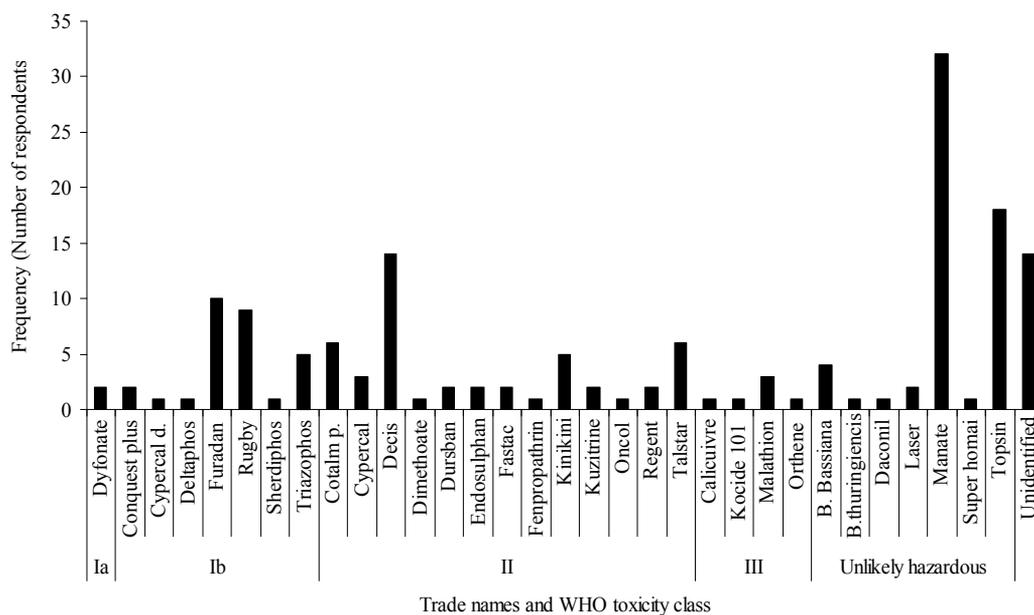


Fig. 3. Pesticides used by the respondents

Synthetic pesticides	Vegetable	ToT	FFS	NP	C	PB	ToT	FFS	NP	C	PB	
		Frequency					Quantity					
		(times/crop season)					(l or g/ha)					
Decis (l)	Cabbage	27	-	33	10	7	19	-	138	46	31,9	
	Gboma	2	2	3	3	5	2	2	-	6	7	
	Lettuce	2	1	4	3	-	1	-	10	8	-	
	Carrot	3	-	-	-	1	3	-	-	-	3,5	
Talstar (l)	Cabbage	3	6	-	-	12	5	13	-	-	14,91	
	Gboma	2	2	-	3	2	3	2	-	6	7	
	Lettuce	2	1	4	3	2	1	-	10	8	7	
	Carrot	1	-	-	-	-	3	-	-	-	-	
Manate (g)	Cabbage	11	-	-	-	-	6	-	-	-	-	
	Gboma	2	3	2	3	2	4	30	7	10	13,9	
	Lettuce	2	2	2	3	2	3	3	4	13	19,9	
	Carrot	-	-	-	-	-	-	-	-	-	-	
Fenprothrin (g)	Cabbage	-	-	18	-	-	-	-	9	-	-	
	Gboma	-	-	-	-	-	-	-	-	-	-	
	Lettuce	-	-	3	-	-	-	-	5	-	-	
	Carrot	-	-	-	-	-	-	-	-	-	-	
Endosulfan (l)	Cabbage	1	-	-	-	-	0,4	-	-	-	-	
	Gboma	1	-	-	-	-	1	-	-	-	-	
	Lettuce	1	2	-	-	-	1	6	-	-	-	
	Carrot	1	-	-	-	-	3	-	-	-	-	
Furadan (g)	Cabbage	-	-	-	-	-	-	-	-	-	-	
	Gboma	-	1	-	-	3	-	46	-	-	13,9	
	Lettuce	1	-	-	-	-	46	-	-	-	-	
	Carrot	1	1	1	-	2	-	-	-	-	13,9	

Table 1. Average frequency and quantity<sup>1</sup> of synthetic pesticides per growing season

<sup>1</sup>based on the quantity of concentrated synthetic pesticide, not quantity of active ingredients. NP = Non-participants, C = Control, PB = Project baseline data

The project emphasized the use of botanical pesticides as an alternative to synthetic pesticides. Forty-seven per cent of the ToT respondents, 26% of the non-participants, 22% of the FFS respondents and 9% of the control respondents said that they used neem, but during the time of this survey, no vegetable producers were observed preparing botanicals. The reason for not using neem extract was the time-consuming and labour intensive preparation as one needs large quantities of leaves or fruits. If they could buy neem extract commercially, many may use it because they had experience with it and appreciated that it was not harmful to the environment or to the individuals who applied it. In the project, knowledge about biological control was introduced. *B. bassiana* was used to control diamondback moth, and the vegetable producers could request it from IITA. Many of the vegetable producers were pleased with *B. bassiana* as it saved them money and labour, and their interest in the product was expressed by a ToT participant: *“When you use bassiana (B. bassiana) you are sure to succeed in growing cabbage. If I didn’t have bassiana I would have stopped growing cabbage”*. At the time, *B. bassiana* was given for free, but if the product is commercialized, the question remains whether the price will be competitive with respect to synthetic pesticides so the producers can afford it. However, increasing evidence of resistance in the diamondback moth may force producers to use alternatives or to abandon cabbage as a crop. Crop rotation was traditional knowledge used by all the producers, but they became more aware of its importance because of the ToT/FFS training. Even if some vegetable producers were not able to explain in scientific terms what was happening in their crops, they improved their practices based on experience, like one control respondent said: *“Normally you only have to apply pesticides three times, but if you grow the same type of vegetable two times after each other you need five pesticide applications”*. All the ToT respondents, 94% of the non-participants, 78% of the FFS respondents and 73% of the control respondents practiced intercropping, but the main reasons for this practice were economic gains and land shortage. Also most of the respondents chose the planting time for economic motives considering market prices, while none considered plant health issues.

### **3.2.2 Awareness of health hazards from synthetic pesticides and proper handling practices**

Awareness of negative effects of synthetic pesticides was quite high among the respondents, with the most known effect being hazards to the farmers’ health as one respondent noted: *“It is not good to apply pesticides as it makes my eyes burn”*. The control group had a more limited view of the negative effects of synthetic pesticides, and only mentioned human, consumer and farmer health, but not environmental and long term effects.

The overall awareness about protection while spraying synthetic pesticides was high (Fig. 4), as one respondent said: *“You should cover all the parts of your body. You may not feel anything today, but the pesticides will accumulate in your body and in some years cause heart problems and headache”*, but the proportion actually using such equipment was low. Among the control respondents, 46% only wore shorts and T-shirts while spraying synthetic pesticides, but virtually none of the other respondents could show any of their protection gear. The most common reason for not using any protection device was expense, while another common reason in the tropics is the heat. The most common post-spray activity among the producers was to take a bath, done by most of the ToT respondents, while the control respondents were more likely to only wash their hands, legs and face. Most of the vegetable producers

stored their synthetic pesticides buried in the vegetable field, hid it in the bushes nearby, or at home. Only the ToT respondents from ONEPI stored synthetic pesticides in a storage room, which could be locked, as they had a common storage room in the garden. Among the respondents buying original pesticide packages, all the FFS and control respondents, 62% of the non-participants and 22% of the ToT respondents sometimes stored the synthetic pesticides in empty soft drink bottles. When using pesticide bottles without labels or storing it in soft drink bottles, other people in the household may use the content in the belief that it is something else. Many threw the pesticide cans in the garbage or bushes where pesticide may leach out in the ground or accidents may happen if children find them. The most common way to apply pesticides was to use a spray sack, but 27% of the control respondents, mixed the synthetic pesticides with water in a bowl and used a bunch of grass to 'paint' the vegetables.

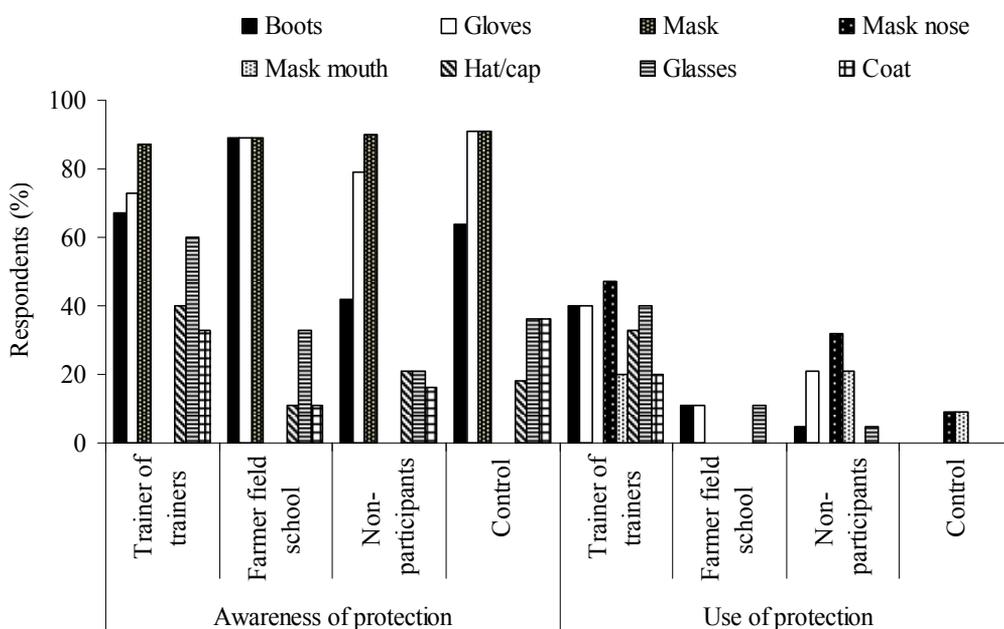


Fig. 4. Awareness and use of protection during application of synthetic pesticides

To scale out IPM knowledge and practices, the ToT participants were expected to carry out FFS sessions on all the four vegetables in the project. However, the FFS respondents' general complaints were that (1) the FFS sessions were often not conducted in both seasons and did not include all the four crops in the project and (2) the quality of the training was lower than that of the ToT sessions. The FFS sessions started the same season as the ToT sessions, thus not allowing the ToT respondents' time to develop their facilitation skills in IPM before commencing as trainers. Initially, the project only had IPM plots, but had no plots where the farmers' existing practice was demonstrated, which indicated a poor emphasis of the project on the vegetable producers' own experimentation and knowledge-creating processes.

#### 4. Conclusion

The project increased the ToT participants' knowledge about IPM, reflected in their good concepts of beneficial insects, improved knowledge about plant health and pest management tools, improved ability to take management decisions based on pest occurrence in the field and increased experimentation with knowledge gained from the project. Increased knowledge and awareness about IPM may be one of the reasons for the participants' change in attitude towards synthetic pesticides. While some claimed having adopted biological IPM tools, the participants had not changed their practices significantly regarding the use of synthetic pesticides and cocktails, and most of the producers did not apply correct pesticides to the target pests. The FFS participants had gained considerably less knowledge than the ToT participants, which may be due to less intensive FFS sessions led by trainers with less developed facilitating skills. Experiential learning reflects and allocates meaning to that experience and develops knowledge from it. There were several examples of this issue in the project area, where vegetable producers, who did not know scientific terms such as IPM or nematodes, had nevertheless learned IPM practices based on experience. In the project, however, access to information about beneficial insects was mainly through theory, thus the participants did not experience what 'beneficial insect' meant in practice, and consequently did not transform this information into meaningful knowledge.

The practical use of concepts such as 'IPM' and 'agro-ecosystem' requires an understanding of complex interactions, which takes time to develop. Lack of monitoring of activities in their own fields is one reason why very few participants had a holistic and good understanding of those concepts. While it remains important to bring in scientific information to improve the vegetable producers' understanding, this information should be built on the participants' local knowledge to make sense to them and be relevant. The results show that the surveyed participants in the project did not adopt a complete package of IPM tools and concepts, but rather experimented with the new information and thereafter adapted parts of what they learned into their production systems. While Mancini (2006) found a strong correlation between knowledge level and the reduction in pesticide use among Indian farmers attending cotton IPM-FFS, also other studies show that farmers with a good understanding of how the field ecosystem works perform better crop management than those who get discrete and simplified pest management instructions (Mangan & Mangan, 1998, Price, 2001). However, in Benin, even the project participants with a good understanding of beneficial insects used superfluous pesticides, which might kill the beneficial insects. This shows that it requires more than a good understanding to change usual practices.

There are many factors in the vegetable producers' environment hindering them in using IPM and using synthetic pesticides more safely, including lack of ecological knowledge and access to product information, little availability of the right products and their relatively high cost. Many participants wished to produce good quality vegetables with safer use of synthetic pesticides. However, limited access to land and wish to make higher profits, were strong driving forces leading some vegetable producers to unsafe practices, such as not respecting the harvest interval and using forbidden synthetic pesticides. Also, normative considerations influence the vegetable producers' practices. As the laws on pesticide use are not enforced in Benin, these have limited impact on people's behaviour, but as seen from the survey, the awareness rising from various NGOs and research institutions has changed the vegetable producers' attitudes towards respecting the pre-

harvest interval. Even more awareness rising is needed to change the producers' attitude towards synthetic pesticides and make dangerous practices unacceptable. Maybe even analysis of pesticide residues of products from individual beds to personalize the information could be an input that is an asset for producers to fully understand the problem of residues in the products.

The results indicate possible trade-offs between health and economic effects, with the latter weighing more heavily. The awareness of pesticide hazards and proper protection gear was generally high, and although many producers had experienced negative health effects of synthetic pesticides, most producers still did not use protection gear due to expense. The project did not have any impact on the safety in storage and handling of pesticides as the practices were rather influenced by what was more convenient for the vegetable producers. This study shows that there is a need to focus more on the vegetable producers' own knowledge creation by emphasizing experiential learning, as well as to enable the producers to realize their role as potential knowledge generators (Simpson & Owens, 2002). This is in line with other studies emphasizing the importance of establishing a dynamic process where the participants take control over the experimentation (Braun et al., 2004) and on developing a 'learning style' (Pretty, 1995) that enables 'exploration, evaluation and adaptation of technological alternatives' (Lee, 2005 : 1332). Post-IPM-FFS activities would probably allow the participants in the Cotonou vegetable IPM-FFS to develop their ideas and concepts about IPM and agro-ecosystems as they practice these in their fields. There were many competent and knowledgeable vegetable producers in the studied areas concerned about the dangerous use of pesticides. In a follow-up of the IPM-FFS activities, these people could be the driving forces of promoting and implementing IPM in their communities. The general results from this study in Benin are in line with other reports (Maumbe et al., 2003, Mancini, 2006), concluding that for IPM to succeed as a proper and reliable plant protection strategy, not only is there a need to consider the educational component involving individual farmers or groups of farmers, but it is also necessary for all the stakeholders involved (farmers, extension, scientists, policy makers and NGOs) to understand the complex nature of IPM. In addition to the educational component, all other factors, such as the need for group versus individual action, farmer's indigenous knowledge, farmer's resource endowments, and last but not least the macroeconomic determinants, do play a significant role in establishing whether IPM can succeed or not. In light of findings from Benin and other IPM-FFS programs, further research is needed on how to facilitate the processes of knowledge creation between the farmers and scientists, and how to involve the ToT participants' in a way that they feel the commitment to continue the learning processes, to share knowledge with their farming communities and to initiate changes in pest management at the community level.

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## The Millardetian Conjunction in the Modern World

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### 1. Introduction

This chapter deals with the review of literature related to the impact of study of biotic interactions on the development of modern methods to control plant diseases. Only diseases caused by fungi and oomycetes, the two major phylogenetic groups of microbial eukaryotic plant pathogens, were considered. To fight these pathogens, the chemical treatment with fungicides is a long-established method and the most usually used still today. The first report of effective chemical control was related to the use of a fungicide. Its discovery results from the conjunction between a double need, that to protect the vineyards from robbers and downy mildew disease, and a gift for observation which led Millardet to a fertile conclusion for vine protection, but also, for the rise of the chemical treatments of crops. Millardet initially observed that the rows of vineyard, in border of road, treated with an aqueous mixture of copper (II) sulphate to protect against the disease and of lime to dissuade the grape thieves, were preserved from mildew. After experimentations he elaborated a treatment based on a combination of these chemicals now known as the Bordeaux mixture which became the first fungicide (Millardet, 1885; Rappilly, 2001). The mixture is nowadays still used, but it was widely supplanted by synthetic fungicides from various chemical natures (carbamates, triazol, amines, amides, quinines, phenol and benzene derivatives, etc...). Today large quantities of fungicides are applied each year to crops and seeds in the agriculture sector. For example, a mean of 40 000 tons of industrial fungicides are now used each year in France (Aubertot et al., 2005) .

Until the 1980s, the productivist and intensive injunction allowed to nourish the vast majority of the human populations in the developed countries. Because of their low cost and their efficiency, fungicides were used in most countries without restrictions to maximize yield profitably and protected crops. From a phytopathological point of view, plants were mainly looked like simple receptacles, both for the pathogens and the fungicidal molecules. Regarded as a nutritive soup for the first ones and as a simple excipient for the second ones, the protected crop plants laid their fruits with abundance. These last decades, the ecological

imperativeness succeeded the productivist one. This has contributed to impose a radically different view of plants in Science and in Agriculture. Host plants are now self-defensing organisms, endowed of an innate immune system, and able to develop various strategies against infections, from the burned ground to the targeted striking. In the same way, substantial knowledge has been gained on the biology of plant pathogens, the epidemiology of diseases and the co-evolution between a host plant and a pathogen. This knowledge constitutes a remarkable sink for genetic and ecological innovations in plant protection. Such alternatives to chemical control have become imperative.

The use of fungicides as well as of the other pesticides (insecticides, herbicides, rodenticides) is now questioned. Their efficiency to control plant pests is counterbalanced by their undesirable and various effects on human health, on sustainability of ecosystems and on biodiversity. There is also the problem of the rapid adaptation of plant pathogenic populations in response to systematic use of pesticide molecules. Within the sustainable development framework, countries and international organizations have a stated political aim of reducing use of pesticides. In France, the Ecophyto 2018 plan constitutes the engagement of the recipients to reduce by 50% the use of the pesticides at the national level within a deadline of ten years, if possible ([http://agriculture.gouv.fr/IMG/pdf/PLAN\\_ECOPHYTO\\_2018.pdf](http://agriculture.gouv.fr/IMG/pdf/PLAN_ECOPHYTO_2018.pdf), 2008). Several fungicides have already been judged like harmful substances which can cause acute or chronic toxicity. In some cases the marketing authorizations of the preparations containing alarming active substances are withdrawn; their distribution and their use are prohibited. In the European Union the directive 2009/128/EC establishes a framework for community action to achieve the sustainable use of pesticides (<http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2009L0128:20091125:EN:PDF>, 2009). The pesticide program helps government of the Organisation for Economic Co-operation and Development to reduce the risks associated with pesticide use, through a variety of actions to supplement pesticide registration and further reduce the risks that may result even when registered pesticides are properly used ([http://www.oecd.org/departement/0,3355,en\\_2649\\_34383\\_1\\_1\\_1\\_1\\_1,00.html](http://www.oecd.org/departement/0,3355,en_2649_34383_1_1_1_1_1,00.html)). In the same time, the main challenge in agriculture is to increase crop yields for feeding seven billion individuals today and about nine billion on the horizon 2050 ([http://km.fao.org/fileadmin/user\\_upload/fsn/docs/SUMMARY\\_2050.pdf](http://km.fao.org/fileadmin/user_upload/fsn/docs/SUMMARY_2050.pdf), 2009). The impact of the absence of fungicidal protection in plant diseases may reduce crop quality and quantity. The limitation of the fungicide use beyond the optimisation may be harmful for some crops (Butault et al., 2010) ([http://www.inra.fr/l\\_institut/etudes/ecophyto\\_r\\_d/ecophyto\\_r\\_d\\_resultats](http://www.inra.fr/l_institut/etudes/ecophyto_r_d/ecophyto_r_d_resultats)). The development of integrated pest management, linking all appropriate options including, but not limited to, the judicious use of pesticides, as well as the development of organic food production, limiting the use of pesticides to those that are produced from natural sources, require to prospect new biological resources for plant protection.

The necessity to reduce pesticide use while maintaining high crop yields is today the double need of what we name in this review the millardetian conjunction. What is today the substrate(s), the scientific fields from which could emerge a seminal(s) observation(s) that would supplement the conjunction? Of course it is advisable to say at once how much is hard to anticipate that today. This chapter is focused on the control of plant diseases caused by fungi and oomycetes (Figure 1). Fungi and oomycetes are today mainly and effectively controlled by fungicide applications. We review our knowledge in three domains that we

consider as potentially fruitful for such emergence and for rupture in phytoprotection. In the context of studies on plants-pathogens interactions, we underline in the section 2 how this knowledge may help to reduce fungicide use. We also highlight in the section 3 how rapidly expanding investigations on interactions between cells of a pathogen, and between a pathogen and microbial species living in the same biotope may promote environmental friendly innovations in plant protection.

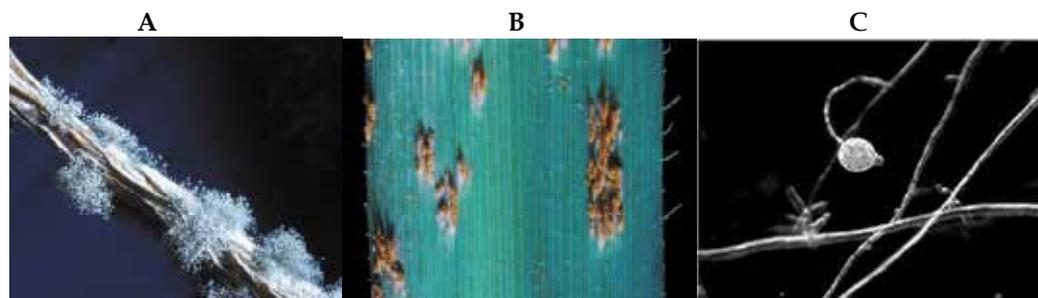


Fig. 1. Examples of eukaryotic pathogens and plant symptoms

(A) Sporulation of the ascomycete *Botrytis cinerea* on a kalanchoe stem. (B) Oat crown rust pustules (basidiomycete) on an oat leaf. (C) Sporangium and mycelium of a polyphagous oomycete, *Phytophthora parasitica*.

## 2. The plant-pathogen interaction

Beside constitutive physical and chemical barriers preventing infection, plants use their innate responses to ward off pathogens. Plants have evolved the ability to detect microbes through the recognition of conserved microbial leitmotifs which are referred to as Pathogen- or Microbe-Associated Molecular Patterns (PAMPs or MAMPs). The molecular responses are mediated by Pattern-Recognition Receptors (PRRs), a class of innate immune response-expressed proteins that respond to PAMPs. This recognition level initiates MAP kinase signaling and PAMP-triggered immunity (PTI), a key aspect of plant innate immunity which contributes to prevent microbial growth (Nurnberger et al., 2004). Pathogens may suppress PTI responses by secreting effectors in the apoplast or directly into the cytoplasm of host cells, leading to effector-triggered susceptibility (Gohre and Robatzek, 2008). Through evolution and by the driving force of natural selection, plant *R* gene function has emerged resulting in direct or indirect recognition of specific effectors by *R* proteins. This second level of microbial recognition, specific to certain races or strains of a pathogen, leads to effector-triggered immunity (ETI). ETI is associated in the host with a local programmed cell death, a response which is referred to as the hypersensitive response (HR), and with the establishment in the whole plant of systemic acquired resistance (SAR) which is long lasting and effective against a broad spectrum of pathogens (Chisholm et al., 2006; Dodds and Rathjen, 2010; Jones and Dangl, 2006; Zipfel, 2009).

### 2.1 Recognition by plants of molecular signatures from pathogens

One way to prevent crop diseases, and in the same time to reduce frequency of chemical treatments, is to enhance the ability of plants to stimulate their own innate immune system. Understanding how plant receptors recognize molecular signatures from pathogens is important to approach such a goal. Over the past 20 years many *R* genes

have been discovered and evaluated to engineer disease resistance in crop (Hammond-Kosack and Parker, 2003). On the other hand, only a few plant PRRs have been identified up to now, and our knowledge of the molecular mechanisms underlying PTI is limited. Nevertheless, new agricultural applications could ensue from recent studies on pattern-recognition receptors. A PRR gene from the cruciferous plant *Arabidopsis thaliana*, occurring only in the Brassicaceae family, was transferred into two plants, *Nicotiana benthamiana* and *Solanum lycopersicum*, in order to determine if adding new recognition receptors to the host arsenal would lead to better resistance (Lacombe et al., 2010). This EFR gene encodes a surface-exposed leucine-rich repeat receptor kinase EFR, and mediates recognition of the bacterial pathogen-associated molecular patterns EF-Tu (elongation factor Tu). It was chosen by the authors because the high level of conservation of EF-Tu protein sequences across bacteria offered the possibility that EFR could confer resistance against a wide range of bacterial pathogens. Based on triggering of an oxidative burst and on induction of defense-marker genes, expression of EFR in *N. benthamiana* and *S. lycopersicum* transgenic plants was found to confer responsiveness to bacterial elongation factor Tu. The heterologous expression of EFR makes also transgenic lines more resistant to a range of phytopathogenic bacteria from different genera (*Pseudomonas*, *Agrobacterium*, *Ralstonia*, *Xanthomonas*). These results were obtained with host plants and pathogens growing in controlled laboratory conditions. Nevertheless, they constitute a first step for the evaluation of the deployment of new PAMP-recognition specificities in crop species. This strategy could be used to engineer pathogen broad-spectrum resistance in crop plants, potentially enabling more durable and sustainable resistance in the field (Dodds and Rathjen, 2010; Gust et al., 2010; McDowell and Stacey, 2008).

## 2.2 Exogenous application of natural compounds stimulating plant defense responses

As mentioned above, one of the main change in the philosophy of plant disease management has been these last twenty years to abandon the systematic use of biocide treatments against pathogens for alternate solutions among which the bio-activation of plant innate immune system. In some cases it has become reasonable to prevent crop diseases by exogenous application of natural compounds used as elicitors of immune defence responses or of systemic acquired resistance (Vallad and Goodman, 2004). This constitutes a potential alternative or a complement to the intensive use of chemical fungicides with the view to reduce their negative effects on environment and human health. Conventional fungicides are metabolic inhibitors (of electron transport chain, of enzymes, of sterol synthesis of nucleic acid metabolism or protein synthesis) while in contrast elicitors have no direct effect on pathogens. Most of elicitors are natural compounds extracted from microorganisms, algae, and crustacean. Due to their biodegradability and to the low doses applied, the risk of environmental contamination by residues appears weak. Also, they don't show, a priori, a profile to present dangers to human health (Lyon et al., 1995). They appear particularly attractive in the case of integrated production and are evaluated in the frame of the organic farming which lacks anti-fungus substances.

The screening for such natural compounds has led to the characterization of some active molecules now used in the field as a supplement to classic fungicidal treatments. Laminarin, a beta-1→3 glucan, derived from the blue green algae, *Laminaria digitata*, elicits defense

responses and resistance to disease in different plants (Aziz et al., 2003; Joubert et al., 1998). Several countries have approved its use particularly on diseases of wheat and barley. Chitosan, another polysaccharide (a deacetylated derivative of chitin, beta-1,4-linked glucosamine) has also been approved by the food and drug administration of the USA first as a wheat seed treatment (El Ghaouth et al., 1994; Hadwiger, 1995). Because of its properties to activate various plant defense responses (phenyl ammonia lyase and peroxidase activities, phytoalexins synthesis, cell wall lignifications) and to trigger resistance, it is considered as an interesting alternative for enhancing natural resistance against *Botrytis cinerea* and other pathogens (Aziz et al., 2006; Povero et al., 2011). Harpin is a proteinaceous stimulator of plant defenses, produced by the plant pathogenic bacterium, *Erwinia amylovora*. When applied to plant surfaces by conventional means, harpin may elicit resistance to pathogens and insects and also enhances plant growth (Wei and Beer, 1996; Wei et al., 1992). Its use is approved in United States on a series of diseases for a wide range of plants: cotton, citrus, wheat, tomatoes, cucumbers, rice, strawberries, peppers, tobacco.

While these elicitors interfere or are suspected to interfere with the early step of recognition by plants of microbial molecular signatures, downstream events of defense signaling pathways have also been subjected to molecular dissection as well as technological evaluation for improving plant resistance to diseases. Two molecular entities have been particularly studied: the NPR (for Nonexpressor of PR genes) gene family and the salicylic acid (SA), two key positive regulators of systemic acquired resistance (Cao et al., 1994; Vernooij et al., 1994). Salicylic acid has been identified by several lines of evidence as a positive component playing an essential role in the SAR transduction pathway. SA levels are elevated at the onset of SAR in cucumber (Mettraux et al., 1990; Rasmussen et al., 1991), tobacco (Malamy et al., 1990), and *Arabidopsis* (Uknes et al., 1993). The exogenous application of SA to leaves of tobacco or *Arabidopsis* induces resistance against the same spectrum of pathogens and activates the same set of SAR genes, as with pathogen-induced SAR (Ward et al., 1991). Transgenic plants expressing a bacterially derived gene that encodes salicylate hydroxylase (*nahG*), an enzyme that converts SA to catechol, are unable to induce SAR (Delaney et al., 1994; Gaffney et al., 1993). The observation that treatment of plants by exogenous SA induces resistance to viral, bacterial and fungal, particularly biotrophic, pathogens has led to application of SA-induced defense responses in plant protection. A SA derivative, the BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester, is mainly used. BTH activates the same set of defense genes and induce similar wide spectrum resistance with lower phytotoxic effect than SA (Gorlach et al., 1996; Lawton et al., 1996). BTH treatment protects against a broad spectrum of pathogens in several fruit, vegetable crops and ornamental plants (Abo-Elyousr et al., 2009; Brisset et al., 2000; Godard et al., 1999; Hukkanen et al., 2007; Iriti et al., 2005; Małolepsza, 2006; Narusaka et al., 1999).

Members of the NPR gene family are also key positive regulators of systemic acquired resistance (Cao et al., 1994; Tada et al., 2008). Genetic studies in *Arabidopsis* have demonstrated that *AtNPR1* encodes an ankyrin repeat protein which is involved in SA perception and downstream SAR responses (Cao et al., 1994; Cao et al., 1997; Ryals et al., 1997). Nuclear localization of NPR1 is essential for SA-induced gene expression (Kinkema et al., 2000). Upon pathogen infection accumulation of SA triggers a change in cellular reduction potential, resulting in partial reduction of NPR1 oligomer to monomers, and then in their translocation in the nucleus where they interact with members of the TGA family of basic Leucine zipper transcription factors (Després et al., 2000; Kinkema et al., 2000) that bind to *PR1* promoter elements. NPR1-mediated DNA binding of TGA factors appears to be

critical for activation of defense genes (Fan and Dong, 2002; Jupin and Chua, 1996; Lebel et al., 1998; Qin et al., 1994) among which *PR* genes, which encode antimicrobial effectors (Van Loon and Van Strien, 1999). The potential of over-expression of *AtNPR1* from *Arabidopsis thaliana* or of its orthologues in crop species is a current approach for the development of more resistant cultivars. Over-expression of the *AtNPR1* gene in citrus and of the *MpNPR1* gene in apple increases resistance to citrus canker (Zhang et al., 2010) and to fire blight (Malnoy et al., 2007), respectively. In some cases negative impacts of the *NPR1* expression have been observed in transgenic plants. In apple, the overexpression of *Malus NPR1* does not create detrimental morphological changes, but side effects of overexpression of *NH1* (rice homolog of *AtNPR1*) have been noted in rice. The *NH1* overexpression leads both to constitutive activation of defense genes and developmentally controlled lesion-mimic phenotype (Chern et al., 2005; Fitzgerald et al., 2004). On the other hand, overexpression of *AtNPR1* in *Arabidopsis* not only potentiates resistance to different pathogens, but also enhances plant response to BTH and effectiveness of three Oomycete fungicides: metalaxyl, fosetyl, and  $\text{Cu}(\text{OH})_2$  (Friedrich et al., 2001). The authors suggest that a combination of transgenic and chemical approaches may lead to effective and durable disease-control strategies.

Despite their great potential for control of diseases, treatments of crops with elicitors are not however considered as the panacea for replacing fungicide application. It can be rather considered as a fungicide supplement when fungicide application may be reduced. Indeed treatments with elicitor provide between 20 and 85% disease control and in several cases their application provides no significant level of resistance. To improve their efficiency in the field, information of the influence of the environment, plant genotype, and crop nutrition on plant responses leading to effective resistance remains required (Walters et al., 2005).

### **2.3 Disease management and plant developmental resistance**

In this section we have paid particular attention on knowledge on plant developmental resistance. An increasing number of studies show that induction of resistance to disease during plant development is widespread in the plant kingdom (see for review Develey-Riviere and Galiana, 2007; Panter and Jones, 2002; Whalen, 2005). The scientific community that has investigated this question has used enough diversified approaches, from genetics to epidemiology, to delineate possible and robust contributions of this field for reducing fungicide uses in crop protection.

#### **2.3.1 A parameter for modeling epidemics and to minimize chemical use**

One important exciting and difficult challenge in plant protection is to define epidemiologic state both to ensure high crop yields and to manage chemical treatments. A precise definition of the defense and resistance potential of each host plant throughout its life cycle is a key element for the control of pathogen infection. In the context of the ecological awareness, developmental resistance may be considered as a very important factor in the rationalization of cultural practices, the main statement being to reduce fungicide application to shorter periods of high host susceptibility. To achieve this, at least two time parameters have to be properly defined: the precise time point at which establishment of developmental resistance occurs and the length of time during which resistance is effective against the disease. Thus the time required for a plant or for new leaves to acquire developmental resistance is now often integrated as one of variables used in modeling plant diseases (Ficke et al., 2002; Gadoury et al., 2003; Kennelly et al., 2005). For example modeling

of the dynamics of infection caused by sexual and asexual spores during *Plasmopara viticola* epidemics considers that only young grape leaves are receptive to infection because of developmental resistance (Burie et al., 2010; Rossi et al., 2009). Such considerations are also explored for powdery mildew of strawberries. Young leaves, flowers and immature green fruits are much more susceptible to the powdery mildew, caused by the biotrophic fungus *Podosphaera aphanis*, than mature tissues. The high susceptibility to powdery mildew at the early developmental stages seems coincident with the succulent nature of the fruits at this stage, making it easy for penetration and establishment of mildew (Asalf et al., 2009; Carisse and Bouchard, 2010). Control measures targeting at these critical windows of fruit susceptibility are likely to reduce yield loss. The authors of these studies concluded that timing fungicide sprays based on periods of high leaf and berry susceptibility should greatly improve management of strawberry powdery mildew. These few examples illustrate how studies on developmental resistance may help for the development of decision-making tools to minimize environmental and public health risk of fungicide application while maintaining high crop yields.

### 2.3.2 Genetic tools for breeders

The excavation of various and new genetic resources constitutes an additional window opened by studies on plant developmental resistance. This form of resistance has been now reported for a large number of crop plants. An increasing number of studies have shown that disease resistance governed by major genes (*R* genes) or minor genes (quantitative trait loci, QTLs) may be plant stage-specific. When it occurs the persistence of the phenomenon throughout the rest of the plant life cycle once it has been induced is of clear agronomic interest. The influence of development on race-specific resistance genes has been first studied in detail in rice and wheat, to assist breeders in their decision-making processes (for review Develey-Riviere and Galiana, 2007). A recent finding indicates that QTLs controlling constitutive expression of defense-related genes co-localizes with QTLs for partial resistance of rice to *Magnaporthe oryzae* (Vergne et al., 2010). Such studies also concern other crop plants. Fruits from several cucurbit crops were tested for the effect of fruit development on susceptibility to the oomycete *Phytophthora capsici*. The seven crops tested represent four species: melon (*Cucumis melo*), butternut squash (*Cucurbita moschata*), watermelon (*Citrullus lanatus*), and zucchini, yellow summer squash, acorn squash, and pumpkin (*Cucurbita pepo*). For all of these fruits, a pronounced reduction in susceptibility accompanied the transition from the waxy green to green stage (Ando et al., 2009). The importance to consider developmental resistance for breeding has been underlined in a review on genetic approaches to the management of blister rust (*Cronartium ribicola*) in white pines. The authors have defined developmental resistance, *R*-gene resistance and partial resistance as the three broad categories of resistance that breeders have to take into account for resistance in North American white pines (King et al., 2010).

### 2.3.3 A putative source for bio-fungicides

Researches on developmental resistance also provided opportunities for characterizing new host molecules influencing pathogen growth *in planta*. Metabolite compounds accumulating in late phases of host plant development may enable the plant to inhibit the infectious cycles of pathogens (Hugot et al., 1999; Kus et al., 2002). However the nature of these compounds remains unknown and it is difficult to define their interest as adaptive resources for plant

protection and for their application to crop fields. It has been merely observed that in *Arabidopsis* the intercellular accumulation of SA is critical for antibacterial activity associated with developmental resistance to *Pseudomonas syringae* (Cameron and Zaton, 2004).

#### 2.4 The pathogen in interaction with its host

During the current decade the main research effort on eukaryotic plant pathogens has been and still is the release of genome sequences for pathogens causing the most devastating crop diseases (Dodds, 2010). As a result, an increasing number of gene collections involved in regulation of the interaction with host plants as putative PAMP or effectors have been identified. The identification within these collections of effectors that are crucial for virulence offers the opportunity to select plant targets for more durable resistance (Houterman et al., 2008). In a functional genomics studies Vleeshouwers and coll. (2008) developed an effector-based method for identification of late blight resistance gene in potato. They used a repertoire of secreted and translocated effectors. The putative effectors were predicted computationally from the oomycete *Phytophthora infestans* genome for the presence of a signal peptide and of a RXLR translocation motif into plant cell (Birch et al., 2006; Kamoun, 2006). In an initial set of 54 candidates, two variants of the effector *ipiO*, *ipiO1* and *ipiO2*, were found to trigger HR-associated responses in *Solanum bulbocastanum*, a species carrying the late blight resistance gene *Rpi-blb1*. Both effectors were also found to induce HR responses in *Solanum stoloniferum*, which is the source of the *Rpi-blb1* homologs *Rpi-sto1* and *Rpi-pta1*. The resistance to *P. infestans* cosegregated with response to *IpiO* in *S. stoloniferum*, and *IpiO* was found to be the avirulence gene of the *Rpi-blb1* resistance gene. Based on these results and on the hypothesis that the resistance genes were orthologous or at least members of the same family, the authors cloned *Rpi-sto1* from *S. stoloniferum* and *Rpi-pta1* from *S. papita* by gene-capture PCR (Polymerase chain reaction). Both genes were found to be functionally equivalent to *Rpi-blb1* and are now used for selective breeding (Pankin et al., 2010).

Comparative genomics of phylogenetically proximal species helps to delineate genome evolution and should also be useful in designing rational strategies for plant disease management. The analytical potential of this approach was illustrated on these two aspects by several articles published in the Science review in 2010 (Dodds, 2010). One of these studies was based on the resequencing of six genomes of four sister species *Phytophthora infestans*, *P. ipomoeae*, *P. mirabilis* and *P. phaseoli* (Raffaele et al., 2010). These species infect diverse plants and form a tight clade of pathogens sharing 99.9% identity in their ribosomal DNA internal transcribed spacer region. The aim of the study was to determine how host jumps affect pathogen genome evolution. Genome sequencing allowed the identification of gene-sparse regions and gene-dense regions. Most pathogen genes and genome regions were found highly conserved. But more than 44% of the genes located in the gene-sparse regions showed high diversity suggesting signature of a rapid evolution, when only 14.7% of remaining genes show such signatures. Gene-dense regions were enriched in genes induced in sporangia. Gene-sparse regions were highly enriched in genes induced during plant infection, especially those encoding the predicted RXLR-containing effectors. This is in accordance with the hypothesis that genes induced *in planta* are supposed to evolve faster in a context of a co-evolution with the host. A similar strategy was developed to reveal pathogenicity determinants in two maize smut fungi, *Ustilago maydis* and *Sporisorium reilianum* (Schirawski et al., 2010). These two closely related Basidiomycetes species present an example of differentiation of two closely related pathogens parasitizing the same host. Both genomes were compared and variable genomics regions were identified. These regions

were supposed to contain genes encoding virulence proteins since one could expect that pathogen secreted effectors should rapidly evolve. On the other hand, both genomes comprise conserved effector genes as expected for pathogens infecting the same host. Eighty nine percent of the *U. maydis* putative effectors are conserved in *S. reilianum*. This statement could enable to target genomics regions involved in virulence on the same host plant and common to the Basidiomycetes. These studies illustrate how comparative genomics allow identifying the biological functions that are evolutionarily the most stable and that could be targeted to create more durable resistance.

Comparative genomics of more distal pathogenic species within a clade, that of the oomycetes, was also fruitful to define signatures associated with adaptation to a particular trait of life, the obligate biotrophy (Baxter et al., 2010; Spanu et al., 2010). The genome of the obligate biotrophic pathogen *Hyaloperonospora arabidopsidis* was sequenced. The identified gene functions were compared to those of three hemibiotrophic *Phytophthora*, *P. infestans*, *P. sojae* and *P. ramorum* (Baxter et al., 2010). Among a total of 14,543 predicted genes in *H. arabidopsidis*, 6882 had no identifiable orthologs in sequenced *Phytophthora* species. Those genes are potentially involved in biotrophic functions. On the other hand the genome of *H. arabidopsidis* showed a drastic reduction in the number of genes encoding enzymes for assimilation of inorganic nitrogen and sulfur, and proteins associated with zoospore formation and motility. Unsurprisingly, the drastic reduction also concerns genes involved in pathogenicity encoding for degradative enzymes (such as secreted proteinases or cell-wall degrading enzymes), for necrosis and ethylene-inducing (Nep1)-like proteins (NLPs) and for PAMPs. The *H. arabidopsidis* genome also exhibited no more than 134 potential effector proteins with RXLR cell translocation motifs that likely function to suppress host defenses while they have been found to be hundred in the *Phytophthora* genomes (Jiang et al., 2008; Tyler et al., 2006; Whisson et al., 2007). Only 36% of them showed significant similarity percentages with *Phytophthora* effectors. With the aim of obtaining specific targets of biotrophic oomycetes these genes could represent good candidates.

### 3. Ex planta biotic interactions and plant health

In Phytopathology the plant-pathogen interaction has caught for a long period the attention of most studies at the molecular level. The aim for controlling disease was to develop the scientific bases for genetic engineering of crops (breeding, genetically modified plants). However at least two other kinds of interactions occur at the host plant surface and are crucial for the disease outcome and also for the development of alternative crop protection strategies. Still today, too few studies deal with these two biotic interactions: (i) the cell-cell interaction governing, within the pathogenic species, the biology of the microorganism; (ii) the diverse interactions between the pathogen and the microbial community in their shared habitat. In support to this observation we investigated the features of literature on biotic interactions and plant disease outcome based on bibliometric means. The MEDLINE database was searched via the PubMed access for articles indexed under the publication type "Plant Fungus". Growth of the literature and thematic distribution were addressed. From 1980 to 2010, a total of 35,767 citations were retrieved dealing with a plant-fungus interaction. The literature growth rate is gradually and exponentially growing (Figure 2A). Throughout this period, studies on microbial community and on cell signaling in pathogenic fungi are scarce. These two topics represent respectively 1 % and 1.9% of the whole analyzed literature (Figure 2A and 2B), and for the topic "cell signaling", most of

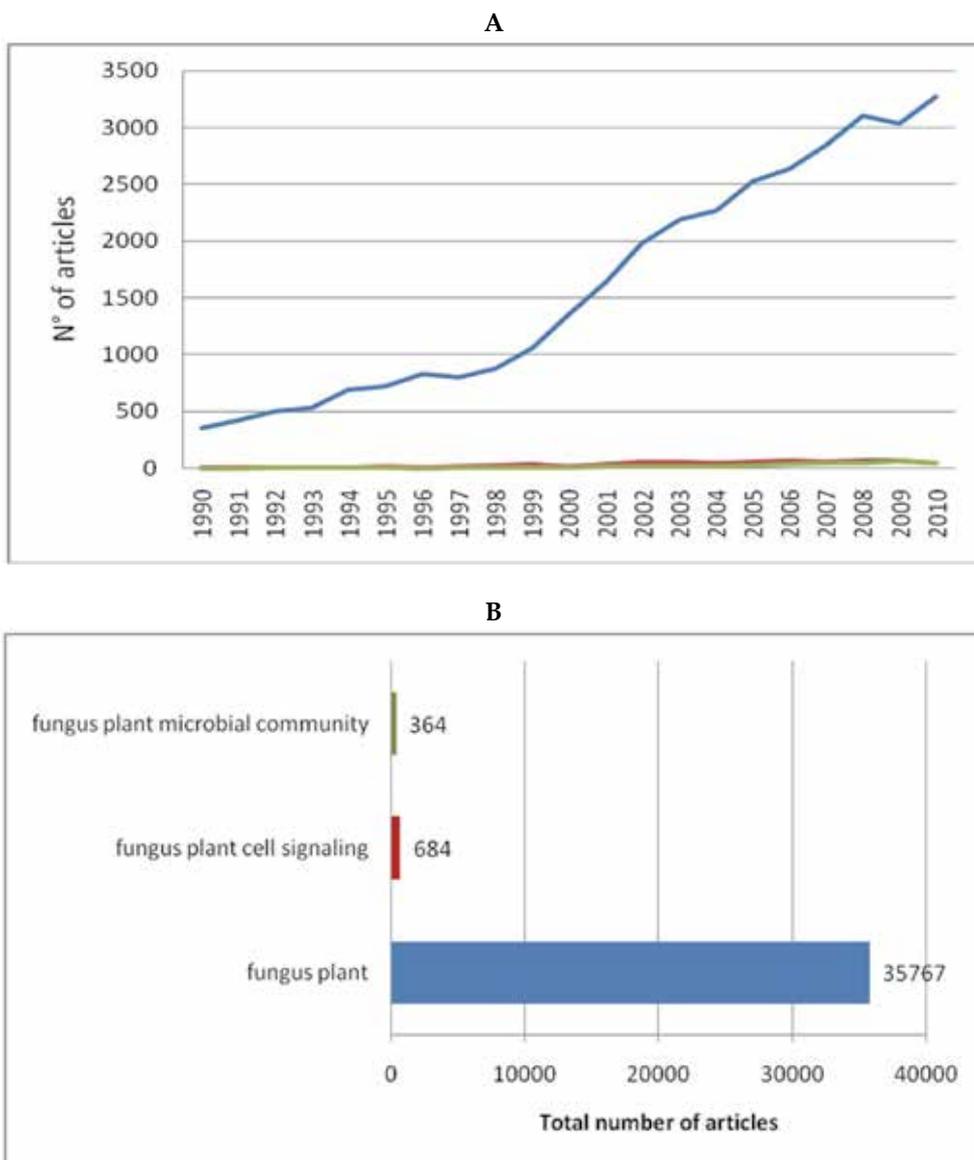


Fig. 2. Bibliometric of the literature on plants-fungi interactions (19. 02. 2011).

PubMed was used to access to the MEDLINE database for searching articles under the publication category “Fungus Plant” (blue), “Fungus Plant Microbial Community” (red), “Fungus Plant Cell Signaling” (red). The retrieved articles were counted and analyzed using Microsoft Excel. (A) growth of the Plant Fungus literature, 1999–2010. (B) Overall number of articles per category. The volume of the literature related to the “Fungus Plant Microbial Community” category is surely underestimated (a search for “Plant Fungus biological control” led to retrieve 1,729 articles but most of them however did not question microbial community as an entity). Similar results were obtained when the data was screened for the publication category “Plant Oomycete” (data not shown).

studies concern the host or fungus responses during the interaction with 46% of them strictly dealing with the plant responses. Thus investigations on interactions between cells of a pathogen and between pathogenic species and the other microorganisms sharing the same biotope are insufficient. Nevertheless, biology and microbial ecology of pathogens must offer opportunities to extend our knowledge on causal relationships between biotic interactions and the epidemiology of a disease. They also open new ways for development of new sustainable agro-ecosystems that should have both agricultural value, by preventing disease, and ecological value, by reducing environmental risks.

### **3.1 The cell-cell interaction within a pathogenic species**

#### **3.1.1 Target cell-to-cell signaling to slow microbial adaptation to treatment**

Before infection, cells, spores, zoospores, or mycelia from an eukaryotic pathogen live mainly in groups attached to surfaces, each biological entity interacting with its neighborhood. The influence of these interactions within the species have been until recently neglected, the pathogen being considered at the unicellular level for investigating the interaction with the host. However, prokaryotes and microbial eukaryotes naturally form multicellular aggregates in particular on the surface of putative hosts. The study of these aggregates has shown that microorganisms are capable of complex differentiation and behaviors. The cells communicate and cooperate to perform a wide range of multicellular behaviors, such as dispersal, foraging, biofilm formation, quorum sensing (Atkinson and Williams, 2009; West et al., 2007), these behaviors contributing to the virulence as well as to the dynamics of interactions with the host.

For phytopathogenic bacteria, it has been shown that aggregation promotes virulence in *Ralstonia solanacearum* (Kang et al., 2002), that quorum sensing regulates a variety of virulence factors in *Pectobacterium atrosepticum* (Liu et al., 2008) and that the transition from an aggregated lifestyle to a planktonic lifestyle promotes dissemination in *Xanthomonas campestris* (Dow et al., 2003). As a consequence, the molecular machinery for cell-to-cell signaling constitutes a novel target for the design of antagonists able of attenuating virulence through the blockade of bacterial cell-cell communication (Williams et al., 2000). As mentioned above, cell-to-cell signaling is not limited to the bacterial kingdom. Oomycetes produce and use molecules to monitor population density of biflagellate motile cells, the zoospores. These cells coordinated their communal behaviors by releasing, detecting, and responding to signal molecules (Kong and Hong, 2010). In the species *Phytophthora parasitica*, zoospores may form biofilms on the host surface, using a quorum sensing-like phenomenon to synchronize behavior (Galiana et al., 2008; Theodorakopoulos et al., 2011). Whether such cell-to-cell interactions contribute to the virulence of oomycetes or fungi is not known. However, the fact that, as bacteria, cells of eukaryotic pathogens cooperate to perform multicellular behaviors, indicate that from the dissection of related transduction pathways could emerge new tools for the management of cellular populations on the surface of host plants. Treatments against disease targeted to cell-cell signaling machinery could have an additional benefit and not the least, that to circumscribe the problem of pathogen resistance to fungicides for a larger period, to some extent at least. By performing modeling of multicellular organization in bacteria as a target for drug therapy to predict the speed of resistance evolution, André and Godelle (2005) concluded that this adaptation may be several orders of magnitude slower than in the case of resistance to usual antibiotics. The hypothesis of the authors makes sense in the context of the hierarchical selection theory (Gould, 2002). By targeting treatments

against adaptive properties of groups instead of individuals, the relevant unit of organization generating resistance and submitted to selection shifts one level up. Instead of facing billions of cells with a very rapid evolutionary rate, these alternate treatments face a reduced number of larger organisms with lower evolutionary potential (André and Godelle, 2005). Nevertheless, to our knowledge the molecules for such treatments are not yet available for eukaryotic pathogens, and anyway it would be advisable to be sure that they have not pleiotropic or toxic effects.

### **3.1.2 Biomimetism to trap pathogens**

The formation of biofilms is a widely spread property of microbial life governed by cell-cell signaling (Costerton et al., 1999; Danhorn and Fuqua, 2007; Hall-Stoodley et al., 2004; Harding et al., 2009). Biofilm generation is a high spot of research because these structures represent for pathogens an important influence on the virulence as well as on the dynamics of interactions with hosts (Costerton et al., 1999; Hall-Stoodley and Stoodley, 2005). They constitute microbial communities living in co-operative groups attached to surfaces and embedded in a self-producing polymeric matrix. Their formation involves first that planktonic (free-swimming or free-floating) cells become attached to a solid surface, leading to the formation of microcolonies, which then differentiate into exopolysaccharide-encased and fluidfilled channel-separated mature sessile biofilms. Biofilms confer several advantages to pathogens promoting attachment, dissemination or virulence and protecting cells against host defenses and biocide treatments. For human pathologies the failure to eradicate them by standard antimicrobial treatments results in several cases in development of chronic and nosocomial diseases (Costerton et al., 1999; Davies, 2003). The impact of biofilm persistence is not really appreciated for the epidemiology and management of plant diseases (Ramey et al., 2004). To our knowledge nothing is known about potential antimicrobial resistance mechanisms to thwart the efficiency of treatments with fungicides or bactericides. But researches on biofilm may offer an attractive option to diversify biologically-based alternatives to systematic treatments with synthetic fungicides. During the biogenesis of biofilms by an eukaryotic plant pathogen concomitant cellular processes are mobilized to synchronize cell behaviour: chemotaxis, adhesion and aggregation (Galiana et al., 2008; Theodorakopoulos et al., 2011). The elucidation of molecular aspects of these processes should help to elaborate biomimetic materials for the development of trapping systems for pathogens, exactly on the same principles than for the design of insect traps used for many years to monitor or reduce insect populations and based on behavioural confusion techniques (Silverstein, 1981).

### **3.2 The interactions of the pathogen within a microbial community**

In an ecosystem, a plant pathogen evolves within a microbial organized community which has a great influence on the local environment and disease. Before infection various species interact with the pathogen on the host surface shaping the distribution, density and genetic diversity of the inoculum. Such a community is considered and studied as a driving force for natural selection and pathogenicity (Kuramitsu et al., 2007; Siqueira and Rocas, 2009). Concomitantly present metagenomics studies of soils provide pictures of a community structure. The abundance distribution and total diversity can be deciphered. The analyses of the released datasets open a great opportunity to explore into the enormous taxonomic and functional diversity of environmental microbial communities (Simon and Daniel, 2011). By

combining studies on function and structure of soil communities, it becomes possible to increase our ability to modify disease states and to question practices of fungicides.

We considered here two levels.

The first one is to re-evaluate the analyses of suppressive soils. Pathogen-suppressive soils have been defined as soils in which the pathogen does not establish or persist, establishes but causes little or no damage, although the pathogen may persist in the soil (Cook and Baker, 1983). Examination of the microbial community compositions in soils possessing various levels of suppressiveness has been referred as a population-based approach (Borneman and Becker, 2007). The strategy leads to establish positive correlation between the population densities of some species and suppressiveness levels, suggesting that they may be involved in the disease suppressive process. The exploration of available metagenomic data will change the dimension of such analyses. As transcriptome analysis reveals gene networks for particular cellular functions, Metagenomics may help now to characterize microbial species networks for ecosystemic functions such as pathogen-suppressive properties of soils. This should help to reveal the huge potential of suppressive soils for managing soilborne pathogens. Characterization of the potential may be “easy” when biological nature of the suppression is known as illustrated by studies of soils with known chitinase and antifungal activities (Hjort et al., 2010). Metagenomics may also lead to screen uncultured microorganisms from soil which represent a potentially rich source of useful natural products. During the screening of seven different soil metagenomic libraries for antibacterially active clones, long-chain N-acyltyrosine-producing clones were found in each library. Of the 11 long-chain N-acyl amino acid synthases that were characterized, 10 were unique sequences. The heterologous expression of environmental DNA in easily cultured hosts as *Escherichia coli* has then been used by the authors to illustrate the access to previously inaccessible natural products (Brady et al., 2004).

The second level is more prospective. It consists in screening the functional diversity of microorganisms within communities in which pathogenic species evolve in respect to the disease outcome. In soil as in the other biotopes there is a myriad of microorganisms interacting with each other or with the environment, and performing a wide range of functions (organic decomposition, reduction/oxidation of different forms of elements, nitrogen-fixation...). The set of biotic interactions involving a pathogen constitutes a key factor for the natural population dynamics and emergence of pathogenic clones. In most cases this set remains uncharacterized and one great challenge for improving disease control is to identify in it the biotic interactions which contribute to the negative and also positive control of a pathogenic population. For this aim methods for screening microbial communities to select species associated with a pathogen and impacting the related host disease are missing and must be developed. As a contribution to resolve this problem we have developed a selection method and applied it to a soilborne plant pathogen, *Phytophthora parasitica*, for screening the microbial community from the rhizosphere of the host plant *Nicotiana tabacum*. Two of the selected microorganisms interfered with the oomycete cycle. An ascomycete strongly suppressed the tobacco shank disease and a ciliate promoted the disease (Galiana et al., unpublished results). In this case the efficiency of the method must be further tested by characterizing other species that affect the tobacco disease. It must also be evaluated for other eukaryotic pathogens before giving food for thought on disease control in two directions. Firstly, the identification of the key suppressive microorganisms will help to diversify material for biological control, a method which have been recommended to replace chemical control methods since it is more

economical and environmentally sustainable (Fravel, 2005; Herrera-Estrella and Chet, 1999; Shennan, 2008; Weller et al., 2002). The molecules supporting the suppressive activity of microbial species should be analyzed for their bio-fungicide properties and for their impact on human health and on the rest of the microbial environment. Secondly, the produced information will gradually allow revealing the set of species interacting with a pathogen. In the same time, their abundance in each soil, in each biotope could be easily determined through metagenomic approaches. Thus the combination of both parameters, richness and identity of microbial species affecting a disease cycle, should be an important consideration to define the status of the biotic environment with respect to the occurrence of an epidemic. It could be fruitful to define new decision-making tools that will have to be considered by farmers to decide serenely to restrict fungicide applications or not if required.

#### 4. Conclusion

How protect crops against diseases caused by fungi and oomycetes with both agricultural and ecological value? The treatment of this complex question combines a lot of parameters (crop rotation diversification, crop diversity, rationalization of N-fertiliser application, environment, climate, farmers practices...) mainly treated in the frame of integrated plant disease management. This chapter focuses only on what could emerge from studies on biotic interactions in plant pathology for contributing to the reduction of fungicide use, the development of alternative methods and the selection of crops more tolerant to diseases (Figure 3).

The concern about reduction of fungicides came forward very early from the advent of their use. Based on experimentations, in the lab first and then in the field, Millardet and Gayon (1888) recommended to winegrowers to use a Bordeaux mixture less rich in copper (II) sulphate and lime than in the first formulation. The new mixture was at once more adhesive on leaves than the former, without danger for the vineyard (which did not present any more foliage injuries), and more effective against the mildew. Today a trend to achieve significant fungicide reduction is to diminish frequencies rather than doses. The use of forecasting epidemics systems to assist in the timing of fungicide applications may be one of the appropriate tools. Fungicide treatments would be performed only when necessary. But this may be acceptable by farmers if the risk of an epidemic development of the disease is very low. With the increasing resources on several biotic parameters (timing for establishment of plant developmental resistance, dynamics of clones within a pathogenic population, presence and richness of microbial species affecting a disease cycle) there is an urgent need to associate and integrate the related number of variables to develop more refined and integrated models. They could serve as a starting point to carefully decide in which timing and in which biotic environment the gain from a reduced number of fungicide applications will not alter the potential risk of loss resulting from an incorrect control strategy. Another way of decreasing the frequencies is to combine fungicide treatments with exogenous applications of natural compounds stimulating plant defense responses. Studies on this subject appear scarce and few pieces of information are available on the efficiency of such approach.

Different aspects may also be considered for the development of alternative methods. In the field of genetics, greater possibilities now result from determination of crop plants and pathogens genomes for selecting new varieties of plants capable of resisting to eukaryotic pathogens. Functional and comparative genomics programs have expanded the resource of genes that can be used into crop species, as it has been recently illustrated through the development of new PAMP-recognition specificities in agricultural species or through

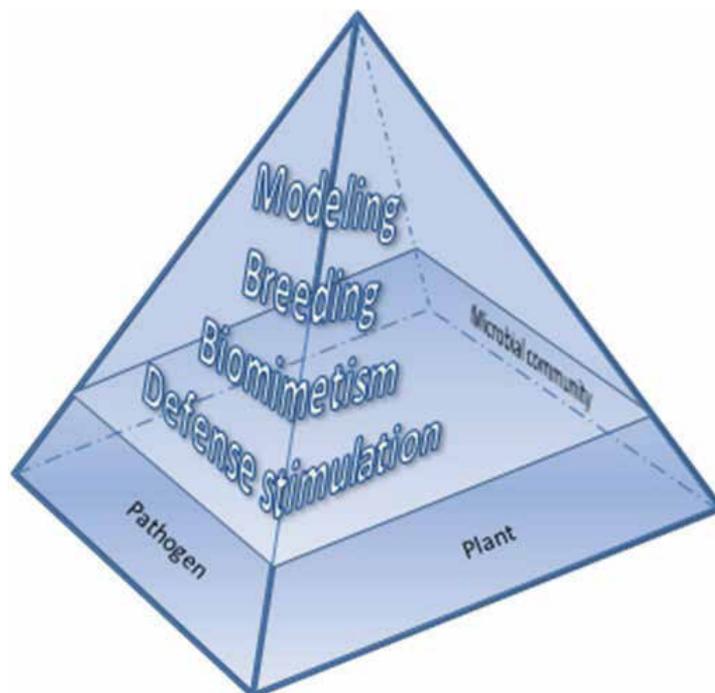


Fig. 3. Schematic representation of the interrelationships between studies of biotic interactions and innovations for crop protection.

The representation by a pyramid symbolizes the integration of different knowledge to develop and properly articulate plant disease management strategies with a low impact on environment and human health. At the bottom the quadrilateral frustum represents the different knowledge of biotic interactions on which may be built crop protection innovations. Three of four base edges mention the biological entities involved in these interactions and which were discussed in this chapter: plant, pathogen, microbial community. The fourth one represents the other biological entities which are important in the biotic environment of a plant (plant community, insects, nematodes,...) and that we did not consider here in the context of the control of disease caused by fungi or oomycetes. At the top of the pyramid are mentioned the topics of emergence of innovations in crop protection. There is no particular consideration for the location of each topic except for modeling at the apex of the pyramid. To our mind this means that robust mathematical models must integrate several biotic variables, often not still parameterized, for building exploitable forecast in terms of rationalization of crop protection.

screening of wild relatives of crop plants to identify new sources of resistance. In the field of biology of organisms, the possibility to elaborate biomimetic materials for the development of behavioural confusion techniques against pathogens must emerge from the molecular elucidation of chemotaxis and aggregation processes. This could lead to design local traps for pathogens associating molecules with specific attractive, aggregative and biocide properties. What is effective to control the populations of insects (pheromone-based trap, sticky fly traps,... ) and what was made possible by studies of molecular bases of the behavior of pest insects, must also be effective and possible for the control of pathogens. The validity of disease management by this way should be easily evaluated at the crop scale in hydroponic systems. Hydroponics as an agricultural production system is one of the fastest growing sector, which is more and more used to produce flowers, fruits or vegetable. "Sticky" pathogen traps could contribute to the sanitary quality of the nutrient circulating

solutions that is crucial in hydroponic systems. In the field of ecology, new ways could also emerge from exploitation of genomics and metagenomics data to manage pathogenic population in the greenhouse or in the field. Based on appropriate screening of microbial communities they will help to develop and to vary biological control strategies. Beyond the challenge to develop new strategies for crop protection, the biggest defy remains to associate, to articulate them in an adequate way in order to conciliate environmental concerns, safety for human health and agricultural imperativeness.

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# Comparative Study of the Mobility of Malathion, Attamix and Thiodan as Obsolete Pesticides in Colombian Soil

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## 1. Introduction

The organizations responsible for carrying out control and surveillance of pesticides in Colombia stored large quantities of them many years ago. They didn't know exactly the impact generated in that storage, but for the nature of the pesticides it is highly possible the incorporation of these hazardous substances in the soil, due the mobility throughout the leaching of the residue. This study worked with soils of the Colombian Agricultural Institute (ICA) because it is the organization in charge of the management of pesticides in Colombia. The research identified the mobility process by leaching of waste from the obsolete pesticides Thiodan, Attamix and Malathion, which according to previous researches (Duarte and Guerrero 2006 & Gonzalez 2011) these chemicals were stored in large quantities and the containers were deteriorated by weathering conditions.

In the investigation was simulated a spill at two concentrations: Event 1 (non diluted or commercial presentation), and Event 2 (agricultural recommended doses). Measures of the concentrations of leach pesticides at different depths into the soil (10, 17 and 25 cm) were determined using gas chromatography. The soils studied were taken from two sources Mosquera and Villavicencio branches of ICA.

## 2. Pesticides<sup>1</sup>

**Malathion 57% EC:** Malathion is an organophosphate insecticide that inhibits cholinesterase from insects. It is used worldwide to control a wide range of crop pests and control campaigns insect vectors that transmit diseases to humans: mosquitoes, bedbugs and mosquitoes. It is a brownish-yellow liquid that smells like garlic. (Proficol 2008), (ATSDR, 2008)

Molecular Formula:  $C_{10}H_{19}O_6PS_2$

Molecular Weight: 330.36

Active ingredient: Malathion S1, 2-di (etoxycarbonil) ethyl O, O-dimetilfosforoditioato, 604 g per liter of formulation to 20 oC.

**Attamix:** Its active ingredient is Chlorpyrifos. Also the pesticide is known as Dursban 23, which belongs to the family of pesticides, insecticides and organophosphates. It is white

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<sup>1</sup>This study was developed also by Ricardo Campos as a Co-Author (Faculty Member at La Salle University) and the research was funded by La Salle University

crystal like solid and strong aroma. In the United States, Attamix is used for residential use and it was allowed until 2000 when it was restricted by the United States Environmental Protection Agency (U.S. EPA). (ATSDR, 1997)

Molecular Formula:  $C_9H_{11}Cl_3NO_3PS$

Chemical Name: O, O – diethyl O – (3,5,6 – trichloro – 2.Pyridinyl) phosphorothioate

**Thiodan E.C.:** Is a pesticide that smells like turpentine, but it does not burn. It is used to control insects on crops and non-food grocery, and as a wood preservative.

Chemical Name: 6,7,8,9,10,10 – hexachloro – 1, 5,5 a, 6,9,9 a – hexahydro – 6, 9 – methane – 2,4,3 – benzodioxatienpin – 3 – Oxia

### 3. Leaching and mobility tests

Leaching is defined as the removal of a substance in a solid phase by a liquid phase which is in contact with it. The determination of the toxic characteristics of waste depends on the analytical method and therefore cannot generalize results if they have not established criteria for evaluating mobility of toxic compounds by experimental analysis.

The factors or variables that limit the leaching method to make related to hazardous waste are given by:

- Surface area of waste
- Nature of the fluid extractor
- Value leachate / waste

From the variables mentioned above have been developed leaching tests classified according to the renewal of the exhaust fluid into two categories: the extraction tests and dynamic tests. The extraction tests were used in this research.

#### 3.1 Mobility tests of a residue

The mobility test of a residue simulates infiltration conditions in the environment (soil). In the test: the waste infiltrates the soil and it can reacts with the components of that environment, causing public health risks due to environmental pollutants it absorbs; or do not react, but to infiltrate in large numbers so that the scope of such sources of groundwater. The methodology used for the mobility test was the procedure of the Colombian Institute Agustin Codazzi (IGAC) because it has a Reference Laboratories in this field in Colombia. It implies:

1. Sampling of the soil object of study.
2. Selection of specific method of sampling for the study. For the present study the sampling method can be seen in Figure 1. The method used was zig-zag due the number of samples to analyze at the laboratory.
3. Making a stripping of topsoil covering the ground.

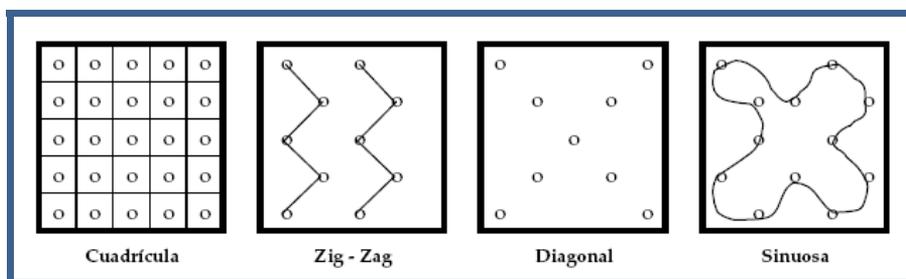


Fig. 1. Methodologies for soil sampling, Source. Codazzi. 2005.

### 3.2 Sampling for Mobility Analysis

To analyze the mobility, blocks of soil were taken from the fields of ICA. The soil block's dimensions were 25x25x25 cm. This height of the blocks was determined with a leaching test with water. In this test was spilled 1000 ml of water to calculate the deepest length. The next set of pictures in figure 2 shows the process to take the samples of soils.



Fig. 2. Soil Sampling.

### 3.3 Experimental Units

The areas of those units, according to the heights determined in the field test for the assembly were: 25 x 25 cm, and a maximum depth of 25 cm with a free margin of 3 cm. All had the same dimensions and were evaluated at three different depths, being able to observe the behavior of the insecticide into the soil. The units were made in acrylic material to observe the movement of the pesticide. The lower surface was perforated for the collection of the leaching material. Figure 3 shows the experimental units.



Fig. 3. Experimental Units.

### 4. Simulated spill of the insecticide

As was mentioned in the previous section was performed spills for each of the events: Event 1 (non diluted or commercial presentation), and Event 2 (agricultural recommended doses). For each depth were spilled different volumes. They were obtained according to pilot tests to collect representative volumes. Below are the photographs of spills, as well as tables 1 to 6, which have the volumes applied and volumes collected after the spills for each pesticide.



Fig. 4. Spill.

#### 4.1 Malathion

Sample Height (cm)	Volume Applied (ml)	Volume Obtained (ml)
25	1000	32.8
25	1000	42.4
25	1000	54.5
17	680	45.6
17	680	73.4
17	680	58.7
10	400	51.4
10	400	46.8
10	400	68.1

Table 1. Event 1

Sample Height (cm)	Volume Applied (ml)	Volume Obtained (ml)
25	1000	48.6
25	1000	43.4
25	1000	63.7
17	680	72.4
17	680	64.2
17	680	56.6
10	400	76.5
10	400	70.4
10	400	62.8

Table 2. Event 2

**4.2 Attamix**

Sample Height (cm)	Volume Applied (ml)	Volume Obtained (ml)
10	1002	35,2
10	1002	9,5
10	1002	14,7
17	1003,4	21,5
17	1003,4	20,5
17	1003,4	16,8

Table 3. Event 1

Sample Height (cm)	Volume Applied (ml)	Volume Obtained (ml)
10	1002	43,3
10	1002	-
10	1002	20,5
17	1003,4	47,5
17	1003,4	-
17	1003,4	48,5

Table 4. Event 2

### 4.3 Thiodan

Sample Height (cm)	Volume Applied (ml)	Volume Obtained (ml)
25	400	142
25	400	152.5
25	400	115
17	680	95
17	680	45
17	680	92
10	1000	100
10	1000	18
10	1000	110

Table 5. Event 1

Sample Height (cm)	Volume Applied (ml)	Volume Obtained (ml)
25	400.8	110
25	400.8	46.5
25	400.8	98
17	681.36	200
17	681.36	177
17	681.36	164
10	1002	184
10	1002	240
10	1002	201

Table 6. Event 2

## 5. Initial concentrations

### 5.1 Malathion

The maximum concentration at which the insecticide is in the market is 650000 mg/L. For the second event, was taken as the concentration the agricultural use specifications provided by the supplier, which was 604 mg/L.

### 5.2 Attamix

The commercial concentration of the pesticide had a value of 480000 mg/L. The concentration used for the second event was 960 mg/L of active ingredient chlorpyrifos.

### 5.3 Thiodan

The first event had a concentration of 350000 mg/L. The second event had as the concentration the value provided by the supplier as 0.7 mg/L.

## 6. Extraction

For all the samples obtained it was used a liquid - liquid extraction to isolate the pesticide from the samples and analyzed by gas chromatography. The methodology used was the described in the EPA 3510C method adjusted to the conditions of the Environmental Engineering Laboratory at the University of La Salle. The technique uses the dichloromethane ( $\text{CH}_2\text{Cl}_2$ ) as a solvent and the process is characterized by separating the active ingredient of impurities and water content. To carry out the procedure first was stabilized the solution at pH 7.0 with sodium hydroxide; then, the solution was mixed with three portions of 90 ml of dichloromethane and the extract was separate in a gravity separation funnel. The figure 5 presents the extraction procedure:



Fig. 5. Extraction

### 6.1 Gas chromatography

The following chromatographic conditions were established for the analysis of the pesticides:

Injection volume: 2.0 mL (splitless mode)

Injector temperature: 280 °C

Detector temperature: 280 °C

Carrier gas, nitrogen at a constant flow of 1.0 mL / min.

Fuel Gas: Air: 300 mL / min.

Hydrogen: 30 mL / min

Make up gas: helium: 35 mL / min.

Oven temperature: 140 ° C start for 1 min. then a gradient of 20 ° C / min. 220 ° C and a stay at this temperature for 2 min. then again performed a gradient of temperature at 5 ° C / min. to 280 ° C with a stay at this temperature for 5 min. for a total analysis time of 24 min.

## 7. Results

The next tables present the results obtained from the chromatography analysis:

### 7.1 Malathion

Event	Height (cm)	$\bar{X}$ Concentration (mg/L)
1	25	581475
1	17	600825
1	10	615600
2	25	439.66
2	17	452
2	10	466

Table 7. Malathion Results

According to the above table, were constructed Figures 6 and 7; each of them corresponding to events 1 and 2. The correlation shows that the concentration decreases in proportion to the height of soil analyzed. The soil has a greater retention of material than the liquid phase, which implies that the impact will be much more evident in the solid phase than in the liquid. On the other hand the concentrations are quite high.

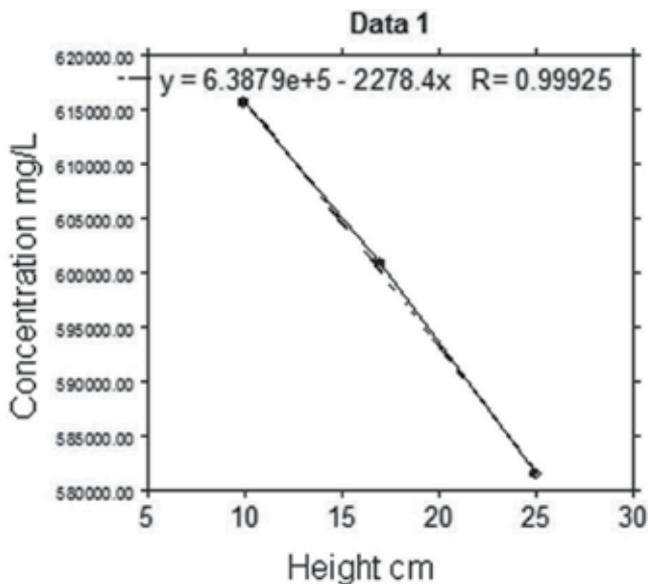


Fig. 6. Event 1

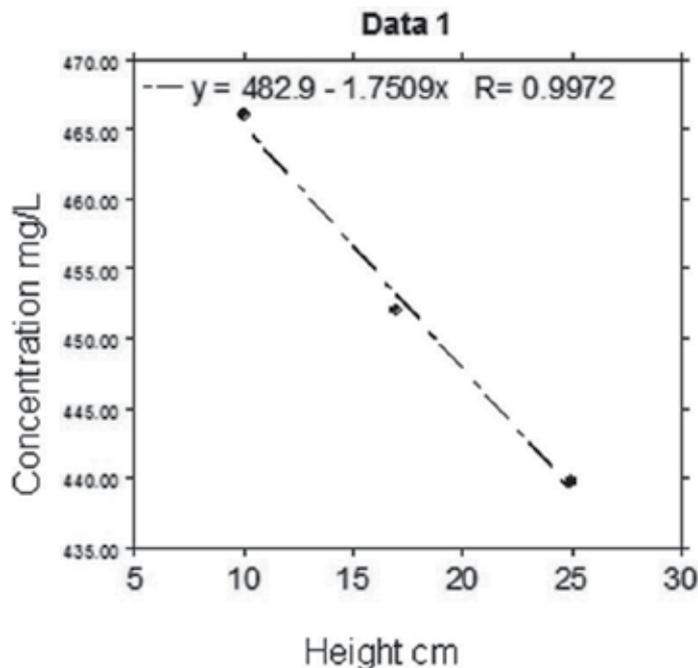


Fig. 7. Event 2

It is observed from the previous graphs that the degree of change in concentration of the pesticide in function to soil depth is significant. The graphs shows that the soil does not catch the pesticide enough. It means the pesticide has the ability to attack the biota present and generating highly toxic levels. For example, the LD50 is 1470 mg / kg oral rat, which implies that the current dose of stroke, 1 (one) liter of pesticide spilled, may die 600 rats of 600 g in weight. In the case of an adult rabbit weighing 4 kg, Malathion, which has a dermal LD50 of 5428, 28 animals die. Since the biotic point view these data have a strong importance.

## 7.2 Attamix

Event	Height (cm)	$\bar{X}$ Concentration (mg/L)
1	17	32.67
1	10	21.58
2	17	17.82
2	10	14.31

Table 8. Attamix Results

According to the above table were constructed the Figures 8 and 9, each corresponding to the different events. The concentration increases as the depth of soil sampled, quite contrary to the previous case. But the concentration is pretty low compared each event.

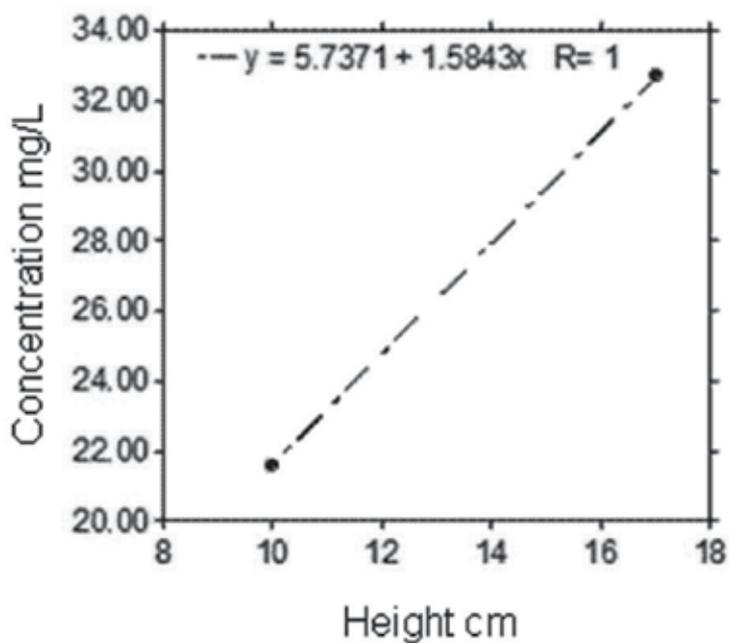


Fig. 8. Event 1

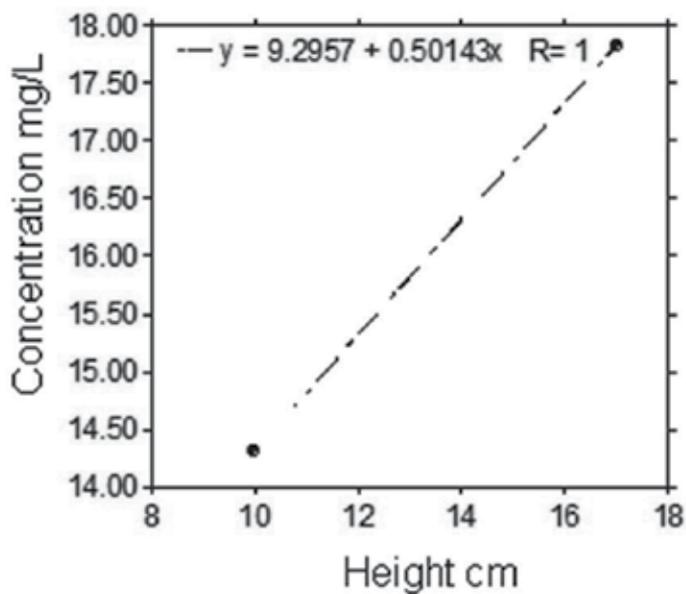


Fig. 9. Event 2

It is observed that the change in concentration of pesticide from events in function to soil depth is not significant. This suggests that the pesticide is highly related to soil because the pure pesticide leaching concentration is only almost twice as high concentration of diluted

pesticide. According to its data sheet, this pesticide is highly toxic to aquatic organisms (LC50/EC50 <0.1 mg/L in most sensitive species). The concentrations obtained by these two events generate mortality. For terrestrial organisms, where the pesticide is held, the first event with an approximately concentration of 900000 mg/L, the product is highly toxic to birds based on a diet (LC50 between 50 and 500 mg/L), causing their death.

### 7.3 Thiodan

Event	Height (cm)	$\bar{X}$ Concentration $\alpha$ -endosulfan (mg/L)	$\bar{X}$ Concentration $\beta$ -endosulfan (mg/L)
1	25	63.3	39.10
1	17	45.9	24.93
1	10	30.9	13.6
2	25	5.29	4.3
2	17	11.11	6.84
2	10	19.88	12.02

Table 9. Thiodan Results

Thiodan has as active ingredients the- and  $\beta$ - endosulfan. According to the above table were constructed figures 10 and 11. The coefficients show that the concentration increases in the first event in proportion to the height of soil analyzed, meaning that deepens as the ground has been a greater release of material, which implies that the impact will be much more evident in the liquid phase in the solid, which implies an impact on water resources.

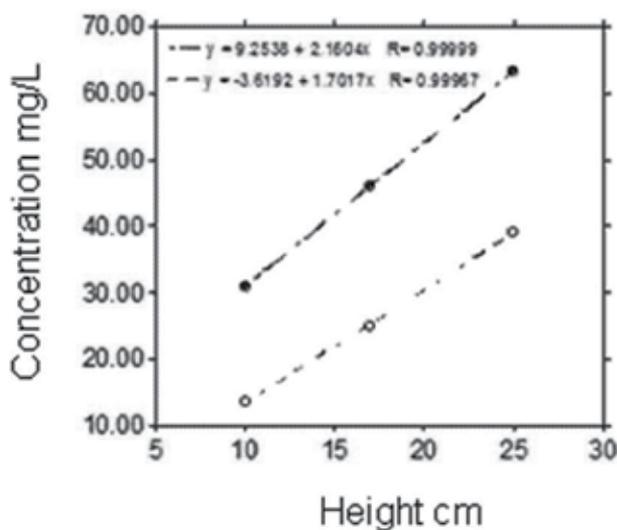


Fig. 10. Event 1. Black circle  $\alpha$ - Transparent circle  $\beta$ -

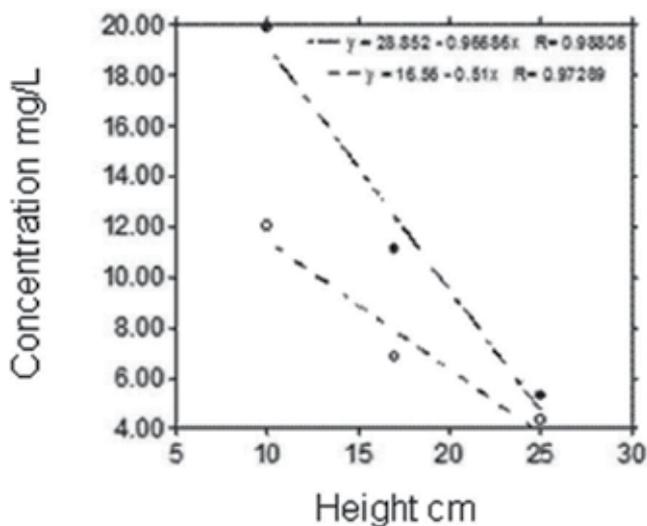


Fig. 11. Event 2. Black circle α- Transparent circle β-

The data from the second event presents a different phenomenon; it means that when the pesticide has more water content will favor retention in the solid phase as it delves into the less leach field.

Taking into account the concentration values reported in the leachate and the datasheet of the material, the lethal dose for biomarkers is 10 mg/kg. This indicates that all concentrations exceed the limits, except for the 25 cm diluted, so evidence that the potential risk of material, which in fact is prohibited in its entirety.

When is compared the leachate concentration with the initial concentration; is observed that the effect will be macro on soil because the material will be deposited into the soil. But the portion of leach is highly toxic yet.

## 8. Conclusions

Was evaluated the mobility of pesticide residues (Malathion, Thiodan And Attamix) in soils of the properties of ICA Mosquera and Villavicencio. For Malathion was found that the concentration decreases in proportion to the depth of the soil analyzed. It implies that the impact will be much more evident in the solid phase than in the liquid phase. The degree of change in concentration of the pesticide in function of the soil depth is representative, because the soil is not enough to catch all of the pesticide and it attacked the biota generating highly toxic levels.

In the case of Attamix the concentration increases as soil depth increases in a contrary way as the Malathion. But the retention of the pesticide is higher than from Malathion. It means that the leaching concentration is very lower than the initial concentration.

Were established concentrations of organochlorine and organophosphorus pesticides in the leachate showing differences between them. For the first event, Malathion about 600,000 mg/L, 28 mg/L for Attamix and 50 mg/L for Thiodan. For the second event 450 mg/L for Malathion, Attamix 15 mg/L and Thiodan and 10 mg/L. This indicates that Malathion is easily leached, while Attamix is preferably on the soil, as well as Thiodan.

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# Biodegradation of Pesticides

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## 1. Introduction

The rapidly growing industrialization along with an increasing population has resulted in the accumulation of a wide variety of chemicals. Thus, the frequency and widespread use of man-made "xenobiotic" chemicals has led to a remarkable effort to implement new technologies to reduce or eliminate these contaminants from the environment. Commonly-used pollution treatment methods (e.g. land-filling, recycling, pyrolysis and incineration) for the remediation of contaminated sites have also had adverse effects on the environment, which can lead to the formation of toxic intermediates (Debarati et al., 2005). Furthermore, these methods are more expensive and sometimes difficult to execute, especially in extensive agricultural areas, as for instance pesticides (Jain et al., 2005). One promising treatment method is to exploit the ability of microorganisms to remove pollutants from contaminated sites, an alternative treatment strategy that is effective, minimally hazardous, economical, versatile and environment-friendly, is the process known as bioremediation (Finley et al., 2010).

Thereafter, it was discovered that microbes have the ability to transform and/or degrade xenobiotics, scientists have been exploring the microbial diversity, particularly of contaminated areas in search of organisms that can degrade a wide range of pollutants.

Hence, biotransformation of organic contaminants in the natural environment has been extensively studied to understand microbial ecology, physiology and evolution due to their bioremediation potential (Mishra et al., 2001). The biochemical and genetic basis of microbial degradation has received considerable attention. Several genes/enzymes, which provide microorganisms with the ability to degrade organopesticides, have been identified and characterized.

Thus, microorganisms provide a potential wealth in biodegradation. The ability of these organisms to reduce the concentration of xenobiotics is directly linked to their long-term adaptation to environments where these compounds exist. Moreover, genetic engineering may be used to enhance the performance of such microorganisms that have the preferred properties, essential for biodegradation (Schroll et al., 2004).

About 30% of agricultural produce is lost due to pests. Hence, the use of pesticides has become indispensable in agriculture.

The abusive use of pesticides for pest control has been widely used in agriculture. However, the indiscriminate use of pesticides has inflicted serious harm and problems to humans as

well as to the biodiversity (Gavrilescu, 2005; Hussain et al., 2009). The problem of environmental contamination by pesticides goes beyond the locality where it is used. The agricultural pesticides that are exhaustively applied to the land surface travel long distances and can move downward until reaching the water table at detectable concentrations, reaching aquatic environments at significantly longer distances. Therefore, the fate of pesticides is often uncertain; they can contaminate other areas that are distant from where they were originally used. Thus, decontaminating pesticide-polluted areas is a very complex task (Gavrilescu, 2005).

Organochloride pesticides are synthetic and were widely used in the 1970s, mainly in the United States (<http://www.epa.gov/history/topics/ddt/02.htm>, accessed in May 2011). Although their use has been banished in many countries, they are still used in developing countries. Organochloride pesticides are cumulative in the organisms and pose chronic health effects, such as cancer and neurological and teratogenic effects (Vaccari et al, 2006). Many xenobiotic compounds are recalcitrant and resistant to biodegradation, especially the organochloride pesticides (Diaz, 2004; Dua et al., 2002; Chaudhry & Chapalamadugu, 1991). In general, these highly toxic and carcinogenic compounds persist in the environment for many years.

Organophosphorus pesticides are actually more widely used in the United States ([http://www.chemicalbodyburden.org/cs\\_organophos.htm](http://www.chemicalbodyburden.org/cs_organophos.htm), accessed in May 2011). These pesticides affect the nervous system of insects and humans, in addition to influencing the reproductive system (Colosio et al., 2009; Jkanovic & Prostran, 2009). These chemical agents block the prolonged inhibition of the cholinesterase enzyme activity. These chemical agents block prolonged inhibition the activity of the enzyme cholinesterase (ChE), responsible for the nervous impulse in organisms (Yair et al., 2008). The excessive use of organophosphorus in agriculture has originated serious problems in the environment (Singh & Walker, 2006). Although, these pesticides degrade quickly in water, there is always the possibility that residues and byproducts will remain, in relatively harmful levels in the organisms (Silva et al., 1999; Ragnarsdottir, 2000).

Carbamate pesticides are important in the agriculture due to their broad activity spectrum. In addition to a wide range of compounds, they are relatively degraded and generally have a low degree of toxicity to humans (Wolfe et al., 1978). However, they inhibit the enzyme acetylcholinesterase, therefore they are considered toxic to humans. The inhibition of the hydrolysis reaction of acetylcholine (ACh) results in the accumulation of ACh, causing various symptoms, such as sweating, lacrimation, hypersalivation and convulsion of extremities (Suzuki & Watanabe, 2005).

Decontamination of pesticide-infested environments is a difficult matter and can be very costly. In fact, the damages from pesticides in the environment are practically irreparable. Any measure used to decrease the effects of pesticides in the environment will always be a palliative solution and never definitive for the problems caused. Regrettably, there is always irreparable damage to the organisms and the environment, as for instance, the extinction of bird species and microorganisms in the world.

The biological methods are advantageous to decontaminate areas that have been polluted by pesticides. These methods consider the thousands of microorganisms in the environment that in order to survive seek for alternatives to eliminate the pesticides that were sprayed. Many native microorganisms develop complex and effective metabolic pathways that permit the biodegradation of toxic substances that are released into the environment. Although the

metabolic process is lengthy, it is a more viable alternative for removing the sources of xenobiotic compounds and pollution (Diaz, 2004; Schoefs et al., 2004; Finley et al., 2010).

On account of the grave risks synthetic pesticides pose to the organisms, there is an incessant search for pesticide safety and for the development of sustainable agriculture. The biological pesticides are based on natural compounds that effectively control the infestation of pests in agriculture. The advantage is that, contrary to synthetic pesticides, they are efficient and do not cause collateral damage (Fravel, 2005; Gerhardson, 2002; Raaijmakers et al., 2002).

The scope of this work demonstrates the use of the degradation of pesticides using microorganisms. This topic is inexhaustible and we are going to underscore the most recent points, including studies on the biodegradation of organochloride, organophosphorus and carbamate pesticides by microbiological process. Afterwards, in perspective, this chapter will show the use of natural pesticides in the biological control of pests.

## 2. Biodegradation

According to the definition by the International Union of Pure and Applied Chemistry, the term biodegradation is "Breakdown of a substance catalyzed by enzymes *in vitro* or *in vivo*. This may be characterized for the purpose of hazard assessment such as:

1. Primary. Alteration of the chemical structure of a substance resulting in loss of a specific property of that substance.
2. Environmentally acceptable. Biodegradation to such an extent as to remove undesirable properties of the compound. This often corresponds to primary biodegradation but it depends on the circumstances under which the products are discharged into the environment.
3. Ultimate. Complete breakdown of a compound to either fully oxidized or reduced simple molecules (such as carbon dioxide/methane, nitrate/ammonium and water). It should be noted that the biodegradation products can be more harmful than the substance degraded." (<http://sis.nlm.nih.gov/enviro/glossaryb.html>, accessed in May 2011; <http://www.epa.gov/OCEPATERMS/bterms.html>, accessed in May 2011).

Microbial degradation of chemical compounds in the environment is an important route for the removal of these compounds. The biodegradation of these compounds, i.e., pesticides, is often complex and involves a series of biochemical reactions. Although many enzymes efficiently catalyze the biodegradation of pesticides, the full understanding of the biodegradation pathway often requires new investigations. Several pesticide biodegradation studies have shown only the total of degraded pesticide, but have not investigated in depth the new biotransformed products and their fate in the environment.

### 2.1 Organochlorine pesticides

#### 2.1.1 Introduction

The organochlorine pesticides are known to be highly persistent in the environment. This class of pesticides includes the chlorinated derivatives of diphenyl ethane (dichlorodiphenyltrichloroethane - DDT, its metabolites dichlorodiphenyldichloroethylene - DDE, dichlorodiphenyldichloroethane - DDD, and methoxychlor), hexachlorobenzene (HCB), the group of hexachlorocyclohexane ( $\alpha$ -HCH,  $\beta$ -HCH,  $\gamma$ -HCH,  $\delta$ -HCH, or lindane), the group of cyclodiene (aldrin, dieldrin, endrin, chlordane, nonachlor, heptachlor and

heptachlor-epoxide), and chlorinated hydrocarbons (dodecachlorine, toxaphene, and chlordecone), (Menone et al., 2001; Patnaik, 2003). Figure 1 shows some structures of organochlorine pesticides.

Unlike the organophosphate and the carbamate pesticides, the toxic properties of the organochlorine pesticides are not very similar (Matolcsy et al., 1988). Although the toxicological properties are analogous to organochlorines with similar structures, like heptachlor and chlordane, the toxicological degree can vary by substituting a chlorine in the molecule. For instance, the substitution of chlorine atoms in the DDT ring for a methoxide group decreases the toxicity (Patnaik, 2003).

DDT is the most well known pesticide from the organochlorine group. The use of organochlorine pesticides started in 1939, when Paul Hermann Müller realized that the DDT, first synthesized by Othmar Zeidler in 1874, was an efficient insecticide (Matolcsy et al., 1988). The DDT's high efficiency, its low water solubility, its high persistence in the environment and its mode of action, unknown until that moment, contributed to the increasing use of DDT (Konradsen et al., 2004).

The industrial manufacture of DDT is based on the synthesis described by Zeidler. Chloral, chloral alcoholate or chloral hydrate is reacted with chloro-benzene in the presence of sulfuric acid, oleum or chlorosulfonic acid. The products obtained from the synthesis reaction contain several impurities, including the *ortho-para* [1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane] and *ortho-ortho* [1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*o*-chlorophenyl)ethane] isomers of DDT, and 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethane (*p,p'*-DDD), its *ortho-para* isomer, 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane (*o,p'*-DDD). The 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane (*p,p'*-DDT), is about 70% of the product mixtures (Matolcsy et al., 1988).

The *p,p'*-DDT is resistant to light, atmospheric oxygen and weak inorganic acids, but is rapidly decomposed to the biologically inactive *p,p'*-DDE (Matolcsy et al., 1988; Ahrens & Weber, 2009). During the World War II, powder DDT was pulverized on the population's skin to prevent epidemics of typhus transmitted by lice. The insecticide was also used in other countries to control the malaria-bearing mosquitoes (Konradsen et al., 2004). The use of DDT to control malaria bearing mosquitoes earned Müller the 1948 Nobel Prize in Medicine. After the war, the use of DDT was adopted as an agricultural pesticides (Benn & McAuliffe, 1975; Ottaway, 1982; Mariconi, 1985), the results were so impressive, that its use continued for 25 to 30 years in most countries. The problem occurred when DDT, like most organochlorine, reduced its efficiency, forcing the use of higher dosages. Consequently, large specialized laboratories sought to develop formulas which were characterized by greater efficiency and biodegradability (Turk, 1989).

At the end of the 1950s, the biologist Rachel Carson began to gather examples of environmental damages attributed to DDT (D'Amato et al., 2002). Between 1970 and 1980, DDT agricultural use was banned in most developed countries. In 2004, the Stockholm Convention, outlawed several persistent organic pollutants, such as aldrin, dieldrin, endrin, toxaphene, mirex and heptachlor, and restricted DDT use to vector control (Ahrens & Weber, 2009; Arisoy & Kolankaya, 1998). In 2009, lindane and chlordecone were added to the outlawed list by the Fourth Conference of the Parties (Ahrens & Weber, 2009).

Although most organochlorine were banned from some countries, organochlorine pesticides are still widely studied due to their recalcitrant nature, that is, even after years since the use has been banned, organochlorine contaminated sites are not rare. Not to mention, that the DDT use is still allowed to control malaria bearing mosquitoes, even though, narrowly.

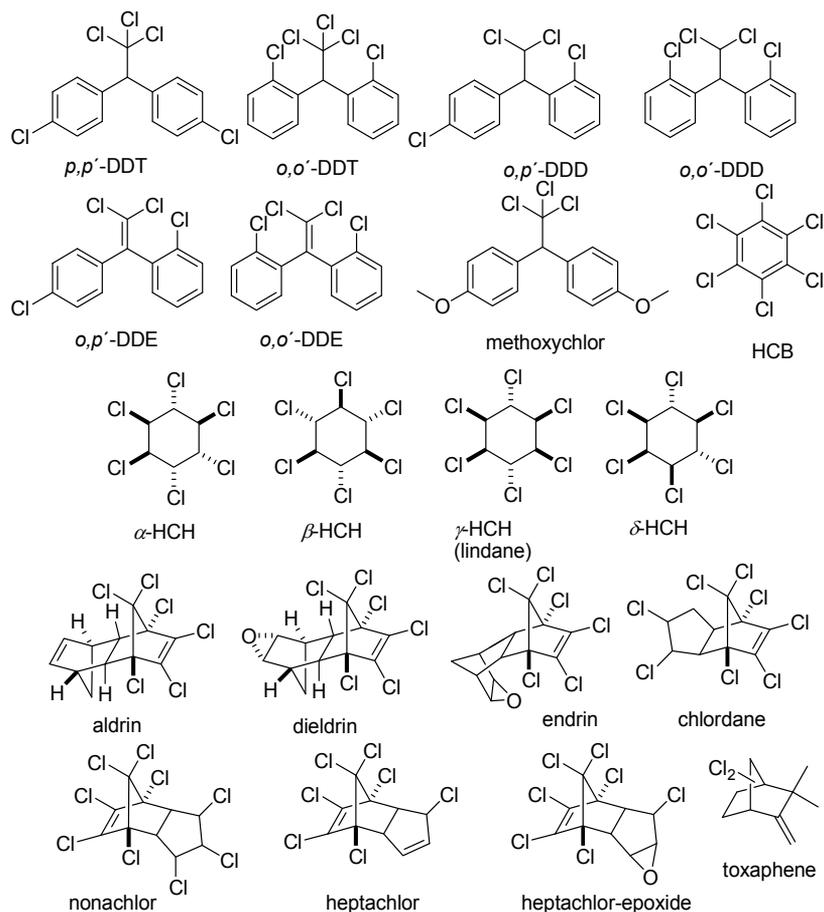


Fig. 1. Structures of organochlorine pesticides

### 2.1.2 Microbial degradation of organochloride pesticides

The fate of pesticides in the environment is determined by both biotic and abiotic factors. The rate at which different pesticides are biodegraded varies widely. Some pesticides such as DDT and dieldrin have proven to be recalcitrant. Consequently, they remain in the environment for a long time and accumulate into food chains for decades after their application to the soil (Kannan et al., 1994).

Most of the studies involving the biodegradation of organochlorine pesticides are done in pure cultures. The culture is usually isolated from a soil sample, generally contaminated with organochlorine pesticides. The strains are characterized and tested with different concentrations of the pesticide studied. DDT-metabolising microbes have been isolated from a range of habitats, including animal feces, soil, sewage, activated sludge, and marine and freshwater sediments (Johnsen, 1976; Lal & Saxena, 1982; Rochkind-Dubinsky et al., 1987).

The degradation of organochlorine pesticides by pure cultures has been proven to occur *in situ*. Nature magazine published one of the pioneer works. Matsumura et al. (1968) were able to evidence the breakdown of dieldrin in the soil by a *Pseudomonas* sp. The bacteria strain was isolated from a soil sample from the dieldrin factory yards of Shell Chemical

Company near Denver, Colorado. Later, in 1970, authors showed the biodegradation of aldrin, endrin and DDT with bacteria that were shown to be able to degrade dieldrin (Patil et al., 1970).

Biodegradation of DDT residues largely involves co-metabolism, that is, it requires the presence of an alternative carbon source, in which microorganisms growing at the expense of a substrate are able to transform DDT residues without deriving any nutrient or energy for growth from the process (Bollag & Liu, 1990).

Under reducing conditions, reductive dechlorination is the major mechanism for the microbial conversion of both the *o,p'*-DDT and *p,p'*-DDT isomers of DDT to DDD (Fries et al., 1969). The reaction involves the substitution of an aliphatic chlorine for a hydrogen atom. Using metabolic inhibitors together with changes in pH and temperature, Wedemeyer (1967) found that discrete enzymes were involved in the metabolism of DDT by *Aerobacter aerogenes*. The suggested pathway for the anaerobic transformation of DDT by bacteria is shown in Figure 2. Degradation proceeds by successive reductive dechlorination reactions of DDT to yield 2,2-bis(*p*-chlorophenyl)ethylene (DDNU), which is then oxidised to 2,2-bis(*p*-chlorophenyl)ethanol (DDOH). Further oxidation of DDOH yields bis(*p*-chlorophenyl)acetic acid (DDA) which is decarboxylated to bis(*p*-chlorophenyl)methane (DDM). DDM is metabolized to 4,4'-dichlorobenzophenone (DBP) or, alternatively, may undergo cleavage of one of the aromatic rings to form *p*-chlorophenylacetic acid (PCPA). Under anaerobic conditions DBP was not further metabolized (Pfaender & Alexander, 1972). Through an investigation of the co-metabolism of DDT metabolites by a number of fungi (Subba-Rao & Alexander, 1985) were able to substantiate the pathway proposed by Wedemeyer (1967). There has been one report describing the conversion of DDE to 1-chloro-2,2-bis(*p*-chlorophenyl)ethylene - DDMU by bacteria (Masse et al., 1989).

Some studies have presented notable results on the biodegradation of organochlorine pesticides. Table 1 presents some of the microorganisms that were able to degrade organochlorine pesticides. Among microorganisms, bacteria comprise the major group involved in organochlorine degradation, especially soil inhabitants belonging to genera *Bacillus*, *Pseudomonas*, *Arthrobacter* and *Micrococcus* (Langlois et al., 1970). In order to predict some of the factors that influence the capacity of biodegradation of DDT by a *Sphingobacterium* sp., Fang et al. (2010), studied the biodegradation at different temperatures, pHs, concentrations of DDT and, with an additional source of carbon. Results of the experience showed that the degradation rates were proportional to the concentrations of *p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDD and *p,p'*-DDE ranging from 1 to 50 mg.L<sup>-1</sup>. The ability of *Sphingobacterium* sp. to degrade DDTs was somewhat inhibited by DDTs at the level as high as 50 mg.L<sup>-1</sup>. According to the authors, this may be due to the fact that DDTs at high concentration are toxic to *Sphingobacterium* sp. and inhibit degradation. The experiment was also tested for different pHs, it was tested for pH 5, 7 and 9. The results indicated that a neutral condition is favorable for the degradation of DDT by *Sphingobacterium* sp., whereas higher or lower pH inhibits degradation. The influence of the temperature on the biodegradation was investigated by performing the experiments at temperatures of 20, 30 and 40 °C. The results indicated that the optimum temperature for the biodegradation of DDTs by a *Sphingobacterium* sp. in pure culture was at 30 °C. Ultimately, the biodegradation was available with an additional carbon source and results showed that the degradation half-lives of DDTs in the presence of glucose, yeast extract, sucrose, and fructose were significantly shorter than those in the treatment without an additional carbon source; and that the presence of glucose generates the fastest degradation of DDTs (Fang et al., 2010).

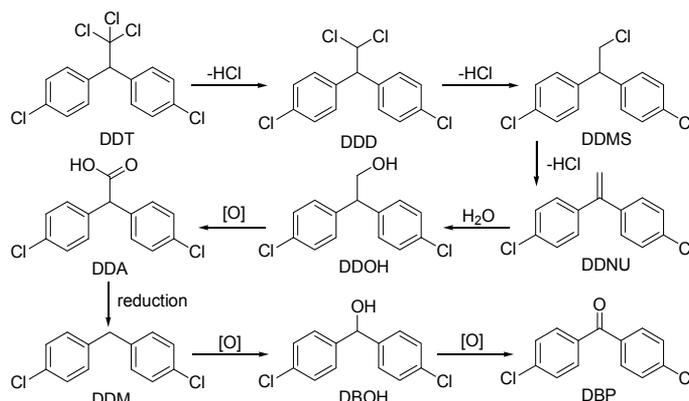


Fig. 2. Proposed pathway for bacterial metabolism of DDT (Adapted from Aislabie et al., 1997).

Results obtained *in vitro* can be applied to contaminated sites for further investigation on the capacity of a microorganism to degrade an organochlorine pesticide. As a continuation of the study proposed by Fang et al. (2010), the bacteria that was evidenced to degrade DDT was applied to field soils after different treatments. The soil known to be contaminated with DDT was studied in four different conditions, the control, which did not receive any treatment; PV, the same soil, only with pumpkin vegetation; DI received inoculation of the *Sphingobacterium* sp. and; PVDI which was the contaminated soil with the pumpkin vegetation and inoculation with *Sphingobacterium* sp. The concentration of *p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDD and *p,p'*-DDE was measured from each soil sample after 90 days, and was then compared to the initial concentration. Analysis indicated that the removal percentages of *o,p'*-DDT and *p,p'*-DDE in the PVDI treatment were statistically significantly higher (Fang et al., 2010).

According to Aislabie & Jones (1995) and Aislabie et al. (1997), the microbial degradation of DDT in soil apparently proceeds by a pathway analogous to that proposed by Wedemeyer (1967), (Figure 2). Under anaerobic conditions the first and major biotransformation product of DDT is DDD, with minor levels of DDA, DDM, DDOH, DBP, and DDE being detected (Guenzi & Beard, 1967; Mitra & Raghu, 1988; Xu et al., 1994; Boul et al., 1994). Reports of biodegradation of DDE in soil are rare, although, Agarwal et al. (1994) described the isolation of DDMU as a biotransformed product of DDE.

Studies with fungi have also evidenced the biodegradation of organochlorine pesticides. Ortega et al. (2011) evaluated marine fungi collected off the coast of São Sebastião, North of São Paulo State, Brazil. The fungi strains were obtained from marine sponges. The fungi *Penicillium miczynskii*, *Aspergillus sydowii*, *Trichoderma* sp., *Penicillium raistrickii*, *Aspergillus sydowii* and *Bionectria* sp. were previously tested in solid culture medium containing 5, 10 and 15 mg of DDD. The tests were also carried out with liquid medium in a rotary shaker, with the same amount of DDD per 100 mL liquid medium. The results showed that the fungi *P. miczynskii*, *A. sydowii* and *Trichoderma* sp. presented good growth in the presence of the pesticide. For further experiments *Trichoderma* sp. was selected as the standard microorganism, as it showed the best resistance to DDD in both solid and liquid medium (Ortega et al., 2011). In the experiments where DDD pesticide was concomitantly added into the growth of *Trichoderma* sp., 21% of the pesticide was degraded. The addition of H<sub>2</sub>O<sub>2</sub> in the experiment promoted a degradation increase (75%). In the experiments where DDD was added after 5 days of *Trichoderma* sp. growth, and with the addition of H<sub>2</sub>O<sub>2</sub>, the total biodegradation occurred (Ortega et al., 2011). Many factors can affect the biodegradation, as described earlier, as for instance the presence of H<sub>2</sub>O<sub>2</sub> increases the efficiency of the DDD degradation by *Trichoderma* sp.

Pesticides	Toxicity <sup>1</sup>	Microorganisms	References
PCP	Class Ib	<i>Arthrobacter</i> sp. <i>Flavobacterium</i> sp.	(Stanlake & Finn, 1982) (Crawford & Mohn, 1985)
1,4-Dichlorobenzene	Class II	<i>Pseudomonas</i> sp.	(Spain & Nishino, 1987)
DDT	Class II	<i>Aerobacter aerogenes</i> <i>Trichoderma viridae</i> <i>Pseudomonas</i> sp. <i>Micrococcus</i> sp. <i>Arthrobacter</i> sp. <i>Bacillus</i> sp. <i>Pseudomonas</i> sp. <i>Sphingobacterium</i> sp.	(Wedemeyer, 1966) (Patil et al., 1970) (Patil et al., 1970) (Kamanavalli & Ninnekar, 2005) (Fang et al., 2010)
Lindane	Class II	<i>Basa thiooxidans</i> <i>Sphingomonas paucimobilis</i> <i>Streptomyces</i> sp. <i>Pleurotus ostreatus</i>	(Pesce & Wunderlin, 2004) (Pesce & Wunderlin, 2004) (Benimeli et al., 2008) (Rigas et al., 2005)
DDE	n.i.	<i>Phanerochaete chrysosporium</i>	(Bumpus et al., 1993)
DDD	n.i.	<i>Trichoderma</i> sp.	(Ortega et al., 2011)
Heptachlor epoxide	n.i.	<i>Phlebia</i> sp.	(Xiao et al., 2011)
Heptachlor	O	<i>Phanerochaete chrysosporium</i> <i>Phlebia</i> sp.	(Arisoy & Kolankaya, 1998) (Xiao et al., 2011)
Toxaphene	O	<i>Bjerkandera</i> sp. <i>Trichoderma viridae</i>	(Lacayo et al., 2006) (Patil et al., 1970)
Aldrin	O	<i>Pseudomonas</i> sp. <i>Micrococcus</i> sp. <i>Bacillus</i> sp. <i>Trichoderma viridae</i> <i>Pseudomonas</i> sp.	(Patil et al., 1970) (Patil et al., 1970) (Patil et al., 1970) (Patil et al., 1970) (Patil et al., 1970)
Endrin	O	<i>Micrococcus</i> sp. <i>Arthrobacter</i> sp. <i>Bacillus</i> sp.	(Patil et al., 1970) (Patil et al., 1970) (Patil et al., 1970)
Dieldrin	O	<i>Pseudomonas</i> sp.	(Matsumura et al., 1968)

Table 1. Microorganisms involved in degradation of organochlorine pesticides.

<sup>1</sup>WHO Recommended classification of pesticides by Hazard. Class I is subdivided into two other classifications: Class Ia (extremely hazardous) and Class Ib (highly hazardous), Class II are the moderately hazardous, Class III are slightly hazardous and Class u, are unlikely to present acute hazard. The use of some pesticides have been discontinued and are classified as obsolete (O), (WHO, 2009).

n.i. - not informed

Studies involving the biodegradation of polychlorinated biphenyls (PCBs), used as a pesticide extender, have also been conducted, several isolated microorganisms have been proven to be capable of aerobically degrade PCBs, preferentially degrading the more lightly chlorinated congeners. These organisms attack PCBs via 2,3-dioxygenase pathway, converting PCBs to the corresponding chlorobenzoic acids. These chlorobenzoic acids can then be degraded by indigenous bacteria, resulting in the production of carbon dioxide, water, chloride, and biomass (Abramowicz, 1995). Anaerobic bacteria attack more highly chlorinated PCB congeners through reductive dechlorination. In general, this microbial process removes preferentially the *meta* and *para* chlorines, resulting in a depletion of highly chlorinated PCB congeners with corresponding increases in lower chlorinated, *ortho*-substituted PCB congeners (Abramowicz, 1995).

Despite the evidence that microorganisms with the ability to degrade DDT are resident in soil, its residues persist. The studies here presented showed that anaerobic conditions are beneficial to dechlorination of DDT, and additional carbon and hydrogen peroxide favors the biodegradation of some organochlorines. The decomposition rate depends on conditions in the soil and the bonding of the pesticide to soil surfaces. For most pesticides, aerobic decomposition proceeds much faster than anaerobic decomposition; however, there are classic exceptions to this, for instance DDT, whose decomposition proceeds ten times faster under anaerobic conditions (Scott, 2000).

Farm management practices also affect the rate at which pesticides are degraded. Irrigation of soils has been shown to enhance degradation of DDT to DDD, thought to be due to the creation of anaerobic microsites (Aislabie & Jones, 1995).

Due to the recalcitrant nature of most organochlorines, many such pesticides are still widely studied in order to find mechanisms that enhance their biodegradation in the environment. Genetic techniques can contribute to elucidate biochemical pathways involved in the microbial degradation of organochlorines, which represent promising alternatives towards developing highly efficient strains as well as the isolation and application of enzymes potentially involved in biodegradation.

## **2.2 Organophosphate pesticides**

### **2.2.1 Introduction**

Currently, among the various groups of pesticides that are used worldwide, organophosphorus pesticides form the major and most widely used group that accounts for more than 36% of the total world market. The most used among these is methyl parathion. Its accumulation has many health hazards associated to it, hence, its degradation is very important (Ghosh et al., 2010).

The organophosphorus pesticides (OP) are all esters of phosphoric acid and are also called organophosphates, which include aliphatic, phenyl and heterocyclic derivatives (Figure 3). Owing to large-scale use of OP compounds, contaminations of soil and water systems have been reported from all parts of the world. In light of this, bioremediation provides a suitable way to remove contaminants from the environment as, in most cases, OP compounds are totally mineralized by the microorganisms. Most OP compounds are degraded by microorganisms in the environment as a source of phosphorus and /or carbon. Classification of Pesticides. Thus, the OP pesticides can be hydrolyzed and detoxified by carboxylesterase and phosphotriesterase enzymes.

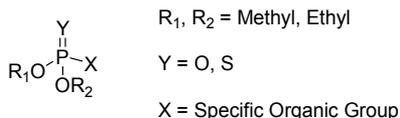


Fig. 3. General structure of organophosphate pesticides

Organophosphates are used to control a variety of sucking, chewing and boring insects, spider mites, aphids, and pests that attack crops like cotton, sugarcane, peanuts, tobacco, vegetables, fruits and ornamentals. OP pesticides are marketed by many of the world's major agrochemical companies. Some of the main agricultural products are parathion, methyl parathion, chlorpyrifos, malathion, monocrotophos, diazinon, fenitrothion and dimethoate (Figure 4).

The organophosphates possess an efficient insecticide activity, due to its characteristic of irreversibly inhibiting the enzyme acetylcholinesterase in the nervous system, which acts in both insects and in mammal. In man, the organophosphates are absorbed through all routes, reaching high concentrations in fatty tissues, liver, kidneys, salivary glands, thyroid, pancreas, lungs, stomach, intestines and, at smaller proportions, in the central nervous system (SNC) and muscles. However, the organophosphates do not accumulate in the human organism, as it is readily biotransformed in the liver. The excretion of these compounds and of their metabolites is quite fast, taking place mostly in the urine and, at small proportions, in the feces, usually within 48 h. The largest excretion levels occur within 24 h after absorption (Oga, 2003; Griza et al., 2008).

Due to the above mentioned health hazards and other problems associated with the use organophosphorus pesticides, early detection and subsequent decontamination and detoxification of the polluted environment is essential. The present subject examines applications and future use of OP-degrading microorganism cultures from agricultural fields and enzymes for bioremediation (Karpouzas & Singh, 2006).

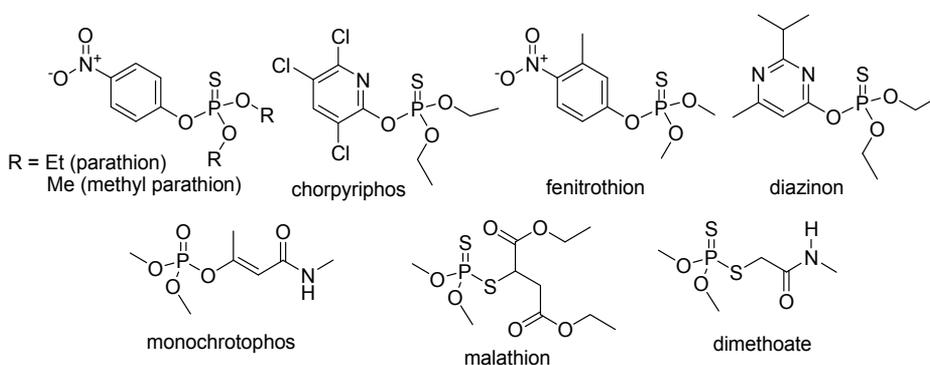


Fig. 4. Structures of organophosphate pesticides

### 2.2.2 Microbial degradation of organophosphate pesticides

Methyl parathion (*O,O*-dimethyl-*O*-(*p*-nitro-phenyl)phosphorothioate) is one of the most used organophosphorus pesticides. This product is widely used throughout the world and its residues are regularly detected in a range of fruits and vegetables. Investigation of microbial degradation is useful for developing insecticide degradation strategies using microorganisms. Bacteria with the ability to degrade methyl parathion have been isolated worldwide (Liu et al., 2003; Hong et al., 2005).

Multiplex tendencies characterize pesticide applications in farming. A number of pesticide mixtures, especially pyrethroid and organophosphorus pesticide mixtures have been formulated as an improvement over individual pesticides (Moreby et al., 2001). Construction of a genetically engineered microorganism (GEM), which can simultaneously degrade these two kinds of pesticides, could benefit the study and application of bioremediation in multiple pesticide-contaminated environments (Yuanfan et al., 2010). Methyl parathion hydrolase gene, *mpd*, which is responsible for hydrolyzing methyl parathion to *p*-nitrophenol and dimethyl phosphorothioate, has also been cloned from these strains. Sequences are effectively conserved in these strains (Yuanfan et al., 2010).

A fenpropathrin-degrading bacterium, *Sphingobium* sp. JQL4-5, was isolated and characterized. A stable, genetically engineered strain, JQL4-5-*mpd*, capable of simultaneously degrading fenpropathrin and methyl parathion was constructed by random insertion of the methyl parathion hydrolase gene (*mpd*) into the chromosome of strain JQL4-5. Soil treatment results indicated that JQL4-5-*mpd* is a promising GEM in the bioremediation of multiple pesticide contaminated environments (Yuanfan et al., 2010).

Organophosphorus hydrolase (OPH), isolated from both *Flavobacterium* sp. ATCC 27551 (Mulbry & Karns, 1989) and *Pseudomonas diminuta* MG (Serdar et al., 1989), is capable of hydrolyzing a wide range of oxon and thion OPs. However, OPH has already been shown to lack any hydrolytic activity toward numerous dimethyl OPs (Horne et al., 2002). The *mpd* gene encoding an organophosphate degrading protein was isolated from a methyl parathion (MP) degrading *Plesiomonas* sp.

The methyl parathion hydrolase gene (*mpd*) and enhanced green fluorescent protein gene (*egfp*) was successfully coexpressed using pETDuet vector in *Escherichia coli* BL21 (DE3). The coexpression of methyl parathion hydrolase (MPH) and enhanced green fluorescent protein (EGFP) were confirmed by determining MPH activity and fluorescence intensity. The recombinant protein MPH showed high enzymatic degradative activity of several widely used OP residues on vegetables. Subsequently, a dual-species consortium comprising engineered *E. coli* and a natural *p*-nitrophenol (PNP) degrader *Ochrobactrum* sp. strain LL-1 for complete mineralization of dimethyl OPs was studied. The dual-species consortium possesses the enormous potential to be utilized for complete mineralization of PNP-substituted OPs in a laboratory-scale bioreactor. These studies demonstrated that MP could be degraded via the MP → PNP → hydroquinone → Krebs cycle (Figure 5) by the dual-species consortium. The data confirm that the mineralization process of MP is initiated by hydrolysis leading to the generation of PNP and dimethylthiophosphoric acid, and PNP degradation, then, proceeds through the formation of hydroquinone. The accumulation of PNP in suspended culture was prevented (Zhang et al., 2008).

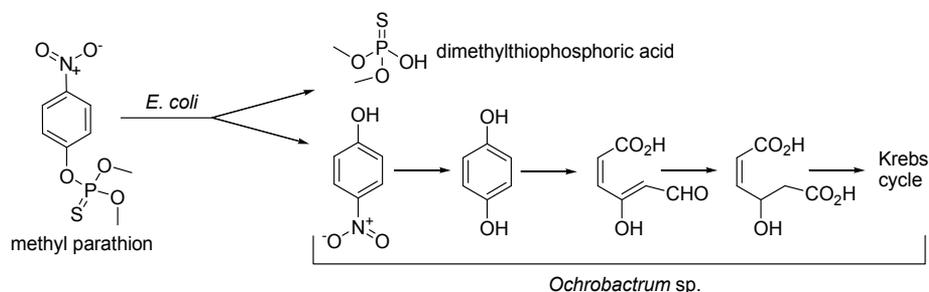


Fig. 5. Proposed pathway for the biodegradation of MP by microbial consortium (Zhang et al., 2008)

Thus, there is an increasing need to develop new methods to detect, isolate, and characterize the strains/enzymes playing a part in these degradation processes (Vallaey et al., 1996). Successful detoxification of recalcitrant organic chemicals may require the concerted effort of multispecies consortia.

Fenamiphos (FEN), ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate, is an organophosphate nematicide used in protected horticultural crops. In soil, FEN is gradually oxidized to its sulfoxide (FSO) and sulfone (FSO<sub>2</sub>), which also possess high nematicidal activity and is equally toxic to non-target vertebrates (Figure 6). Degradation studies of FEN in a range of soils showed half-life values ranging from 12 to 87 days. FEN and its oxidation products FSO and FSO<sub>2</sub> showed low to moderate affinity for soil adsorption and their soil accumulation may result in their eventual downward movement into groundwater. Indeed, previous studies have suggested that under favorable environmental conditions FEN could leach to groundwater where it could persist (Franzmann et al., 2000). Therefore, tools are needed for the decontamination of natural resources by the residues of chemicals such as FEN and its oxidation derivatives.

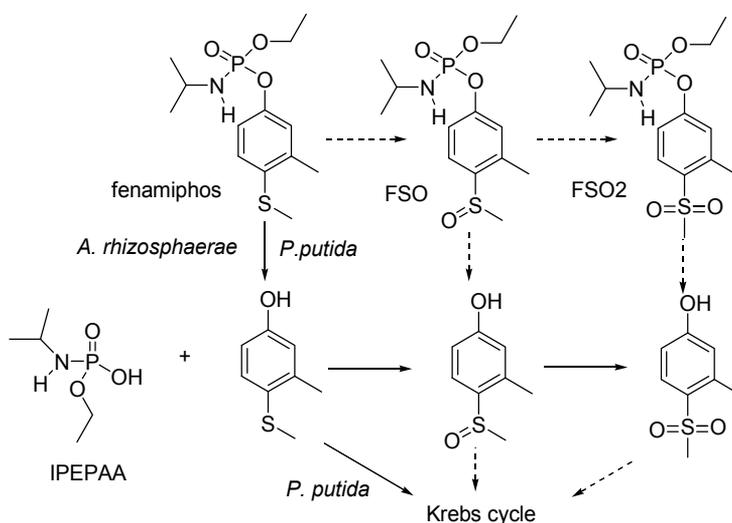


Fig. 6. Metabolic pathway of FEN by the isolated bacteria (Chanika et al., 2011)

Two bacteria identified as *Pseudomonas putida* and *Acinetobacter rhizosphaerae*, able to rapidly degrade the organophosphate fenamiphos, were isolated. Denaturing gradient gel electrophoresis analysis revealed that the two isolates were dominant members of the enrichment culture. Clone libraries further showed that bacteria belonging to  $\alpha$ -,  $\beta$ -,  $\gamma$ -Proteobacteria and Bacteroidetes were also present in the final enrichment, but were not isolated. Both strains hydrolyzed FEN to fenamiphos phenol and ethyl hydrogen isopropylphosphoramidate (IPEPAA), which was further transformed, only by *P. putida*. The two strains were using FEN as C and N source. Cross-feeding studies with other pesticides showed that *P. putida* degraded OPs with a P-O-C linkage (Chanika et al., 2011). Thus, both bacteria were able to hydrolyze FEN, without prior formation of FSO or FSO<sub>2</sub>, to FEN-OH which was further transformed only by *P. putida* (Figure 6), suggesting elimination of environmentally relevant metabolites. In addition, *P. putida* was the first wild-type bacterial isolate able to degrade OPs. All the above characteristics of *P. putida* and its

demonstrated ability to remove aged residues of FEN highlight its high bioremediation potential (Chanika et al., 2011).

Herein, it was shown that the construction of genetically engineered microorganism (GEM) and the dual-species consortium has the potential to be used in the degradations of different kinds of pesticides. These studies show the benefits of bioremediation in multiple pesticide-contaminated environments and mineralization of toxic intermediates in the environment, which can lead to complete bioremediation of contaminated sites that have an adverse effect.

## 2.3 Carbamate pesticides

### 2.3.1 Introduction

Carbamates were introduced as pesticides in the early 1950s and are still used extensively in pest control due to their effectiveness and broad spectrum of biological activity (insecticides, fungicides, herbicides). High polarity and solubility in water and thermal instability are typical characteristics of carbamate pesticides, as well as high acute toxicity. The carbamates are transformed into various products in consequence of several processes such as hydrolysis, biodegradation, oxidation, photolysis, biotransformation and metabolic reactions in living organisms (Soriano et al., 2001).

Chemically, the carbamate pesticides are esters of carbamates and organic compounds derived from carbamic acid (Figure 7). This group of pesticides can be divided into benzimidazole-, *N*-methyl-, *N*-phenyl-, and thiocarbamates. The compounds derived from carbamic acid are probably the insecticides with the widest range of biocide activities (Sogorb & Vilanova, 2002).



Fig. 7. General structures of carbamate pesticides

Highly toxic acetylcholinesterase (AChE)-inhibiting pesticides, organophosphates and carbamates are intensively used throughout the world and continue to be responsible for poisoning epidemics in various countries (De Blecker, 2008). The carbamates are inhibitors of AChE and are responsible for the greatest number of poisonings in the rural environment. The use of pesticides in the Brazilian rural environment has brought a series of dire consequences to the environment as well as to the health of rural workers (Oliveira-Silva et al., 2001). The clinical effects of carbamate pesticides depend on the dose, route of exposure, type of carbamate involved, use of protective gear, and the premorbid state of the victim (Rosman et al., 2009).

The study of pesticide degradation is usually beneficial, since the reactions that destroy pesticides convert most pesticide residues in the environment to inactive, less toxic, harmless compounds (Lan et al., 2006).

The enzymatic hydrolysis of carboxyl esters by carboxyl esterases (CbEs) is based on the reversible acylation of a serine residue within the active centre of the protein (Gupta, 2006). Firstly, the substrate must gain access to the active site and this acylation causes a nucleophilic attack by the serine on the carboxyl carbamate producing a transition state

formation, in addition to forming a stable acylated enzyme (Figure 8). This acyl-enzyme intermediate is hydrolysed by nucleophilic attack of water that releases the corresponding carbamine acid, plus the free active enzyme again ready to initiate a new catalytic cycle (Reed & Fukuto, 1973; Sogorb & Vilanova, 2002; Hemmert & Redinbo, 2010).

Moreover, the investigation of biodegradation pathways are quite complex. In addition to the complexity of the (bio)degradation of the pesticides there are also other factors, such as pesticide nonextractable residues in soils. The definition of bound residues was described as “bound residues represent compounds in soils, plants, or animals which persist in the matrix in the form of the parent substance or its metabolite(s) after extraction. The extraction method must not substantially change the compounds themselves or the structure of the matrix” (Barriuso et al., 2008).

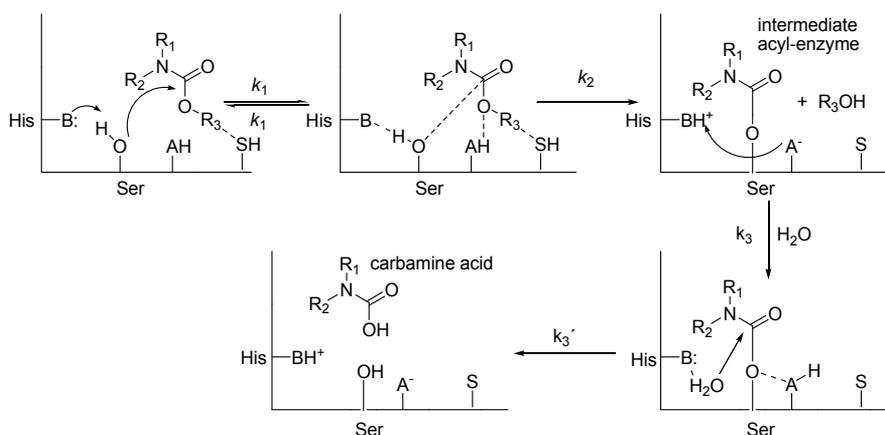


Fig. 8. Catalytic mechanism for carbamate hydrolysis by carboxyl esterases,  $k_1$  defines the affinity of the enzyme for a given substrate and  $k_2$  describes how quickly the acyl-enzyme intermediate is formed (Reed & Fukuto, 1973).

### 2.3.2 Microbial degradation of carbamate pesticides

The biodegradation of carbamates has been investigated by different microorganisms that metabolize carbamate pesticides. In most cases, the studies did not eliminate the possibility that abiotic processes are involved in the degradation.

A number of bacteria capable of degrading carbofuran (*Pseudomonas*, *Flavobacterium*, *Achromobacterium*, *Sphingomonas*, *Arthrobacter*) have been isolated and characterized in an effort to better understand the bacterial role to remove carbofuran from the environment. Carbofuran is one of the pesticides belonging to the *N*-methylcarbamate class used extensively in agriculture. It exhibits high mammalian toxicity and has been classified as highly hazardous. Carbofuran was degraded first to carbofuran phenol and the result was degraded to 2-hydroxy-3-(3-methylpropan-2-ol) phenol by *Sphingomonas* sp. (Kim et al., 2004) and *Arthrobacter* sp. (De Schrijver & De Mot, 1999), (Figure 9).

Carbendazim is a widely used broad-spectrum benzimidazole fungicide to control a wide range of fungal pathogens on cereals and fruits, it is also used in soil treatment and foliar application on the appearance of disease. The fungicide carbendazim was degraded by a microbial consortium obtained from several soil samples in Japanese paddy fields with continuous culture enrichment. Biodegradation using immobilized bacterial consortium was

investigated in various parameters, as temperature, pH, and nutrient concentration. The degradation ability of the consortium was increased by immobilization on loofa (*Luffa cylindrica*) sponge, in comparison with that of free-living consortium. This immobilized consortium on loofa sponge is a promising material for bioremediation of polluted water with these pesticides in paddy fields (Pattanasupong et al., 2004).

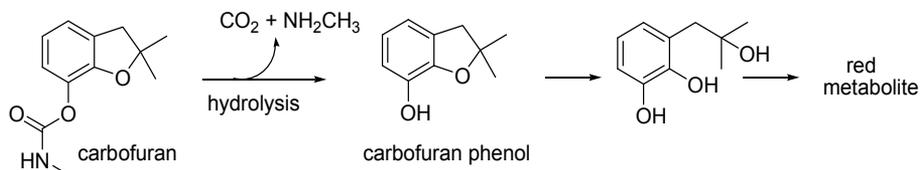


Fig. 9. Biodegradation of carbofuran by *Sphingomonas* sp.

Afterwards, the carbamate carbendazim was converted to 2-aminobenzimidazole by *Pseudomonas* isolates (Figure 10). In general, a limited number of xenobiotic pesticides are metabolized by single strain, but usually consortia of microorganisms are catalyzed for complete degradation. Several Actinomycetes that metabolize carbamate pesticides were isolated. In most cases, this is initiated by hydrolysis of the carbamate at the ester linkage. (De Schrijver & De Mot, 1999).

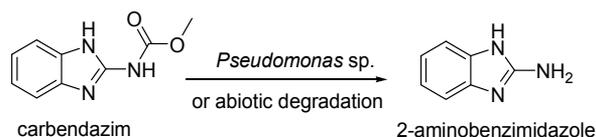


Fig. 10. Biodegradation of carbendazim by *Pseudomonas* sp.

Juvenoids are efficient pesticides with relatively low toxicity to humans (Figure 11). However, few studies have evaluated the effect of degradation by soil microorganisms on their toxicity. The effects of bacterial, fungal and yeast isolates on aerobic decomposition of ethyl *N*-{2-[4-(2,2-ethylenedioxy-1-cyclohexylmethyl)phenoxy]ethyl} carbamate during eight weeks were determined. Higher degradation activity was observed during the first week of the experiment and a substantial decrease in the rate of degradation occurred during the following seven weeks. This can be described both to the accumulation of degradation products and to impaired physiological state of microbial cultures during the long-term experiments (Novák et al., 2003).

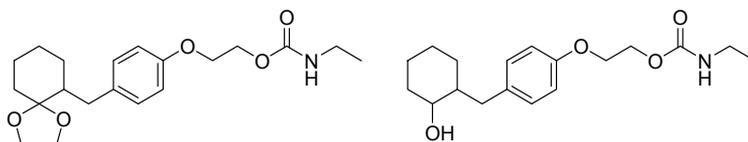


Fig. 11. Juvenoid pesticides

Ethylenethiourea is an important degradation product of ethylenebisdithiocarbamate fungicides (maneb, zineb, mancozeb), which are widely used in different kinds of crops (Figure 12). The ethylenebisdithiocarbamates are not highly toxic and degrade rapidly in the presence of moisture and oxygen, forming different types of compounds such as the polar

ethylenethiourea, which is relatively stable and is a potential contaminant for groundwater. Experiments conducted under biotic and abiotic conditions, showed complete degradation of ethylenethiourea in the presence of microbial nitrate reduction with pyrite, which occurs in deeper parts of the aquifers (Jacobsen & Bossi, 1997).

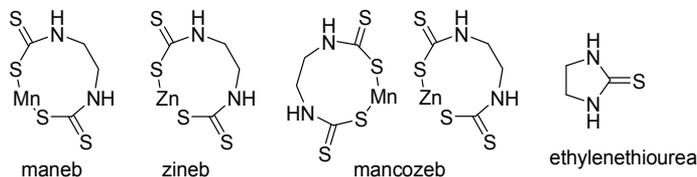


Fig. 12. Ethylenethiourea pesticides

In general, pesticide-degrading microorganisms are isolated via enrichment cultures. A novel strategy has been reported using a coexpression vector for the purpose of developing bacteria that can detoxify different pesticides. The organophosphate hydrolase gene from *Flavobacterium* sp. and carboxylesterase B1 gene (b1) from *Culex pipiens* were cloned in the coexpression vector. A single microorganism was capable of producing both enzymes for degradation of organophosphate (parathion), carbamate (pirimicarb) and pyrethroid pesticides (deltamethrin), (Figure 13). The technical capability of genetically engineering bacteria with more enzymes should open up new opportunities for extending the wide range of pesticides that can be biodegraded in the future (Lan et al., 2006).

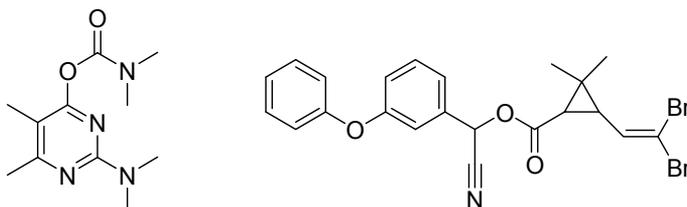


Fig. 13. Pirimicarb (left) and deltamethrin (right) pesticides

Recently, the isolation of a soil bacteria able to hydrolyze organophosphate and carbamate pesticides was performed. Cross-feeding studies with other pesticides showed that *Pseudomonas putida* degraded organophosphates with a P-O-C linkage (fenamiphos), and oxamyl (Figures 6 and 14) and carbofuran carbamates (Chanika et al., 2011). In addition, the biodegradation of insecticidal organophosphates and carbamates has been described by human brain esterases, which actively degraded 1-naphthyl acetate and other substrates (Sakai & Matsumura, 1971).

Contamination of surface water by organophosphate and carbamate compounds is of concern because of the potential toxicity to aquatic organisms, especially those at lower trophic levels. Many organophosphate and carbamate compounds have acute and chronic toxicity to fish and aquatic invertebrates. Bondarenko et al. (2004) showed that the persistence of diazinon and chlorpyrifos was much longer than for malathion and carbaryl in freshwater, and was further prolonged in seawater. Afterwards, microbial degradation contributed significantly to the dissipation of diazinon and chlorpyrifos in freshwater, but was inhibited in seawater. In contrast, degradation of malathion and carbaryl was rapid and primarily abiotic. The interactions of pesticide persistence with water location, temperature,

and type of pesticides suggest that site, and compound-specific, information is needed when evaluating the overall ecotoxicological risks of pesticide pollution in a watershed.

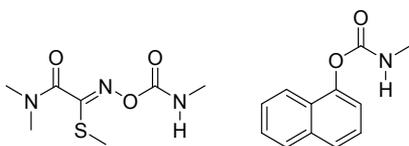


Fig. 14. Oxamyl (left) and carbaryl (right) pesticides

Kaufman and Blake (1973) have selected soil microorganisms capable of degrading isopropyl carbanilate (propham), 3',4'-dichloropropionanilide (propanil), 3'-chloro-2-methyl-*p*-valerotoluidide (solan), and methyl 3,4-dichlorocarbanilate (swep), (Figure 15). Degradation of the pesticides in enrichment solutions, and by pure cultures of effective microbial isolates (*Pseudomonas striata*, *Achromobacter* sp., *Aspergillus ustus*, *Aspergillus versicolor*, *Fusarium oxysporum*, *Fusarium solani*, *Penicillium chrysogenu*, *Penicillium janthinellu*, *Penicillium rugulosum* and *Trichoderma viride*) were demonstrated by the production of the corresponding aniline, chloride ion liberation and disappearance of the original compounds. Each organism demonstrated unique substrate specificity and was capable of degrading other aniline-based pesticides of the acetamide, acylanilide, carbamate, toluidine and urea classes.

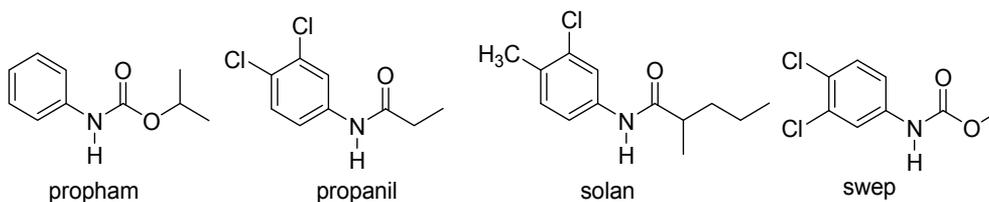


Fig. 15. Carbamate pesticides degraded by soil microorganisms

As described here, the carbamate pesticides are easily degraded by different types of microorganisms (fungi and bacteria). The degradation of these pesticides by enzymatic systems of microorganisms has contributed to the total removal of xenobiotics from soils, hence avoiding the contamination of waters and the environment.

## 2.4 Biological pesticides

Synthetic chemical pesticides provide many benefits to agriculture and food production, however, as previously discussed, they also present toxicity to non-target organisms and cause environmental pollution, therefore efforts to find new pest control alternatives have been studied, essentially due to the increasing concern about the effects of these compounds on human health and on the environment. Biodegradation and bioremediation of synthetic pesticides have been used as alternative green technologies to solve the problems related to the accumulation of these contaminants in soil and water. Another proposal to reduce the environmental impact of pesticides is the use of biological-derived products also known as biopesticides.

According to the Environmental Protection Agency (EPA), biopesticides are defined as naturally occurring pest control substances. They are classified into three groups (Joshi, 2006):

- a. Microbial pesticides: in which a microbial living organisms (bacteria, fungi, viruses, protozoans) is the active control agent;
- b. Plant pesticides: pesticidal substances produced by plants from introduced genetic material (plant incorporated protectants);
- c. Biochemical pesticides: naturally occurring substances that control pests by non-toxic mechanisms. These include substances that interfere with growth or mating such as pheromones.

The main advantage of biopesticides is their safety to non-target organism, biodegradability and their specificity, which permits the use of small dosages and power exposure, hence avoiding pollution caused by conventional pesticides (Rosell et al., 2008). In addition to being less harmful than chemicals, biopesticides have been of great value in integrated pest management (IPM) strategies where the use of biopesticides greatly decreases the use of chemicals, maintaining crop yields. The specificity of biopesticides contrasts with the broad spectrum of chemical counterparts. In contrast, biopesticides are also slow acting, have relatively critical application times, most suppress rather than eliminate the target population, have limited field persistence and short-shelf life.

Despite the range of biopesticides that have been described, our discussion focuses on microbial pesticides.

### **2.4.1 Microbial pesticides**

Microbiological control is sustained by beneficial interactions resulting from competition, antagonism and parasitism of microorganisms against plant pathogens, insects and weeds (Montesinos, 2003). In general, microorganisms are able to suppress pests by producing a toxin, causing a disease or preventing the establishment of other organisms. Currently, several microorganisms involved in such processes are the active ingredient of microbial pesticides.

#### **2.4.1.1 Bacteria**

Most biopesticides available in the market are bacterial-based products. The well-known and widely used bacterial biopesticide comprises the gram-positive, spore-forming bacteria belonging to the genus *Bacillus* that are commonly found in soil. The majority of commercial microbial insecticides are preparations based on strains of *Bacillus thuringiensis* (Bt) that produces a crystalline inclusion body during sporulation (Frankenhuyzen, 2009). The crystal proteins (Cry proteins) are toxic to many insects and are defined as endotoxins (Bt toxin) that are generally encoded by bacterial plasmids (Gonzales & Carlton, 1980). Both spores and inclusion bodies are released upon lysis of the parent bacterium at the end of the sporulation cycle and if ingested, the spores and crystals act as poisons in certain insects. The protein is activated by alkaline conditions and enzyme activity of insect's gut hence, Bt is referred as a stomach poison (Chattopadhyay et al., 2004). The toxicity of the activate protein is dependent on the presence of receptor sites on the insects gut wall. This match between toxin and receptor sites determines the range of insect species killed by each Bt subspecies and isolates (Frankenhuyzen, 2009).

Cry proteins are produced as protoxins that are proteolytically converted into a combination of up to four smaller toxins upon ingestion. These proteins bind to specific receptors in the larval midgut epithelium causing the formation of large cation-selective pores that increase the water permeability of the cell membrane. A large uptake of water then causes cell

swelling and rupture of the midgut. Poisoned insects can die quickly from the toxin activity or may die within 2-3 days from septicemia due to the entering of gut contents into the bloodstream. Bt strains containing mixtures of up to 6-8 Cry proteins have been used as microbial pesticides since Bt var. kurstaki have been commercially available since 1961 (Montesinos, 2003). Formulations are active against insect order Lepidoptera (moths and butterflies); Diptera (flies and mosquitoes); Coleoptera (beetles and weevils) and Hymenoptera (bee and wasps) larvae (Frankenhuyzen, 2009). Of the recognized subspecies of Bt, var. kurstaki is toxic to gypsymoth, cabbage looper, and caterpillars (order Lepidoptera), var. israelensis is toxic to fungus gnat larvae, mosquitoes (species of *Aedes* and *Psorophora*), coffee berry borer (Mendez-Lopez et al., 2003), back fly, and some midges (order Diptera), var. san diego is effective against potato beetle, elm leaf beetle, and boll weevils (Whalon & McGaughey, 1998), var. aizawai is effective against wax moth larvae and diamondback moth caterpillar and var. morrisoni is toxic against moth and butterfly caterpillars (order Lepidoptera) (Chattopadhyay et al., 2004). A number of Bt-derived products were used in Europe to control Lepidoptera pests in vegetables, tomatoes, top fruit, vines, olives and forestry (Butt et al., 1999).

Besides Cry proteins (crystal delta endotoxins), Cyt proteins (cytolysins) have been described as another class of insecticidal protein produced by Bt (Yokoyama et al., 1998). Cytolysins interact with phospholipid receptors on the cell membrane in a detergent-like manner (Gill et al., 1987). The hydrophobic portion of the cytolysins bind the amphipathic phospholipids; transmembrane pores are formed and cells are lysed by osmotic lysis (Knowles & Ellar, 1987). The spore inclusions contain many proteins, which sometimes possess distinct activities and may act in a synergistic manner (Yokoyama et al., 1998).

With regard to toxicity, Cry proteins are non toxic to vertebrate species even at doses higher than  $1 \times 10^6$   $\mu\text{g} / \text{kg}$  body weight, while dosages acutely toxic to susceptible insects are about  $\mu\text{g}/\text{kg}$  body weight (Rosell et al., 2008), however Bt formulations can cause skin and eye irritation (Siegel & Shaddock, 1990). The acidic environment of the mammalian stomach does not favor solubilization and activation of the Cry proteins. These proteins are degraded very fast (often in some seconds), from 60-130 kDa to polypeptides less than 2 kDa that corresponds to peptides with 10 amino acids in length. The rapid degradation of these proteins by proteases in the mammalian gastrointestinal tract precludes their toxicity in mammals. Several studies in vertebrates have failed to find high affinity Cry protein binding sites on gut epithelial cell membranes (Rosell et al., 2008). Bt has thus become a bioinsecticide of great agronomical importance and is classified as toxicity class III pesticide (slightly toxic).

The commercial Bt products are powders comprised of a mixture of dried spores and toxin crystal proteins and these are applied to areas like leaves and roots where insects feed. The commercial Bt product contains about  $2.5 \times 10^{11}$  viable spores per gram. Bt products are marketed worldwide and they account for about 1% of the total agrochemical market. Bt products are known to lose their effectiveness to some extent when stored for longer than six months (Joshi, 2006).

Other species of *Bacillus*, including *B. sphaericus*, *B. popilliae*, *B. subtilis*, *B. lentimorbus*, *B. pumilus* and *B. firmus* have been applied as biopesticides (Schisler et al., 2004).

Bacteria belonging to other genera such as *Pseudomonas fluorescens*, *P. syringae*, *P. putida*, *P. chlororaphis*, *Burkholderia cepacia* and *Streptomyces griseoviridis* have also been used as biopesticides (Montesinos, 2003). However, these bacteria generally lose viability when

stored for several weeks, a disadvantage when compared with spore-forming *Bacillus* that demonstrates better shelf-life and facilitates the development of commercial products.

Insect resistance to Bt toxins has led to pursue suitable alternatives. Two more bacteria that are also known to produce insecticidal toxins are *Xenorhabdus* and *Photorhabdus* (both of these belong to the family *Enterobacteriaceae*). Both bacteria are entomopathogens, *Xenorhabdus luminescens* is found to occur in a specialized intestinal vesicle of the nematode *Steinernema carpocapsae* (Akhurst & Dunphy, 1993) with which it maintains a symbiotic relationship. *Photorhabdus luminescens* maintains a symbiotic relationship with nematodes of the family Heterorhabditidae (Poinar, 1990) and is present throughout the intestinal tract of these nematodes. In both mutualistic associations, the nematodes and the bacteria complement each other: the nematode acts as a vector and transports the bacteria into the target insect larva where it bores holes in the intestinal walls of the insect and releases the bacteria in the hemolymph. In the absence of the nematode, the bacteria cannot penetrate into the hemocoel (Tanada & Kaya, 1993). Both the nematode and the bacteria release insecticidal toxins, which eventually kill the insect (Poinar et al., 1977). The bacteria causes septicemia in the insect, the insect is killed and its tissues are used as nutrients (Kaya & Gaugler, 1993). Moreover, bacteria are required by the nematodes for their development into the infective juvenile stage and thus are required for efficient completion of the nematode life cycle. In the absence of the bacterium the nematode cannot reproduce (Tanada & Kaya, 1993). With emerging resistance to Bt among insects, *Xenorhabdus* and *Photorhabdus* are considered the next generation of microbial insecticides.

#### 2.4.1.2 Fungi

Fungi often act as important natural control agents against insects, pathogenic fungi, nematodes and as herbicide. Many fungi utilized as biopesticides are pathogenic to insect hosts, therefore they are referred as entomopathogenic fungi; among them, members of Entomophthorales (Zygomycota) and Hyphomycetes are currently under research (Srivastava et al., 2009). Fungal strains are considered suitable for biopesticide development because, unlike other microorganisms, the infectious propagules (conidia) do not need to be ingested and contact with cuticle permits the fungi to penetrate the insect body (Thomas & Read, 2007).

Fungi can act as insecticide by two ways:

- a. Infection: most of the fungi species cause death to the insect through asexual spores called conidia. The conidium is the infective unit of entomopathogenic fungi and binds to the host cuticle by nonspecific interaction mediated by cuticle degrading enzymes present on the conidia or by fungal lectins. These conidia enter through the body wall of the host pest by dissolving the body wall by the combined action of enzymes, i.e., chitinase and protease, secreted by the fungi. Fungal penetration is further enhanced by mechanical force. The site of invasion is often between the mouth parts, at intersegmental folds or through spiracles, where locally high humidity promotes germination and the cuticle is nonsclerotized and more easily penetrated. Under favorable environmental conditions (>95% humidity) the fungus will break out through the cuticle and sporulate; it may grow profusely in the blood and give the carcass a characteristic mummified appearance. Therefore, insect death is probably the result of obstruction of blood circulation, starvation or physiological/biochemical disruption brought about by the fungus. The whole procedure takes 3–14 days for insect death (Roy et al., 2006).

- b. Mycotoxins: another fungi mode can cause death of the host by the production of mycotoxins, which can interfere in the nervous system of insects. Mycotoxins such as aflatoxin B, trichothecenes, patuline and ochratoxin are reported to be toxic to insects, (Figure 16), (Srivastava et al., 2009).

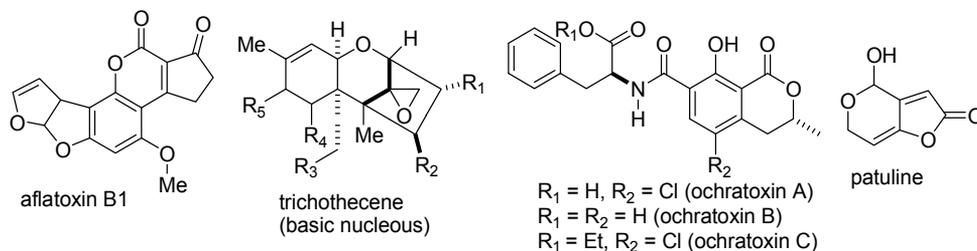


Fig. 16. Examples of mycotoxins produced by fungi

Fungi are known to infect a broader range of insects belonging to orders Lepidoptera, Homoptera, Hymenoptera, Coleoptera and Diptera. *Beauveria bassiana*, *Beauveria brongniari*, *Metarhizium anisopliae*, *Metarhizium flavoviride* and *Lagenidium giganteum* are examples of commercially available mycoinsecticides (Rosell et al., 2008).

*Trichoderma harzianum*, *T. viride*, *Talaromyces flavus*, *Gliocladium virens*, *Phytium oligandrum* shows fungicide activity against soil-borne pathogenic fungi (Montesinos, 2003). The application of a biopesticide containing the fungus *Verticillium lecanii* was reported to suppress the growth of plant pathogens as well as insect pests (Koike et al., 2005).

A formulation containing the unicellular fungi *Candida oleophila* O is used as a post-harvest biofungicide to control the pathogens *Botrytis cinerea* (gray mold) and *Penicillium expansum* (blue mold) which cause deterioration of apples and pears (Environmental Protection Agency-EPA, 2009).

The main difficulties to be overcome for applying entomophagous fungi in pest control are: (i) scant production of mycotoxins; (ii) carcinogenic mycotoxicosis in non-target organisms; and (iii) slow effectiveness of entomophagous conidia. The combination of fungi formulations with plant extracts exploring their synergistic action is an alternative strategy to overcome these problems (Srivastava et al., 2009).

### 2.4.1.3 Viruses

Virus-based biopesticides have been used as insect control agents. The larvae of many insect species are vulnerable to viral diseases. Baculoviruses are a large virus group belonging to the family *Baculoviridae* and can infect different insect orders, particularly Lepidoptera and Diptera (Theilmann et al., 2005; Moscardi, 1999). Theilmann et al., 2005; Moscardi, 1999). Baculoviruses are classified into two genera: nuclear polyhedrovirus (NPV) and granulovirus (GV), (Cory & Hails, 1997; McCutchen & Flexner, 1999). Two morphologically distinct forms of infectious particles are generated in the baculovirus cycle, the occlusion derived virus (ODVs), comprising enveloped virions embedded within a crystalline matrix of protein (polyhedrin for NPVs and granulins for GVs), and budded virus (BVs), consisting of a single virion enveloped by a plasma membrane. Due to their specificity and high virulence to a number of insect pest species, they have been used worldwide to control lepidopteran pests in many crops (Moscardi, 1999). BVs are responsible for the systemic or cell-to-cell spread of the virus within an infected insect. ODVs, in turn, are responsible for the larva-to-larva transmission of the virus (Inceoglu et al., 2006).

Viruses, like bacteria, must be ingested to infect the insect hosts. During infection the host larvae is debilitated, resulting in reduced movement and increased exposure to predators. Post larval effects include the reduction in reproductive capacity and longevity. Disease and death insects serve as inoculums for virus transmission which may occur by rain and movement of insects on plants (Rosell et al., 2008). The commercial formulations that have been used include Granulosis virus to control *Byctiscus betulae*, Pine sawfly NPV to control *Diprion similis*, *Heliothis* NPV to control *Helicoverpa zea*, Gypsy moth NPV to control *Lymantria dispar*, *Mamestria brassicae* NPV to control *Heliothis* (Montesinos, 2003).

Insect viruses are safe to vertebrates, plants and non-target organisms. Limitations on the use of virus formulations include narrow spectrum of biological activity, slow mode of action (5–7 days after ingestion of NPVs and 7–14 days in the case of GV infections), and photolability (solar radiation), (Rosell et al., 2008). The major success of microbial control with viruses takes place in forestry. Forest pests are good targets for viral pesticides because the permanence in forest environment contributes to the pathogen cycle and the forest canopy also helps to protect viral particles from radiation. There have been different approaches directed to enhance the role of baculovirus as effective biopesticides. For instance, the effect of baculovirus may be enhanced by the synergistic action of specific chemical insecticides, such as the pyrethroids deltamethrin and permethrin (McCutchen & Flexner, 1999). To improve the potency and rapid action, recombinant baculovirus have been developed (Bonning & Hammock, 1996).

#### 2.4.1.4 Protozoa

Some protozoan pathogens can kill insect hosts; however, many of them cause chronic infections with debilitating effects (Lacey & Goettel, 1995). One important consequence of protozoan infection is the reduction in the number of offsprings by the infected insects. Species of the genera *Nosema sp.* and *Vairimorpha necatrix* offer the greatest biopesticide potential. *Nosema locustae* is a specie of *Microsporidium* commercially available to control grasshoppers and crickets. It is most effective when ingested by immature grasshoppers (early nymphal stages). The spore formed by the protozoan is the infection stage in susceptible insects; it germinates in the midgut and causes a slow progress infection where the pathogen causes death three to six weeks after initial infection (Rosell et al., 2008). *Ostrinia nubilalis* that causes important damages to corn was controlled by *Nosema pyrausta* infection, which reduced the egg production per female 53 and 11% at the 16 and 27°C temperature, respectively (Bruck et al., 2001). *Nosema locustae* has been used to reduce grasshopper population in rangeland areas; although not all insects are killed, the infected grasshoppers consume less forage and the females produce fewer eggs. However, the utility of *N. locustae* as biopesticide remains questionable because of the difficulty to determine the treatment efficacy in this highly mobile insect.

#### 2.4.2 Challenges of microbial pesticides

The main problems that should be solved regarding the widespread use of microbial pesticides include their specificity, once they are not effective against a wide range of pests. Although specificity is considered an advantage, it also limits the potential market and increases costs when compared to synthetics. Another important aspect is that biopesticide preparations are sensitive to heat, desiccation and ultraviolet radiation, reducing their effectiveness. Special formulations and storage conditions are necessary; this in turn can complicate the distribution and application of products. Molecular genetics of

microorganisms and genetic engineering technology will help in the development of new strategies for biopesticide improvement and its use. More work should be done to enhance shelf-life, to increase the speed of kill, the biological spectrum and the field efficacy of biopesticides.

### 3. Conclusion

The pollution of the environment by pesticides is a consequence of the continuous agricultural expansion, combined with the population increase. Pesticides are used in sizeable areas and applied to soil surfaces and accumulate beneath the ground surface, reaching rivers and seas. The natural microbiota is continuously exposed to pesticides therefore, it is no surprise that these microorganisms, that inhabit in polluted environments, are armed with resistance by catabolic processes to remove the toxic compounds. Biological degradation by organisms (fungi, bacteria, viruses, protozoa) can efficiently remove pesticides from the environment, especially organochlorines, organophosphates and carbamates used in agriculture. The enzymatic degradation of synthetic pesticides with microorganisms represents the most important strategy for the pollutant removal, in comparison with non-enzymatic processes. Regarding the use of biopesticides, their main advantage is their environmental-friendly nature when compared to chemicals.

To improve the use of microbe-based processes some questions still have to be answered, such as the long term impact of introducing microorganisms into the environment, as well as the narrow range of applications (particularly in the case of biopesticides).

The degradation of persistent chemical substances by microorganisms in the natural environment has revealed a larger number of enzymatic reactions with high bioremediation potential. These biocatalysts can be obtained in quantities by recombinant DNA technology, expression of enzymes, or indigenous organisms, which are employed in the field for removing pesticides from polluted areas.

The microorganisms contribute significantly for the removal of toxic pesticides used in agriculture and in the absence of enzymatic reactions many cultivable areas would be impracticable for agriculture.

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# Pesticide-Soil Interaction

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## 1. Introduction

Modern pesticides have to fulfill two requirements: they have to control weeds, pests, diseases, etc. while not to load the environment. These agents should put out their plant protective effect before their degradation. It results in an apparent contradiction because the stable chemicals are able to become environmental contaminants concentrated mostly in the soil. The retention of the pesticides in the soil is important otherwise they can reach the groundwater by leaching. It means that the vertical mobility of these compounds is strongly correlated with their adsorption behavior. If we want to predict the leachability of a pesticide the first step is the investigation of its chemical stability under the given conditions (soil type, pH etc). It is followed by the second step being the study of its adsorption on the soil.

## 2. The degradation of pesticides in the soil

Pesticide breakdown in the soil is classified as chemical and biological processes influenced by physical factors leading to very complicated pathways. The decomposition is chemical when it takes place in the absence of living organisms. In this case the reaction is activated by thermal, photochemical, radiochemical, electrochemical factors as well as by the interaction with the soil. Photochemical degradation occurs at the soil surface where the energy of sunlight can be absorbed either directly by the pesticide or indirectly by soil components working as photocatalyst. Since atmospheric ozone absorbs solar radiation below 290 nm only chemicals that absorb above this wavelength can be decomposed directly by sunlight. The interaction with the soil means either the catalytic effect of its constituents (e.g. clay minerals) or reactions with its organic matter content (e.g. redox reactions).

In the presence of living organisms biological degradation can take place. In these pathways mainly the enzyme system of microorganisms (bacteria, algae, fungi etc.) works as biocatalyst. Also other species can contribute to the transformation of the parent compound. For example the roots of plants produce fluids which assist the decomposition.

The microorganisms in the soil are either pesticide susceptible or not. In the latter case they will be killed by the compound and no degradation occurs while only a part of the resistant communities can degrade the pesticide (Pierzynski et. al., 1994). The non-biodegradable compounds are persistent and their presence in the soil results in contamination.

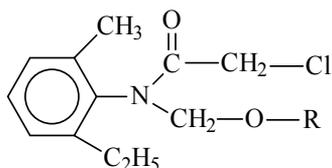
The most notorious example for the persistent pesticides is DDT being one of the chlorinated hydrocarbon type insecticides. This compound and their degradation products (DDD, DDE) can be detected in the soil and sediment even after about 30 year of the contamination (Skibniewska et.al., 2003).

As the soil is contaminated in highest amounts by herbicides present study is focused on the fate of different types of these agents in the soil.

The degradation of pesticides takes place in most cases either on (by oxygen, light) or near to the soil surface (by oxygen, microbial activity). For this reason the residence time of the agent in this active layer is a determinant factor. The conditions in deeper active layer are considered mainly anoxic. It is represented by the laboratory experiments (aeration just during the sampling, 25 °C, and natural light in the lab) in the following sections which are numbered according to the type of the herbicide investigated.

### 2.1 Chloroacetanilide type herbicides (acetochlor and propisochlor)

The chloroacetanilide type herbicides acetochlor and propisochlor (Fig. 1) are widely used in the world. This is the same in Hungary where they were produced in high amounts until the beginning of the XXI. century. In the literature more information can be found about the decomposition of the active ingredient **1** than about **2** which is an original Hungarian product.



1. R=ethyl
2. R=isopropyl

Fig. 1. The structure of acetochlor (**1**) and propisochlor (**2**)

Chloroacetanilides are stable at environmentally relevant pH and temperature in buffer solutions. In the soil, plants and animals more than 30 degradation products of these herbicides have been already identified. Two different dechlorination reactions of acetochlor have been proved in the soil: a. hydrolysis, b. glutathione conjugation (Roberts, 1998a). Both pathways are very important in the detoxification of this compound.

The aim of the study presented here was to check the stability of the compounds **1** and **2** in different types of soils under the conditions detailed above. The decay of these herbicides was followed in the liquid phase (pH=7) of the suspension made from soil : buffer= 1 : 10 ratio. Figure 2 shows that both compounds proved to be rather stable but the degradation of acetochlor (Fig. 2 a.) seemed to be slightly slower than that of propisochlor (Fig. 2 b.). Comparing the soils the decomposition was the fastest in the presence of chernozem having the highest organic carbon content (25.18 mg TOC/g). In the case of propisochlor the order of the breakdown rates follows the TOC values of the soils, which can be seen on Fig 2b. The decay of acetochlor proved to be rather similar in the soils having lower organic carbon content (brown forest and sandy soils).

These results emphasize that the degradation of the chloroacetanilide type compounds need the presence of soil organic matter which is the living space of different species. It means that the degradation is governed by biological processes.

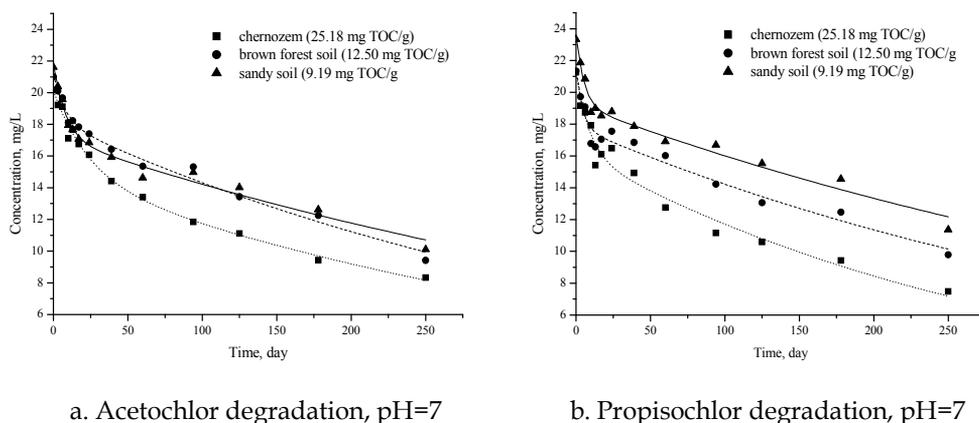


Fig. 2. Degradation of acetochlor and propisochlor in the presence of different soils

Field measurements in China soils resulted in  $t_{1/2} \approx 5$  days for propisochlor (Wang et al., 2007). In experiments carried out with compound **1** and **2** in soils of temperate zone relatively long half-life time was determined under field conditions: for acetochlor approx. 17 days while for propisochlor approx. 10 days (Ferenczi, 1998). According to these results it can be pointed out that the climate (temperature, moisture, pH of the soil etc.) significantly influences the degradation of these compounds.

We must mention that first order kinetic is commonly used for pesticide decomposition even in the case when the fitting is not the best at the well-known linearization method.

$$c = c_1 \cdot e^{-k_1 \cdot t_1} + c_2 \cdot e^{-k_2 \cdot t_2} \quad (1)$$

$c_1$  and  $c_2$  : initial concentrations;

$k_1$  and  $k_2$ : rate constants;

$t_1$  and  $t_2$ : reaction time in the two parallel steps.

In our case neither the equation of the simple first order nor that of second order kinetic reaction resulted in relatively good fitting ( $R^2$  should be at least ca. 0.9). It suggests a complex degradation pathway that cannot be described by one process alone. The application of two parallel reactions with first order kinetic (1) resulted in quite good  $R^2$  value (Table 1). The half life time ( $t_{i(1/2)}$ ) was calculated in both steps ( $i=1,2$ ) by equation (2) as it is generally calculated in first-order kinetic reactions.

$$t_{i(1/2)} = \frac{\ln 2}{k_i} \quad (2)$$

where  $k_i$  means the rate constants in step 1 and 2.

According to the calculated  $t_{i(1/2)}$  of these two reactions it can be pointed out that one of the reactions (see  $t_{1(1/2)}$  in Table 1) is much faster than the other (see  $t_{2(1/2)}$  in Table 1). Regarding the total degradation rate these data are in accordance with the explanations given for Fig. 2., however, the acetochlor decomposition on brown forest soil is faster than it was understand on the basis of Fig. 2 a.

Our results and the field experiments carried out by other authors (Ferenczi, 1998; Konda & Pasztor, 2001; Wang et al., 2007) indicate that the organic matter content of the soil is

essential in the breakdown of chloroacetanilides but the composition of this organic matter and also other factors can influence this process.

Soil	Chernozem		Brown forest		Sandy soil	
Compound	1	2	1	2	1	2
Parameters						
$k_1$ (1/day)	0.0426	0.04426	0.13133	0.24598	0.11934	0.18978
$t_{1(1/2)}$ (day)	16.27	15.66	5.28	2.82	5.81	3.65
$k_2$ (1/day)	0.00238	0.00273	0.00243	0.00225	0.00189	0.00183
$t_{2(1/2)}$ (day)	291.24	253.90	285.25	308.07	366.74	378.77
$R^2$	0.9940	0.9704	0.9874	0.9684	0.9848	0.9774

Table 1. Calculated parameters of equation (1) for chloroacetanilide degradation

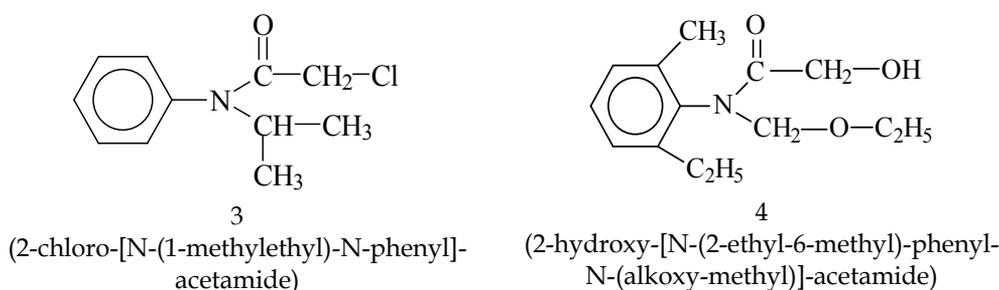


Fig. 3. Chloroacetanilide degradation products identified in the soil

The types of degradation products (Fig. 3) identified in the soil at the end of the experiments support the idea of two ways of pesticide breakdown. (Of course the decomposition pathway can be much more complicated.) After appropriate preparation of the samples compound 3 ( $m/z$ : 211,  $M^+$ ) was determined by GC-MS in the case of both chloroacetanilides. The structure of this acetamide indicates an isomerisation reaction. Product 4 determined by DRIFT FT-IR spectroscopy is the result of the hydrolysis of acetochlor.

Isomerization generally needs catalyst that is likely an enzyme of any microorganism in the soil. The hydrolysis can occur in any aquatic medium even under sterile conditions but it can be faster when living organisms are present.

## 2.2 Urea type herbicides (isoproturon)

The urea type compounds are herbicides with intermediate persistence in aerobic soils. Enhanced rates of their degradation have not been observed even under anaerobic conditions. Isoproturon (see compound 5 in Fig. 5) is applied nowadays most frequently in combination with other active ingredients but even at the end of the XX. century its consume was about 500 tons/year in Europe. Since the information about the decomposition of this compound is rather incomplete (Roberts, 1998b) the present section focuses on this representative of urea type herbicides.

The decay of isoproturon was compared in different buffered solutions (pH=5, pH=7, pH=8) in the absence as well as in the presence of soils. This active ingredient proved to be a rather persistent compound because its concentration in the buffer decreased only by 5.5-12 % until

the 350th days of the study. It can be pointed out that the decomposition of isoproturon hardly depends on pH but it is faster in the presence of the soils. Comparing the degradation in the presence of different soils at neutral pH the experience was similar as it was found in the case of chloroacetanilide type herbicides: the organic content of the soil plays leading role in the decay of the compound but also other factors affect the breakdown (Fig. 4, Table 2).

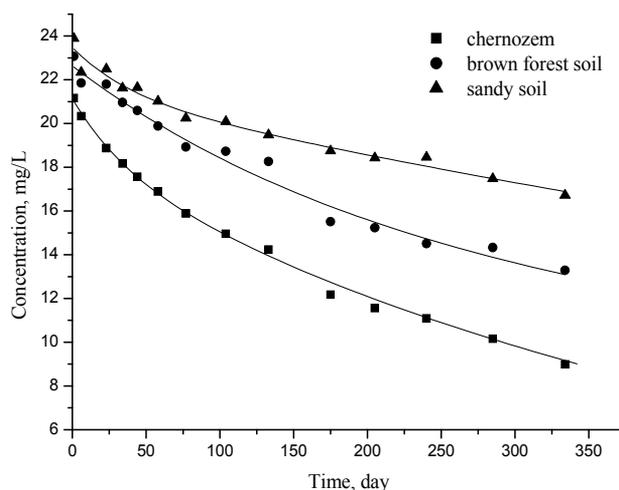


Fig. 4. Degradation of isoproturon in the presence of different soils, pH=7

The fitting according to equation (1) and the determination of the half-life times (Eq. (2)) were carried out as it was described for chloroacetanilide herbicides (Section 2.1.). Calculated parameters show that both steps as well as the total reaction were the slowest in the presence of brown forest soil.

Parameters	Soil		
	Chernozem	Brown forest	Sandy soil
$k_1$ (1/day)	0.02669	0.0049	0.02517
$t_{1(1/2)}$ (day)	25.97	141.46	27.54
$k_2$ (1/day)	0.00206	0.00057	0.0007
$t_{2(1/2)}$ (day)	336.48	1216.05	990.21
$R^2$	0.9980	0.9817	0.9774

Table 2. Calculated parameters of equation (1) for isoproturon degradation

The investigation of the degradation products, however, indicates other type of reactions, too. According to GC-MS the liquid phase (pH=7) over chernozem contained two degradation products (6 and 7 in Fig. 5). 6 was identified earlier in aquatic solution using UV light (pH>7) as well as in soil while 7 formed only under UV radiation at pH=7 (Roberts, 1998c).

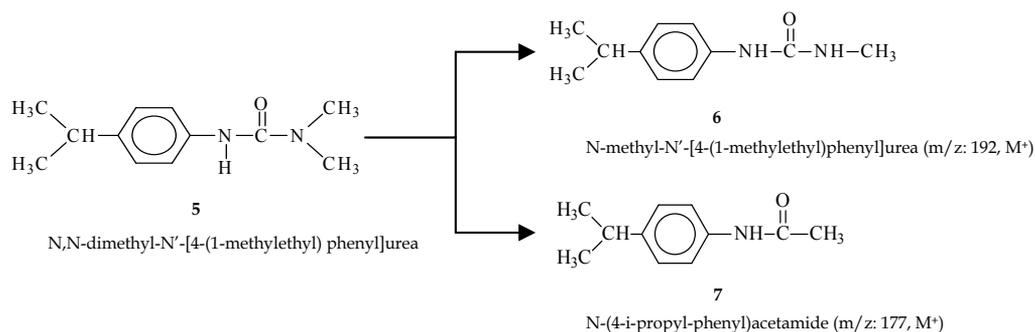
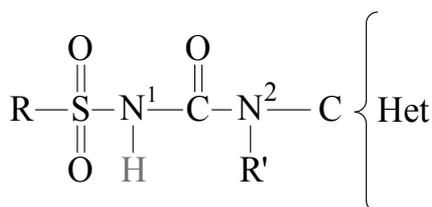


Fig. 5. Isoproturon (5) degradation regarding the products (6, 7) of photo catalytic reaction

This refers to the photosensitization effect of humic substances in the decomposition of isoproturon. This conclusion is in accordance with the results of Gerecke et al. who observed the photocatalytic role of DOM (Gerecke et al., 2001) in the degradation of phenylurea herbicides. This process can work on the soil surface after application of the herbicide, and in surface waters in case of their pollution by this compound.

### 2.3 Sulfonyl urea type herbicides

The sulfonyl urea type herbicides (8) are relatively young compounds among the pesticides. Their stability and other chemical as well as physical properties (e.g. solubility) strongly depend on their structure. This is in correlation with the dissociable proton (signed with red color in Fig. 6) that results in weak acidic character of these compounds (Roberts, 1998d). The importance of this reaction is emphasized by pK<sub>a</sub> values. It can be seen e.g. in the comparison of two sulfonyl urea type compounds having just one difference: tribenuron methyl (9 in Fig. 8) has a methyl substitution on N<sup>2</sup> while metsulfuron methyl (10 in Fig. 9) has a proton in this position. The pK<sub>a</sub> values of compounds 9 and 10 are 5.0 and 3.3, respectively. For this reason the investigations were focused on the comparison of degradation of these two compounds on two different soils: on the rather basic chernozem (pH=8.34) and on the acidic brown forest (pH=5.92) soils. The aquatic medium equilibrated with the surface was buffered and had the same pH like the soil.



8

R=Me, substituted Ph, substituted heterocycle; C{Het= substituted pyrimidine or triazine, R'=H, Me

Fig. 6. General structure of sulfonylurea herbicides

As it is found the tribenuron methyl is relatively stable over chernozem but not over brown forest soil (Fig. 7). The reaction rates differ much more significantly than in the other cases when the pH was the same and only the TOC content of the soils varied.

The fitting of the measured data for chernozem was carried out according to zero order kinetic being characteristic in heterogeneous system, and it is described with a simple linear equation.

Soil	Chernozem	Brown forest	
Equation	$c = -kt + c_0$	$\ln(c/c_0) = -kt$	$c = c_1 e^{-k_1 t_1} + c_2 e^{-k_2 t_2}$
Parameters	$k$ (1/day) 0.2207	$k$ (1/day) 0.2343	$k_1$ (1/day) 0.2080
			$t_{1(1/2)}$ (day) 3.33
	$t_{1/2}$ (day) 102.70	$t_{1/2}$ (day) 2.96	$k_2$ (1/day) 3374.74
			$t_{2(1/2)}$ (day) 0.0002
$R^2$	0.9887	0.9934	0.9971

Table 3. Fitting parameters of tribenuron methyl decomposition supposing different kinetic

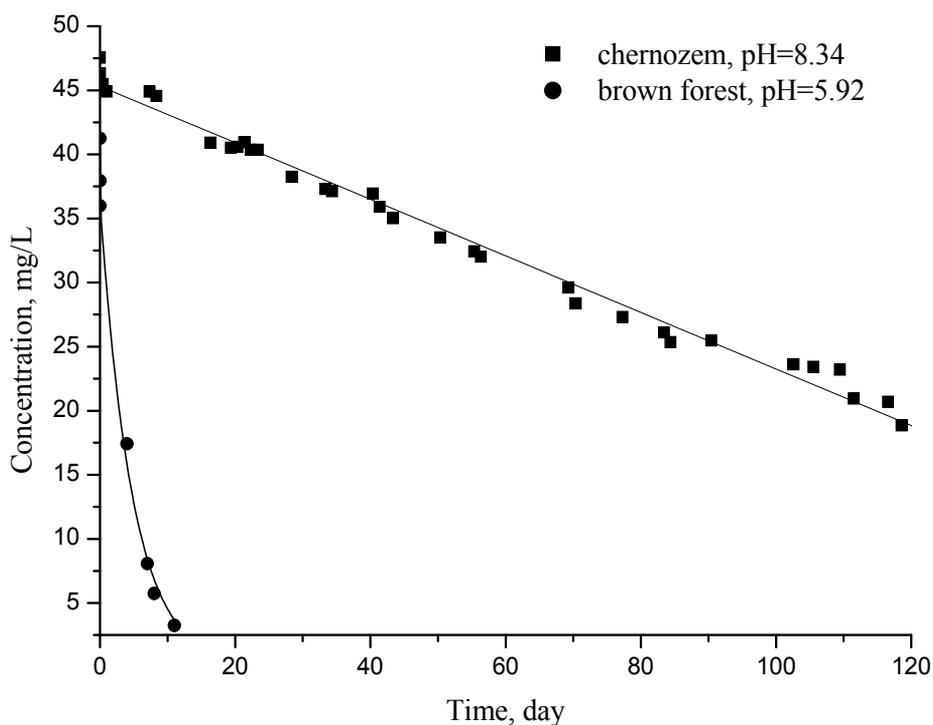


Fig. 7. Degradation of tribenuron methyl in the presence of two different soils

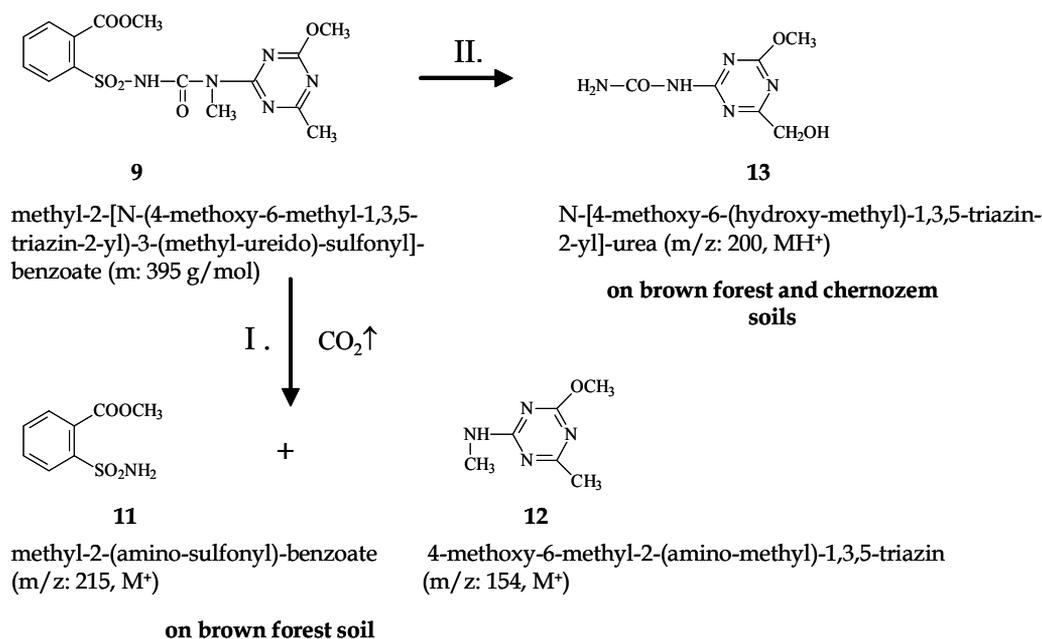


Fig. 8. The scheme of tribenuron methyl (**9**) decomposition concerning the identified degradation products

In the case of brown forest soil the first order kinetic was applied for the fitting when either one (using linearization method) or two parallel reactions (Eq. (1)) were supposed. Every counted parameter is summarized in Table 3 where the  $R^2$  values are rather good but regarding the brown forest soil the fitting for two parallel reactions was even better than only for one step. According to these results it has to be emphasized that the reaction pathways are always much more complicated than they are commonly simplified.

The degradation of metsulfuron methyl (**10** in Fig. 9) followed zero order kinetic. This compound proved to be persistent under the laboratory conditions because its half life time was over chernozem 500, and over brown forest soil 515 days indicating that the pH did not influenced its decomposition. Since the decay rate of this compound over the soils did not show significant difference from that in the buffer solution it seems that the degradation of this herbicide is slightly assisted by organic matter of the soils used in this experiment.

The degradation products were investigated after extraction of the equilibrated solution as well as of the soil and were compared to the blanks studied without soil. According to GC-MS spectra both extracts (obtained from the solution or from the soil) had the same components. In the case of the acidic sample (brown forest soil investigations) the identified degradation products of tribenuron methyl (**9**) were: **11**, **12** and **13** (Fig. 8). The blank sample contained **11** and **12** indicating the hydrolytic cleavage of the sulfonylurea linkage (see I. in Fig. 8). It can take place even without the presence of the soil.  $\text{CO}_2$  is produced during this reaction which is often hundreds of times faster under acidic conditions (Roberts, 1998d).

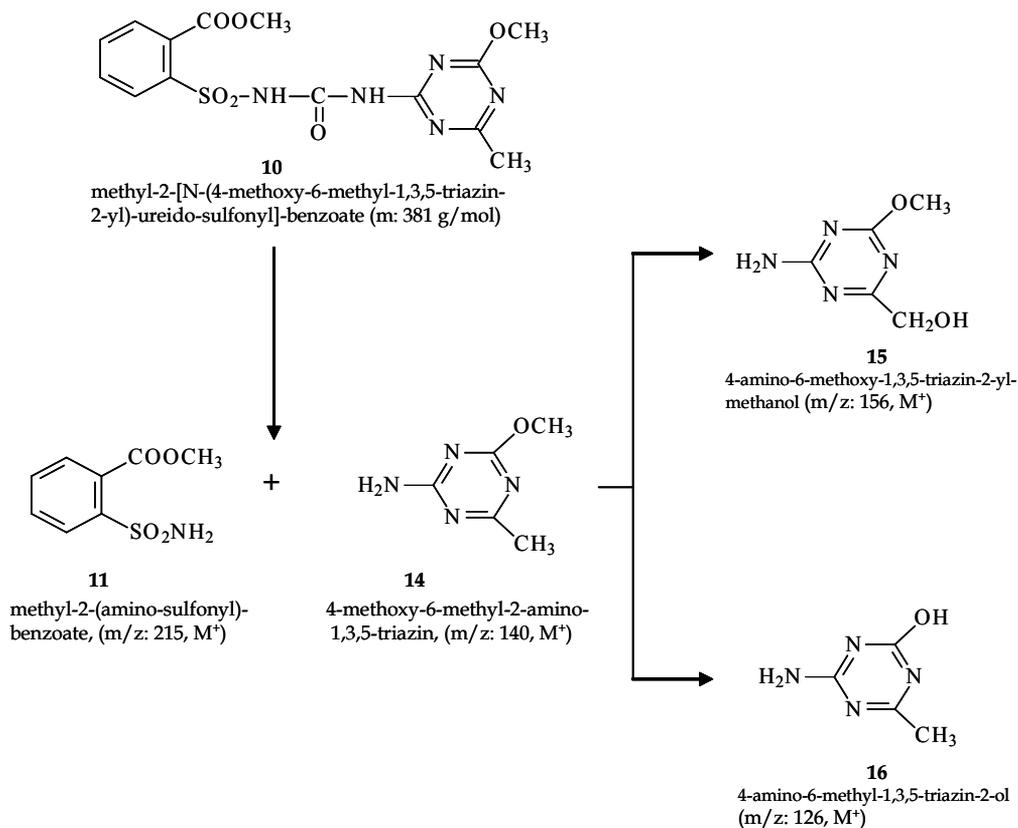


Fig. 9. The scheme of metsulfuron methyl (10) decomposition concerning the identified degradation products

Extracts obtained from the chernozem investigations contained only **13** being a newly determined compound and no hydrolysis products were identified. This result suggests another decomposition mechanism (II.) attributable to the presence of the soil (e.g. humic substances, living organisms). Reaction I. proved in the presence of brown forest soil does not need any organic matter.

Investigations regarding the metsulfuron methyl degradation also resulted in hydrolytic cleavage products of the sulfonylurea linkage (see compounds **11**, **14** in Fig. 9). The other degradation products (**15**, **16** Fig. 9) were found only in the presence of the chernozem soil. These compounds indicate, that the organic matter did not increase the decay rate of metsulfuron methyl, however, it must have a special role in the decomposition.

### 3. Pesticide sorption on the soil

The sorption on the solid matrix of the soil is one of the most important processes which control transport, persistence, bioavailability and degradation of organic pesticides on soil. Many theories and models have been presented in the literature to describe the different types of sorption isotherms. The most commonly used adsorption isotherm equations for organic contaminants on soil are the Langmuir and the Freundlich isotherms.

The soil as an adsorbent has various active sites leading to rather complicated adsorption mechanisms with the environmental pollutants like pesticides.

### 3.1 Adsorption described by multi-step isotherm

Bioavailability and environmental transport of pesticides in soil–water system are controlled by their adsorption properties. The extent of adsorption is one of the most important chemical parameter entering into the differential material balance equations of the hydrogeochemical transport models (Weber et al., 1991; Klein et al., 1997; Kovács, 1998). Equilibrium distribution of the studied solute is usually described by some specific form of the general equation (3):

$$q = f(c) \quad (3)$$

where  $q$  is the amount of adsorbed solute per gram of sorbent and  $c$  is the concentration of the solute in the equilibrium solution phase. Depending on the type of interactions contributing to the adsorption mechanism equation (3) can take on various specific forms. The non-classical nature of the adsorption of hydrophobic organic compounds by soils is taken into consideration in the so-called distributed reactivity model developed by Weber et al. (1992). Soil is basically considered here as a composite material containing inorganic and two types of organic constituents each characterized by its local sorption isotherm. Adsorption of organic contaminants on the exposed surface of the inorganic mineral components is described by the Langmuir isotherm. One part of the soil organic constituent is the geologically older (hard) organic fraction (kerogen, coals etc.) that presents a relatively hydrophobic surface upon which the retention of the contaminant is described by the Freundlich isotherm. The other type of organic soil component is represented by the evolutionary immature (soft) material (humic substances etc.) which is more likely to function as partitioning media upon which the retention of solute is characterized by a linear Henry type of isotherm equation. The distributed reactivity model accommodates these linear and non-linear local adsorption isotherms and the total sorption is approximated as the sum of the isotherms (Weber et al., 1992):

$$q_r = \sum_{i=1}^m x_i \cdot q_i \quad (4)$$

where  $q_r$  is the overall solid-phase concentration of the solute,  $q_i$  is the part of  $q_r$  attributable to the  $i$ th of  $m$  individual local isotherms (in this case it is expressed per unit mass of the solid-phase component responsible for the  $i$ th local reaction) and  $x_i$  is the mass fraction of that solid-phase component. Different types of isotherms suggested for the description of the adsorption of hydrophobic organic contaminant on soils were summarized and discussed by several recent reviews (Voice & Weber, 1983; Weber et al., 1983; Samiullah, 1990; McBride, 1994; Wolt, 1994; Mader et al., 1997; Carmo et al., 2000). Two-step isotherms were reported for the adsorption of the tenside sodium dodecylsulfate on graphitized carbon (Zettlemoyer & Micale, 1971). At low concentrations the tenside is adsorbed at some equilibrium orientation when a longer length of the hydrocarbon chain is in contact with the surface. At higher concentrations, however, the orientation changes to a new equilibrium position leading to the enhancement of adsorption shown as a second plateau of the isotherm. Systematic study of the adsorption of different surfactants by Clunie & Ingram (1983) and Jönsson et al. (1998a) also revealed the occurrence of two-step isotherms. Sodium dodecyl sulfate (SDS) is a widely used and also well investigated anionic surfactant.

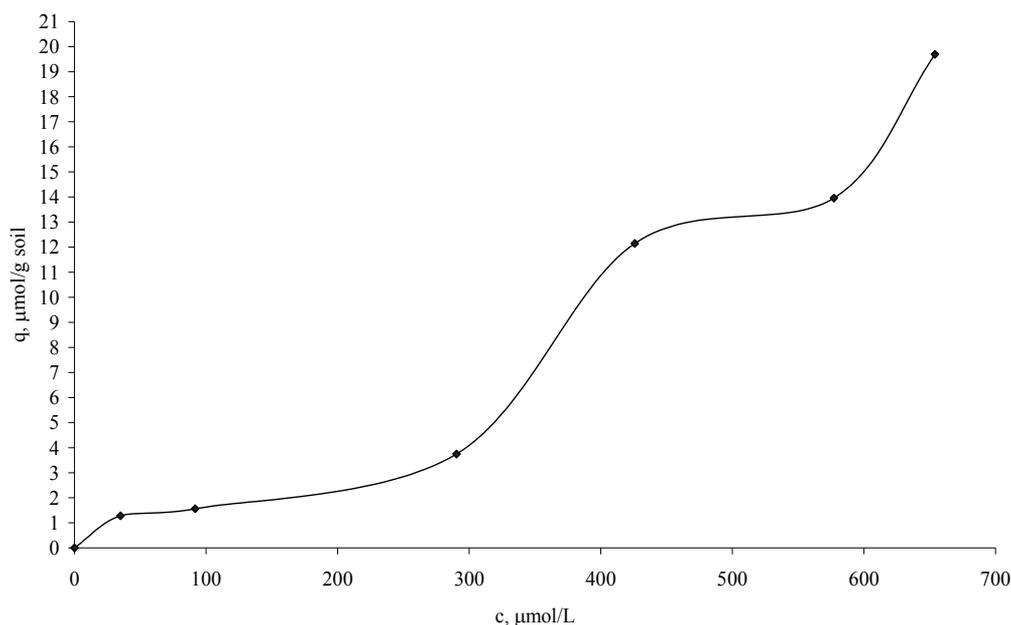


Fig. 10. Adsorption of SDS on sandy soil

Since it can be used even in pesticide formulation the adsorption of SDS was carried out on sandy soil (see Fig. 10). The isotherm has three steps indicating the micelle formation of the molecules either on the surface or in the solution. The critical micelle concentration (cmc) of this surfactant is  $830 \mu\text{mol/L}$  at  $20^\circ\text{C}$  (Jönsson et. al., 1998b). It can be seen on Fig. 10 if the equilibrium concentration is less than  $100 \mu\text{mol/L}$  the curve forms a Langmuir-type isotherm which suggests the filling of active sites by single molecules. When the specific adsorbed amount ( $q$ ) is  $1.6 \mu\text{mol/g soil}$  the surface is covered by single SDS molecules (maximal coverage:  $q_T$ ). In the solution of this surfactant the micelle formation starts above  $800 \mu\text{mol/L}$ , however, the molecules adsorbed already on the surface assist the formation of associates even at lower equilibrium concentration values. The second step of the isotherm having  $14 \mu\text{mol/g soil}$  maximal adsorption capacity ( $q_T$ ) means if the ratio of the  $q_T$  values of the second and first steps ( $14/1.6$ ) is calculated, its result is 9. This allows us to suppose that 9 levels of SDS molecules can cover the surface.

Our experiments with the chloroacetanilide as well as with other type of herbicides usually resulted in similar isotherms to those reported for the adsorption of surfactants. These plots show at least two well defined adsorption steps (see section 3.3), which could not be fitted to the Freundlich or Langmuir equations.

### 3.2 The derivation of the equation of multi-step isotherm

The aim of the present section is to describe these isotherms by a suitable equation because the steps received little attention in the adsorption studies so far.

In the system reaction (5) is supposed:



S: empty sites of the soil surface, A: solute,  $SA_n$ : surface complex,  $n \geq 1$ : a non-integer number representing the average degree of association of the solute molecules.

The equilibrium constant ( $K$ ) of the reaction:

$$K = \frac{[SA_n]}{[S] \cdot [A]^n} \quad (6)$$

where  $[S]$ ,  $[A]$ ,  $[SA_n]$  are the equilibrium concentrations of S, A and  $SA_n$ , respectively.

The available total concentration of the surface sites  $[S]_T$  is given by the mass balance equation (7), and  $[S]$  can be substituted:

$$[S]_T = [S] + [SA_n] \quad (7)$$

$$[S] = [S]_T - [SA_n] \quad (8)$$

After the substitution of  $[S]$  the value of  $K$  can be given by equation (9):

$$K = \frac{[SA_n]}{([S]_T - [SA_n]) \cdot [A]^n} \quad (9)$$

$$[SA_n] = \frac{[S]_T \cdot K \cdot [A]^n}{1 + K \cdot [A]^n} \quad (10)$$

$$q = \frac{q_T \cdot K \cdot [A]^n}{1 + K \cdot [A]^n} \quad (11)$$

where  $q$  and  $q_T$  are the surface concentrations of  $[SA_n]$ /mass adsorbent and  $[S]_T$ /mass adsorbent, respectively.

If associates form above a certain concentration limit of the solute, the concentration variable  $[A]$  is replaced by the following relationship:

$$[A] = \frac{c - b + |c - b|}{2} \quad (12)$$

$c$ : controlled value of the equilibrium concentration of solute,  $b$ : critical concentration limit of associates.

If  $b = 0$  the control variable is given by the concentration of the non-associated solute ( $c = [A]$ ) and associates can be formed only on the surface. If  $b > 0$  associates can be formed either on the surface or in the solution and the concentration of these associates appears in equation (13). Its rearrangement results in equation (14) describing one step of the isotherm.

$$q = \frac{q_T \cdot K \cdot \left( \frac{c - b + |c - b|}{2} \right)^n}{1 + K \cdot \left( \frac{c - b + |c - b|}{2} \right)^n} \quad (13)$$

$$q = \frac{q_T \cdot K \cdot (c - b + |c - b|)^n}{2^n + K \cdot (c - b + |c - b|)^n} \quad (14)$$

Similarly to the so-called distributed reactivity model which allows the addition of various isotherm equations (Weber et al., 1992) the multi-step isotherm is calculated as the sum of the equation of the  $i$ th steps:

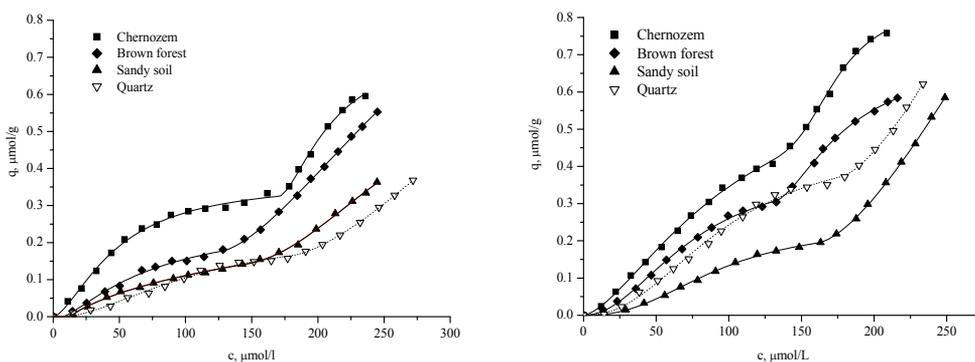
$$q = \sum_{i=1}^s \left\{ \frac{q_{Ti} \cdot K_i \cdot (c - b_i + |c - b_i|)^{n_i}}{2^{n_i} + K_i \cdot (c - b_i + |c - b_i|)^{n_i}} \right\} \quad (15)$$

where  $q$  means the specific adsorbed amount,  $s$  is the number of steps of the isotherm,  $q_{Ti}$  and  $K_i$  are the adsorption capacity and the equilibrium constant, while  $b_i$  and  $n_i$  are the concentration limit and the average degree of association relevant to the  $i$ th step of the curve.

### 3.3 Application of multi-step isotherm equation for the adsorption of certain herbicides on soils and its components

The adsorption properties of those compounds were studied which proved to be stable under conditions being appropriate in static equilibrium experiments. Equation (15) was used to evaluate all of the isotherms. Parameters characteristic for the adsorption curves were calculated by using the nonlinear least square curve fitting procedure of the "Origin" scientific graphing and analysis software.

The adsorption isotherms of chloroacetanilide type herbicides on soils and quartz are shown in Fig. 11. a and b. The two steps of the curves can be clearly seen, and fitting was successful.



a. Adsorption of acetochlor, pH=7

b. Adsorption of propisochlor, pH=7

Fig. 11. Adsorption of acetochlor (1) and propisochlor (2) on different soils and quartz

The mechanism of the adsorption is very similar in the case of these compounds. More propisochlor is bounded by the soils as well as by quartz than acetochlor. The main force of the adsorption is the hydrophobic interaction.

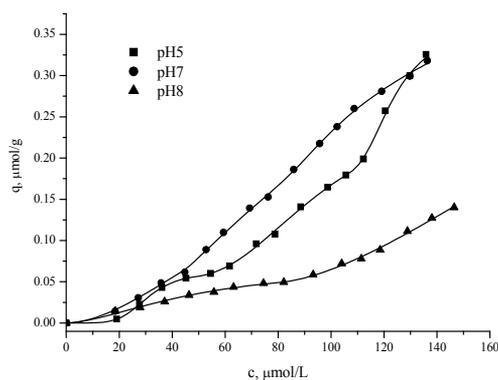


Fig. 12. Adsorption of isoproturon (5) on quartz at different pH values

The adsorption of isoproturon on quartz is introduced here (Fig. 12). The static equilibrium experiments were carried out at different pH values. The points were fitted only once by two- (pH8) and twice by three-step isotherm equation. Since quartz has neutral siloxane surface that functions as a very weak Lewis base (Johnston & Tombácz, 2002) under basic conditions the possibly negatively charged isoproturon is adsorbed in lower amounts than at neutral as well as at acidic pH where the adsorption must be governed by hydrophobic interaction as it was found at chloroacetanilides, too.

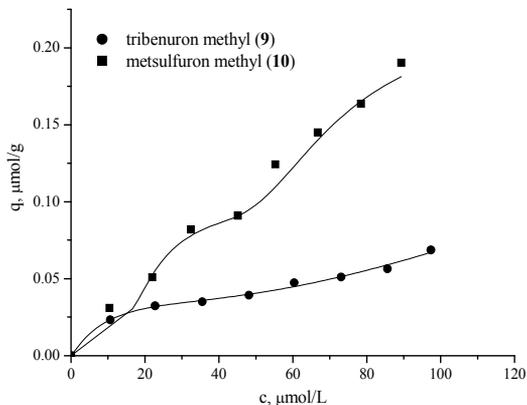


Fig. 13. Adsorption of two sulfonylurea herbicides on chernozem, pH=8.3

Since tribenuron methyl proved to be a rather instable compound under acidic conditions its adsorption studies can give us exact results only at  $\text{pH} \geq 7$ . Fig. 13 shows the isotherm of two sulfonylurea herbicides (tribenuron methyl and metsulfuron methyl) on chernozem. The pH of the liquid phase was equal to that of the soil. Metsulfuron methyl (10) has three steps of isotherm and adsorbs in higher amounts on chernozem than the tribenuron methyl (9) having a two-step graph. The structure of 10 results in a more stable and less polar compound than 9, and the hydrophobic interaction works more effectively leading to more extent adsorption.

#### 4. Buffering of pesticide by soil

The soil has a very important function that is the buffering ability. It means that the soil can adsorb some pesticide as contaminant but it also means if the concentration in the soil solution decreases the pesticides can go to the liquid phase by desorption.

In the literature of soil science and agricultural chemistry the term “buffer capacity” is used mainly for the phosphorus availability and pollution problems. This is the reason that the estimation of the pesticide buffering ability of soils starts here with the equations applied for the better known soil-P system.

The buffer capacity is measured either from the adsorption or from the desorption isotherms and the equilibrium buffer capacity function  $B$  is calculable as (16):

$$B = \frac{\partial q}{\partial c} \quad (16)$$

The value  $B$  can also be described in the following function of the equilibrium concentration specifically in the case of the Langmuir isotherm (Rattan, 2005):

$$B = \frac{A}{(1 + k \cdot c)} \quad (6)$$

The buffer capacity of the soil-P system can be calculated by means of the phosphorus adsorption isotherm. As a result of the differentiation of the adsorption equation, the equilibrium buffer capacity at any concentration could be calculated as:

$$B = \frac{dP_{ads}}{dc} = \frac{1}{3} \cdot \frac{k}{\sqrt[3]{c^2}} \quad (17)$$

It can be assumed that the adsorption reduces exponentially according to the Freundlich isotherm with  $n = 1/3$  exponent.

The advantage of using  $Q/I$  relationships ( $Q$ : quantity factor – nutrient (P) in the solid phase,  $I$ : intensity factor – nutrient (P) in the soil solution) is that they allow the prediction of both P retention and release in soils (Kpombekou & Tabatabai, 1997). The P-buffering capacity of a soil is its ability to resist a change in the P concentration of the solution phase. Phosphorus-buffering capacities of soils can be related to both plant nutrition and environmental pollution. The  $Q/I$  model can be applied to either adsorption or desorption experiments (Yaobing & Michael, 2000). Results showed that  $Q/I$  parameters (intercept labile P,  $a$ ; equilibrium buffering capacity,  $B$ ; and equilibrium P concentration,  $EPC$ ) varied significantly between and within sites for the cropping systems studied.

As it is detailed above (see section 3.) the adsorption of herbicides as solutes can not be exactly described neither by simple Langmuir nor by Freundlich equation because these compounds resulted in two- or multi-step isotherms on soils and quartz. Using these isotherms (see equation (15)) the equilibrium buffering capacity ( $B$ ) was calculated by the derivative function (18):

$$B = \frac{\partial}{\partial c} \left\{ \sum_{i=1}^s \left\{ \frac{q_{T_i} \cdot K_i \cdot [(c - b_i) + abs(c - b_i)]^{n_i}}{2^{n_i} + K_i \cdot [(c - b_i) + abs(c - b_i)]^{n_i}} \right\} \right\} \quad (18)$$

The model parameters ( $q_{T_i}$ ,  $K_i$ ,  $b_i$  and  $n_i$ ) were determined by non-linear regression using sequential simplex optimization.

The mechanical calculation of the derivative of function (18) is impossible because of the break point of abs function. We know, however, that the abs function is just needed due to negative (c-b) data which are not taken into account. It means we can make the derivative function as the sum of single Langmuir isotherms in every x region. Therefore the following function can be applied as the equilibrium buffering capacity of multi-step adsorption isotherms:

$$B = \sum_{i=1}^s \left\{ \frac{2^{n_i} \cdot q_{T_i} \cdot K_i \cdot n_i \cdot [(c - b_i) + \text{abs}(c - b_i)]^{n_i - 1}}{\left\{ 2^{n_i} + K_i \cdot [(c - b_i) + \text{abs}(c - b_i)]^{n_i} \right\}^2} \right\} \quad (19)$$

Before the interpretation of the calculation of equilibrium buffering capacity of the soil the adsorption isotherms of some pesticides are shown in Figure 14.

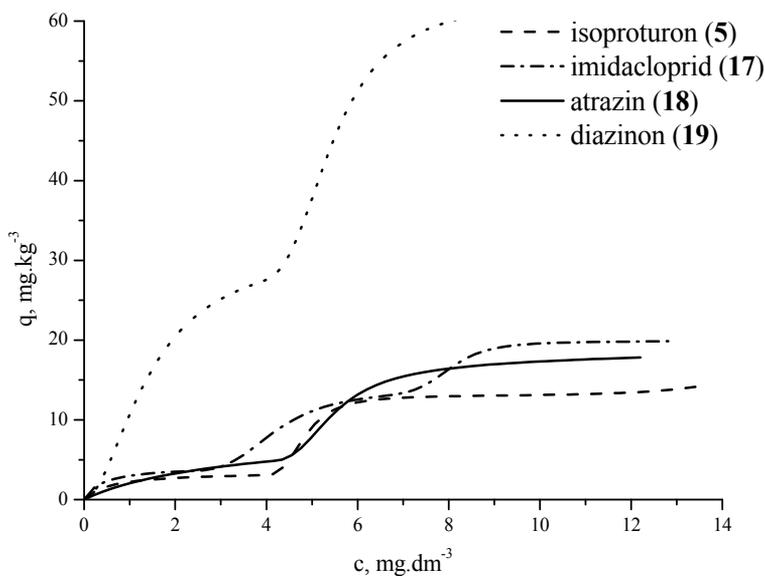


Fig. 14. Adsorption of the investigated pesticides on soil

Sign	$q_{T_1}$	$q_{T_2}$	$q_{T_3}$	$K_1$	$K_2$	$K_3$	$b_1$	$b_2$	$b_3$	$n_1$	$n_2$	$n_3$
5	9.29	9.93	4.42	0.30	3.79	1.47	0	4.57	13.16	0.45	0.55	3.11
17	31.73	31.89	0.00	0.51	0.28	1.00	0	3.86	1.00	1.84	2.67	1.00
18	9.26	10.97	0.00	0.28	0.49	1.00	0	4.16	1.00	0.95	2.46	1.00
19	4.22	9.82	6.01	2.41	0.14	0.01	0	2.33	5.67	1.00	3.00	6.00

Table 4. The fitted parameters of the equilibrium buffering capacity data ( $R^2 \geq 0.9960$ )

Using the given parameters the equilibrium buffering capacity function was calculated but the larger error of derivative function was fitted on the differentiate data of the original measurement result.

We calculated the new parameters generally (these values are not exactly equal to the original sorption isotherm parameters due to the new fitted weighting points) which are summarized in Table 4.

Calculated functions are shown in Figure 15 where very big differences can be seen in the  $B$  values at relatively small equilibrium concentration differences. This phenomenon seems to be almost a periodical change in the case of some compounds.

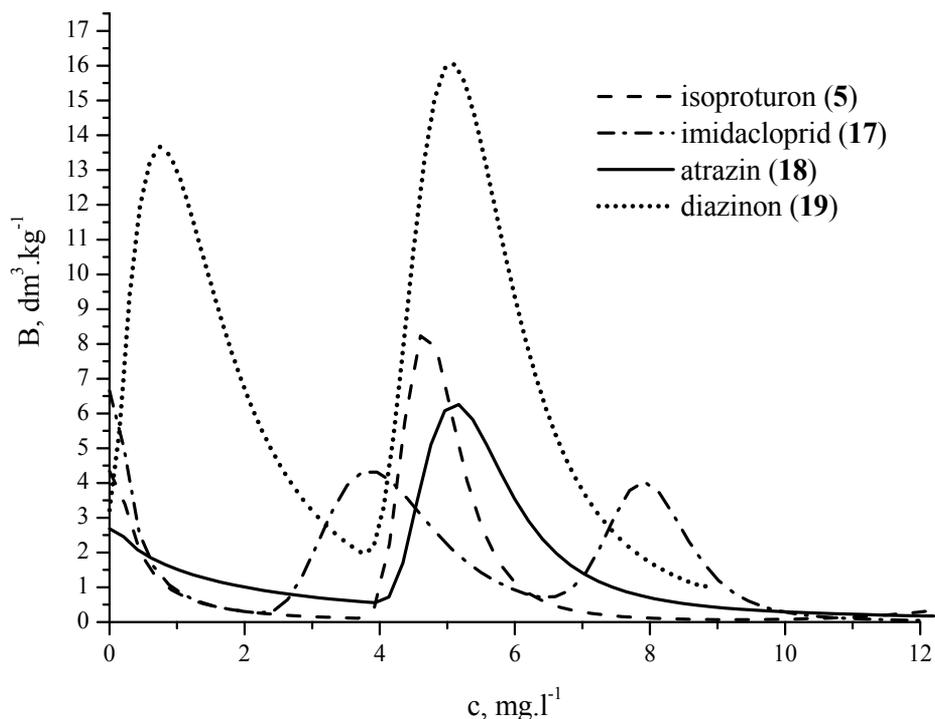


Fig. 15. Equilibrium buffering capacity functions of different pesticides

#### 4.1 An example: The buffering of isoproturon by soil

Adsorption of isoproturon (5) on the soil resulted in three steps of isotherm. In this case the adsorption is described by equation (20) while the buffering capacity by equation (21).

$$q = \frac{[q_{T_1} \cdot K_1 \cdot c]^{n_1}}{[1 + K_1 \cdot c]^{n_2}} + \frac{q_{T_2} \cdot K_2 \cdot [(c - b_2) + \text{abs}(c - b_2)]^{n_2}}{2^{n_2} + K_2 \cdot [(c - b_2) + \text{abs}(c - b_2)]^{n_2}} + \frac{q_{T_3} \cdot K_3 \cdot [(c - b_3) + \text{abs}(c - b_3)]^{n_3}}{2^{n_3} + K_3 \cdot [(c - b_3) + \text{abs}(c - b_3)]^{n_3}} \quad (20)$$

$$B = \frac{q_{T_1} \cdot K_1 \cdot n_1 \cdot c^{n_1-1}}{\left\{1 + K_1 \cdot [c]^{n_1}\right\}^2} + \frac{2^{n_2} \cdot q_{T_2} \cdot K_2 \cdot n_2 \cdot [(c-b_2) + \text{abs}(c-b_2)]^{n_2-1}}{\left\{2^{n_2} + K_2 \cdot [(c-b_2) + \text{abs}(c-b_2)]^{n_2}\right\}^2} + \frac{2^{n_3} \cdot q_{T_3} \cdot K_3 \cdot n_3 \cdot [(c-b_3) + \text{abs}(c-b_3)]^{n_3-1}}{\left\{2^{n_3} + K_3 \cdot [(c-b_3) + \text{abs}(c-b_3)]^{n_3}\right\}^2} \quad (21)$$

After substitution of calculated parameters, we can write the equation of the isoproturon adsorption and buffering capacity of examined soil samples as follows (see equation (22) and (23):

$$q = \frac{[9.29 \cdot 0.3 \cdot c]^{0.45}}{[1 + 0.3 \cdot c]^{0.45}} + \frac{9.93 \cdot 3.79 \cdot [(c-3.57) + \text{abs}(c-3.57)]^{0.55}}{2^{0.55} + 3.79 \cdot [(c-3.57) + \text{abs}(c-3.57)]^{0.55}} + \frac{4.42 \cdot 1.47 \cdot [(c-13.16) + \text{abs}(c-13.16)]^{3.11}}{2^{3.11} + 1.47 \cdot [(c-13.16) + \text{abs}(c-13.16)]^{3.11}} \quad (22)$$

$$B = \frac{9.29 \cdot 0.3 \cdot 0.45 \cdot c^{-0.55}}{\left\{1 + 0.3 \cdot [c]^{0.45}\right\}^2} + \frac{2^{0.55} \cdot 9.93 \cdot 3.79 \cdot 0.55 \cdot [(c-3.57) + \text{abs}(c-3.57)]^{-0.45}}{\left\{2^{-0.45} \cdot 3.79 \cdot [(c-3.57) + \text{abs}(c-3.57)]^{0.55}\right\}^2} + \frac{2^{3.11} \cdot 4.42 \cdot 1.47 \cdot 3.11 \cdot [(c-13.16) + \text{abs}(c-13.16)]^{2.11}}{\left\{2^{3.11} + 1.47 \cdot [(c-13.16) + \text{abs}(c-13.16)]^{3.11}\right\}^2} \quad (23)$$

Figure 16 shows the graph of equation (23) emphasizing the special change of  $B$  value as a function of the equilibrium concentration.

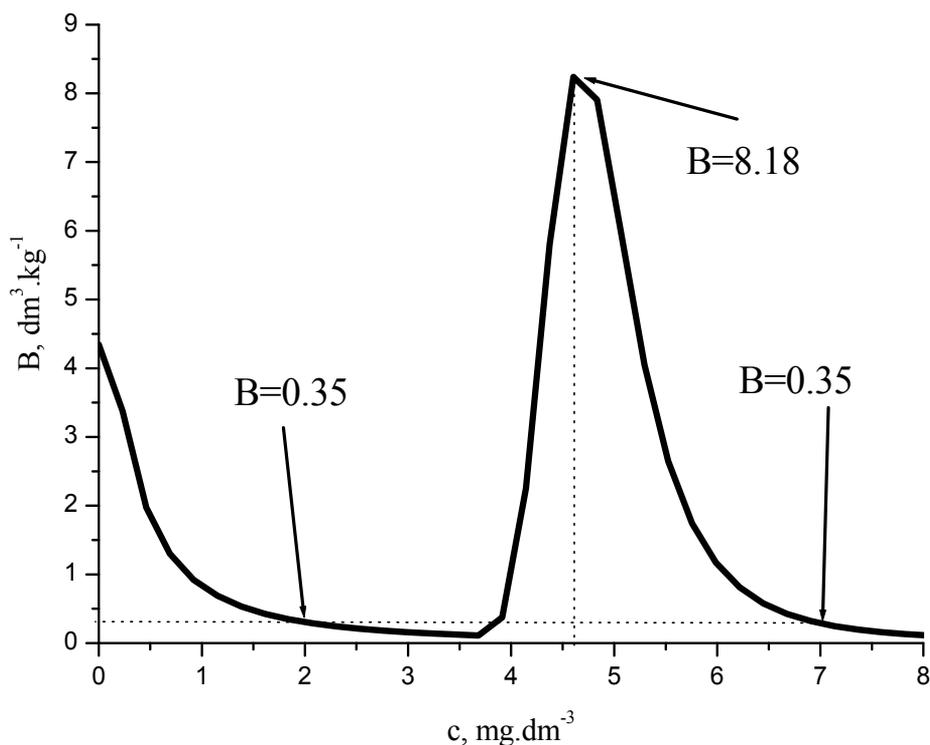


Fig. 16. Equilibrium buffering capacity function of isoproturon

The adsorption isotherm is shown in Figure 17 with some lines for better understanding. The 0.1 size interval of  $c$  is marked three times at very important values. The dotted lines show the adsorbed amount intervals connected to the marked equilibrium concentrations. In the  $B$  function one maximum and two minimum points can be found (Fig. 16). At the low equilibrium concentration the adsorbed amount of pesticide increased significantly. It means that the slope of the curve ( $B$ ) is relatively high. Then the surface concentration is increasing and the system is approximating the surface saturation, while the slope ( $B$ ) is decreasing. The  $B$  value reaches its minimum when the surface is almost saturated: in this area the slope of the adsorbed amount function is almost zero ( $c$  is approx. 3.5 mg.dm<sup>-3</sup>). Then the second layer is forming, and the surface concentration will be zero again. New empty sites are present meaning a new type of surface. The adsorbed amount is increasing in function of equilibrium concentration. When the new small adsorbed regions are evolved, the slope of the curve reaches its maximal value:  $B$  has its maximum at 4.5 mg.dm<sup>-1</sup> equilibrium concentration. After this point the process explained above takes place again: the surface is saturating, and the  $B$  value is decreasing.

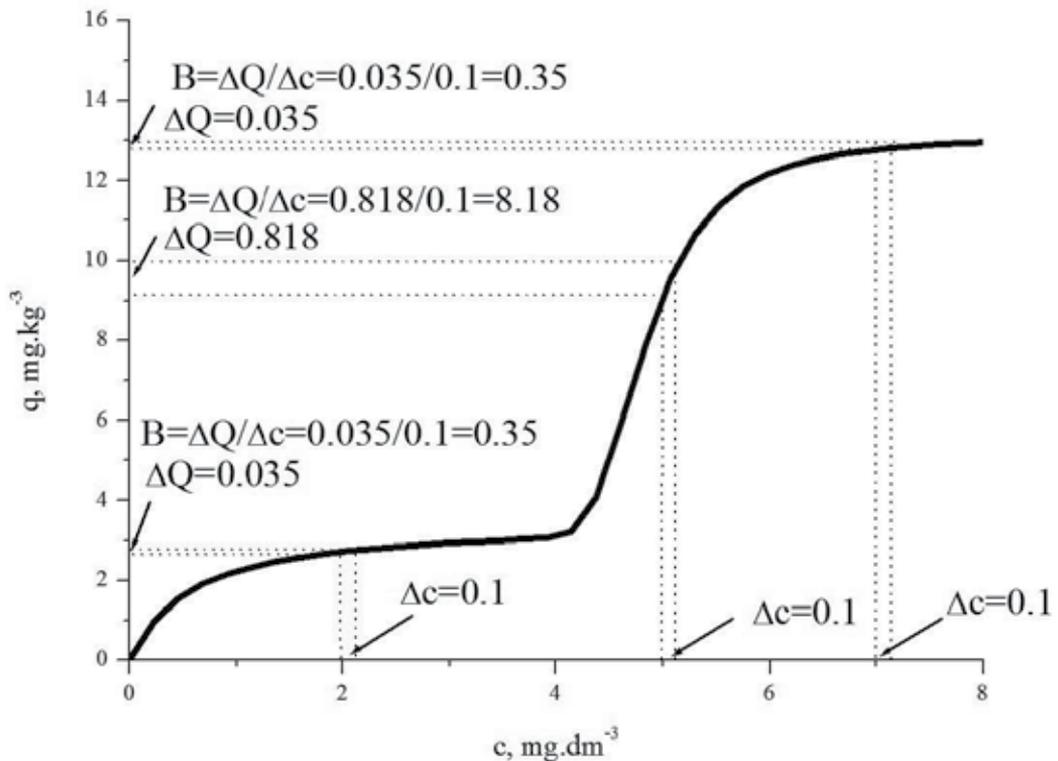


Fig. 17. The explanation of equilibrium buffering capacity function of isoproturon

The parameters and  $B$  can be used for calculating of the equilibrium concentration of a contaminated soil while the total amount of a given compound can be calculated from its concentration measured. But this is more complicated, it needs some additional calculations.

If we know the water content of the soil we can give the ratio of adsorbed and dissolved amount of the investigated compound on the basis of the buffering capacity. In the analytical and monitoring practice the change of the concentration in the liquid phase is measured, and it is generally concluded that this is proportional to the changing in the extent of pollution. According to our research this is not true. The suggested calculation method is detailed below step by step.

In this example the amount of the pesticide is present in one unit (1 kg) solid phase, and also one unit (1 dm<sup>3</sup>) liquid phase. The ratio of solid/liquid phase has to be calculated in the case of known water content of the soil. If the water content  $\Theta$  is given in dm<sup>3</sup>.dm<sup>-3</sup>, the volume of solid phase is  $1-\Theta$  in dm<sup>3</sup>.dm<sup>-3</sup>. In this case the following relationship can be written:

$$Q = \frac{1-\Theta}{\Theta} \cdot q \quad (24)$$

where  $Q$  is the extent of pollution in solid phase of wet soil, mg.kg<sup>-1</sup>,  $q$  is extent of pollution in clear solid phase of soil (specific adsorbed amount of pollutant), mg.kg<sup>-1</sup>,  $\Theta$  is the water content of the soil, dm<sup>3</sup>.dm<sup>-3</sup>.

The changing of the extent of pollution in solid phase at given water content of the soil can be calculated by the application of equation (24).

$$dQ = \frac{1-\Theta}{\Theta} \cdot B \cdot dc \quad (25)$$

where  $dQ$  is the change of the extent of pollution in solid phase, mg.kg<sup>-1</sup>,  $dc$  is the change of the concentration in the solution, mg.dm<sup>-3</sup>,  $B$  is the equilibrium buffering capacity, dm<sup>3</sup>.kg<sup>-1</sup>. In order to calculate the total amount of the compound in milligrams in the soil we must summarize its amount in the solid as well as in the liquid phase.

$$dQ = \frac{1-\Theta}{\Theta} \cdot V_{soil} \cdot \rho_{soil} \cdot B \cdot dc + \frac{\Theta}{1-\Theta} \cdot V_{soil} \cdot dc \quad (26)$$

where  $V_{soil}$  is the total volume of examined soil dm<sup>3</sup>,  $\rho_{soil}$  is density of solid phase of soil, mg.dm<sup>-3</sup>.

In the present example let us assume that we have got one hectare of soil with 25 cm plugged layer, and we calculate total amount of adsorbed material in kilograms.

$$dQ = 10^{-6} \cdot \left( \frac{1-\Theta}{\Theta} \cdot \rho_{soil} \cdot B + \frac{\Theta}{1-\Theta} \right) \cdot V_{soil} \cdot dc \quad (27)$$

where  $\rho_{soil}$  is the density of solid phase, about 2.6 kg.dm<sup>-3</sup>;  $V_{soil}$  is the volume of soil, in this case 2 500 000 dm<sup>3</sup>;  $dQ$  is the change of the extent of pollution in the given soil area, kg.

Substituting the derived function into  $B$  we can calculate the changing buffering effect of the soil in the function of changing equilibrium concentration of the given pesticide. In order to make an approximate calculation, we do not need to use the total complicated function. How we can decide which part should be used from this sum? We have to compare the equilibrium concentration with  $b_i$  parameters and we have to choose that part of sum, where  $b_i$  is less than the equilibrium concentration, but  $b_i+1$  is bigger than  $c$ .

If the equilibrium concentration is less than 3.5 mg.dm<sup>-3</sup> the substitution of the known data into equation (27) results in equation (28):

$$dQ = 10^{-6} \cdot \left( \frac{1-0.2}{0.2} \cdot 2.6 \cdot \frac{9.29 \cdot 0.3 \cdot 0.45 \cdot c^{-0.55}}{\{1 + 0.3 \cdot [c]^{0.45}\}^2} + \frac{0.2}{1-0.2} \right) \cdot 2500000 \cdot dc \quad (28)$$

where  $\rho_{soil}$  is the density of solid phase, about 2.6 kg.dm<sup>-3</sup>;  $V_{soil}$  is the volume of soil, in this case 2 500 000 dm<sup>3</sup>;  $\Theta$  is the water content of the soil, dm<sup>3</sup>.dm<sup>-3</sup>, in this case 0.2.

If the value of  $c$  is: 13.5 mg.dm<sup>-3</sup> >  $c$  > 3.5 mg.dm<sup>-3</sup>, the substitution of the known data into equation (27) results in equation (29):

$$dQ = 10^{-6} \cdot \left( \frac{1-0.2}{0.2} \cdot 2.6 \cdot \frac{2^{0.55} \cdot 9.93 \cdot 3.79 \cdot 0.55 \cdot [(c-3.57) + \text{abs}(c-3.57)]^{-0.45}}{\{2^{-0.45} \cdot 3.79 \cdot [(c-3.57) + \text{abs}(c-3.57)]^{0.55}\}^2} + \frac{0.2}{1-0.2} \right) \cdot 2500000 \cdot dc \quad (29)$$

The calculations above emphasize how big difference can be calculated depending on the equilibrium concentration. It is the reason for using the whole buffering capacity equation and the soil parameters given in equation 28.

Based on the isoproturon curve (Fig. 17.) it can be seen if the equilibrium solution concentration increased from 2 mg.dm<sup>-3</sup> to 2.1 mg.dm<sup>-3</sup>,  $B$  is 0.35 dm<sup>3</sup>.kg<sup>-1</sup>. The change in the total isoproturon content is 0.1 kg. If the equilibrium solution concentration is increasing from 4.5 mg.dm<sup>-3</sup> to 4.6 mg.dm<sup>-3</sup> under the same conditions (water content and isoproturon buffer function),  $B$  is 8.18 dm<sup>3</sup>.kg<sup>-1</sup>. If  $c$  changes from 7 mg.dm<sup>-3</sup> to 7.1 mg.dm<sup>-3</sup>, the value of  $B$  is 0.35 dm<sup>3</sup>.kg<sup>-1</sup> again. The change in total isoproturon content is 21 kg.

In opposite point of view, for example let's suppose 20 kg.ha<sup>-1</sup> isoproturon added into the soil, presumably for plant protection activity. If the original equilibrium solution concentration was 2 mg.dm<sup>-3</sup> it is increasing by 2 mg.dm<sup>-3</sup> and results in 100 % arising. If the original equilibrium solution concentration was 4.5 mg.dm<sup>-3</sup>, the increasing is 0.1 mg.dm<sup>-3</sup> which means just 2 %. In the case of 7 mg.dm<sup>-3</sup> of original equilibrium solution concentration the increasing is 2 mg.dm<sup>-3</sup> (35 % arising).

## 5. Conclusion

The chloroacetanilide type herbicides acetochlor and propisochlor proved to be rather stable compounds under the given conditions. Degradation can occur due to the presence of soil microorganisms. Tribenuron methyl being a sulfonyl urea type compound is stable only under basic conditions and it is decomposed in acidic soils fast. Three degradation products were identified by GC-MS: two of them are formed through acidic hydrolysis and the third newly investigated compound is attributable to the presence of organic matter content of the soil (e.g. humic substances). The structure of metsulfuron methyl is very similar, however, its breakdown is slow. Isoproturon is a rather persistent compound. Its degradation hardly depends on pH but it is faster in the presence of the soil. Two degradation products were identified which can prove the photo catalytic effect of humic substances.

New equation has been derived by making use of the usual mass balance and equilibrium relationships of the adsorption and by considering the possibility of the formation of

associates of the solute molecules. The characteristic model parameters of each step of the adsorption isotherm were estimated for the studied systems by a non-linear least square regression.

Its applicability is proved by the fitting of the isotherms of herbicides having different structures. The calculated curves fit well to the experimentally obtained multi-step isotherms. The parameters of the model can be used for the characterization of the pesticide–soil interactions. On the basis of the new equation formulas were derived to calculate the equilibrium buffering capacity ( $B$ ) of soil. These buffer capacity equations show special curves with one or more peaks. The measured and fitted values were recalculated by using real soil parameters. These results indicate that we must redefine our original contamination assessment methods in order to avoid even a magnitude error. Not only the equilibrium solution concentration measurement is very important for assessing the real amount of contaminants but also the equilibrium buffering capacity ( $B$ ) function has to be used.

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# Application of Fenton Processes for Degradation of Aniline

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## 1. Introduction

Fenton process is one of advanced oxidation processes (AOPs) which are considered as alternative methods for treatment of non-biodegradable and toxic organic compounds. Fenton process has been widely used in the treatment of persistent organic compounds in water. In general, the mechanism of Fenton reaction included the formation of hydroxyl free radicals, which has  $E^\circ$  of 2.8 V, that can oxidize and mineralize almost all the organic carbons to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  (Glaze et al., 1987), by the interaction of hydrogen peroxide with ferrous ions (Walling C., 1975). The Fenton's reagent is generally occurred in acidic medium between pH 2 -4 (Rodriguez et al., 2003). The advantage of Fenton process is the complete destruction of contaminants to harmless compounds, for instance, carbon dioxide and water (Neyen E. et al., 2003). However, its application has been limited due to the generation of the excess amount of ferric hydroxide sludge that requires additional separation process and disposal (Chang P.H., 2004). Therefore, electro-Fenton (EF) process is developed for minimizing the disadvantages of conventional Fenton process.

In the electro-Fenton method, the Fenton's reagent was utilized to produce hydroxyl radical in the electrolytic cell, and ferrous ion was regenerated via the reduction of ferric ion on the cathode (Zhang et al., 2007). The regenerated ferrous ion will react with hydrogen peroxide and produce more hydroxyl radicals that can destroy the target compounds. However, the electro-Fenton reaction still faces several obstacles that must be overcome first such as the formation of ferric hydroxide sludge. Therefore, the new method which can promote the ferrous ion regeneration was focused in this part of experiment. The efficiency of pollutant removal and the reduction of ferric hydroxide sludge can be improved by using UV-radiation.

The photoelectro-Fenton process involves the additional irradiation of the solution with UVA light. Due to the generation of additional hydroxyl radical from the regeneration of ferrous ion and the reaction of hydrogen peroxide that reacted with UV light, so-called photoelectro-Fenton process (Brillas et al., 2000). Under UV-vis irradiation, the overall

efficiency of the process increases due mainly to the regeneration of ferrous ions and formation of additional hydroxyl radicals. UVA light can favor (1) the regeneration of ferrous ion with production of more amount of hydroxyl radical from photoreduction of  $\text{Fe}(\text{OH})^{2+}$ , which is the predominant ferric ion species in acid medium (Sun and Pignatello, 1993) and (2) the photodecomposition of complexes of ferric ion with generated carboxylic acids (Flox et al., 2007). The maximum adsorption wavelength of  $\text{Fe}(\text{OH})^{2+}$  species is less than 360 nm, visible irradiation may not drive the reaction of equation (1). An interesting and potentially useful modification of the photoreduction reaction takes advantage of the photo-lability of  $\text{Fe}(\text{III})$ -oxalate complexes, which has efficiency up to 500 nm (Pignatello et al., 2006).

Aniline has been used as a target chemical. It is one of the main pollutants in wastewater, mainly from the chemical processes of dye, rubber industry, pesticide manufacture and pharmaceutical sectors (Song et al., 2007). In this study, the effects of the initial concentration of 2,6-dimethylaniline concentration,  $\text{Fe}^{2+}$  concentration,  $\text{H}_2\text{O}_2$  dosage on 2,6-dimethylaniline and COD removal efficiency were explored. Moreover, this experiment intended to provide important information on the kinetic study of various Fenton processes (Fenton process, electro-Fenton process and photoelectro-Fenton process) on the aniline degradation.

## 2. Materials and methods

All chemicals used in this study were prepared using de-ionized water from a Millipore system with a resistivity of  $18.2 \text{ M}\Omega \text{ cm}^{-1}$ . Aniline, perchloric acid, ferrous sulfate and hydrogen peroxide were purchased from MERCK. Sodium hydroxide was purchased from Riedel-da Haën. All the preparations and experiments were realized at the room temperature. Synthetic wastewater containing 1 mM of aniline was dissolved with de-ionized water and then adjusted pH with perchloric acid. After pH adjustment, a calculated amount of catalytic ferrous sulfate was added as the source of  $\text{Fe}^{2+}$  in this experiment. Then, the  $\text{H}_2\text{O}_2$  was added into the reactor. The electrical current (for electro-Fenton process and photoelectro-Fenton process) and UVA lamps (for photoelectro-Fenton process) were delivered through out the experimental period. The samples taken at predetermined time intervals were immediately injected into tubes containing sodium hydroxide solution to quench the reaction by increasing the pH to 11 (Anotai et al., 2006). The samples were then filtered with  $0.45 \mu\text{m}$  to remove the precipitates formed, and kept for 12 hours in the refrigerator before chemical oxygen demand (COD) analysis was conducted. This work has been carried out to investigate the effect of the concentration of hydrogen peroxide on the COD value.

The samples were also analyzed for aniline and COD. Aniline was analyzed by a gas chromatography (HP 4890II) equipped with a flame ionization detector (FID) and a SUPELCO Equity™ - 5 Capillary Column (length: 15m; id:  $0.15 \mu\text{m}$ ) with the rate  $65 \text{ (}^\circ\text{C/min)}$ , initial temperature  $85^\circ\text{C}$  and flow 10 psi. The solution pH was monitored using a SUNTEX pH/mV/TEMP (SP-701) meter. COD was measured by a closed-reflux titrimetric method based on Standard Methods (APHA, 1992). All experimental scenarios were duplicated. All experiments were carried out at batch mode using an acrylic reactor with a working volume of 5 liters. For the photoelectro-Fenton process, the anodes and cathodes used were mesh-type titanium metal coated with  $\text{IrO}_2/\text{RuO}_2$  and stainless steel, respectively. The electrodes were connected to a Topward 33010D power supply operated at the desired electric current. The

reactor was also equipped with two mixers to ensure appropriate agitation and the UVA source was turned on to initiate the reaction. The irradiation source was a set of twelve 0.6 W UVA lamps (Sunbeamtech.com) fixed inside a cylindrical Pyrex tube (allowing wavelengths  $\lambda > 320$  nm to penetrate). In addition to all the experimental conditions mentioned above, UV light with maximum wavelength of 360 nm was irradiated inside the reactor, supplying a photoionization energy input to the solution of 7.2 W.

### 3. Results and discussion

In this step, the degradation of aniline was examined by various processes. Electrolysis, Photolysis, UV + hydrogen peroxide, Fenton, electro-Fenton, photo-Fenton and photoelectro-Fenton experiments in order to investigate the synergistic effect of Fenton's reagent combined with photo and electrochemical methods. As shown in Figure 1, the results show that electrolysis can remove 10% of 2,6-dimethylaniline within one hour. In the electrolysis method, aniline would be destroyed by reaction with adsorbed hydroxyl radical generated at the surface of a high oxygen-overvoltage anode from water oxidation. The same tendency can be found in the research of Brillas et al (Brillas et al., 1998).

Photolysis has lower degradation efficiency compared to electrolysis. The removal efficiency by photolysis was only 8% when using UVA lamps (12 lamps) at pH 3. The degradation of aniline was 12% when using UV+hydrogen peroxide after 60 minutes. Aniline was not well degraded by electrolysis, photolysis and UV+hydrogen peroxide. When using Fenton process, the degradation of aniline increased significantly compared to that when using direct photolysis, electrolysis and UV+hydrogen peroxide. For Fenton process, the degradation of aniline was 88% within one hour. This is due to the fast reaction of ferrous ion and hydrogen peroxide producing hydroxyl radicals (equation 1).

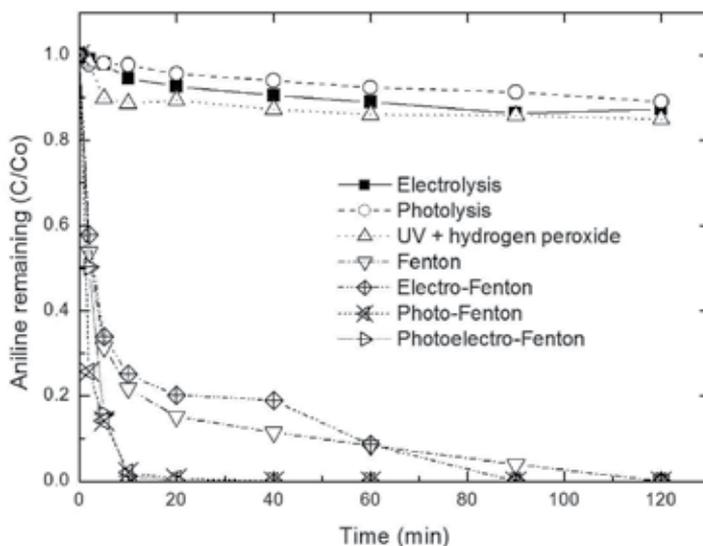


Fig. 1. Effect of different processes on aniline removal efficiency. [Aniline] = 1 mM;  $[\text{Fe}^{2+}] = 0.5$  mM;  $[\text{H}_2\text{O}_2] = 20$  mM; pH = 3; I = 1 A, UVA lamps = 12.

The 90% removal efficiency was achieved by the electro-Fenton process. The reason that electro-Fenton process can remove aniline more than the Fenton process is due to the regeneration of ferrous ion from equation (2) on the cathode side:



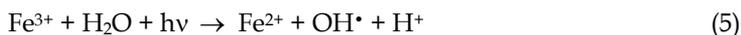
and the ability of electricity that can produce hydroxyl radicals from water oxidation as described in equation (3).



In photo-Fenton process, 100% of aniline degradation was observed at 60 min. Hence, photo-Fenton process is more efficient than Fenton process and electro-Fenton process. It is obvious that the photo-Fenton system enhanced the photooxidation of aniline. The hydroxyl radical which is a strong oxidant that can degrade aniline occurs by the following equation:



Degradation of aniline is mainly due to hydroxyl radical generated by photochemical reaction. In photo-Fenton process in addition to the above reaction the formation of hydroxyl radical (equation 1) also occurs by equation (4) and (5)



Meanwhile, the utmost removal efficiency was found when applied photoelectro-Fenton process, aniline was removed completely during the first 20 minutes. The degradation of aniline was due to the formation of hydroxyl radical from Fenton's reaction (equation 1) and the ferric ion would be reduced to ferrous ion from photoreduction (equation 5) and the cathode (equation 2). This would induce the formation of hydroxyl radicals efficiently.

The relative efficiencies of the above processes are in the following order: Photoelectro-Fenton > Photo-Fenton > Electro-Fenton > Fenton > UV + hydrogen peroxide > Electrolysis > Photolysis.

The degradation of aniline was monitored by measuring the COD removal. For COD removal, by electrolysis, photolysis, UV+hydrogen peroxide, Fenton, electro-Fenton, photo-Fenton and photoelectro-Fenton processes, it was followed the same trend as aniline degradation. The results revealed that electrolysis could remove COD only 9%, while photolysis was able to remove about 8% as shown in Figure 2. When UVA was combined with hydrogen peroxide, the COD removal increased to 14%. For Fenton process, the COD removal was 43% which is higher than using direct photolysis, electrolysis and UV+hydrogen peroxide. This is due to the fast reaction of ferrous ion and hydrogen peroxide producing hydroxyl radicals (equation 1).

The COD removal efficiency of aniline by the electro-Fenton process and photo-Fenton process were 49% and 52%, respectively. The reason might be due to the formation of hydroxyl radical from Fenton's reaction (equation 1) and the ferric ion would be reduced to ferrous ion from photoreduction (equation 5) in photo-Fenton process and the regeneration of ferrous ion on the cathode (equation 2) in electro-Fenton process. The highest COD removal was found when applied photoelectro-Fenton. It was about 70%. The decrease of COD can be attributed to the mineralization of aniline by the hydroxyl radicals from Fenton's reaction, from the electrochemically generated Fenton's reagent during electro-

Fenton and photoelectro-Fenton processes and the production of hydroxyl radical from photoreduction of  $\text{Fe}(\text{OH})_2^{2+}$  in photoelectro-Fenton process.

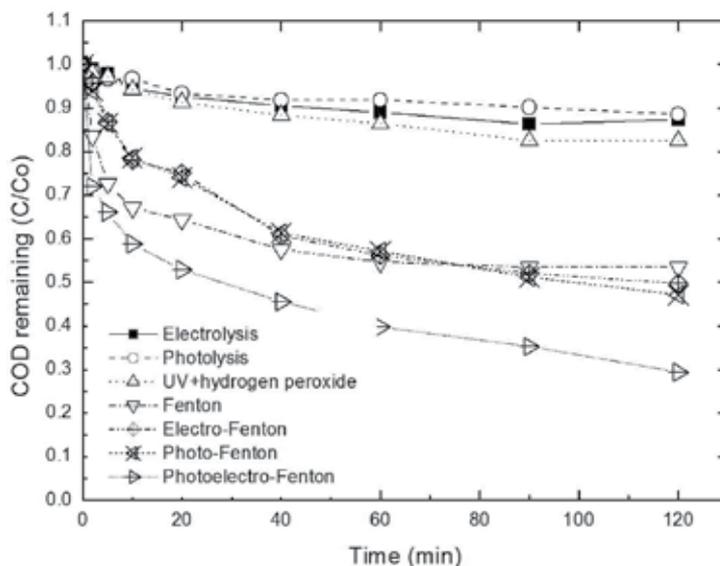


Fig. 2. Effect of different processes on COD removal efficiency.  $[\text{Aniline}] = 1 \text{ mM}$ ;  $[\text{Fe}^{2+}] = 0.5 \text{ mM}$ ;  $[\text{H}_2\text{O}_2] = 20 \text{ mM}$ ;  $\text{pH} = 3$ ;  $I = 1 \text{ A}$ , UVA lamps = 12.

From this part of experiment, the photoelectro-Fenton was found to be the most efficient treatment to oxidize aniline. Thus, the following part in this chapter will focused on this process.

### 3.1 Kinetic study of aniline degradation by photoelectro-Fenton process

The kinetic study of aniline degradation by the photoelectro-Fenton was investigated. In this study, the kinetic was performed using initial rate techniques (at the first 10 minute of the reaction) in order to eliminate the interferences from intermediates that might occur during the study period. The test range of each parameter was chosen according to the reality of photoelectro-Fenton process application and the needs of kinetic study in this experiment. The effects of the initial concentration of aniline, initial concentration of ferrous ion and hydrogen peroxide on the kinetic study of aniline degradation will be separately discussed in the following sections.

#### 3.1.1 Effect of initial aniline concentration

The pollutant concentration is one of the important factors in photoelectro-Fenton process. The effect of initial aniline concentration on the removal efficiency of aniline by photoelectro-Fenton processes is shown in Figure 3. The figure clearly reveals that increasing the aniline concentration decreases the removal efficiency of aniline. When increasing the aniline concentration from 0.5 to 5 mM, the degradation of aniline decreased from 100%, 100% to 60%. The main reason for this phenomenon is the hydroxyl radical. Increasing in aniline concentration increases the number of aniline molecules, however, there is not enough hydroxyl radicals to degrade 2,6-aniline, then the removal efficiency decreases.

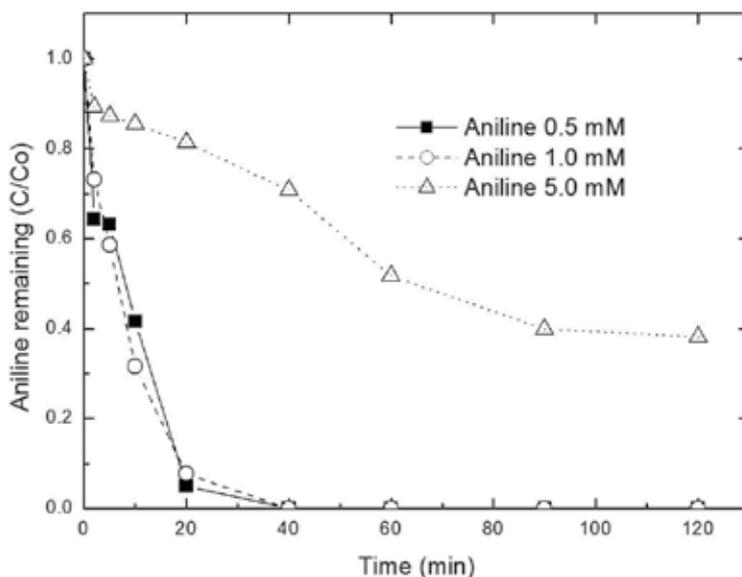


Fig. 3. Effect of initial aniline concentration on the removal efficiency of aniline by Photoelectro-Fenton process .  $[\text{Fe}^{2+}] = 0.5 \text{ mM}$ ,  $[\text{H}_2\text{O}_2] = 20 \text{ mM}$ ,  $I = 1 \text{ A}$ ,  $\text{pH} = 3$ , UVA lamp = 12

The effect of the initial aniline concentration on the kinetic was studied by varying the initial concentration from 0.5 to 5 mM and at the experimental condition of 0.5 mM of ferrous ion, 20 mM of hydrogen peroxide, pH 3, an electric current of 1 amperes for and UVA 12 lamps. The study shows the aniline degradation kinetics at the given test condition. The second-order behavior appeared to fit the degradation of aniline by the photoelectro-Fenton process when plot the relationship between aniline degradation and initial aniline concentration was found in a good linear ( $R > 0.98$ ).

[Aniline] ( $\times 10^{-3}$ ) M	Initial degradation rate
	Photoelectro-Fenton process ( $\text{mMs}^{-1}$ )
0.5	0.0006
1	0.0008
5	0.0010

Table 1. Initial rate of aniline degradation by varying initial concentration of aniline using photoelectro-Fenton process.

The results on Table 1 shows that when the aniline concentration was increased from 0.5 to 5 mM, the initial degradation rates increased. The high initial degradation rate at a high concentration of aniline was probably due to the excess amount of aniline to react with hydroxyl radical which produced during the process that destroyed the aniline.

For that reason, the initial rate for the higher concentration of aniline increased. These results are similar to the study of *p*-nitroaniline degradation by Fenton oxidation process by Sun and others (Sun et al., 2007) and the results are also similar to the removal of nitroaromatic explosives with Fenton's reagent (Liou and Lu, 2007).

For the effect of initial aniline on the removal efficiency, the results show that increasing the initial concentration of aniline will decrease the removal. The plot between the initial rate and aniline concentration showed a straight line with the slopes of 0.21 for the photoelectro-Fenton process. Therefore, the reaction rate equation became:

$$-\left(\frac{d[\text{Aniline}]}{dt}\right)_{\text{photoelectro-Fenton}} = r_{\text{PEF,Aniline}} [2,6\text{-DMA}]^{0.21} \quad (6)$$

where  $r_{\text{PEF,Aniline}}$  is rate constant for the photoelectro-Fenton processes with respect to aniline.

### 3.2 Effect of ferrous ion concentration

The concentration of the catalyst is another important parameter for Fenton processes. The ferrous ion plays the catalyst's role in this Fenton's reaction. Normally, the rate of degradation increases with an increase in the concentration of ferrous ions. The effect of the ferrous ion concentration on the kinetic study of aniline degradation was studied by varying the ferrous ion concentration from 0.1 to 1 mM. The result showed that the removal efficiency of photoelectro-Fenton method was promoted when the ferrous ion concentration increased from 0.1 to 1 mM, as shown in Figure 4. The removal efficiency increased from 94% to 100% in 40 minutes.

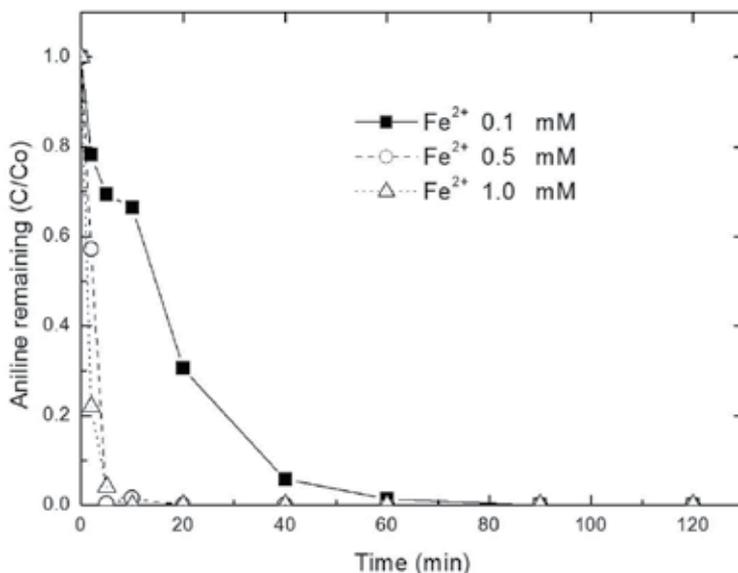


Fig. 4. Effect of initial ferrous ion concentration on the removal efficiency of aniline by Photoelectro-Fenton process . [Aniline] = 1 mM, [H<sub>2</sub>O<sub>2</sub>] = 20 mM, I = 1 A, pH = 3, UVA lamp = 12

For the initial degradation rate of aniline, the initial rate increased when the initial ferrous ion concentration increased from 0.0005 to 0.0016 mM s<sup>-1</sup> in the photoelectron-Fenton process as listed in Table 2. Usually, when increasing the initial ferrous ion concentration, it will increase the generation rate of hydroxyl radical due to equation (1).

Therefore, this will enhance the degradation rate of aniline. Accordingly, the results underwent a similar direction. An improvement in removal efficiency by ferrous ion should also consider the supplement of hydrogen peroxide through out the experiment which might lead to more regeneration of ferrous ion from ferric ion. Therefore, the total regenerated hydroxyl radicals and the removal efficiency could increase as the ferrous ion concentration increased.

[Fe <sup>2+</sup> ] (x10 <sup>-3</sup> ) M	Initial degradation rate
	Photoelectro-Fenton process (mMs <sup>-1</sup> )
0.1	0.0005
0.5	0.0014
1.0	0.0016

Table 2. Initial rate of aniline degradation by varying initial concentration of ferrous ion using photoelectro-Fenton process.

In photoelectro-Fenton process, the applied electricity and UVA lamps during experiment will enhance the regeneration of ferrous ion from ferric ion via equation (3) and (5). The supplied electrons from electrical current can regenerate ferrous ions rapidly and, thus, can react with hydrogen peroxide as long as hydrogen peroxide is still available in the reactor (Anotai et al., 2006). For that reason, the degradation of aniline continuously proceeded without the inadequacy of ferrous ions in the solution which in turn increased the efficiency of hydroxyl radical production.

The aniline degradation kinetics at the given test conditions was second-order behavior. The degradation kinetic for the photoelectro-Fenton process when plot the relationship between aniline degradation and aniline concentration was in a good linear ( $R > 0.95$ ) when using second-order kinetic model. It was seen from the results that the rate of aniline degradation increased with the increase of ferrous ions concentration. The relationship between the initial rate and the ferrous ion concentration in the photoelectron-Fenton process was determined. From the calculation, it showed that the slope of photoelectro-Fenton processes was 0.5289. Thus, the kinetics for aniline degradation on the effect of ferrous ion concentration can be described by following equation:

$$-\left(\frac{d[\text{Aniline}]}{dt}\right)_{\text{photoelectro-Fenton}} = r_{PEF, Fe^{2+}} [Fe^{2+}]^{0.5289} \quad (7)$$

where  $r_{PEF, Fe^{2+}}$  is the rate constant for photoelectro-Fenton process with respect to the ferrous ion. The results indicated that ferrous ions play an important role in degradation of aniline by reacting with hydrogen peroxide to generate hydroxyl radicals. However, higher ferrous ion concentration supplementation by using photoelectro-Fenton processes is not recommended due to the large amount of ferric hydroxide sludge that could occur.

### 3.3 Effect of hydrogen peroxide concentration

Initial concentration of hydrogen peroxide plays an important role in the photoelectro-Fenton process. It is an oxidizing agent in the Fenton reaction. It has been observed that the percentage degradation of the pollutant increases with an increase in the concentration of

hydrogen peroxide (Pignatello, 1992). The effect of initial hydrogen peroxide concentration on the removal efficiency and kinetic rate of aniline degradation was investigated by varying the initial hydrogen peroxide concentration from 1 to 40 mM under the experiment condition of initial aniline concentration 1 mM, ferrous ion 0.5 mM, pH 3, electrical current 1 A and UVA 12 lamps.

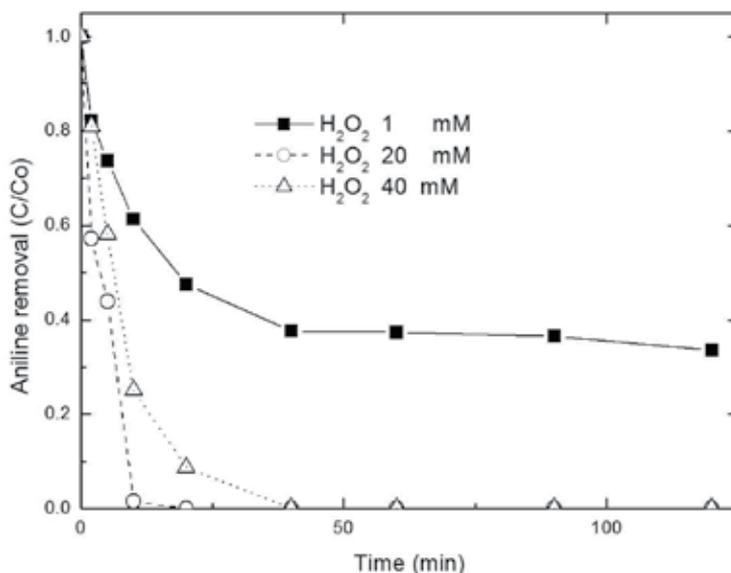


Fig. 5. Effect of initial hydrogen peroxide concentration on the removal efficiency of aniline by Photoelectro-Fenton process. [Aniline] = 1 mM, [Fe<sup>2+</sup>] = 0.5 mM, I = 1 A, pH = 3, UVA lamp = 12

It can be seen from the results that the removal efficiency of photoelectro-Fenton processes increased when increasing the initial concentration of hydrogen peroxide as shown in Figure 5. The removal efficiency increased from 63 % to 100 % as the hydrogen peroxide concentration increased from 1 to 40 mM in 60 minutes. This was from the production of hydroxyl radicals produced during the experiment by the reaction of ferrous ion and hydrogen peroxide in the solution. Similar result was obtained by Lin et al.,1999.

[H <sub>2</sub> O <sub>2</sub> ] (x10 <sup>-3</sup> ) M	Initial degradation rate
	Photoelectro-Fenton process (mMs <sup>-1</sup> )
1	0.0006
20	0.0015
40	0.0012

Table 3. Initial rate of aniline degradation by varying initial concentration of hydrogen peroxide using photoelectro-Fenton process.

Table 3 shows that the initial rate of aniline degradation increased when the hydrogen peroxide concentration was increased from 1 to 20 mM. The initial rate increased from 0.0006 to 0.0015 mMs<sup>-1</sup> by photoelectro-Fenton process. This increase in the initial rate was

due to the availability of hydrogen peroxide to react with ferrous ions in the solution. However, with the continuous increase in the initial hydrogen peroxide concentration to 40 mM, the increase of hydrogen peroxide leads to the decline of the initial rate. This phenomenon was probably due to the scavenging of hydroxyl radicals by hydroxyl radical as described via equation (5) (Lu et al., 1999). The accumulation of hydroperoxyl radicals also consumed hydroxyl radicals (Sun et al., 2007; Kang et al., 2002).

It was found that the second-order kinetic model is applicable to the aniline degradation quite well under photoelectro-Fenton process with  $R > 0.98$ . The relationship between the initial rate and hydrogen peroxide is linear and the slope is 0.2232 for the photoelectro-Fenton process, therefore:

$$-\left(\frac{d[\text{Aniline}]}{dt}\right)_{\text{photoelectro-Fenton}} = r_{PEF, H_2O_2} [H_2O_2]^{0.2232} \quad (8)$$

where  $r_{PEF, H_2O_2}$  is rate constant for the photoelectro-Fenton methods with respect to hydrogen peroxide.

### 3.4 The overall reaction rate equation for aniline degradation by photoelectro-Fenton

From the previous sections, the reaction rate equation of aniline degradation is proposed and it varied with the respect to aniline, ferrous ion and hydrogen peroxide concentrations in photoelectro-Fenton process. The overall degradation kinetic for aniline by the photoelectro-Fenton process can be summarized as shown below:

$$-\left(\frac{d[\text{Aniline}]}{dt}\right)_{\text{photoelectro-Fenton}} = r_{PEF} [\text{Aniline}]^{0.21} [Fe^{2+}]^{0.5289} [H_2O_2]^{0.2232} \quad (9)$$

where  $r_{PEF}$  is the overall rate constant for the photoelectro-Fenton method. From the equation (9), it can be seen that the degradation rate of aniline by photoelectro-Fenton process depended on Fenton's reagent both ferrous ion and hydrogen peroxide. However, from equation (9), the reaction shows that the aniline degradation, when applied with an electrical current and UVA lamp, the degradation rate is still depend on hydrogen peroxide. Moreover, the ferrous ion is also the important species for the degradation of this chemical so the Fenton's reagent still be the major key for this process.

The  $r_{PEF}$ , from equation (9) can be calculated using a non-linear least squares method which minimizes the sum of the error squares between the observed initial rates attained from the study and from the calculated initial rates. Accordingly, the  $r_{PEF}$  can be proposed by calculated using concentration of aniline, ferrous ion and hydrogen peroxide in millimolar (mM) units. From the calculation, the  $r_{PEF}$  was 1.32. Therefore, the final reaction rate equations can be described as:

$$-\left(\frac{d[\text{Aniline}]}{dt}\right)_{\text{photoelectro-Fenton}} = 1.32[\text{Aniline}]^{0.21} [Fe^{2+}]^{0.5289} [H_2O_2]^{0.2232} \quad (10)$$

#### 4. Conclusions

Fenton, electro-Fenton and photoelectro-Fenton processes were able to oxidize aniline. However, in presence of electrical current and UVA lamps, the removal of aniline was higher. The results show that relative efficiencies of the AOPs processes on aniline degradation are in the following order: Photoelectro-Fenton > Photo-Fenton > Electro-Fenton > Fenton > UV + hydrogen peroxide > Electrolysis > Photolysis. These processes could degrade this compound. The photoelectro-Fenton process was found to give the highest degradation efficiency than the other processes. 100% degradation of 1 mM aniline was achieved in 40 minutes when 1 mM of ferrous ions, 20 mM of hydrogen peroxide, pH 3, 1 A of electric current and 12 lamps of UVA were applied. This was due to the regeneration of ferrous ion from ferric ion that can improve the performance of overall degradation. The overall rate equations for the degradation of aniline by photoelectro-Fenton process was evaluated as shown in equation (10). The kinetics established in this experiment was mathematically determined by considering the three important parameters including aniline, ferrous ion and hydrogen peroxide. The initial rate of aniline was increased with the increase of aniline concentration in all processes and increased with the increase of initial ferrous ion and hydrogen peroxide concentration. It can be indicated that aniline, ferrous ion and hydrogen peroxide have strong influences on the kinetic rate constants for aniline degradation.

#### 5. Acknowledgements

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# Bioremediation of Hexachlorocyclohexane Contaminated Soil: Field Trials

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## 1. Introduction

Hexachlorocyclohexane or the abbreviation HCH is identified as a monocyclic chlorinated hydrocarbon. HCH was discovered in 1825 by Faraday, who just had discovered benzene. By reacting benzene with chlorine in bright sunlight, formation of HCH was observed. Neither Faraday nor the Dutch chemist Van der Linden, who in 1912 isolated the pure  $\gamma$ -isomer from a HCH mixture, realized the insecticidal potential of the compounds they produced (Amadori, 1993). The insecticidal properties of HCH were however first mentioned by Bender in a patent paper. HCH was first patented in the 1940s. Dupire conducted detailed investigations on the insecticidal properties in 1940, and HCH was first used to combat the Colorado beetle (Stoffbericht, 1993). In 1942 Slade proved that  $\gamma$ -HCH was the sole carrier of the insecticidal properties of technical HCH (Stoffbericht, 1993). HCH production started commercially since 1947 in Germany. The common name of Hexachlorocyclohexane is "benzene hexachloride", which is incorrect according to the IUPAC rules (Galvan, 1999). Nevertheless it is still widely used, especially in the form of its abbreviation "BHC"

Technical HCH consists mainly of a mixture of various stereo-isomers, which are designated by Greek letters. Only one of these stereoisomers,  $\gamma$ -HCH, is the carrier of the insecticidal properties, while the other isomers are sometimes collectively referred to as "inactive isomers". The raw product from the chlorination of benzene contains about 14%  $\gamma$ -HCH and 86 % of inactive isomers, i.e. 65-70%  $\alpha$ -, 7-10%  $\beta$ -, 14-15%  $\gamma$ -, approximately 7%  $\delta$ -, 1-2%  $\epsilon$ -HCH, and 1-2% other components. Therefore, in the production of one ton of technical HCH, 140 kg is  $\gamma$ -HCH and 860 kg is "inactive isomers". The latter is potentially waste and predestined for disposal. It is possible to extract and purify the active  $\gamma$ -HCH. If the purity is 99.0% or more it may be called "Lindane", which is an accepted common name for this substance. Lindane is also called  $\gamma$ -HCH, or  $\gamma$ - BHC and by FAO  $\gamma$ - BHC (technical grade). Technical grade HCH and fortified HCH (FHCH) containing a varying mixture of at least 5 isomers, with a minimum of 40%  $\gamma$ -isomer was available commercially. HCH is no longer produced in USA and few other European countries and cannot be sold for domestic use by EPA regulation as well as many other countries (FCH, 1984). However, in some developing countries HCH especially  $\gamma$ -isomer continues to be used because of economic purposes and also in public health programmes. Thus Technical grade HCH continues to be produced and

all isomers except  $\gamma$ -isomer continue to be dumped unutilized. The production of Lindane creates huge amounts of isomers waste. The total quantity of waste will be about 8 times the Lindane output (Bodenstein, 1972), i.e. for each ton of Lindane produced 8 tons of waste will be generated. The large environmental consequences that are created can be imagined.



Photo from the mid-1990s of a temporary storage site for 200 000 tons of soil contaminated with waste HCH isomers.

Considering every ton of lindane produced generates approximately 6 -10 tons of other HCH isomers, a considerable amount of residues would be generated during the manufacture of this insecticide. For decades, the waste isomers were generally disposed off in open landfills like fields and other disposal sites near the HCH manufacturing facilities. After disposal, degradation, volatilization, and run off of the waste isomers occurred. If the estimate of global usage of lindane of 600,000 tons between 1950 and 2000 is accurate, the total amount of possible residuals (if it is assumed that a mean value of 8 tons of waste isomers are obtained per ton of lindane produced) amounts to possibly 4.8 million tons of HCH residuals that could be present worldwide giving an idea of the extent of the environmental contamination problem. Air releases of lindane can occur during the agricultural use or aerial application of this insecticide, as well as during manufacture or disposal. Also, lindane can be released to air through volatilization after application.

### 1.1 Fate of lindane

Once released into the environment, lindane can partition into all environmental media. Hydrolysis and photolysis are not considered important degradation pathways and reported half-lives in air, water and soil are: 2-3 days, 3-300 days and up to 2 - 3 years, respectively. A half-life of 96 days in air has also been estimated. Lindane can bioaccumulate easily in the food chain due to its high lipid solubility and can bio-concentrate rapidly in

microorganisms, invertebrates, fish, birds and mammals. The bio-concentration factors in aquatic organisms under laboratory conditions ranged from approximately 10 upto 4220 and under field conditions, the bio concentration factors ranged from 10 upto 2600.

Lindane is listed as a "substance scheduled for restrictions on use". This means that products in which at least 99% of the HCH isomer is in the  $\gamma$ -form (i.e. lindane, CAS: 58-89-9) are restricted to the following uses: 1. Seed treatment. 2. Soil applications directly followed by incorporation into the top soil surface layer 3. Professional remedial and industrial treatment of lumber, timber and logs. 4. Public health and veterinary topical insecticide. 5. Non-aerial application to tree seedlings, small scale lawn use, and indoor and outdoor use for nursery stock and ornamentals. 6. Indoor industrial and residential applications. Lindane is one of the listed priority hazardous substances for which quality standards and emission controls will be set at EU level to end all emissions within 20 years. Lindane is banned for use in 52 countries, restricted or severely restricted in 33 countries, not registered in 10 countries, and registered in 17 countries.

Lindane can be found in all environmental compartments, and levels in air, water, soil sediment, aquatic and terrestrial organisms and food. Humans are therefore being exposed to lindane as demonstrated by detectable levels in human blood, human adipose tissue and human breast milk in different studies in diverse countries. Exposure of children and pregnant women to lindane are of particular concern.  $\gamma$ -HCH has been found in human maternal adipose tissue, maternal blood, umbilical cord, blood and breast milk. Lindane has also been found to pass through the placental barrier. Direct exposure from the use of pharmaceutical products for scabies and lice treatment should be of concern. Exposure from food sources is possibly of concern for high animal lipid content diets and subsistence diets of particular ethnic groups. Occupational exposure at manufacturing facilities should be of concern, because lindane production implies worker exposure to other HCH isomers as well, for example the  $\alpha$ - isomer is considered to be a probable human carcinogen.

Hepatotoxic, immunotoxic, reproductive and developmental effects have been reported for lindane in laboratory animals. The US EPA has classified lindane in the category of "Suggestive evidence of carcinogenicity, but not sufficient to assess human carcinogenic potential". The most commonly reported effects associated with oral exposure to  $\gamma$ -HCH are neurological. Most of the information is from case reports of acute  $\gamma$ -HCH poisoning. Seizures and convulsions have been observed in individuals who have accidentally or intentionally ingested lindane in insecticide pellets, liquid scabicide or contaminated food (WHO/Europe, 2003). Lindane is highly toxic to aquatic organisms and moderately toxic to birds and mammals following acute exposures. Chronic effects to birds and mammals measured by reproduction studies show adverse effects at low levels such as reductions in egg production, growth and survival parameters in birds, and decreased body weight gain in mammals, with some effects indicative of endocrine disruption.

Comparing to other POPs and hazardous waste problems, the HCH-residuals differ significantly as the extent of the problem is huge and as an environmentally sound disposal method will be necessary. However the enormous financial burden needed to achieve this will be a main barrier. On the other hand, the former practice of simple encapsulation is considered far from sustainable and will leave a huge number of time bombs in the global landscape. Hence bioremediation is the best option of removing these isomers from the contaminated environments. Microbial degradation of chlorinated pesticides such as HCH

is usually carried out by using either pure or mixed culture systems. The main goal of the laboratory studies is to predict the biodegradation rates in the environment. But it is very difficult to extrapolate the results obtained in the laboratory systems to predict their fate in the environment [Spain et al 1990]. The microbial degradation of HCH isomers in liquid cultures has been studied using pure microbial cultures such as *Clostridium rectum*, *Pandoraea* [Ohisa et al 1990, Okeke et al 2002], mixed native soil microbial population (undefined consortium) [Bachmann et al 1988 a, Sahu et al 1995], *Phanerochaete chrysosporium* [Kennedy et al 1990, Mougín et al 1997] and sewage sludge under aerobic and anaerobic conditions [Bachmann et al 1988b, McTernan, and Pereira 1991, Buser, and. Mueller 1995]. The degradation of  $\gamma$ - and  $\alpha$ -isomers was almost complete and  $\beta$ - and  $\delta$ -isomers showed more resistance to degradation. At this stage it is imperative to develop technologies where all isomers of tech-HCH are degraded completely. In this communication we describe the degradation of tech-HCH in artificial plots and also in actual fields using a microbial consortium.

## 2. Materials and methods

### 2.1 Substrate

$\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -HCH isomers (99% pure) were procured from Sigma-Aldrich Chemical Company, St. Louis, MO, USA. Technical grade HCH was obtained from Hindustan insecticides, Mumbai, India. Other chemicals and the reagents used in this study were of analytical grade and were purchased from standard chemical companies. *Rhizobium* and *Azospirillum* were obtained from IMTECH, Chandigarh. Soil used in small plot studies was collected from CFTRI campus without history of HCH usage.

### 2.2 Microbial consortium

The microbial consortium capable of degrading Tech-HCH was developed in our laboratory by long term enrichment of HCH contaminated soil and sewage according to Manonmani et al. [2000]. The Tech-HCH degrading consortium that got enriched was acclimated with increase in concentration of Tech-HCH from 5 to 25 ppm. The consortium thus obtained was maintained as liquid culture in minimal medium containing 10 ppm of Tech- HCH and in minimal agar medium containing 10 ppm of Tech- HCH.

### 2.3 Culture medium

Wheat bran hydrolysate used for growth of microbial consortium was prepared by acid hydrolysis of wheat bran. Minimal medium used in degradation studies consisted of  $\text{KH}_2\text{PO}_4$ , 0.675g;  $\text{Na}_2\text{HPO}_4$ , 5.455g and  $\text{NH}_4\text{NO}_3$ , 0.25g and 1L of water.

### 2.4 Degradation of Tech-HCH using inoculum grown on different carbon sources

#### 2.4.1 Biomass build-up and pre exposure of the inoculum

Individual isolates of the microbial consortium were grown in wheat bran hydrolysate/ peptone- glycerol medium and the cells were harvested after 72 h of growth, washed well and pre-exposed to 25ppm of individual isomers and Tech-HCH for 72 h separately with addition of individual isomers and Tech- HCH every 24 h. The induced cells were harvested by centrifugation at 10,000rpm, 4°C, 10min, washed well and used as inoculum.

### 2.4.2 Essentiality of individual members of the consortium for degradation of Tech-HCH

Degradation of higher concentration (25ppm) of Tech-HCH was taken up to test the performance of different combinations of the members as well as individual isolates of the HCH-degrading consortium. The combinations as given in Table 2 were tested for their ability to degrade tech-HCH. Samples were harvested after known period of incubation and analysed for residual HCH isomers.

Microbial combination used	Degradation (%)			
	$\alpha$ -isomer	B-isomer	$\gamma$ -isomer	$\Delta$ -isomer
1	15.70	4.62	48.41	4.66
2	6.21	7.19	40.21	1.66
3	6.82	2.52	33.33	6.04
4	5.06	10.27	23.16	5.21
5	6.66	4.73	11.58	5.91
6	8.14	3.05	18.58	3.07
7	1.21	6.27	28.63	4.32
8	21.86	8.77	47.55	6.27
9	8.5	6.85	28.55	5.58
10	17.92	7.68	16.32	6.00
1-2	7.06	5.31	51.16	4.68
1-3	11.25	9.11	56.20	8.5
1-4	34.35	20.66	62.41	13.82
1-5	48.62	50.14	70.73	26.24
1-6	64.36	62.18	72.48	46.86
1-7	68.24	74.28	76.88	58.46
1-8	70.11	76.32	84.14	63.46
1-9	80.12	81.32	100	74.32
1-10	94.23	95.16	100	85.11

Table 2. Essentiality of individual members of the consortium for degradation of Tech-HCH

### 2.4.3 Preparation of inoculant formulations and their stability

Inoculant formulations containing the HCH-degrading microbial consortium and *Rhizobium* and *Azospirillum* were prepared using *Sphagnum* mass and wheat bran. The inoculum used contained  $10^7$  cells/g of the substrate. The consortium was mixed with *Sphagnum* mass. *Rhizobium* and *Azospirillum* were added to these separately. The inoculant formulation was prepared as both pellet and wet powder. The formulations were checked at regular intervals for survivability of the consortial members and their degradation capacity.

### 2.5 Degradation of Tech-HCH in different soils

Different soils such as clay soil, red soil, garden soil, soils from coconut, coffee, turmeric and tomato plantations were studied to evaluate the degradation of Tech- HCH. The substrate and inoculum were used at 25  $\mu\text{g g}^{-1}$  and 500  $\mu\text{g protein g}^{-1}$  soil respectively. All the experiments were done in replicates of ten for each parameter studied.

### 2.6 Degradation of technical hexachlorocyclohexane in small artificial plots

Four artificial plots of 2ft x 2ft x 0.5ft were prepared. Red loamy non- sterile soil containing 1-1.5% organic carbon and 32% water holding capacity and pH 7.0, collected from CFTRI campus, was taken in these plots. Each plot was spiked with 25 ppm of tech- HCH and mixed well. Two plots were inoculated with 72 h HCH- induced microbial consortium. Two plots were maintained as abiotic (uninoculated) controls. All plots were kept wet by regular sprinkling of water and the soil in the plots was mixed regularly. Samples from each plot were collected at 10 different locations selected randomly at intervals of 24 h (around 5 g each). Collected soil was mixed thoroughly. One-gram soil was used for recording colony-forming units (cfu) and other set was used for residue analyses.

### 2.7 Degradation of HCH in artificial test plots (large size)

Individual isolates of the HCH- degrading consortium were grown individually in 25 L carbuoys, containing 20 L wheat bran hydrolysate medium (containing 0.75 % sugars) for 72 h. The cells were harvested and mixed at equal  $\text{OD}_{600}$ . The reconstituted consortium was induced with 25 ppm of Tech- HCH for 72 h in minimal medium, centrifuged, washed well in minimal medium and used as inoculum after resuspending the cells in known volume of minimal medium. Six artificial plots of 2m x 1m x 0.5ft were prepared and red loamy soil collected from CFTRI campus was taken in these plots. Each plot was spiked with 25 ppm Tech- HCH and mixed well. Four plots were inoculated with 72 h HCH- induced microbial consortium. The inoculum was added at levels containing  $10^7$  to  $10^8$  cells/ g soil. Two plots were maintained as uninoculated controls (containing only HCH). The soil was mixed well at regular intervals and was kept moist by regular sprinkling of water. At intervals of 24 h, samples were collected at 25 different areas of the plot (10 g each). The collected soil was mixed thoroughly and used for both the analysis of growth and residual substrate.

### 2.8 Degradation of Tech-HCH in actual fields

8 plots measuring 2m<sup>2</sup> were chosen at CFTRI campus (Plate 1). The plots were prepared by tilling and toeing. The individual members of the microbial consortium were grown separately in a 25 L carbuoy containing 20 L of wheat bran hydrolysate medium (containing 0.75% reducing sugars). After 72 h of growth the cells were harvested and pooled at equal  $\text{OD}_{600}$ . The reconstituted consortium was induced with 25 ppm of Tech- HCH for 72 h. Then the microbial mass was separated by centrifugation, washed well and resuspended in required quantity of minimal medium. Spiking of Tech- HCH to the soil was repeated for four times on alternative days. The final concentration of Tech- HCH added to the soil was 25 ppm. Six of the plots received Tech- HCH while two remaining were maintained as unspiked, uninoculated controls. Two plots were inoculated with microbial consortium and two more plots received the one-month-old inoculant formulation prepared using *Sphagnum* mass. The soil was mixed at regular intervals and kept wet by regular sprinkling of water. The inoculum at 0 h was added at the level containing  $10^7$ -  $10^8$  cells. Sampling was done at 24 h intervals. 25 samples were removed randomly from each plot and analysed for residual HCH isomers and survivability of microbial consortium.



Plate 1. HCH degradation experiments in open plots/actual fields

### 2.9 Bioassay of the remediated soil

Seeds of *Raphanus sativus* (radish) and *Aeblumuschnus esculantus* (ladies finger), members of *Brassicaceae* and *Malvaceae* which showed very high toxic effects of HCH towards seed germination were used as indicator plants to study the degradation of HCH in bioremediated soil. All the four bioremediated plots and the HCH- spiked but uninoculated controls and non- HCH- spiked controls were all sown with seeds of both radish and ladies finger. The seeds were allowed to germinate and grow completely. The plants were checked for growth, deformalities etc.

### 2.10 Degradation of other organochlorine pesticides by the microbial consortium

Soil was spiked with different organochlorine pesticides such as endosulphan, heptachlor, endrin, dieldrin at 10ppm level. Microbial consortium induced with respective organochlorine pesticide was used as inoculum. Pesticide spiked soil was inoculated with microbial consortium at inoculum 500  $\mu\text{g}$  protein /g soil. Moisture was maintained at 15 % by sprinkling water daily. Randomized sampling (1g soil from 2 different locations) was done at every 3h interval and analysed for residual spiked pesticide.

### 2.11 Degradation of Tech-HCH in native soil

The Tech-HCH degrading consortium was inoculated to different types of native soils. The types of soil chosen were clay soil; soil from coconut fields; tomato fields; coffee plantations; turmeric fields; red soil and garden soil. These soils were spiked with Tech-HCH and mixed well. It was inoculated with microbial consortium at 500  $\mu\text{g}$  protein /g soil. Moisture was maintained at 15 % by sprinkling water daily. Randomized sampling (1g soil from 2 different locations) was done at every 3h interval and analysed for residual spiked pesticide.

### 3. Analytical

#### 3.1 Growth

Growth of the consortium was determined by estimating total protein in the biomass by modified method of Lowry et al according to Murthy et al (2007). Cells were harvested from a suitable quantity of culture broth, washed with minimal medium, suspended in 3.4 mL distilled water and 0.6 mL of 20% NaOH. This was mixed and digested in a constant boiling water bath for 10 min. Total protein, in cooled sample of this hydrolysate, was estimated by using Folin-Ciocalteu reagent. A total of 0.5 mL of the hydrolysate was taken in a clean test tube. To this was added 5.0 mL of Lowry's C. After 10 min 0.5 mL of Lowry's D [Folin-Ciocalteu reagent (1:2)] was added and mixed well. The colour was read at 660nm after 20.0 min of standing at room temperature, using a spectrophotometer (Shimadzu UV- 160A, Japan). Total amount of protein was computed using the standard curve prepared with BSA (Bovine serum Albumin).

Growth was also measured in terms of colony forming units (cfu) as described by Sahu et al (1995) using appropriately diluted broth.

#### 3.2 Quantification of HCH

Residual HCH was quantified by Thin Layer Chromatography (TLC) and Gas Chromatography (GC).

##### 3.2.1 Extraction of residual HCH

The soil samples (whole cups), were removed after required period of incubation, they were air dried and extracted thrice with equal volumes of dichloromethane. The three solvent extracts were pooled and passed through column containing sodium sulphate (anhydrous) and florisil. These fractions were concentrated at room temperature and resuspended in a known volume of acetone and used for analysis of residue.

##### 3.2.2 Thin Layer Chromatography

Thin layer chromatography (TLC) was done using silica gel G TLC plates. Residual substrate samples dissolved in required quantity of acetone were spotted on TLC plates and these plates were developed in cyclohexane. The residual tech-HCH spots were identified after spraying the air-dried developed plates with *O*-tolidine in acetone. The residual substrate spots were delineated by marking with a needle and the area was measured. The concentration of residual substrate was computed from a standard plot of log concentrations *versus* square root of the area prepared for standard tech-HCH.

##### 3.2.3 Gas chromatography

Concentrated solvent containing residual substrate was resuspended in HPLC grade acetone and gas chromatography was done using Chemito gas chromatograph (GC 1000). After appropriate dilution 1 $\mu$ L sample was injected into gas chromatograph equipped with <sup>63</sup>Ni detector and capillary column DB 5 (30m X 0.25 mm) packed with (5% phenyl)-methylpolysiloxane. The column, injector and detector were maintained at 230<sup>o</sup> C, 230<sup>o</sup> C and 320<sup>o</sup> C respectively with a flow rate of carrier gas IOLAR grade I nitrogen at 1mL/min. The recovery of HCH isomers ranged from 92 to 95% from mineral salts medium. All the data presented in this study are based on triplicate estimations.

Other organochlorine pesticides were also estimated by GC under same conditions.

## 4. Results and discussion

The study is meant to assess the efficiency of microbial consortium to degrade HCH isomers contained in soil. The optimized conditions obtained in our previous study (Murthy and Manonmani, 2007) were adopted in this present study to find out the applicability. The optimized parameters used were: inoculum level 500 µg protein g<sup>-1</sup> soil, pH 7.5 and incubation temperature 30<sup>o</sup>C.

The soil used was red soil with no history of HCH applications. Soil had a particle size less than 0.5 mm. This size was chosen to provide high superficial area for interaction between HCH and microorganisms.

### 4.1 Microbial consortium

HCH-degrading microbial consortium developed by the long-term enrichment of the contaminated soil and sewage samples (Manonmani et al, 2000; Bidlan and Manonamami, 2002). This was reconstituted by mixing the different consortia having the ability to degrade  $\alpha$ -, $\beta$ -, $\gamma$ - and  $\delta$ -HCH isomers. This reconstituted consortium was acclimated with increasing concentration of Tech-HCH from 5ppm through 25 ppm. in shake flasks through three consecutive transfers every 24h. This consortium was used to study the degradation of Tech-HCH. The community structure of the consortium was identified by dilution plating technique. The bacterial of the consortial community were identified by biochemical tests and following Bergey's Manual of Determinative Bacteriology. The identification was confirmed by using Microbact Identification system. The consortium was found to be made of ten bacterial isolates consisting of seven *Pseudomonas* spp.; one species each of *Burkholderia*, *Flavobacterium* and *Vibrio* (Table 1)

Different carbon sources, both simple and complex, were used to optimize biomass production. Molasses supported highest biomass production, followed by glucose, sucrose, rice straw extract supplemented with glucose, rice straw hydrolysate, nutrient broth and wheat bran hydrolysate. The biomass grown on different carbon sources, when inoculated to 25ppm of HCH, showed that biomass grown on wheat bran hydrolysate showed better HCH-degradation. Nearly 80 – 90 % of all the four isomers disappeared by 72h of incubation. Next was molasses grown inoculum, which showed 65 – 70 % of degradation (Fig 1).

Sl. No.	Bacterial isolate	No.
1	<i>Pseudomonas fluorescens</i> biovar II	T <sub>1</sub>
2	<i>Pseudomonas diminuta</i>	T <sub>2</sub>
3	<i>Pseudomonas fluorescens</i> biovar I	T <sub>3</sub>
4	<i>Burkholderi pseudomallei</i>	T <sub>4</sub>
5	<i>Pseudomonas putida</i>	T <sub>5</sub>
6	<i>Flavobacterium</i> sp.	T <sub>6</sub>
7	<i>Vibrio alginolyticus</i>	T <sub>7</sub>
8	<i>Pseudomonas aeruginosa</i>	T <sub>8</sub>
9	<i>Pseudomonas stutzeri</i>	T <sub>9</sub>
10	<i>Pseudomonas fluorescens</i> biovar V	T <sub>10</sub>

Table 1. Composition of the Tech-HCH-degrading microbial consortium

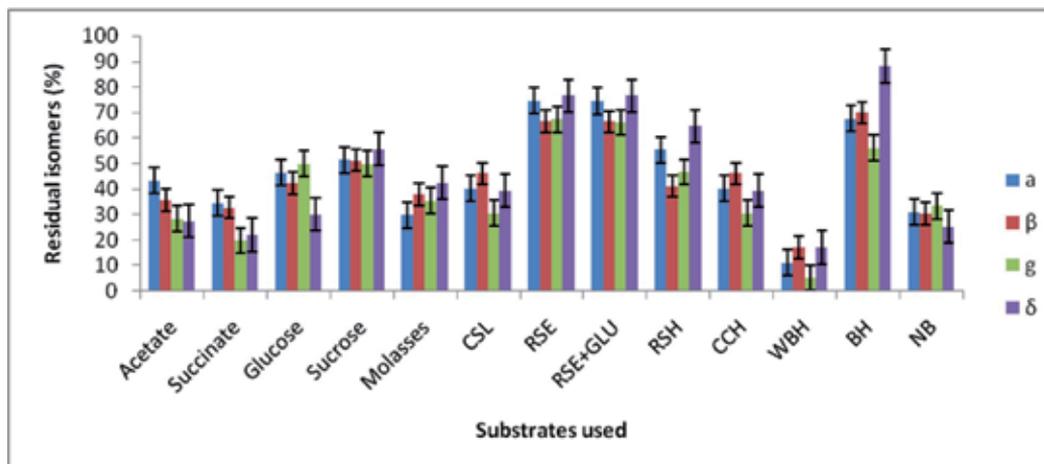
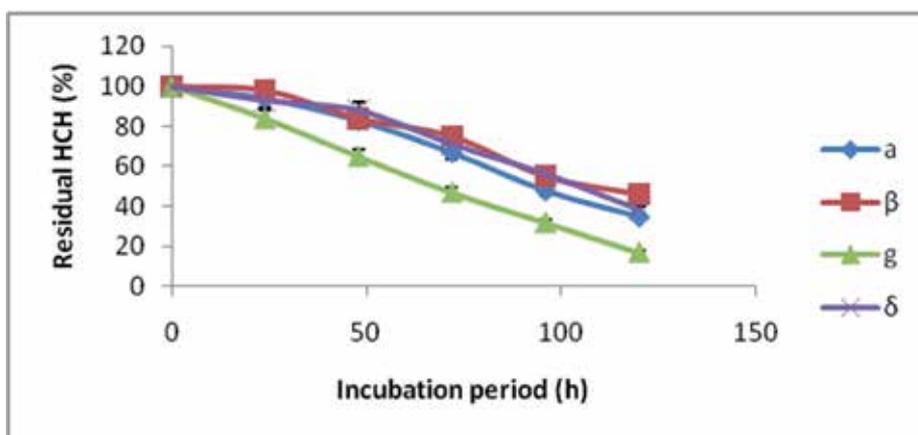


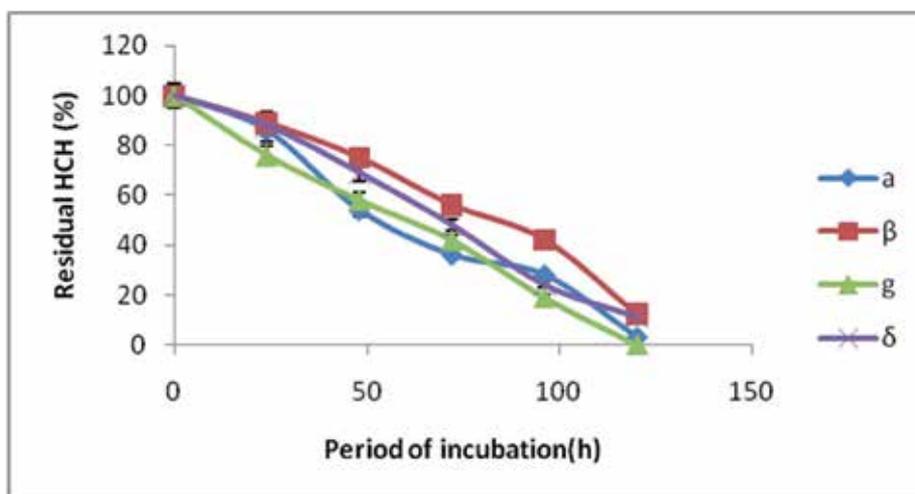
Fig. 1. Degradation of Tech-HCH with inoculum grown on different carbon sources CSL , Corn steep liquor; RSE , Rice straw extract; RSH, Rice straw hydrolysate; CCH, Corn cob hydrolysate; WBH, Wheat bran hydrolysate; BH, Bagassae hydrolysate; NB, Nutrient broth.

#### 4.2 Effect of induction/pre-exposure on the degradation of Tech-HCH

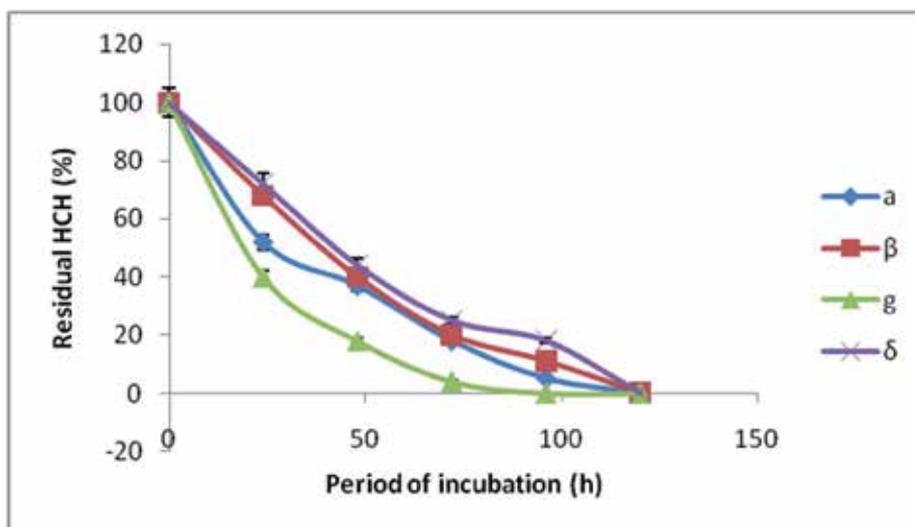
The pre-exposure of the HCH-degrading consortium was tried to understand the adaptation of the developed consortium and to understand the choice of the inducer that could be used for complete mineralization of tech-HCH. By exposing the WBH grown microbial consortium to  $\alpha$ -isomer resulted in the complete degradation of  $\alpha$ -isomer of Tech-HCH by 72 h of incubation.  $\beta$ -,  $\gamma$ - and  $\delta$ -isomers were still present at 2.035%, 6.019% and 24.13% levels by the end of 72 h of incubation Similarly,  $\beta$ -,  $\gamma$ - and  $\delta$ -HCH induced consortium degraded the substrates used for induction better than other isomers. But, Tech-HCH induced consortium degraded all the isomers (Fig 2) and in further experiments Tech-HCH induced inoculum was used unless otherwise stated.



a) 24h pre-exposure



b) 48h pre-exposure



c) 72h pre-exposure

Fig. 2. Effect of Pre-exposure to Tech-HCH on Tech-HCH degradation

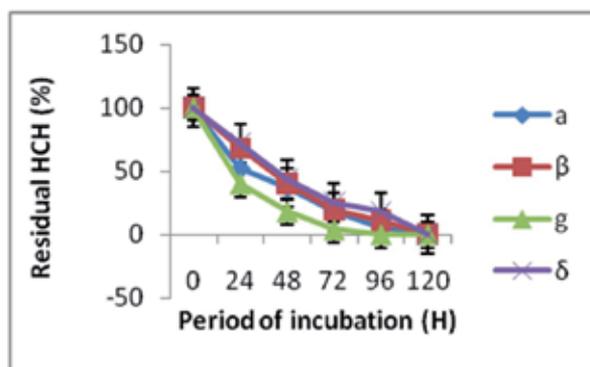
#### 4.3 Essentiality of individual members of the consortium for degradation of Tech-HCH

Degradation of higher concentrations (25ppm) of Tech-HCH was taken up to test the performance of different combinations of the members as well as individual isolates of the HCH-degrading consortium. Table 2 describes the results of this study. It appears obvious that the presence of all the ten strains is necessary for the faster and efficient degradation of higher concentrations of HCH. With  $\alpha$ -isomer as substrate, isolate T8 degraded 21% of the substrate by 120h. No other individual isolates were able to degrade this isomer. With increase in the number of individual members of the consortium the degradation of the  $\alpha$ -isomer increased and when all ten isolates were mixed together 94% degradation of  $\alpha$ -

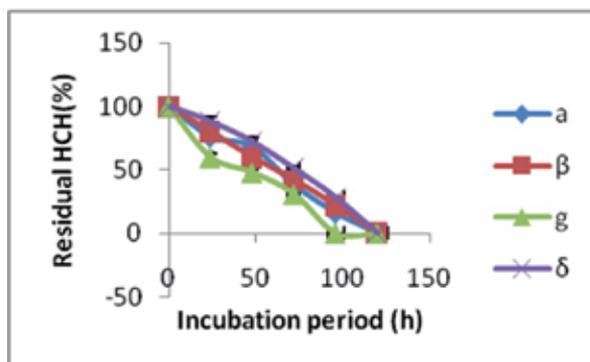
isomer was observed. When  $\beta$ -isomer was used as a substrate, isolate T4 degraded up to 10% of the  $\beta$ -isomer. No other individual isolates were able to degrade this isomer up to 10%. With increase in the number of individual members of the consortium the degradation of the  $\beta$ -isomer increased and when all ten isolates were mixed together 95% degradation of  $\beta$ -isomer was observed. When  $\gamma$ -isomer was used as a substrate, isolates T1, T8 and T2 were able to degrade 48, 47 and 49 % of  $\gamma$ -isomer respectively when inoculated individually. Combination of all T9 and T10 isolates could degrade the  $\gamma$ -isomer completely. With  $\delta$ -isomer as sole substrate, isolate T8 showed highest degradation of 6%. No other isolates were successful in degrading  $\delta$ -isomer. Combination of all individual members of the consortium could degrade 85% of the isomer. Increase with addition of individual members showed increase in the degradation of  $\delta$ -isomer.

#### 4.4 Degradation of Tech-HCH in native soil

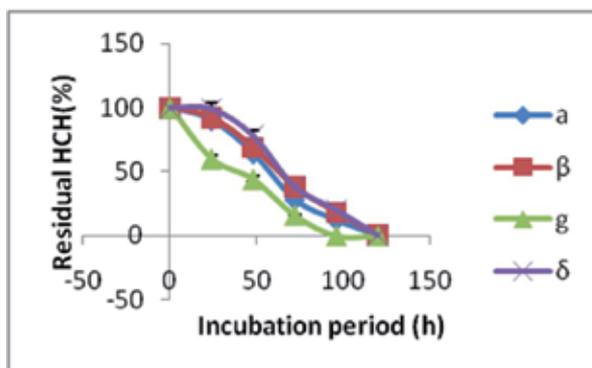
The Tech-HCH degrading consortium was inoculated to different types of native soils. Degradation was complete in all soils, but it was faster in red soil. In all soil types,  $\gamma$ -isomer disappeared faster (Fig 3). Degradation by native microflora was very low. The growth of the individual members of the consortium was also not inhibited by the presence of the native isolates.



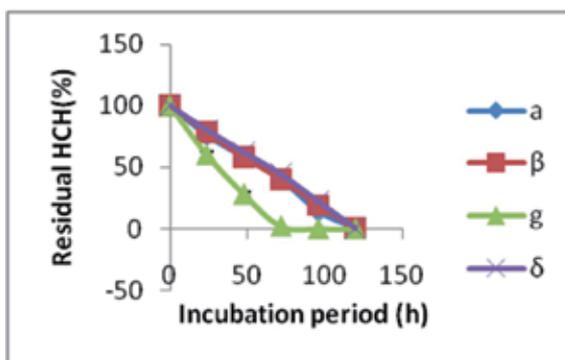
a) Clay soil



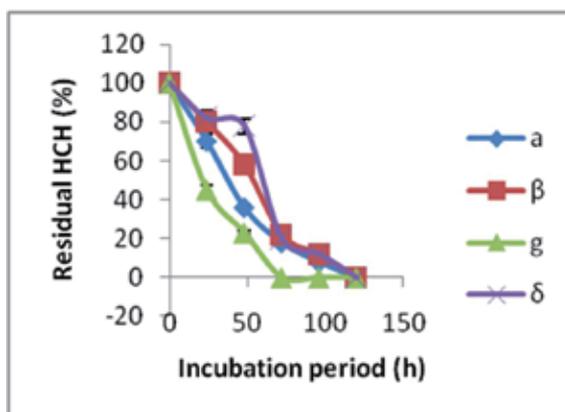
b) Soil from coconut fields



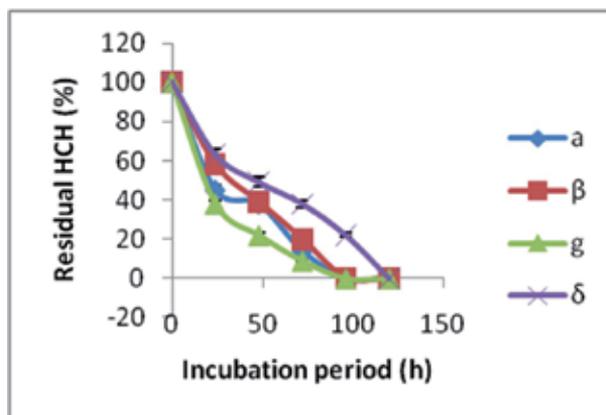
c) Soil from tomato fields



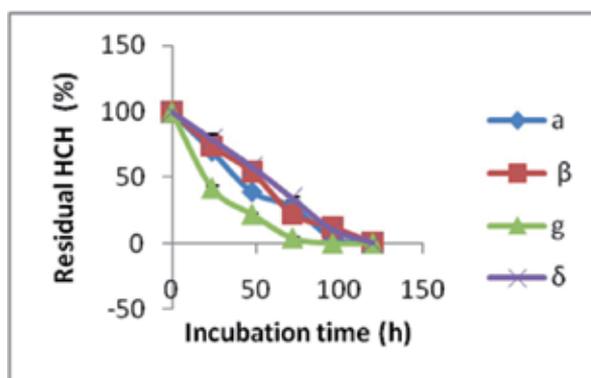
d) Soil from coffee plantations



e) Red soil



f) Soil from turmeric field



g) Garden soil

Fig. 3. Degradation of Tech-HCH in different native soils

#### 4.5 Degradation of other organochlorine pesticides by the microbial consortium

The HCH degrading microbial consortium acclimated with both tech-HCH and different pesticides under study were inoculated separately to respective organochlorine pesticides. The analysis of soil at different periods of incubation time indicated that the degradation was good when the consortium induced with HCH was used. The organochlorine pesticides such as DDT, heptachlor, endrin, dieldrin and endosulphan disappeared with HCH induced consortium (Fig 4). With the consortium induced with respective substrates, all substrates, except endrin were degraded completely.

#### 4.6 Preparation of inoculant formulations and their stability

Inoculant formulations containing the HCH-degrading microbial consortium and *Rhizobium* and *Azospirillum* were prepared using *Sphagnum* mass and wheat bran. The inoculum used contained  $10^7$  cells/g of the substrate. The inoculant formulation was prepared as both pellet and wet powder. The formulation was found to be stable for 60 days at refrigerated conditions. The formulations, when inoculated to soil containing 25 ppm tech-HCH, were found to degrade all the isomers of HCH completely (Fig 5). The formulation prepared in wheat bran took slightly longer time for complete degradation of tech- mixture.

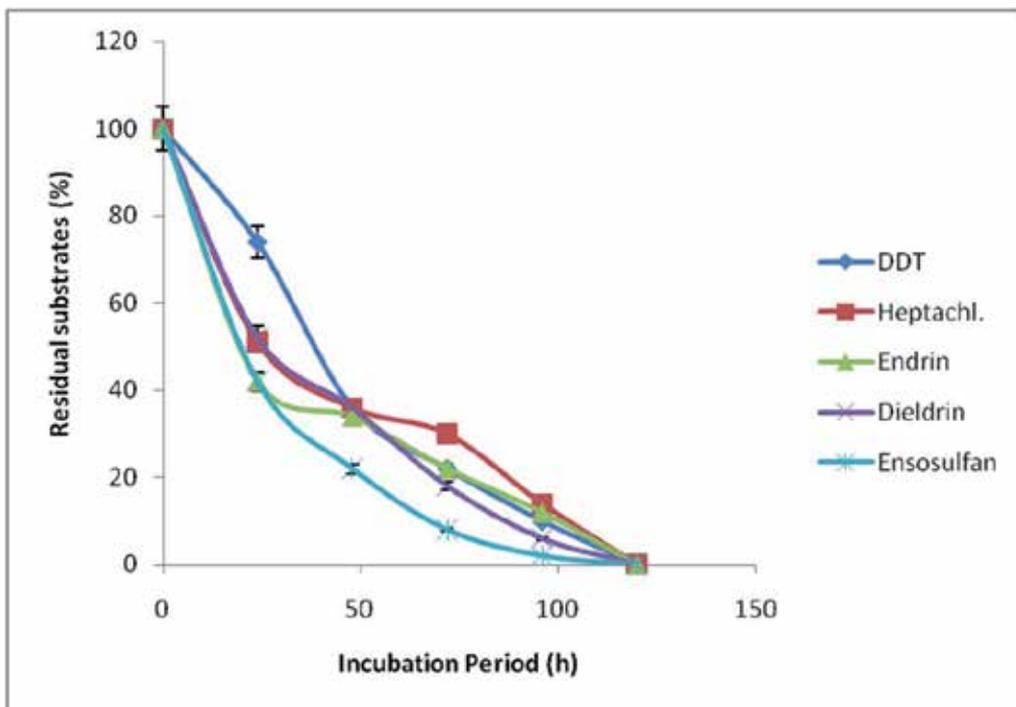


Fig. 4. Degradation of other organochlorine pesticides by Tech-HCH-degrading microbial consortium

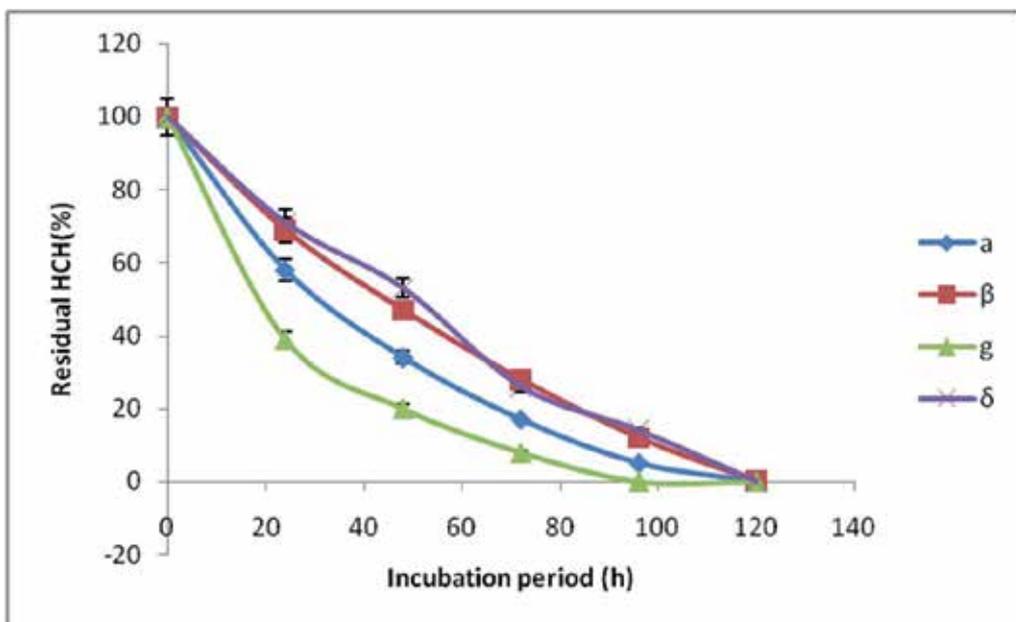


Fig. 5. Degradation of Tech-HCH by inoculant formulation (prepared using *Sphagnum* mass)

#### 4.7 Degradation of technical hexachlorocyclohexane in small artificial plots of 2ft x 2ft x 0.5ft dimensions

Samples from each plot were collected at 10 different locations selected randomly at intervals of 24 h (around 5 g each). Collected soil was mixed thoroughly. One-gram soil was used for recording colony-forming units (cfu) and other set was used for residue analyses. The solvent fractions, passed through anhydrous sodium sulphate and florisil, were analysed for residual HCH isomers by GC. The maximum and minimum temperatures recorded during this period of study were 25- 28°C and 18- 22°C respectively. The degradation of Tech- HCH was complete by 120 h of incubation in inoculated plots (Fig.6).  $\gamma$ -Isomer was degraded faster followed by  $\alpha$ -,  $\beta$ - and  $\delta$ - isomers. The native microflora did not show the formation of any dead end metabolites or inhibition of degradation. The individual members of the consortium showed good survival during degradation. No competition or inhibition from native micro flora was observed towards both the substrate and the added microbial consortium (Table3). Uninoculated plots showed very little degradation of HCH- isomers. Only  $\alpha$ - and  $\gamma$ - isomers were degraded by 2 and 4 % respectively, while  $\beta$ - and  $\delta$ - isomers were not degraded by the native microorganisms even after 120 h of incubation.

!	Log of CPU									
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
0	10.32±0.31	10.32±0.14	9.32±0.32	10.21±0.21	10.76±0.21	9.76±0.06	10.76±0.16	10.31±0.31	10.85±0.36	10.35±0.18
48	9.48±0.16	11.32±0.32	11.24±0.16	10.52±0.24	11.29±0.21	9.20±0.14	9.14±0.11	9.79±0.30	9.79±0.36	9.31±0.18
72	8.52±0.18	10.35±0.24	10.14±0.14	9.24±0.04	9.39±0.28	8.47±0.36	7.59±0.16	8.14±0.10	8.77±0.16	7.31±0.14
120	7.61±0.11	8.39±0.18	8.59±0.18	7.56±0.36	6.12±0.16	7.66±0.16	5.62±0.18	7.69±0.24	7.08±0.14	6.38±0.10

Table 3. Survivability of individual members of the consortium during the degradation of Tech- HCH

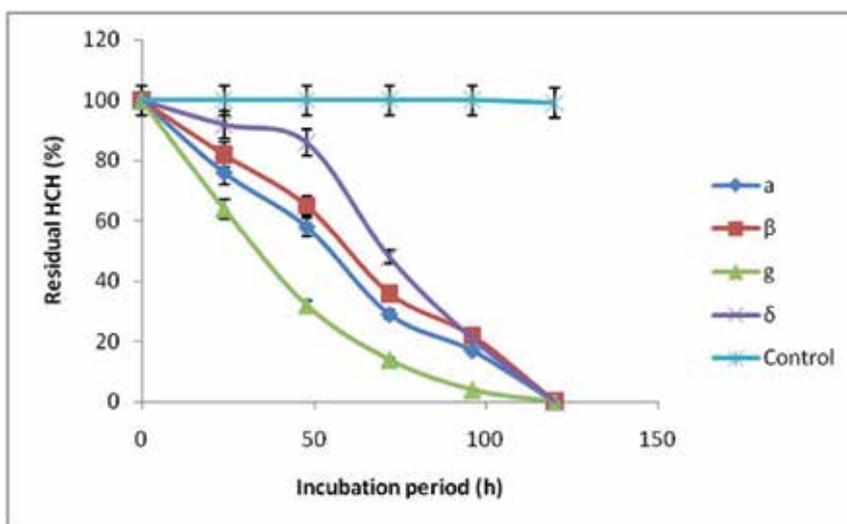


Fig. 6. Degradation of Tech-HCH in small artificial plots of 2ft x 2 ft x 0.5 ft

**4.8 Degradation of HCH in artificial test plots of 2m x 1m x 0.5ft dimensions**

At intervals of 24 h, samples were collected at 25 different areas of the plot (10 g each). The collected soil was mixed thoroughly and used for both the analysis of growth and residual substrate. It was observed that the degradation of all the isomers of HCH was complete with  $\gamma$ - isomer disappearing faster followed by  $\alpha$ -,  $\beta$ -, and  $\delta$ - isomers (Fig.7). The growth of the individual members of the consortium was also good (Table 4). The degradation of HCH-isomers was not observed by the native microflora.

!	Log of CPU									
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
0	10.46±0.34	10.48±0.16	10.97±0.31	10.17±0.16	10.68±0.16	10.58±0.18	10.76±0.19	10.03±0.24	10.42±0.36	10.42±0.08
48	9.66±0.14	8.00±0.11	7.93±0.008	9.14±0.18	8.00±0.24	9.89±0.18	9.10±0.14	79.38±0.16	10.53±0.31	9.91±0.14
72	8.03±0.16	7.93±0.21	6.36±0.16	8.76±0.20	7.47±0.08	9.25±0.14	7.72±0.21	7.72±0.14	9.71±0.26	8.96±0.10
120	8.21±0.18	7.83±0.20	6.05±0.11	8.54±0.11	7.21±0.16	7.79±0.18	7.10±0.30	7.45±0.18	8.89±0.11	8.08±0.26

Table 4. Survivability of individual members of the consortium during the degradation of Tech-HCH

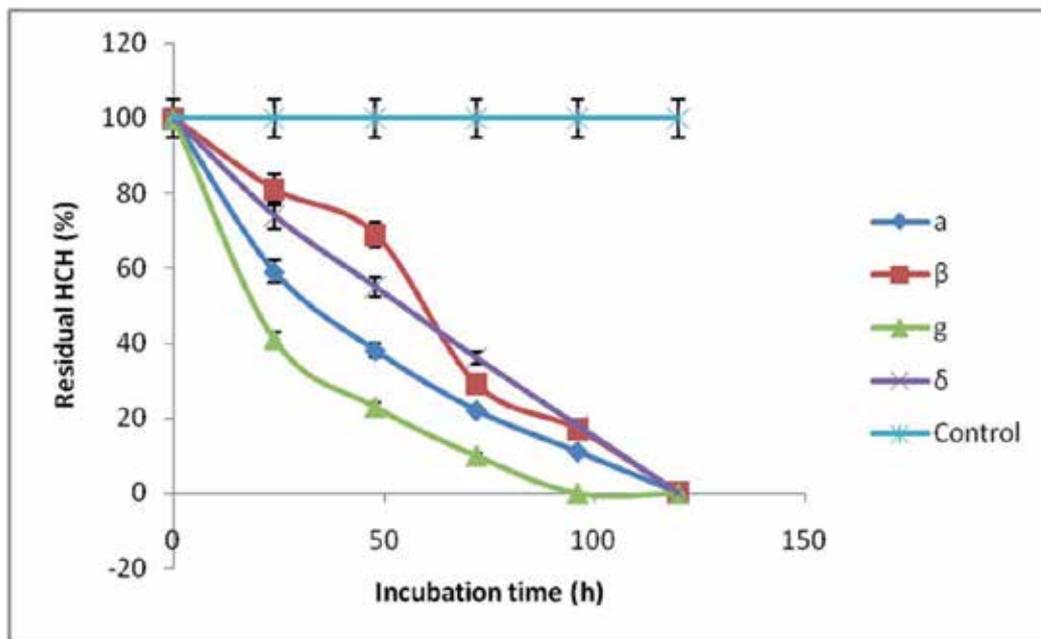
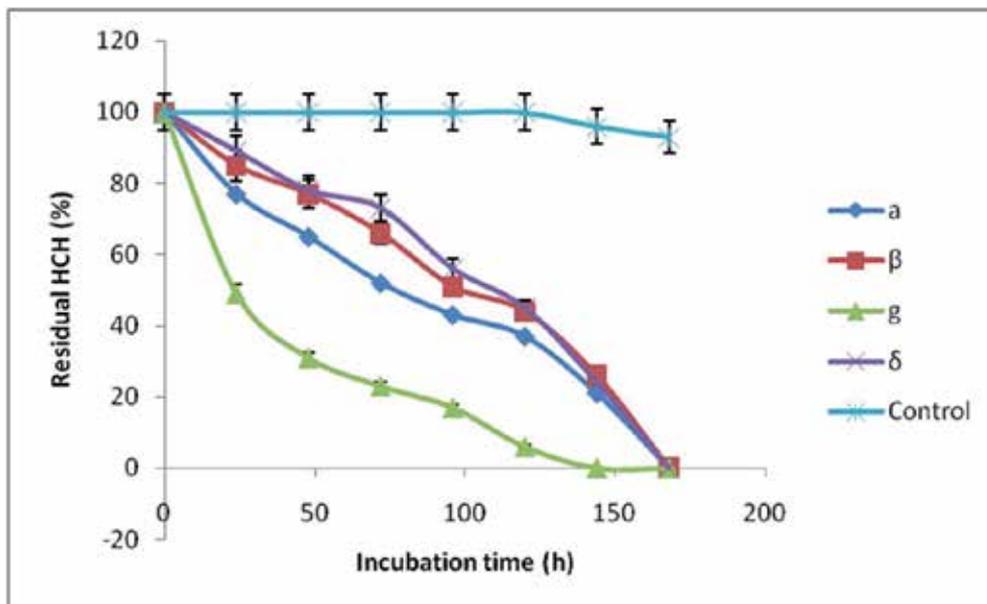


Fig. 7. Degradation of Tech-HCH in plots of 2m x 1 m x 0.15 m dimensions

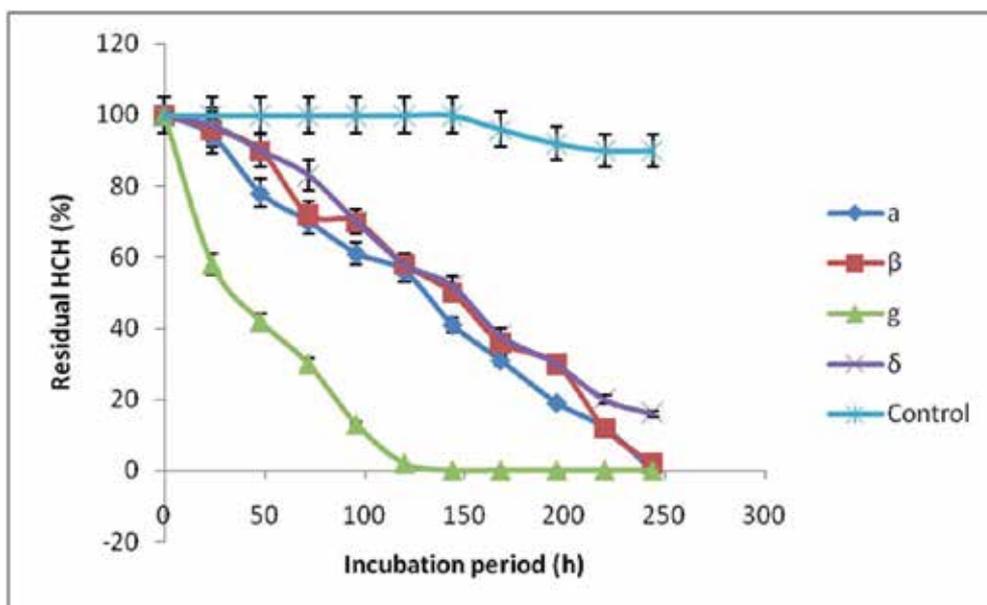
**4.9 Degradation of Tech-HCH in actual fields measuring 2m<sup>2</sup>**

25 samples were removed randomly from each plot. Collected soil from each plot was mixed well and 5 g soil from each sample was extracted. The solvent layers were pooled, passed through florisil and anhydrous sodium sulphate. The solvent fraction was pooled,

concentrated at room temperature and analysed for residual HCH. Compared to laboratory trials, the degradation of all isomers of HCH by the microbial consortium took 168 h for complete degradation (Fig.8). But the inoculant formulation took 10 days for complete degradation of all four isomers of HCH (Fig.9).



8a. Degradation by microbial consortium



8b. Degradation by microbial inoculant formulation

Fig. 8. Degradation of Tech-HCH in actual open fields

#### 4.10 Bioassay of the remediated soil

Seeds of *Raphanus sativus* (radish) and *Aeblumusculus esculantus* (ladies finger), members of *Brassicaceae* and *Malvaceae* which showed very high toxic effects of HCH towards seed germination were used as indicator plants to study the degradation of HCH in bioremediated soil. All the four bioremediated plots and the HCH- spiked but uninoculated controls and non-HCH- spiked controls were all sown with seeds of both radish and ladies finger. The germination of the plants was delayed in seeds sown in HCH- spiked soil. The height of the plants was reduced when compared to controls. Many other growth- related deformalities were also observed (Plate 2 and 3 ). In the bioremediated soil, growth related deformalities were not observed and the plant looked healthy *in par* with controls (Plate 4 and 5 ).

### 5. Discussion

HCH has been used worldwide as general broad spectrum insecticide for a variety of purposes including fumigation of the house hold and commercial storage areas, pest control on domestic animals, mosquito control and to eradicate soil-dwelling and plant-eating insects. Although only lindane has insecticidal property, HCHs as group are toxic and considered potential carcinogens (Walker et al., 1999) and listed as priority pollutants by the US EPA. Due to their persistence and recalcitrance, HCHs continue to pose a serious toxicological problem at industrial sites where post production of lindane along with unsound disposal practices has led to serious contamination. In addition, many countries including India have permitted HCH production (lindane is permitted to be used) and use. This has become a global issue due to problems of volatility and transportation of HCH isomers by air to remote locality (Galiulin et al., 2002; Walker et al., 1999). Due to the toxicity and persistence of HCH, soils contaminated with HCHs have been targeted for remediation. Biodegradation of  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ - isomers of HCH have been extensively studied in the laboratory at individual level. But information is insufficient on pilot or full-scale *in situ* field settings. The HCH-isomers have been shown to differ in their persistence in soil and in their properties like solubility and volatility that determine their rates of biodegradation. Earlier studies suggested that degradation of HCH was faster under anoxic conditions and that microbial degradation was primary route of HCH disappearance from soil (MacRae et al., 1967). Microbial degradation of all the HCH-isomers has since been observed under oxic conditions both in soil (Bachmann et al., 1988 b); Doelman et al., 1985; Sahu et al., 1993) and in pure cultures of microorganisms (Bhuyan et al., 1993; Thomas et al., 1996). We have isolated in our laboratory a microbial consortium consisting of ten bacterial isolates which have got the capacity to degrade HCH (Manonmani et al., 2000; Murthy and Manonmani, 2007) under oxic conditions. Translation of the laboratory scale trials to small plots and actual open fields was studied in soil

The defined microbial consortium used in the degradation of tech-HCH was developed by the long-term enrichment of the contaminated soil and sewage samples. This microbial consortium was acclimated with increasing concentrations of tech-HCH from 5 to 25 ppm. The consortium that got established at 25 ppm level was used in our studies. The initial inoculum used in acclimation is obtained from diverse sources such as HCH contaminated soil and sewage. The advantage of sewage is that it provides sufficient inoculum during acclimation. As the test compound is used as a sole source of carbon and energy the organisms having the machinery for the degradation of the compound would survive and therefore would be able to accomplish the mineralization process. Moss [1980] employed an

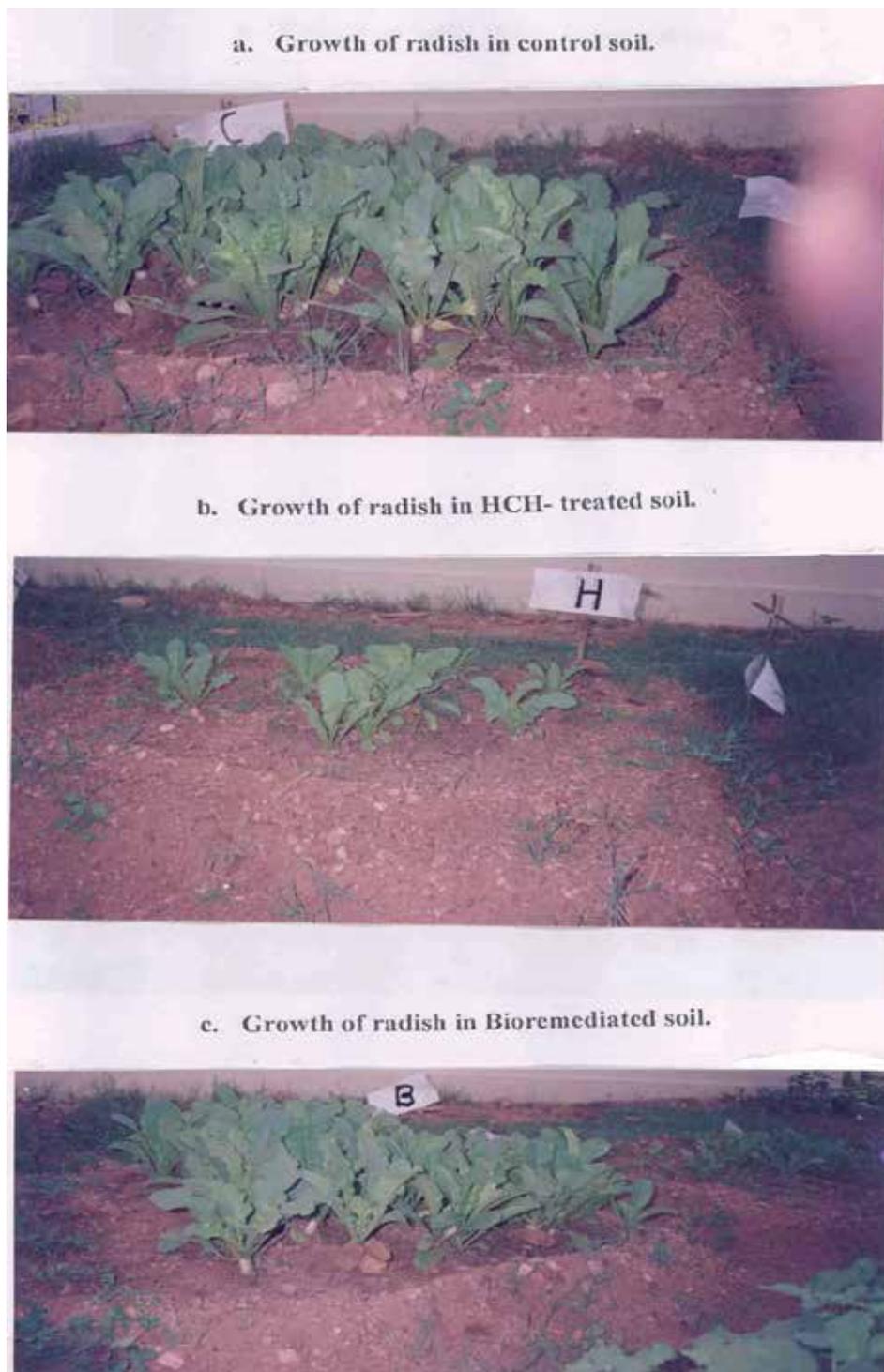


Plate 2. Bioassay in soil

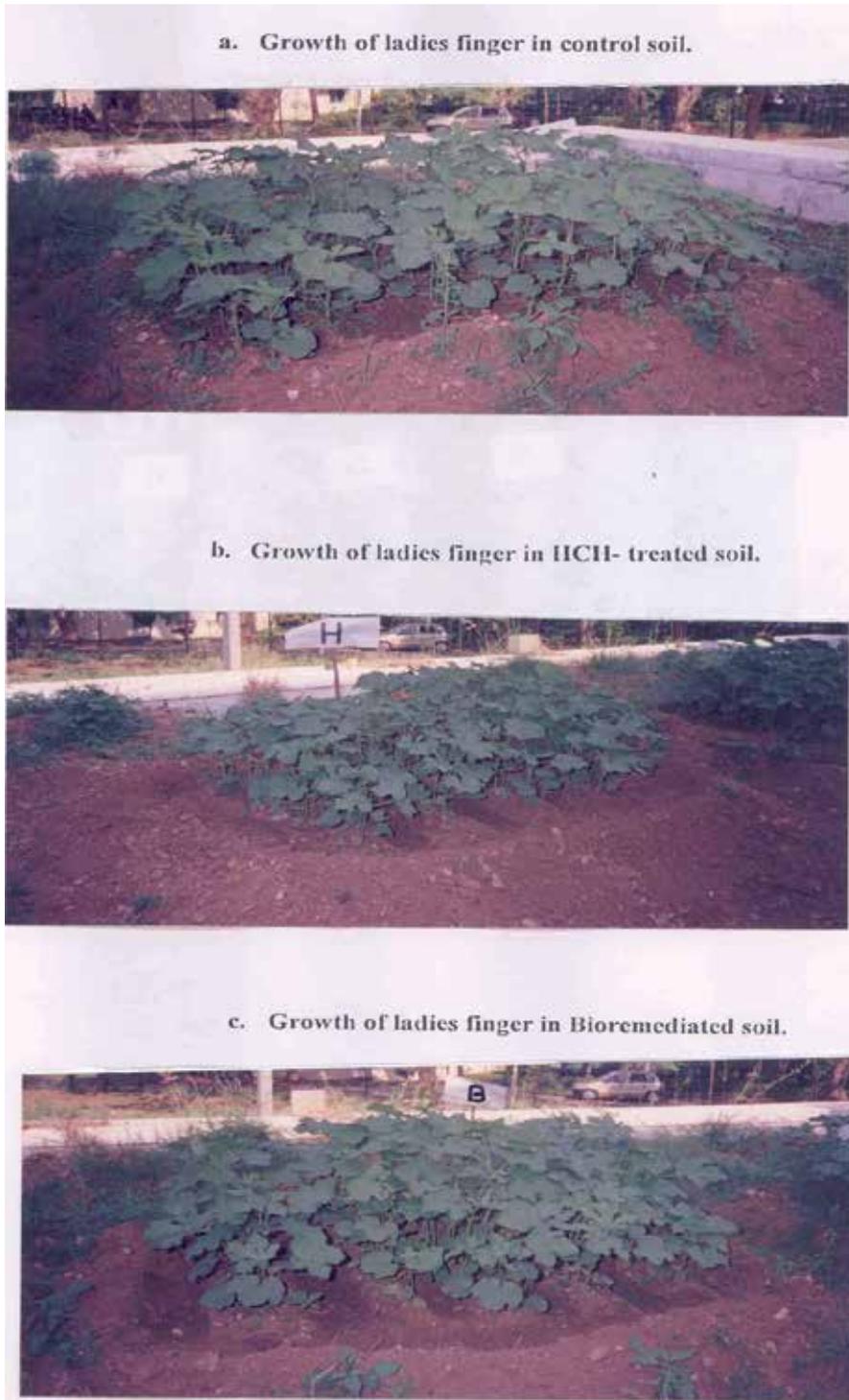


Plate 3. Bioassay

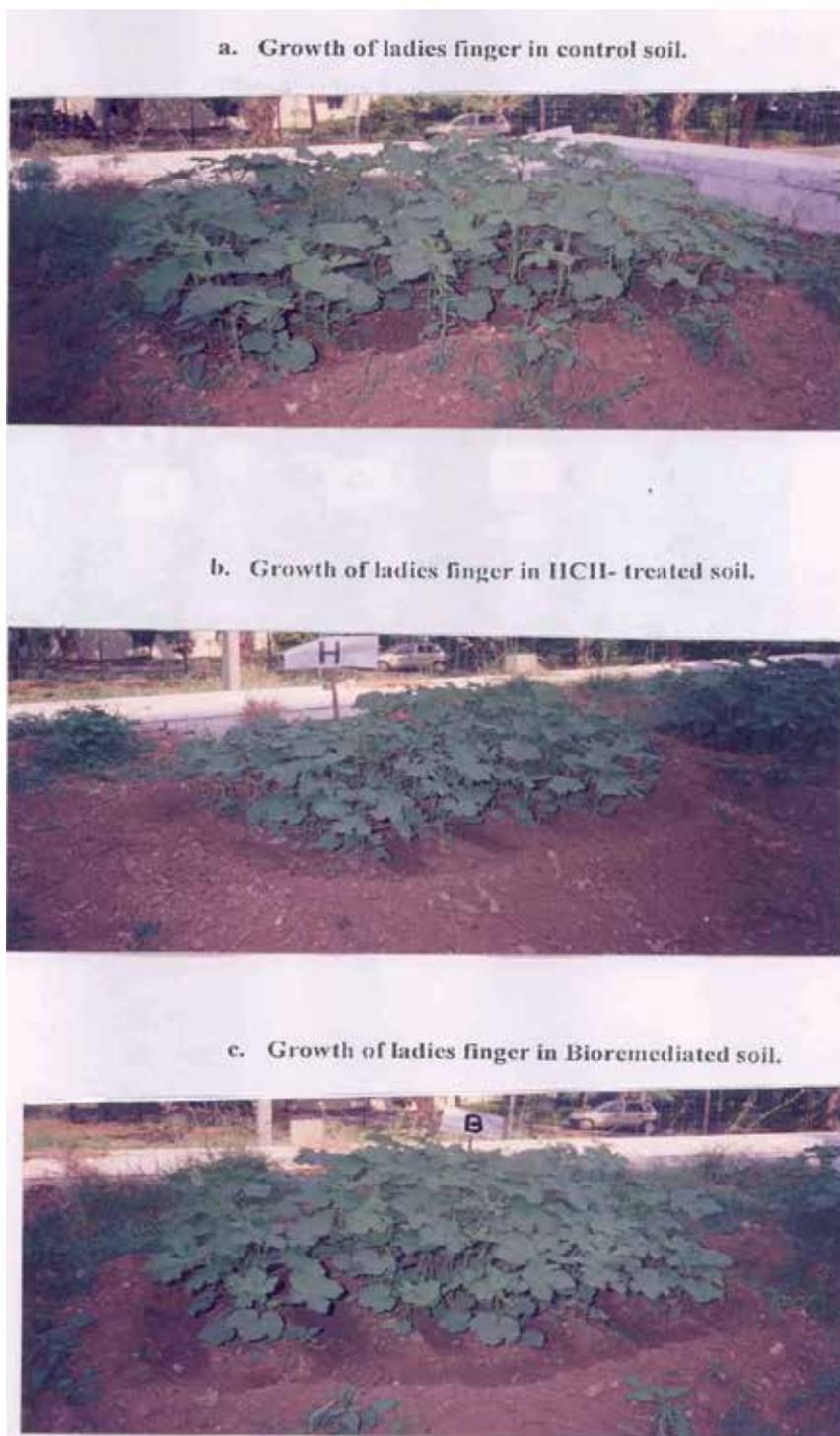


Plate 4. Yields of Ladies finger in bioassay experiments

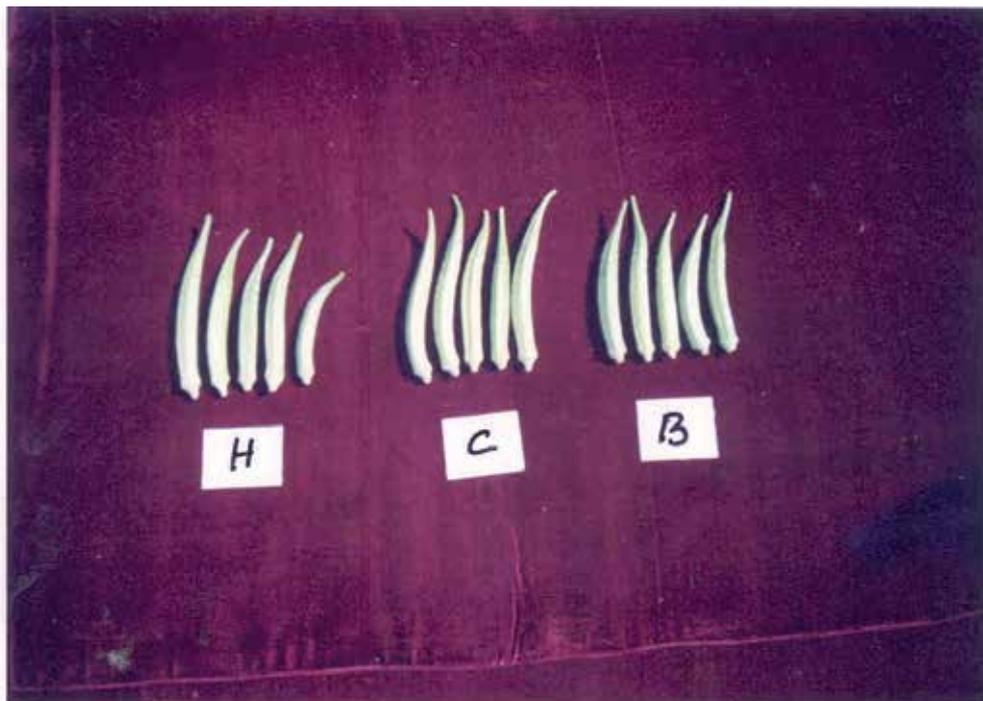


Plate 5. Yields of radish in bioassay experiments

acclimation and enrichment procedure that used a continuous culture of microorganisms growing at very low specific growth rates. The compound being tested was applied continuously at low concentrations and the concentration was increased in a systematic manner. Similar acclimation technique has been used by Bidlan and Manonmani [2002] to isolate DDT degrading microorganisms. Acclimation also would help in evading toxicity prior to the actual degradation and this is an essential part for further degradation studies. Acclimation would result in altered composition of the microbial populations involved in the early stages of degradation. Pre-exposure helped in obtaining faster degradation without any lag. The degradation appeared to have started as soon as inoculum and substrate were together. There was no initial lag in degradation with the inoculum induced with any of the isomers of HCH. In all these cases, the HCH-isomer used for induction was degraded completely and other isomers were partially degraded. This probably could be due to presence of required enzyme that got induced with the particular isomer. The partial degradation could be due to the multiple functions of the enzyme which was able to degrade the substrates only to certain extent. The partial degradation could be due to the enzymes, which might not have got induced by the other isomer used, or there could be inhibition of pathway enzymes by the intermediary metabolites formed during the degradation of non-inducer substrates. Also the complete degradation could be achieved with increase in incubation time. It has been reported that the degradation of  $\alpha$ - and  $\gamma$ -isomers follow the same pathway and the degradation of  $\beta$ -HCH has been deciphered to only one or two steps of biodegradation (Nagata et al 2005). To our knowledge no reports are available on the degradation pathway of  $\delta$ -HCH degradation. In our studies also the degradation of these isomers might be following a different pathway, As tech-HCH is a mixture of all isomers, degradation of different isomers is a complex phenomenon, as these enzymes might face many inhibitory/stimulating effects by the intermediates formed by different isomers present together. Failure to achieve results with consistent complete mineralization, on the other hand, would also suggest that complete biodegradation is not possible in short time or that it is dependent on co-metabolism. Thus, the tech-HCH induced inoculum was used in further studies. The microbial consortium developed in the laboratory was capable of degrading all isomers of HCH and the biodegradation could occur in a particular environment. With all the optimized conditions, the biodegradation of tech-HCH becomes a highly system specific event. These optimized results can be adapted well in the treatment of industrial effluent or water bodies contaminated with HCH.  $\alpha$ - and  $\gamma$ -isomers have been reported to be degraded rapidly under aerobic and anaerobic conditions.  $\alpha$ - and  $\gamma$ -isomers were degraded first in 12 h of incubation (Manonmani et al 2000, Johri et al 1998, Datta et al 2000), where as  $\beta$ -isomer was found to remain undegraded under similar environmental conditions (Bachmann et al 1988a, Beurakens et al 1991). The degradation of different isomers of HCH has been shown to be dependent on many features mainly the type of microorganism used, aeration, the adaptability of these microorganisms to the pollutants (Moreno and Buitron 2004), type of carbon source to cultivate them (Radha et al 2010), pre-exposure of the used organism to the pollutant (Bidlan and Manonmani 2002), etc. The recalcitrancy of the isomers also has been shown to play a key role in the degradation of different isomers of HCH (Bachmann et al 1988a, Haider and Jagnow 1975). In our studies also, the time required for the highly recalcitrant  $\beta$ - and  $\delta$ -isomers was more compared to the other two isomers. The degradation of tech-HCH by the microbial consortium appears to be gratuitous metabolism where in, the substrate, i.e. tech-HCH is used as a sole source of carbon and energy and no other co-substrates are being supplemented. This is evident from the survival of all members of consortial community during degradation. However, no substantial growth was observed, i.e. the cells behaved as resting cells as the

amount of carbon supplied by the substrate is not sufficient to support good growth of the consortium. But the cell count was being maintained during degradation. The initiation of degradation might be by the enzyme system that was already induced during pre exposure cycle, and hence degradation did not show any lag. The pH of the medium had a substantial effect on the survivability of the members of the consortial community. At low pH levels, there was decrease in the microbial population. The degradation was observed to take place over a wide range of pH from 4.0 to 8.0 (Murthy and Manonmani, 2007). Degradation was found to decrease at pH 9.0. The degradation of each isomer was influenced by the presence of other isomer. The HCH isomers, i.e. non-growth substrates have  $\gamma$ -isomer, the more easily degradable and  $\beta$ - and  $\delta$ -isomer very highly recalcitrant. These two isomers thus show resistance toward degradation. It could be that because of recalcitrance, the structure may prevent it fitting into enzyme within the cell when it is likely to accumulate or the transformation product of one substrate may become toxic than the original substrate that might result in slower rate of degradation and also the degradative pathway of each isomer may be different which would be influenced by many factors. However, the different isomers present in the mixture will not associate in their co-metabolic degradation.

As our microbial consortium consisted of aerobic microorganisms, soil was mixed often in small plots to facilitate aeration. Similar degradation of  $\alpha$ -HCH under oxic conditions in either moist soil or soil slurries has been reported (Doelman *et al.*, 1985). Degradation of 23 mg kg<sup>-1</sup> day<sup>-1</sup> of  $\alpha$ -HCH in soil under oxic conditions has been obtained (Bachmann *et al.*, 1988a, b). However, reduction of 13 mg kg<sup>-1</sup> day<sup>-1</sup> was obtained under methanogenic conditions. Van Eekert *et al.* (1998) have reported the removal of  $\alpha$ -HCH from a sandy soil containing low concentration of the isomer in slurries where lactate or sulfide had been added to reduce redox potential. Degradation of HCH was faster under anoxic conditions and that microbial degradation was primary route of HCH disappearance from soil (MacRae *et al.*, 1967). Microbial degradation of all the HCH-isomers has since been observed under oxic conditions both in soil (Bachmann *et al.*, 1988 (a,b); Doelman *et al.*, 1985; Sahu *et al.*, 1993) and in pure cultures of microorganisms (Bhuyan *et al.*, 1993; Thomas *et al.*, 1996). Soil slurry has been adopted for the microbial degradation of pesticides, explosives, polynuclear aromatic hydrocarbons, and chlorinated organic pollutants (Gonzalez *et al.* 2003). In our studies translation of the laboratory scale trials to small reactors was studied in artificial plots and open actual fields. The moisture content in soil has been shown to influence greatly HCH degradation. Chessells *et al.* (1988) have reported a correlation between soil moisture content and removal rates of HCH isomers in field agricultural soils. Enhanced removal of HCH in soils with higher moisture contents has been reported. This has been made possible due to prevailing anoxic conditions during flooding. Thus anaerobic metabolism has been reported to be existing in these soils. In our earlier studies, soil moisture content of 15 to 20% was found to give good biodegradation of HCH-isomers (unpublished data). This was used in the current study in small and actual plots. As our microbial consortium consisted of aerobic microorganisms, mixing at regular intervals was done to maintain oxic condition. Degradation of  $\alpha$ -HCH in glass columns packed with contaminated sediments and held under methanogenic conditions has been reported (Middeldorp *et al.*, 1996), although degrading population of microorganisms appeared not to be methanogens. Degradation of  $\gamma$ -HCH under oxic conditions has also been reported (Yule *et al.*, 1967).  $\beta$ -HCH isomer, an indisputably most recalcitrant isomer, does not undergo biodegradation easily. The concentration did not decrease noticeably in field study under any treatment (moist soil and oxic soil slurries in small pots) (Doelman *et al.*, 1985)

Complete degradation of Tech-HCH was obtained in all the studies carried out. The bioassay of the bioremediated soil was carried out to check the degradation or mineralization of HCH isomers. The growth of Seeds of *Raphanus sativus* (radish) and *Aeblumusculus esculantus* (ladies finger) was poor in HCH spiked soil and their growth in bioremediated soil was *in par* with that in control soils. The crop yield in bioremediated soil was also *in par* with control soils.

## 6. Conclusion

The use of microbes to clean up polluted environments, bioremediation is rapidly changing and expanding the area of environmental biotechnology. Although much work is being done to remediate the polluted environment, our limited understanding of the biological contribution and their impact on the ecosystem has been an obstacle to make the technology more reliable and safer. In our studies a defined microbial consortium was able to degrade HCH (technical grade containing all four major isomers) up to 25 ppm level in soil at ambient temperature and neutral pH. The consortium was able to degrade HCH in artificial plots and also in open fields.

The inhibition of degradation by the presence of other isomers and native microflora was marginal. With the translation of lab trials to large scale trials coupled with process molecular microbiological techniques can make the bioremediation process more reliable and safer technology.

Although HCH removal has been observed under both oxic and anoxic bioremediation treatments, treatments under oxic condition have resulted in the almost complete removal of HCH *via* mineralization. These observations are on par with our results wherein under oxic conditions good degradation of HCH-isomers of technical mixture has been observed. Even though all the four isomers were present together in technical mixture, no adverse or inhibitory effects were observed by either parent compounds or their metabolites. We have tried to address the inadequately addressed topic of bioremediation of HCH contaminated soils in field studies. With the disadvantages of ex-situ bioreactors such as requirements for soil excavation, handling, conditioning and bioreactor construction/operation that typically increase treatment costs compared to most simple bioremediation techniques. The successful results obtained in small scale soil studies need to be addressed during translation further to still larger scale.

## 7. Legends to figures

### Fig. 1. Degradation of Tech-HCH with inoculum grown on different carbon sources

CSL, Corn steep liquor; RSE, Rice straw extract; RSH, Rice straw hydrolysate; CCH, Corn cob hydrolysate; WBH, Wheat bran hydrolysate; BH, Bagassae hydrolysate; NB, Nutrient broth.

Microbial consortium were grown in different carbon sources and induced with Tech-HCH and inoculated to Tech-HCH. Analysis was done as given under Methodology.

All the experiments were done in replicates of ten for each parameter studied.

### Fig. 2. Preexposure and degradation of Tech-HCH

Individual isolates of the microbial consortium were grown in wheat bran hydrolysate/peptone- glycerol medium and the cells were harvested after 72h of growth, washed well and preexposed to 25ppm of individual isomers and Tech-HCH for 72h separately with

addition of individual isomers and Tech- HCH every 24h. The induced cells were harvested by centrifugation at 10,000rpm, 4°C, 10min, washed well and used as inoculum. All the experiments were done in replicates of ten for each parameter studied.

**Fig. 3. Degradation of Tech-HCH in different native soils:**

Different soils such as clay soil, red soil, garden soil, soils from coconut, coffee, turmeric and tomato plantations were studied to evaluate the degradation of Tech- HCH. The substrate and inoculum were used at 25 µg g<sup>-1</sup> and 500 µg protein g<sup>-1</sup> soil respectively. All the experiments were done in replicates of ten for each parameter studied.

**Fig. 4. Degradation of other organochlorine pesticides by Tech-HCH-degrading microbial consortium.**

Soil was spiked with different organochlorine pesticides such as endosulphan, heptachlor, endrin, dieldrin at 10ppm level. Microbial consortium induced with respective organochlorine pesticide was used as inoculum. Pesticide spiked soil was inoculated with microbial consortium at inoculum 500 µg protein /g soil. Moisture was maintained at 15 % by sprinkling water daily. Randomized sampling (1g soil from 2 different locations) was done at every 3h interval and analysed for residual spiked pesticide. All the experiments were done in replicates of ten for each parameter studied.

**Fig. 5. Degradation of Tech-HCH by inoculant formulation (prepared using *Sphagnum* mass)**

Inoculant formulations containing the HCH-degrading microbial consortium and *Rhizobium* and *Azospirillum* were prepared using *Sphagnum* mass. two more plots received the one-month-old inoculant formulation prepared using *Sphagnum* mass. The soil was mixed at regular intervals and kept wet by regular sprinkling of water. Randomized sampling was done at every 3h interval and analysed for residual spiked pesticide.

**Fig. 6. Degradation of Tech-HCH in small artificial plots of 2ft x 2 ft x 0.5 ft**

Four artificial plots of 2ft x 2ft x 0.5ft were prepared. Red loamy non- sterile soil containing 1-1.5% organic carbon and 32% water holding capacity and pH 7.0, collected from CFTRI campus, was taken in these plots. Each plot was spiked with 25 ppm of tech- HCH and mixed well. Two plots were inoculated with 72 h HCH- induced microbial consortium. Two plots were maintained as abiotic (uninoculated) controls. All plots were kept wet by regular sprinkling of water and the soil in the plots was mixed regularly. Samples from each plot were collected at 10 different locations selected randomly at intervals of 24 h (around 5 g each). Collected soil was mixed thoroughly and was used for residue analyses.

**Fig. 7. Degradation of Tech-HCH in plots of 2m x 1 m x 0.15 m dimentions.**

Six artificial plots of 2m x 1m x 0.5ft were prepared and red loamy soil collected from CFTRI campus was taken in these plots. Each plot was spiked with 25 ppm Tech- HCH and mixed well. Four plots were inoculated with 72 h HCH- induced microbial consortium. The inoculum was added at levels containing 10<sup>7</sup> to 10<sup>8</sup> cells/ g soil. Two plots were maintained as uninoculated controls (containing only HCH). The soil was mixed well at regular intervals and was kept moist by regular sprinkling of water. At intervals of 24 h, samples were collected at 25 different areas of the plot (10 g each). The collected soil was mixed thoroughly and used for both the analysis of growth and residual substrate.

**Fig. 8. Degradation of Tech-HCH in actual open fields:**

8a. Degradation by microbial consortium.

8b. Degradation by microbial inoculant formulation:

8 plots measuring 2m<sup>2</sup> were chosen at CFTRI campus. The plots were prepared by tilling and toeing. Spiking of Tech- HCH to the soil was repeated for four times on alternative days. The final concentration of Tech- HCH added to the soil was 25 ppm. Six of the plots received Tech- HCH while two remaining were maintained as unspiked, uninoculated controls. Two plots were inoculated with microbial consortium and two more plots received the one-month-old inoculant formulation prepared using *Sphagnum* mass. The soil was mixed at regular intervals and kept wet by regular sprinkling of water. The inoculum at 0 h was added at the level containing 10<sup>7</sup>- 10<sup>8</sup> cells. Sampling was done at 24 h intervals. 25 samples were removed randomly from each plot and analysed for residual HCH isomers.

#### Plate 1.

8 plots measuring 2m<sup>2</sup> were chosen at CFTRI campus.

#### Plate 2 and 3.

Seeds of *Raphanus sativus* (radish) and *Aeblumuschus esculantus* (ladies finger), members of *Brassicaceae* and *Malvaceae* which showed very high toxic effects of HCH towards seed germination were used as indicator plants to study the degradation of HCH in bioremediated soil. All the four bioremediated plots and the HCH- spiked but uninoculated controls and non- HCH- spiked controls were all sown with seeds of both radish and ladies finger.

#### Plates 4 and 5.

Seeds of *Raphanus sativus* (radish) and *Aeblumuschus esculantus* (ladies finger) grown in HCH-spiked, bioremediated and non-spiked soils.

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# Setting Up of Risk Based Remediation Goal for Remediation of Persistent Organic Pesticides (Pesticide-POPs) Contaminated Sites

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## 1. Introduction

### 1.1 Persistent Organic Pollutants (POPs)

Persistent Organic Pollutants (POPs) is a common name of a group of pollutants that are semi-volatile, bioaccumulative, persistent and toxic. POPs comprising of pesticides, industrial chemicals and unintentionally produced POPs are toxic chemicals that adversely affect human health and the environment around the world (Vallack et al.,1998; Mocarelli & Tallman,1998 & Jones & de Voogt,1999). Since these pollutants can be transported by wind (natural ,human barriers and perturbation , grass in the desert ,cities and thermal updrafts, etc), water (ssurface, rain, ground, pumped and/or anthropogenically modified chemicals) and human intervention (waste-water conduits, drainage ditches, roadways, railways, irrigation, ponding, physical transformation physical collection and concentration, human species transformation) (Sw-846,USEPA,2007) , most POPs generated in one country can affect people and wildlife far from where they are used and released. These chemicals persist for long periods of time in the environment and can accumulate and pass from one species to the next through the food chain.

POPs can be deposited in marine and freshwater ecosystems through effluent releases, atmospheric deposition, runoff, and other means. As POPs have low water solubility, they bond strongly to particulate matter in aquatic sediments. As a result, sediments can serve as reservoirs or "sinks" for POPs. When sequestered in these sediments, POPs can be taken out of circulation for long periods of time. If disturbed, however, they can be reintroduced into the ecosystem and food chain, thereby potentially becoming a source of local, and even global contamination.

To address this global concern, there exists a groundbreaking United Nations' treaty in Stockholm, Sweden (May 2001). Under the treaty, known as the Stockholm Convention, countries agree to reduce or eliminate the production, use, and/or release of 12 key POPs (UNEP Toolkits on POPs,1999,2001,2003,2005) which are shown in **Table 1**.

The Convention specifies a scientific review process that could lead to the addition of other POPs chemicals of global concern. The list of nine new POPs added to the Stockholm Convention in May, 2009 is shown in **Table 2**.

Sr. No.	POPs Identified
POPs-Pesticides	
1	Aldrin <sup>1</sup>
2	Chlordane <sup>1</sup>
3	Dichlorodiphenyl trichloroethane (DDT) <sup>1</sup>
4	Dieldrin <sup>1</sup>
5	Endrin <sup>1</sup>
6	Heptachlor <sup>1</sup>
7	Hexachlorobenzene <sup>1,2</sup>
8	Mirex <sup>1</sup>
9	Toxaphene <sup>1</sup>
POPs-nonpesticides	
10	Polychlorinated biphenyls (PCBs) <sup>1,2</sup>
11	Polychlorinated dibenzo-p-dioxins <sup>2</sup> (dioxins)
12	Polychlorinated dibenzofurans <sup>2</sup> (furans)

1. Intentionally produced.
2. Unintentionally Produced - Result from some industrial processes and combustion.

Table 1. List of 12 POPs identified by the Stockholm Convention of May 2001

POPs	Usage
Alpha hexachlorocyclohexane	Pesticide, produced as byproduct of lindane
Beta hexachlorocyclohexane	Pesticide, produced as byproduct of lindane
Chlordecone	Pesticide, agricultural use
Hexabromobiphenyl ether	Flame retardant
Hexabromodiphenyl ether and heptabromodiphenyl ether	Flame retardant, recycling of articles containing these chemicals is allowed
Lindane (Gamma hexachlorocyclohexane)	Pesticide, for control of head lice and scabies as second line treatment
Pentachlorobenzene	Pesticide, unintentionally produced POPs
Perfluorooctane sulfonic acid, its salts and perfluorooctane sulfonyl fluoride	Industrial chemical: Photo-imaging, photo-resist and anti-reflective coatings for semi-conductor and liquid crystal display (LCD) industries, etching agent for compound semi-conductors and ceramic filters, aviation hydraulic fluids, metal plating (hard metal plating and decorative plating), certain medical devices (such as ethylene tetrafluoroethylene copolymer (ETFE) layers and radio-opaque ETFE production, in-vitro diagnostic medical devices, and CCD colour filters), fire-fighting foam, insecticides for control of fire ants and termites, electric and electronic parts for some colour printers and colour copy machines, chemically driven oil production, carpets, leather and apparel, textiles and upholstery, paper and packaging, coatings and coating additives, rubber and plastics
Tetrabromodiphenyl ether and pentabromodiphenyl ether	Flame retardant, recycling of articles containing these chemicals is allowed

Table 2. Nine new POPs added to the Stockholm Convention in May, 2009

An attempt is now being made to include endosulfun in the list of POPs.

## 1.2 Remediation requirement for obsolete pesticides stockpiles and contaminated sites

Obsolete pesticide stocks refer to pesticides that have been banned or whose shelf life has expired. Many international organizations are working on the issue of obsolete pesticide stocks. These include FAO, UNEP Chemicals and the Secretariat of the Basel Convention, UNIDO (United Nations Industrial Development Organization), industry associations and NGOs (non-governmental organisations) dealing with environment. Approximately 20,000 tons of obsolete pesticides are located in Africa and in the Middle East, often in containers that leak toxic waste into the environment (Fitz,2000). While exact quantities are unknown, large stockpiles also exist in Central Eastern European Countries (CEEC) and the New Independent States (NIS) (UNEP GEF Draft Report, 2002). The risks associated with large-scale storage of compounds pose a particular environmental risk. The principal uncertainty in terms of these obsolete stocks is characterization in terms of POP content. Little is known of the composition of the waste materials and it must be recognized that, within the 'cocktail' of possible chemicals, a variety of substances will be present in unknown amounts. These could represent locally and regionally important on-going primary source inputs of compounds to the environment.

A contaminated site can be defined as an area of the land in which the soil or any groundwater lying beneath it, or the water or the underlying sediment, contains a hazardous waste, or another prescribed substance in quantities or concentrations exceeding prescribed risk based or numerical criteria or standards or conditions.

Article 6 of the Stockholm Convention describes measures to reduce or eliminate releases from stockpiles and contaminated sites.

This chapter will focus on the risk assessment prior to remediation of sites contaminated with persistent organic pesticides [DDT, aldrin, chlordane, dieldrin, endrin, heptachlor, hexachlorobenzene (HCB), mirex, toxaphene, and hexachlorohexane (HCH)]. The pesticide contaminated sites include pesticide manufacturing sites, pesticide formulation sites and other sites, including storage facilities and aerial application facilities (Fitz,2000;NNEMS,2000). Due to the complexity of these sites, it is difficult to make generalizations regarding the risk assessment for the types of contamination present and the remediation activities that were/ are being chosen.

The readers of this chapter are suggested to go through the UNIDO document titled "Persistent Organic Pollutants: Contaminated Site Investigation and Management Toolkit (2010) available on UNIDO site for free download (Contaminated Site Toolkit, 2010). This Toolkit aims to aid developing countries with the identification, classification and prioritization of POPs-contaminated sites, and with the development of suitable technologies for land remediation in accordance with best available techniques/best environmental practices (BAT/BEP). The Toolkit focuses exclusively on the 12 POPs listed in **Table 1**. The nine POPs recently added to the Stockholm Convention (listed in **Table 2**) are not included because there are still significant scientific challenges and unknowns associated with them. The Toolkit could be used both as a training tool and as a self-directed manual and resource document for decision-makers, practitioners and a range of other stakeholders. Pertaining to POPs contaminated site management

The readers may also peruse the Alberta Tier 1 Soil and Groundwater Remediation Guidelines (Alberta Tier 1, 2010) encompassing generic remediation guidelines for achieving equivalent land capability. Site-specific guidelines for achieving equivalent land capability can be developed using a Tier 2 approach (Alberta Tier 2,2010).

## 2. Site prioritization for risk assessment

### 2.1 Contaminated site prioritization

The purpose of the site prioritization is to classify contaminated sites based on risk assessment. In this section, two semi-quantitative tools are presented that allow the user to determine which sites should be assessed and then to prioritize sites based on their potential for causing unacceptable risks to humans and/or to natural environment. These are pre-screening tool and prioritization tools.

*The pre-screening tool determines, through inventorization approach, as to whether the site has a history of activity leading to pesticide-POPs contamination or whether there are other reasons to believe that contaminants have been present at the site*

The tool aims to gather contaminant characteristics, off-site migration potential, exposure and socio-economic factors. Then, based on the information obtained, the sites should be classified into the following categories:

- Class 1 – High priority for risk assessment
- Class 2 – Medium-high priority for risk assessment
- Class 3 – Medium priority for risk assessment
- Class 4 – Low priority for risk assessment
- Class N – Not a priority for risk assessment

The site prioritization tools are based on principles derived from the Canadian National Classification Tool for contaminated sites (CCME 2008).

The following web resources are helpful for designing a sampling program: [http://www.ccme.ca/assets/pdf/pn\\_1101\\_e.pdf](http://www.ccme.ca/assets/pdf/pn_1101_e.pdf) (PDF file).

While the tools are applicable to any contaminated site, a greater emphasis has to be put on pesticide-POP's related contaminant issues.

### 2.2 Setting up of risk-based standards by regulatory agency

#### 2.2.1 Risk based approach

The assessment before procedures and of contaminated sites is to follow the human health and environmental risk based approaches delineated in the Procedures for the Use of Risk Assessment under Part XV.1 of the Environmental Protection Act, Ontario Ministry of the Environment, Canada. The Contaminated Sites Monograph Series, The Health Risk Assessment and Management of Contaminated Sites, and Australian Standard AS4482 – Guide to the sampling and investigation of potentially contaminated soil and the equivalent National Environment Protection Measure (NEPM) Guidelines (a,b,c,d,e,f and g,1999) are some of the important references for consultants carrying out contaminated site investigations.

Land becomes contaminated when there is spillage, leakage or disposal of pesticide POPs to the ground. Soil at or below the ground surface, and sometimes groundwater, as well, may be contaminated depending on the subsurface conditions. To determine objectively if a piece of land is contaminated, certain standards would need to be put in place. Generally developing countries have no locally-derived standards for land contamination assessment. These countries adopt standards from developed country(ies) which has/have very different conditions. Therefore there is a need to develop contaminated land standards that are tailor-made for local conditions.

The United States (US) Environmental Protection Agency (USEPA) pioneered the application of chemical risk assessment principles and procedures to evaluate contaminated

sites under their Superfund Program in the 1980s. Other countries (mainly Canada, Australia and some European countries including the Netherlands) followed the US footsteps and began developing their own risk-based standards in the 1990s by making reference to the US approach. [http://www.epd.gov.hk/epd/english/boards/advisory\\_council/files/ACE\\_Paper\\_18-2006.pdf](http://www.epd.gov.hk/epd/english/boards/advisory_council/files/ACE_Paper_18-2006.pdf)

The risk-based approach means that contaminated land will be managed by considering the nature and extent of the potential risk it poses as a result of the receptors' exposure to chemicals in the soil and/or groundwater. This basically acknowledges that there is an acceptably low level of exposure to contaminants, which poses negligible risk. Choosing the level of negligible risk is a very important decision in the derivation of risk-based standards. The risk levels usually considered for protection of public health are:

- An excess lifetime cancer risk of 1 in  $10^6$  for carcinogens
- Actual intake must be less than the safe dose for non-carcinogens.

These risk limits are in line with the international practice and are at the conservative end of the range of risk limits adopted worldwide. For example, it is noted that the risk limit of 1 in  $10^6$  has also been adopted by some countries such as the US, the Netherlands and Canada, etc. while the UK has used a higher risk of 1 in  $10^5$ .

### **2.2.2 Health risk assessment**

#### **Establishment of Base Line Human Health Risk Assessment (BHRA)**

The risk assessment procedures, developed and documented by US EPA, are still subject to improving, especially concerning human health (US EPA 1989; 1991a; 1995; 1996a; 1996b; 2001a; 2001b; 2002).

Generally, the purpose of base line human health risk assessment (BHRA) is to:

- Assess potential risks to human health
- Determine the need for remedial action
- Determine measures needed to eliminate or mitigate health and environmental effects.

BHRA is an analysis of the potential adverse health effects caused by exposure to hazardous substances released from a site in the absence of actions to control or mitigate these releases (i.e., under an assumption of no action) (US EPA 1989).

To estimate BHRA, the following steps are undertaken:

- Data collection and evaluation
- Selection of indicator chemicals
- Exposure assessment
- Toxicity assessment
- Risk characterization.

#### **Data collection and evaluation**

An objective of the data collection and evaluation step is to produce data that can be used to assess risks to human health. This step includes:

- Review of available site information
- Consideration of modeling parameter needs
- Collection of background data (samples not influenced by site contamination)
- Preliminary identification of potential human exposure

- Development of an overall strategy for sample collection
- Identification of analytical needs
- Collection and evaluation of data
- Development of a data set that is of acceptable quality for the risk assessment.

### Human health implications of POPs

The implications of chronic and acute exposures to POPs are not fully understood. Laboratory investigations and environmental impact studies in the natural environment have indicated that POPs exposure can result in endocrine disruption, reproductive and immune dysfunction, brain and nervous system disorders, developmental disorders and cancer. Some organochlorine chemicals are likely carcinogenic by promoting the formation of tumors. Six of the 9 pesticide-POPs, identified in the *Stockholm Convention*, are classified as *possibly* carcinogenic to humans. The remaining three - endrin, dieldrin and aldrin are classified by WHO as highly hazardous (class 1b) on the basis of their acute toxicity to experimental animals.

Fetuses and infants are particularly vulnerable to pesticide POPs exposure due to the transfer of these POPs from the mother during critical stages of development. Exposure during development has been linked to reduced immunity (and increased infections), developmental abnormalities, brain and nervous system impairment, and cancer and tumor induction or promotion in infants and children. There may also be a link to human breast cancer.

### Cancer risk

The International Agency for Research on Cancer identifies most of the 12 POPs targeted by the Stockholm Convention as presenting a potential carcinogenic risk to humans, as described in the **Table 3** below.

Sr. No.	IARC Classification	POPs
1	<b>Group 1:</b> The agent (mixture) is carcinogenic to humans	<ul style="list-style-type: none"> <li>• 2,3,7,8-Tetrachlorodibenzo-para-dioxin (TCDD)</li> </ul>
2	<b>Group 2A:</b> The agent (mixture) is probably carcinogenic to humans	<ul style="list-style-type: none"> <li>• Mixtures of polychlorinated biphenyls (PCB)</li> </ul>
3	<b>Group 2B:</b> The agent (mixture) is possibly carcinogenic to humans	<ul style="list-style-type: none"> <li>• Chlordane</li> <li>• DDT</li> <li>• Heptachlor</li> <li>• Hexachlorobenzene</li> <li>• Mirex</li> <li>• Toxaphene (mixtures of Polychlorinated camphenes)</li> </ul>
4	<b>Group 3:</b> The agent (mixture or exposure circumstance) is unclassifiable as to carcinogenicity in humans	<ul style="list-style-type: none"> <li>• Aldrin, Dieldrin and Endrin</li> <li>• Polychlorinated dibenzo-para-dioxins (other than TCDD)</li> <li>• Polychlorinated dibenzofuran</li> </ul>

**Source:** [http://www.chem.unep.ch/gpa\\_trial/02health.htm](http://www.chem.unep.ch/gpa_trial/02health.htm)

Table 3. POPs posing potential carcinogenic risk to humans

### **Possible human exposure pathways**

Humans can be exposed to pesticide-POPs through diet, occupation, accidents and both the indoor and outdoor environments. Exposure to these POPs can either be a short-term exposure to high concentrations (acute) or long-term exposure to lower concentrations (chronic).

Acute exposure to pesticide-POPs can occur during production and application and industrial accidents. In addition, exposure to chlorinated pesticides can occur both from accidental ingestion of treated seeds or via poor handling or application processes. Presently, pesticide poisoning is mainly attributable to aldrin, dieldrin, HCB and chlordane. Chronic exposure occurs most commonly via dietary exposure pathways. Due to their tendency to bio-accumulate, longer-term human exposure to the pesticide- POPs identified in the Stockholm Convention is generally via food. Foods containing the greatest concentrations of POPs include the fatty tissues of animals and edible oils. The contamination of food, including breast milk, by POPs is of worldwide concern (Stober,1998).

### **Toxicity assessment**

Toxicity assessment is based on available scientific data on potential adverse health effects of the contaminants in humans, which are usually compiled in the form of a toxicological profile for each contaminant. This step also includes also identification of important measures of toxicity, i.e., reference doses (RfDs) to evaluate non- carcinogenic effects, and cancer slope factors (CSFs) for carcinogenic effects.

RfDs and CSFs have been developed by the US EPA and published in the Integrated Risk Information System (IRIS) (IRIS 2003), and Health Effects Assessment Summary Tables (HEAST) databases. IRIS is recommended as a preferred source of toxicity information. HEAST is used when data are not available in IRIS (US EPA 1989; 2003).

The US EPA has also developed provisional values of RfDs and CSFs, which are used for specific purposes (US EPA 2003). If no RfDs and CSFs are available, the chemicals can be evaluated only qualitatively.

### **Exposure assessment**

The exposure assessment stage estimates the magnitude of actual and/or potential human exposure, the frequency and duration of exposure, and pathways by which humans are potentially exposed (USEPA 1989).

The exposure assessment proceeds with the following steps:

- Step 1.** Characterization of exposure setting - the physical environment of the site and the potentially exposed populations are characterised.
- Step 2.** Identification of exposure pathways - chemical sources and mechanism of chemical release, transport media (e.g., soil, air, groundwater), exposure points as well as exposure routes (e.g., ingestion, inhalation, dermal contact) are identified in this step; an exposure pathway describes the course a chemical or physical agent takes from the source to the exposed individual (e.g., ingestion of contaminated schoolyard soil by children).
- Step 3.** Quantification of exposure - exposure concentrations of contaminants are estimated and pathway-specific intakes are calculated.

During this step, site-specific exposure scenarios are developed for both current and/or intended future land use patterns (e.g., residential, commercial/industrial, recreational).

Results of the exposure assessment are pathway-specific contaminant intakes, under developed exposure scenarios. Standard intake equations and suggested values of exposure parameters are provided by the US EPA; however, site-specific factors and expert judgment can influence the final selection thereof.

In the classical approach, exposure parameters, such as body weight, exposure duration, ingestion or inhalation rates, can be selected to estimate "reasonable maximum exposure" (RME), defined as the highest exposure that is reasonably expected to occur at a given site (US EPA 1989). The goal of RME is to combine upper-bound and mid-range exposure factors in the equation so that the result represents an exposure scenario that is both protective and reasonable, not the worst possible case (US EPA 1991b).

The quantification of exposure is based on an estimate of the average daily intake, i.e., the average amount of the contaminant entering the receptor's body per day.

The considered human receptors are strictly related to defined land use patterns, e.g., adult receptors under the industrial land use, and children and adults under residential/recreational land uses.

The generic equation for calculating chemical intakes is as follows:

$$DI = C \times (IR / BW) \times (EF \times ED / AT) \quad (1)$$

where:

DI = daily intake of chemical (mg/kg-d)

C = concentration of chemical in an environmental medium (e.g., mg/kg for soil or food, mg/L for water, mg/m<sup>3</sup> for air)

IR = intake rate of the environmental medium (e.g., kg/day for food or soil, L/day for water, m<sup>3</sup>/day for air)

BW = body weight (kg)

EF = exposure frequency (days/yr)

ED = exposure duration (years)

AT = averaging time (days)

It may be noted that the term IR/BW is a description of the basic contact rate with a medium (e.g., L of water per kg body weight per day) and the second term (EF×ED/AT) adjusts for cases where exposure is not continuous. For example, if a person was exposed for 50 days/year for 20 years of a lifetime (70 years), the value of this term would be  $50/365 \times 20/70 = 0.039$ .

There is often wide variability in the amount of contact between different individuals within a population. Thus, human contact with an environmental medium is best thought of as a distribution of possible values rather than a specific value. Usually, emphasis is placed on two different points of this distribution:

#### **Average or Central Tendency Exposure (CTE)**

CTE refers to individuals who have average or typical intake of environmental media.

#### **Upper bound or Reasonable Maximum Exposure (RME)**

RME refers to people who are at the high end of the exposure distribution (approximately the 95th percentile). The RME scenario is intended to assess exposures that are higher than average, but are still within a realistic range of exposure.

As the calculations of CTE and RME risk are done using single numbers (point estimates) for each input value, this approach is usually referred to as the point estimate method. In

some cases, the risk assessor may wish to describe each exposure parameter not by a single number but as a distribution. This is referred to as probabilistic risk assessment (PRA). In this case, computations require computer-based methods (Monte Carlo simulation) and the output is also a distribution rather than a point estimate. This approach provides a more complete description of the range of exposures that occur in the exposed population and also helps increase the accuracy of combining exposure levels across different pathways.

In some cases, human exposure may be measured directly (biomonitoring) rather than calculated based on assumed exposure parameters. For example, exposure to lead is often evaluated by measuring the amount of lead in blood, and exposure to arsenic is often evaluated by measuring the amount of arsenic in urine or in hair. While direct measurement bypasses many of the uncertainties associated with calculating human exposure, this approach is limited by providing data only on current conditions. In addition, if exposure is occurring from more than one source, direct measurement does not distinguish between the sources.

Equations for exposure pathway to contaminated soil for outdoor and indoor workers are in the Tables published by USEPA (USEPA, BJC/OR-271, 2006.) URL: <http://rais.ornl.gov/homepage>.

### **Risk characterization**

Risk characterization combines toxicity assessment with exposure assessment, in order to quantify risks posed by a contaminated site under a given set of conditions.

Risk characterization is considered separately for carcinogenic and non-carcinogenic effects, and includes identification of sources of uncertainty. Chemicals, which produce both non-carcinogenic and carcinogenic effects are evaluated in both groups.

Risks are quantified under the present site conditions for present and/or future exposure scenarios relevant to the land use pattern. Risk characterization should also include a discussion on accompanying uncertainties.

### **Non-cancer risk**

Potential non-cancer risks are evaluated by comparison of the estimated contaminant intakes from each exposure route (oral, dermal, inhalation) with the relevant RfD to produce the hazard quotient (HQ), defined as follows (US EPA 1989):

$$\begin{aligned} \text{HQ-ingestion and dermal} &= \text{CDI/RfD} \\ \text{HQ-inhalation} &= \text{CDI/RfC} \end{aligned}$$

where:

HQ: Hazard Quotient (unitless),

CDI: Chronic Daily Intake (mg/kg/day),

RfD: Reference Dose (mg/kg/day).

RfC : Reference Concentration

The hazard quotient assumes that there is a level of exposure (i.e., RfD/RfC) below which it is unlikely to experience adverse health effects, even for sensitive populations. If the HQ exceeds unity (a value of 1), there may be a concern for potential non-carcinogenic effects.

To assess the overall potential for non-carcinogenic health effects posed by more than one chemical, the HQs calculated for each chemical are summed (assuming additivity of effects), and expressed as a Hazard Index (HI) (US EPA 1989).

$$\text{HI} = \text{HQ}_1 + \text{HQ}_2 + \dots + \text{HQ}_n \quad (2)$$

In cases where the non-cancer HI does not exceed unity ( $HI < 1$ ), it is assumed that no chronic risks are likely to occur at the site (US EPA 1989). If the HI is higher than unity, as a consequence of summing several hazard quotients, the compounds are segregated by effects, target organs, and by mechanism of action and separate HIs are derived for each group.

Because of the potential for different health effects/target organs via oral/dermal and inhalation exposures, these exposures are evaluated separately (US EPA 2002). To assess the overall potential for non-carcinogenic effects, posed by several exposure pathways, HIs for each exposure pathway contributing to exposure of the same individual or subpopulation are summed up and expressed as a total hazard index (HI Tot). When HI Tot exceeds unity, there may be concern for potential non-cancer health effects.

### Quantitative risk assessment

Under the residential and recreational scenarios, i.e., scenarios which refer to different group receptors (children, adults), HIs are generated separately for children and adults.

### Cancer risk

Cancer risks are estimated as the incremental probability of an individual developing cancer over a lifetime as a result of exposure to the potential carcinogen (i.e., incremental or excess individual lifetime cancer risk). The following linear low-dose carcinogenic risk equation is used for each exposure route (US EPA 1989):

$$\text{Excess Lifetime Cancer Risk (ELCR)-ingestion \& dermal} = \text{CDI} \times \text{slope factor (CSF)} \quad (3)$$

$$\text{Excess Lifetime Cancer Risk (ELCR) - inhalation} = \text{CDI} \times \text{unit risk factor (URF)} \quad (4)$$

$$\text{Cancer Risk} = \text{CDI} \times \text{CSF} \quad (5)$$

where:

CDI: Chronic Daily Intake averaged over 70 years (mg/kg/day),

CSF/URF:: Cancer Slope/Unit Risk Factor (mg/kg/day)  $1^{-1}$ ; a plausible upper-bound estimate of the probability of a response per unit intake of a chemical over a lifetime.

CDI and CSF/URF represent the same exposure route (i.e. oral, dermal and inhalation CDIs are multiplied by oral, dermal and inhalation CSFs/URF, respectively). The risk number represents the probability of occurrence of additional cancer cases. For example, if it is expressed as  $1E-06$ , it means that one additional case of cancer is expected in a population of one million people exposed to a certain level of a given chemical over their lifetime.

If a site has multiple carcinogenic contaminants, cancer risks for each carcinogen are added (assuming additivity of effects), and the cancer risk for each exposure pathway is calculated. For multiple exposure pathways, the total cancer risk is calculated by summing up the pathway-specific cancer risks:

Risks in the range of  $1E-06$  to  $1E-04$  are generally accepted by regulatory agencies, e.g., US EPA (US EPA 1990; 1991a; 1991c). A risk-based remedial decision can be superseded by the presence of a non-carcinogenic impact or environmental impact requiring action at the site. Remedial action is generally required at a site, when a cumulative carcinogenic risk exceeds 100 in a million ( $1E-04$ , excess cancer risk) or the cumulative non-carcinogenic HI exceeds 1, based on RME assumptions (US EPA 1991a; 1991c). If the cumulative risk is less than  $1E-04$ , action generally is not required, but may be warranted if a risk-based chemical-specific

standard (e.g., drinking water standards) is violated. Setting up 1E-06 risk level for individual chemicals and pathways should generally lead to cumulative site risks within the range of 1E-06 to 1E-04 for the combinations of chemicals.

Under the scenarios, which refer to both receptors – a child and an adult (i.e., residential and recreational), cancer risks are calculated separately for these receptors, and then summed up to yield the total cancer risk for the aggregate resident/recreational user.

### **2.3 Dealing with biased data**

The basic unit of a risk assessment is an exposure unit, and the key description of exposure is the arithmetic mean concentration within an exposure unit. If the data collected from within an exposure unit are either random or systematic, the methods for computing the mean (and confidence limits around the mean) are relatively straightforward. However, in some cases, the data available are not random or systematic, but are biased. That is, more samples are collected from areas with high concentrations than with low concentrations. This unequal sampling density poses a difficulty in computing the mean, but techniques are available for adjusting for this issue. Important guidance documents on how to make these adjustments include the following:

Spatial Analysis and Decision Assistance (SADA) Software Home Page GeoSEM Software (Syracuse Research Corporation)

### **2.4 Probabilistic Risk Assessment (PRA)**

Equations for computing human exposure contain a number of terms that are inherently variable. For example, not all people have the same body weight. Rather, there is a distribution of body weights across different people. The same is true for intake rates, exposure frequencies, and exposure durations. If data are available to describe the distribution of each of these terms, then a mathematical method is needed to combine the distributions.

While there are a number of different methods available, the most common and convenient is Monte Carlo simulation. In this approach, each term in the exposure model is described by a distribution rather than a single value. The computer draws a value at random from each distribution, computes the exposure, and saves the value. This process is repeated many times, resulting in a distribution of exposure values. This distribution provides a more complete description of exposure than the point estimate approach and helps ensure that values selected for CTE and RME exposures are realistic. Key guidance documents dealing with PRA include the following:

- RAGS III Part A: Process for Conducting Probabilistic Risk Assessment (OSWER 9285.7-45, December 2001)
- Note: In particular, see Chapter 3 - Using Probabilistic Analysis in Human Health Assessment (PDF) (27 pp, 2MB).
- Guiding Principles for Monte Carlo Analysis (EPA/630/R-97/001, March 1997)
- Policy for Use of Probabilistic Analysis in Risk Assessment at the U.S. Environmental Protection Agency (May 1997)

### **2.5 Biomonitoring**

In some cases, biomonitoring may be a useful tool to help evaluate current exposure levels at a site. This requires that a population of humans are present at the site and that there is a

method available for measuring the level of exposure in the population. In general, the results of the biomonitoring may be compared to other (reference) populations to help understand the magnitude of the site-related exposure, and/or may be compared to health-based guidelines for the maximum level of exposure that is considered acceptable. Important guidance documents on planning, performing, and interpreting biomonitoring studies are presented below.

- Criteria for Evaluating Blood Lead (PDF) (Region 8 Guidance RA-07, September 1995) (22 pp, 1.6MB)
- Sample Analysis and Quality Assurance Plan for Urinary Arsenic and Blood Lead Among Residents of VBI70 Neighborhoods (PDF) (Region 8, June 2002) (27 pp, 333K)
- Experience Using Filter Paper Techniques for Whole Blood Lead Screening in a Large Pediatric Population (PDF) (8 pp, 187K) (J.A. Collins and S.E. Puskas, MEDTOX Laboratories, Inc., Saint Paul, MN)

### 3. Development of site-specific Health-based Remedial Goals (HBRGs)

#### 3.1 HBRGs

Health-based remedial goals (HBRGs), termed also risk-based concentrations [RBCs, ([http://www.image-train.net/products/papers/ASC3\\_EW\\_RBA.pdf](http://www.image-train.net/products/papers/ASC3_EW_RBA.pdf))], are concentration levels for individual chemicals that correspond to target risk (TR), i.e., a specific cancer risk level (e.g.,  $1E^{-06}$ ) or hazard quotient (HQ) or hazard index (HI) (e.g., less than or equal to 1) (US EPA 1991a). RBCs are usually calculated under all developed scenarios for the purpose of guiding remedial activities at a site; they are used during analysis and selection of remedial alternatives.

There are two methods for calculating RBCs. The first method (Method 1) is a simplified method based on site-specific exposure data (US EPA 1995). This method uses the ratio between the target risk and calculated risk due to a specific chemical in a given medium:

$$\frac{C}{\text{Calculated Risk}} = \frac{\text{RBC}}{\text{Target Risk}} \quad (6)$$

where:

C: Chemical Concentration in soil or groundwater

RBC: Risk-Based Concentration (oral/dermal or inhalation).

Rearranging this equation, RBC is calculated as follows:

$$\text{RBC} = C \frac{\text{Target Risk}}{\text{Calculated Risk}} \quad (7)$$

RBCs are calculated for both carcinogenic and non-carcinogenic substances, and only for those contaminants for which the calculated site-specific risk is above acceptable risk levels (target risk). For carcinogens, RBCs can be calculated for target cancer risks of  $1E^{-06}$ ,  $1E^{-05}$  or  $1E^{-04}$ . Concerning non-carcinogenic risk, target HQs of 0.1 or 1 can be substituted for target risk, and the calculated HQs substituted for calculated risks.

Under industrial scenario, RBCs are estimated for adult receptors, and under the residential/recreational scenarios - separately for child and adult receptors for non-carcinogenic effects, and for an aggregate resident/recreational user for carcinogenic

effects. According to the US EPA recommendations, RBCs are calculated separately for oral/dermal and inhalation exposures, because of the potential for different health effects (target organs) via these routes (US EPA 2002). If both carcinogenic and non-carcinogenic RBCs are calculated for a given contaminant, and for both oral/dermal and inhalation exposures, then lowest of these values should be applied as the preliminary remedial goal.

Concerning non-carcinogens, if more than one chemical affects the same target organ/system, RBCs calculated for those chemicals should be divided by the number of chemicals present in the group. In that way, RBCs are adjusted to reflect the potential for additive risks:

$$\text{ARBC} = \text{RBC}/n \quad (8)$$

where:

ARBC: Risk-Based Concentration adjusted for exposure to multiple contaminants with the same target organs/effects

RBC: Risk-Based Concentration for an individual non-carcinogen

n: Number of contaminants with the same target organs/effects.

RBCs can also be calculated by rearrangement of standard risk equations, separately for combined oral and dermal exposures, and for inhalation exposure by a receptor within the used scenario (US EPA 2002).

In summary, application of risk-based approach to contaminated land assessment and remediation allows to:

- Determine the needs for remedial action, aimed at reducing risk,
- Determine preliminary remedial goals based on the protection of human health,
- Provide a basis for the selection of an appropriate remedial option
- Facilitate making decision on appropriate corrective actions at the site.

### 3.2 Health concerns

Persistent pesticides pose a threat to the well-being of the environment and to human health. The solid organochloride insecticides are known to accumulate in human adipose tissue. Some of these insecticides, including chlordane, can even be absorbed dermally. Other health problems caused by exposure to the solid organochloride insecticides are convulsions, a hyperexcitable state of the brain and a predisposition to cardiac arrhythmia. Eating wheat treated with hexachlorobenzene, another organochloride insecticide, has been associated with human dermal toxicity, which can result in blistering of the skin. Although not all organochlorine insecticides are considered POPs, many of them are among the compounds on the UNEP's list of persistent organic pollutants, including aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzenes, mirex and toxaphene.

The assessment of health effects of contamination is to be made with reference to the human health-based investigation levels for various settings described in Contaminated Sites Monograph Series No 5, 1996 as incorporated in Appendix 9. The assessment of environmental impacts is to include reference to the ANZECC/NHMRC Environmental Investigation Thresholds in Appendix 9.1.

In urban residential settings where sensitive ecological receptors are not present, assessment should address:

- Potential health risks to occupants
- The capacity of the soil to support a normal ornamental domestic garden without significant phytotoxic effect.

In this process, reference may be made to the contemporary background metal levels for Queensland horticultural soils determined in studies by the Department of Natural Resources (full reference in Appendix 9.2). The chemical form of the contaminant and its mobility characteristics will be essential components of assessments.

Complex health risk assessments are to be undertaken by qualified professionals using nationally accepted health risk assessment methodology when a significant exposure risk exists. A suitable module on Site-Specific Health Risk Assessment and Management of Contaminated Sites is to be referred to, if available.

Environmental risk assessment is to be conducted on a site-specific basis when contamination levels exceed background. The characteristics of the contaminant (including chemical form, mobility, leachability and bioavailability) and the exposure routes to local receptors should be identified.

#### **4. Integrating risk assessment with contaminated site management**

Risk is governed by the contaminants present on the site, pathways through which these contaminants reach the receptors and the receptors who are usually the site users. A conceptual site model, encompassing all the three site attributes, usually helps to focus on risk. Construction of a contaminant-pathway-receptor model is the first step of risk assessment.

Since remediation through risk management deals with eliminating or controlling one or more of the three risk components: (i) contaminant, ii) exposure pathway, and iii) receptor, remediation becomes the proactive risk management solution. The remediation measures include:

- Source control
- Site stabilization and decontamination to the extent required by the Regulatory Agency for a specific site-use purpose(s).
- Alternative forms of risk management on a contaminated site, such as exposure barriers, administrative controls and/or partial remediation, may be acceptable to a regulatory agency in certain cases.

Remediation, either on-site (in-situ) or off-site (ex-situ) can employ one method, or a combination of the available physical, chemical and biological methods. Sometimes, it is not possible to remove the contaminants or exposure routes due to technical or economic or environmental constraints, the last resort is to control the receptor's accessibility by relocations and imposing land use restrictions.

Long-term remediation strategies are intended to implement a comprehensive monitoring program that properly characterizes the baseline (pre-remediation) condition and monitors improvements to be achieved through targeted remediation. Long-term remedial measures focus on compliance with all regulatory standards applicable to all contaminated media (e.g., groundwater, soil, and soil vapour) present at the site.

The readers of this chapter are suggested to go through the UNIDO document titled "Persistent Organic Pollutants: Contaminated Site Investigation and Management Toolkit (2010) available on UNIDO site for free download. This Toolkit aims to aid

developing countries with the identification, classification and prioritization of POP-contaminated sites, and with the development of suitable technologies for land remediation in accordance with best available techniques/best environmental practices (BAT/BEP).

## 5. References

- Alberta Tier 1 Soil and Groundwater Remediation Guidelines Alberta Environment  
December 2010
- Alberta Tier 2 Soil and Groundwater Remediation Guidelines Alberta Environment  
December 2010
- Contaminated Site Toolkit (2010)  
[http://www.unido.org/fileadmin/user\\_media/Services/Environmental  
Management/Stockholm\\_Convention/POPs/toolkit/Contaminated\\_site.pdf](http://www.unido.org/fileadmin/user_media/Services/Environmental_Management/Stockholm_Convention/POPs/toolkit/Contaminated_site.pdf)
- Environmental Sampling (2007) SW-846 Update IV, DRAFT DOCUMENTATION
- Fitz, N. (2000). Pesticides at Superfund Sites. Unpublished Data.
- Jones, K.C. & de Voogt, P. (1999) Persistent Organic Pollutants (POPs): State of the Science.  
*Environmental Pollution* Vol.100 , pp. 209–221.
- Mocarelli, P. & Taalman, R.D.F. (1998) Controlling Persistent Organic Pollutants—What next?  
*Environmental Toxicology and Pharmacology* Vol.63 , pp. 143–175.
- National Environment Protection Measure (NEPM) Guidelines
- a. Schedule B(4): Health Risk Assessment Methodology - Dec 1999  
This Guideline incorporates aspects of the *ANZECC Guidelines for the Laboratory Analysis of Contaminated Soil 1996*, which were prepared in response to a recognised need for consistent procedures of soil analysis for environmental assessment of contaminated land.  
The Guideline covers the philosophy behind the methods selected, it also comprises guidelines on the quality assurance procedures and techniques for sample preparation and describes methods for the analysis of physico-chemical properties, inorganics and organics in soil.  
filename: ASC\_NEPMsch\_03\_Lab\_Analysis\_199912.pdf  
This document provides an approach to site-specific health risk assessment. Due to the complexity and scale of the health risk assessment process a concise 'cookbook' is not practicable. Similarly, the site-specific issues are often sufficiently complex and 'site-specific' for a particular site that a manageable and complete algorithm for decision-making cannot be drafted. The document provides a series of guidelines (and prescriptions) to assist the decision-making process. Where possible, the document is prescriptive about certain aspects of risk assessment.  
filename: ASC\_NEPMsch\_04\_Health\_Risk\_Assessment\_199912.pdf
- b. Schedule B(5): Ecological Risk Assessment - Dec 1999  
The overall aim of this guideline is to promote a consistent, rational approach to ecological risk assessment of site contamination throughout Australia. Specifically, this document aims to provide a clear framework for ecological risk assessment for chemically contaminated soils that can be readily and consistently used by jurisdictional environmental agencies and risk assessors.  
filename: ASC\_NEPMsch\_05\_Ecological\_Risk\_Assessment\_199912.pdf
- c. Schedule B(6): Risk Based Assessment of Groundwater Contamination- Dec 1999

The purpose of this draft Guideline is to provide a framework for the risk based assessment of groundwater that may have been affected by site contamination. The general processes outlined for the assessment of contaminated groundwater are compatible with the Policy Framework and the site assessment processes shown in Schedule A of the NEPM. The aim of this process is to minimise the risk of adverse human health and environmental impacts arising from contaminated groundwater.

filename: ASC\_NEPMSch\_\_06\_Groundwater\_199912.pdf

d. Schedule B(7a): Health-Based Investigation Levels - Dec 1999

e. This guideline was published jointly with the National Environmental Health Forum (NEHF) and is part of a series of NEHF monographs.

This guideline discusses the general principles for deriving guidance values for health-based investigation levels and also explores the process applied to develop health-based investigation levels for soils.

filename: ASC\_NEPMSch\_\_07a\_Health\_Based\_Investigation\_Levels\_199912.pdf

vvvSchedule B (7b): Exposure Scenarios and Exposure Settings - Dec 1999

This guideline was published jointly with the National Environmental Health Forum (NEHF) and is part of a series of NEHF monographs.

This paper focuses on the component of exposure scenarios which may be seen as exposure settings (or standard land uses), with some reference to the characteristics of the populations potentially exposed in those settings. The intention is to define more clearly a standard range of exposure settings which regulators and risk assessors could use as baseline cases, to improve consistency of assessments, and provide a sound basis for land use/planning and remediation decisions based upon such risk assessments.

filename: ASC\_NEPMSch\_\_07b\_Exposure\_Scenarios\_199912.pdf

f. Schedule B(8): Community Consultation and Risk Communication-Dec. 1999

This guideline provides a systematic approach to effective community consultation and risk communication in relation to the assessment of site contamination. It is not intended to be prescriptive but is intended to be used as a tool for effective consultation by consultants and regulators and should also provide a useful reference for all stakeholders including industry, government, landholders and the wider community. It should be noted that, in addition to this Guideline, each State or Territory has its own regulatory requirements regarding notification of pollution to the appropriate regulatory agency.

filename: ASC\_NEPMSch\_\_08\_Community\_Consultation\_199912.pdf

g. Schedule B(9): Protection of Health & the Environment During the Assessment of Site Contamination Dec 1999

NNEMS (2000) The Bioremediation and Phytoremediation of Pesticide-contaminated Sites Prepared by Chris Frazar National Network of Environmental Studies (NNEMS) Fellow Compiled June - August 2000).

Stober, J. ( 1998). Health effects of POPs, *Proceedings of the Subregional Awareness Raising Workshop on Persistent Organic Pollutants (POPs)*, pp. 11-14. Kranjska Gora, Slovenia.

UNEP Toolkits on POPs (1999,2001,2003,2005

Vallack,H.W.; Bakker D. J.; Brandt,I.; Brorström-Lundén,E.; Brouwer,A.; Bull,K.R. (1998); Gough,C.; Guardans,R.; Holoubek,I.; Jansson,B.; Koch,R.; Kuylenstierna,J.; Lecloux,A.; Mackay,D.; McCutcheon,P.; Controlling persistent organic pollutants-What next? *Environ.Toxicol.Pharm.*6(3).143-175.



*Edited by Margarita Stoytcheva*

This book brings together issues on pesticides and biopesticides use with the related subjects of pesticides management and sustainable development. It contains 24 chapters organized in three sections. The first book section supplies an overview on the current use of pesticides, on the regulatory status, on the levels of contamination, on the pesticides management options, and on some techniques of pesticides application, reporting data collected from all over the world. Second section is devoted to the advances in the evolving field of biopesticides, providing actual information on the regulation of the plant protection products from natural origin in the European Union. It reports data associated with the application of neem pesticides, wood pyrolysis liquids and bacillus-based products. The third book section covers various aspects of pesticides management practices in concert with pesticides degradation and contaminated sites remediation technologies, supporting the environmental sustainability.

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