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Insecticides

Development of Safer and More Effective Technologies

Edited by Stanislav Trdan

INSECTICIDES - DEVELOPMENT OF SAFER AND MORE EFFECTIVE TECHNOLOGIES

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http://dx.doi.org/10.5772/3356
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Contributors

Mahdi Banaee, Philip Koehler, Alexa Alexander, Francisco Sánchez-Bayo, Juliana Cristina Dos Santos, Ronald Zanetti Bonetti Filho, Denilson Ferrreira De Oliveira, Giovanna Gajo, Dejane Santos Alves, Stuart Reitz, Yulin Gao, Zhongren Lei, Christopher Fettig, Donald Grosman, A. Steven Munson, Nabil El-Wakeil, Nawal Gaafar, Ahmed Ahmed Sallam, Christa Volkmar, Elias Papadopoulos, Mauro Prato, Giuliana Giribaldi, Manuela Polimeni, Žiga Laznik, Stanislav Trdan, Shehata E. M. Shalaby, Gehan Abdou, Andreia Almeida, Francisco Amaral Villela, João Carlos Nunes, Geri Eduardo Meneghello, Adilson Jauer, Moacir Rossi Forim, Bruno Perlatti, Patrícia Luísa Bergo, Maria Fátima Da Silva, João Fernandes, Christian Nansen, Solange Maria De França, Mariana Breda, César Badji, José Vargas Oliveira, Gleberson Guillen Piccinin, Alan Augusto Donel, Alessandro Lucca Braccini, Gabriel Loli Bazo, Keila Regina Hossa Regina Hossa, Fernanda Brunetta Godinho Brunetta Godinho, Lilian Gomes De Moraes Dan, Maria Lourdes Aldana Madrid, Maria Isabel Silveira, Fabiola-Gabriela Zuno-Floriano, Guillermo Rodríguez-Olibarría, Patrick Kareru, Zachaeus Kipkorir Rotich, Esther Wamaitha Maina, Taema Imo

Notice

First published in Croatia, 2013 by INTECH d.o.o.
eBook (PDF) Published by IN TECH d.o.o.
Place and year of publication of eBook (PDF): Rijeka, 2019.
IntechOpen is the global imprint of IN TECH d.o.o.
Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

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

Insecticides - Development of Safer and More Effective Technologies
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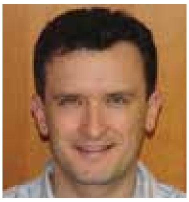
p. cm.

ISBN 978-953-51-0958-7

eBook (PDF) ISBN 978-953-51-5348-1

Meet the editor



Prof. Stanislav Trdan, head of the Chair of Phytomedicine, Agricultural Engineering, Crop Production, Pasture and Grassland Management (Dept. of Agronomy, Biotechnical Faculty, University of Ljubljana, Slovenia), obtained his BSc, MSc and PhD (agricultural entomology) from the University of Ljubljana. Since 2006, he has been the president of the Plant Protection Society of Slovenia; since 2008, he has been an associate professor of plant protection. He is a member of many international and national research societies. He has organised two international symposia and (co)organised four national conferences in the field of plant protection. He has attended almost 30 international and 20 national conferences, workshops and seminars. Until now, he was a leader of four national scientific projects and a member of many national and international project groups. Dr. Trdan has published more than 100 scientific papers, and he or the members of his research group have given approximately 90 presentations at symposia. He was the supervisor of four PhD theses, six MSc theses and approximately 70 undergraduate theses. He was a reviewer of more than 50 scientific papers from the field of agricultural entomology or plant protection. His fields of interest are agricultural entomology and zoology, integrated pest management, biological control and other alternatives for controlling plant pests and the efficacy of insecticides.

Contents

Preface

Insecticides are products that help to minimise the damage to plants, animals and human beings by controlling pest insects. From the point of view of protecting cultivated or wild-growing plants, insects are the most important group of pests because theyrepresent the most abundant animal group. Of the approximately 1.2 million known insect species, 5,000 to 10,000 are economically noxious,and their influence on reduced quantity and quality of plants depends on numerous abiotic and biotic factors. The most important biotic factor is the role of humans, who with appropriate control measures for pest insects can achieve the desired result – the reduction of individual abundance under the economic threshold of damage. However,with unsuitable control measures,humans can also demolish the natural balance in agroecosystems,resulting in larger noxiousness of harmful organisms or a decreased production economy.

Until the Second World War, only some insecticides were known. Some inorganic substances (arsenious, leaden, baric) were used to control biting insects;on smaller scales, plant extracts (tobacco, rotenone) were used against sucking insects; and carbolines or mineral oils were usedfor thewinter spraying of fruit trees. Close to and after the Second World War, organic insecticides were chemically synthesised, and this method spread worldwide in the fifties.These synthesised insecticides were chlorinate carbon hydrogen (DDT, lindane, endrine) and organic phosphor esters, which control biting and sucking insects.The development ofcarbamates, synthetic pyretroids, neonicotinoids, octadiazyonids, antifeedants, and inhibitors and regulators of insect development followed.The last two groups along with natural and plant insecticides are an important part of integrated plant protection and other forms of environmentally friendly production of food, ornamental plants or forage feed. Their efficacies, when compared to the groups of insecticidesfirst mentioned,areseveral times smaller but they can offer protection measures (usage of pheromone traps, colored sticky boards, natural enemies, usage of resistant plant varieties, plant hygiene, etc.)when combined with other plants to attain better synergy and consequently reduce the abundance of pest insects.

Experts and users of insecticides are aware of the great importance of this group of plant protection products in providing sufficient quantities of food for the fast-growing human population and feed for livestock, which isan important food source for the majority of the human population.Still, many negative examples of improper usage of insecticides from the past and present warn us about the great attention necessary when using insecticides. The application of insecticides, especially the improper application, can cause many negative outcomes. The number of selective insecticide products is relatively small; thus, many insecticides demonstrate a non-targeted influence on other insect species includingbeneficialspecies. A smaller number of natural enemies can also influence the larger abundance and noxiousness of other species of insects, which before the usage of nonselective insecticides did not have any important economical meaning in agroecosystems.The second difficulty when unsuitable usage of insecticide occursis the phenomenon of resistance and the fact that,until now, more than 500 species of insects and mites were documented. Althoughthe price of insecticides is quite low when compared to natural enemies, the cost of insecticides increases due to the appearance of secondary pests, the appearance of resistance, government measures and the legal procedures obliged to healthy and integrated food and environment influence.

In this book, experts from different continents present the advantages and problems when applying insecticides and the possibilities for using other measures. The aim of this book is to educateresearchers, scientists, students and end users (farmers, hobby producers)about insecticides and their usage.

This book is dedicated to my family, my wife Milena, daughtersŠpela, Neža and Urška, and sons, Gašper, Miha and Peter, who assisted me in many ways. I extend to them my love and appreciation.

Stanislav Trdan
Head of the Chair of Phytomedicine,
Agricultural Engineering, Crop Production,
Pasture and Grassland Management
Dept. of Agronomy, Biotechnical Faculty,
University of Ljubljana, Slovenia

Section 1

Non-Target Effects of Insecticides

Chapter 1

Side Effects of Insecticides on Natural Enemies and Possibility of Their Integration in Plant Protection Strategies

Nabil El-Wakeil, Nawal Gaafar, Ahmed Sallam and
Christa Volkmar

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/54199

1. Introduction

Recently, plant protection strategy has recommended, minimizing the use of chemical pesticides. Therefore, studying the side effect of insecticides on the natural enemies is highly required to exclude the detrimental effects on the natural enemies. Every crop is infested by various pests; some but not all of them may be controlled by biological means using pathogens, predators, parasitoids and spiders. But to achieve a satisfactory control of complexes of pests, selective pesticides are also indispensable. In fact, they are a prerequisite of Integrated Pest Management.

The integration of chemical and biological control is often critical to the success of an integrated pest management (IPM) program for arthropod pests (Smilanick et al. 1996; El-Wakeil & Vidal 2005; El-Wakeil et al. 2006; Volkmar et al. 2008). In contrast with nonsystemic insecticides, many systemic insecticides and their metabolites are claimed to be fairly safe for beneficial insects because direct exposure to these chemicals occurs when insects feed on plant tissue. However, systemic insecticides can potentially contaminate floral and extrafloral nectar when systemically distributed throughout the plant (Lord et al. 1968) and cause high mortality to nectarfeeding parasitoids for as long as some weeks after insecticide application (Stapel et al. 2000).

Most biological control agents, including predators, parasitoids and spiders, at work in the agricultural and urban environments are naturally occurring ones, which provide excellent regulation of many pests with little or no assistance from humans. The existence of naturally occurring biological control agents is one reason that many plant-feeding insects do not ordinarily become economic pests. The importance of such agents often becomes quite

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apparent when pesticides applied to control one pest cause an outbreak of other pests because of the chemical destruction of important natural enemies. There is great potential for increasing the benefits derived from naturally occurring biological controls, through the elimination or reduction in the use of pesticides toxic to natural enemies.

The main objective of this book chapter studying the insecticide side effects on development, parasitism or predation efficacy and emergence capacity as well as to preserve effective biological control agents is a combination of tactics including an understanding of the biology and behaviour of arthropods (parasitoids, predators and spiders), detailed monitoring of life history and population dynamics of pests and natural enemies, employment of selective pesticides, application only when absolutely necessary, basing chemical control on established economic injury levels and application at the least injurious time.

2. Side effects on parasitoid wasps

Integrated Pest Management (IPM) programs are used worldwide for controlling different agricultural pests. The use of natural enemy agents in combination with selected insecticides, which have no effect on them, is effective in depressing the population density of the pest. Generally, egg parasitoids such as *Trichogramma* have been widely used as biological control agent as reported by Hassan (1982), Bigler (1984) and El-Wakeil & Hussein (2009); who confirmed that 65 – 93% reduction in larval infestations of *Ostrinia nubilalis* in corn fields was achieved following *Trichogramma* releases in Germany and Switzerland as well in Egypt.

2.1. Egg parasitoids

2.1.1. Trissolcus grandis

The scelionid egg parasitoid *Trissolcus grandis* Thompson (Hymenoptera: Scelionidae) had a very important role in reducing *Eurygaster integriceps* (Puton) population (Radjabi 1995; Critchley 1998). However, intensive use of insecticides has caused severe damage to parasitoid populations (Radjabi 1995). It is estimated that egg parasitoids reduce *E. integriceps* pest population by ca. 23% yearly in Iran (Amirmaaif 2000). Presently, chemical control is the main tool used to control the *E. integriceps* populations. The chemicals currently used for controlling this pest are organophosphorous insecticides such as fenitrothion, fenthion, trichlorfon, chlorpyrifos, and pirimiphos methyl (Orr et al. 1989; Kivan 1996; Saber 2002), and synthetic pyrethroids such as deltamethrin, cypermethrin, cyßuthrin, and cyhalothrin (Kivan 1996). Fenitrothion and deltamethrin are the most commonly used insecticides to control the *E. integriceps* in Iran (Amirmaaif 2000; Sheikhi Garjan 2000). There are many studies on the effects of conventional insecticides on *E. integriceps* egg parasitoids (i.e. Novozhilov et al. 1973; Smilanick et al. 1996; Sheikhi Garjan 2000).

Saber et al. (2005) assessed effects of fenitrothion and deltamethrin, on adults and preimaginal stages of egg parasitoid *Trissolcus grandis*. Fenitrothion and deltamethrin reduced the emergence rates by 18,0 and 34.4%, respectively, compared with the control. However, neither insecticide significantly affected the longevity or reproductive capacity of emerged females, or the sex ratio of their progeny. This study revealed that application of these insecticides should be cautiously through season to conserve natural or released populations of *T. grandis*. Adult females of *T. grandis* usually produce the majority of offspring in the first few days after emergence. Proportion of male offspring produced by *T. grandis* in the early life span of the parasitoid is higher in the treatments than control that will result in a higher proportion of males in the insecticides treatments (Fig. 1).

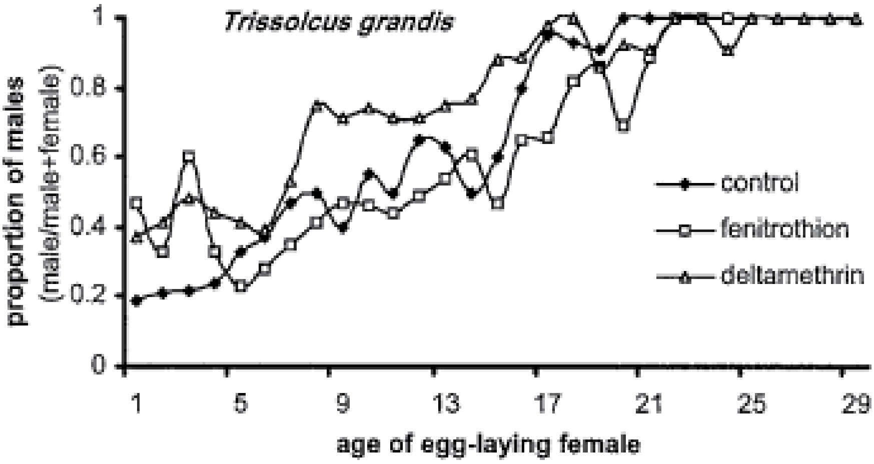

Figure 1. Proportion of male offspring produced by *Trissolcus grandis* adults emerged from treated parasitized eggs at pupal stage and control (after Saber et al. 2005)

2.1.2. *Telenomus remus*

It is very important studying the insecticide side effects on egg parasitoids. The first study on side-effects of neem products on egg- parasitoids was conducted by Joshi et al. (1982) in India. These authors applied a 2% aqueous NSKE (Neem Seed Kernel Extract) on the egg masses of the noctuid *Spodopteru litura.* The egg parasitoid *Telenomus remus* was not repelled from egg laying. When the treatment was carried out before egg laying of the parasitoid, the emergence of adult parasitoids was normal but their duration of life was shorter than that of controls. On the other hand, spraying with NSKE after oviposition of *T. remus* increased the fecundity of the wasps developed in treated eggs and prolonged their life as compared with that of untreated controls; similar results were also reported by Golec (2007).

2.1.3. *Trichogramma species*

Trichogramma genus is a tiny parasitoid and some species are susceptible for chemicals. In both cases using insecticides alone or compatible with *Trichogramma*, there is a side effect on the later as studied by by Shoeb (2010), who mentioned that effect of five insecticides, Profect (w.p.), CAPL- 2 (mineral oil), Lambda-cyhalothrin, Spinosad, and Fenitrothion (Sumithon) were studied on the immature stages of *Trichogramma evanescens* (West.). Longevity of the emerged parasitoid was affected by the tested insecticides. Eggs treatment with chemical insecticides caused death of the emerged adults within few hours post emergence. The number of parasitized eggs was varied according to timing of treatment. Adult emergence rate varied according to the used insecticide and the parasitoid stage. There was no emergence for the parasitoid treated with Lambda-cyhalothrin, spinosad, and fenitrothion (Sumithon) one, two or four days after parasitism. On the other hand, El-Wakeil et al (2006) reported that there was no serious side effect on parasitism and emergence rates of *T. pretiosum* (Riley) and *T. minutum* (Riley) when treated with neem products. Similarly, neem products achieved a good control of *H. armigera* in greenhouse. Therefore, neem products are recommended for controlling *Helicoverpa* and are compatible with mass release of *Trichogramma*.

Assessment of the potential effects that pesticides have on the natural enemies is therefore an important part of IPM programs (Hirai 1993; Hassan 1994; Consoli et al. 1998; Takada et al. 2000). Detailed knowledge of the effects of different pesticides on the immature stages of natural enemies will help to determine the timing of sprays, thus avoiding the most susceptible stages (Campbell et al. 1991; Guifen and Hirai 1997). Mass breeding and release of parasitoids for control of various lepidopterous pests is now a commercial practice in many countries. However, the efficacy of the parasitoid is influenced a great deal by the insecticide spray schedule before and after parasitoid release. Candidate parasitoids for IPM programs should therefore be tested for susceptibility to the insecticides being used for controlling crop pests (Hassan et al. 1987). Egg parasitoids are known to be very effective against a number of crop pests. *Trichogramma dendrolimi* (Matsumura) has been described as a control agent for the pine moth, citrus swallowtail (Hirose 1986), *Spodoptera litura* (Hamada 1992), and other cruciferous insect pests (Dai et al. 1991). The cabbage moth, *Mamestra brassicae* (L.), is an important pest of ca. 20-51 species of plants (Hirata 1960). The use of broad-spectrum insecticides, however, has resulted in a decline in the natural enemies of *M. brassicae.* There are many research dealing with determining the susceptibility of *T. dendrolimi* to several insecticides, and evaluate its potential use for controlling the cabbage moth and other lepidopteran insects (Takada et al. 2000, 2001). Who tested toxicity of six insecticides, acephate, methomyl, ethofenprox, cartap, chlorfluazuron, and *Bacillus thuringiensis* (Bt) on different developmental stages of *Trichogramma dendrolimi* (Matsumura). Ethofenprox showed the highest toxicity and cartap showed relatively higher toxicity compared with the other insecticides. The development of the parasitoids treated with these two insecticides was normal, similar to that of the control group; the same trend of results was also obtained by Vianna et al. (2009) and Shoeb (2010).

Suh et al (2000) investigated effect of insecticides on emergence, adult survival, and fitness parameters of *Trichogramma exiguum*. Insecticides tested were lambda cyhalothrin, cypermethrin, thiodicarb, profenophos, spinosad, methoxyfenozide, and tebufenozide. All insecticides, with the exception of methoxyfenozide and tebufenozide, adversely affected *Trichogramma* emergence from *Helicoverpa zea* (Boddie) host eggs when exposed at different preimaginal stages of development (larval, prepupal, or pupal). However, the mean life span of emerged *T. exiguum* females significantly varied among insecticides, and was significantly affected by the developmental stage when treated.

During the past three decades, *Trichogramma* spp. wasps have been evaluated as biological control agents for heliothine pest suppression in cotton (Knutson 1998; Suh et al. 1998, 2000; El-Wakeil 2003). Results of augmentative releases have been variable and at least some of the variability has been attributed to the use of broad spectrum insecticides in or near release plots during the time releases were made (Varma & Singh 1987; Kawamura et al. 2001; Brunner 2001; Geraldo et al. 2003). These insecticides were generally used to manage boll weevil, *Anthonomus grandis* (Boheman) and sometimes used to salvage *Trichogramma* release plots under extreme heliothine infestations. Numerous laboratory and field studies have shown that *Trichogramma* spp. wasps are highly susceptible to most broad-spectrum insecticides (Bull & Coleman 1985). Consequently, use of insecticides and *Trichogramma* has historically been considered incompatible (Hassan 1983).

Since the successful eradication of *A. grandis* in North Carolina, heliothines [predominantly *Helicoverpa zea* (Boddie)] have emerged as the primary mid to late season insect pest in North Carolina cotton (Bacheler 1998). Thus, most of the foliar insecticide applications (generally pyrethroids) made to cotton in North Carolina are aimed for control of the heliothine complex, *H. zea* and *Heliothis virescens* (F.). Unfortunately, these commonly used insecticides also are toxic to many non target organisms, including predators and parasitoids. Additionally, some heliothine pests (particularly *H. virescens*) have developed resistance to pyrethroids in some cotton growing areas. In an attempt to combat insecticide resistance, conserve arthropod natural enemies, and reduce health risks, several new insecticides (e.g., tebufenozide, methoxyfenozide, spinosad) have been developed and tested against lepidopteran pests in cotton (Bull & House 1983; Stapel et al. 2000; Vianna et al. 2009). Also, there is very important studies regarding the compatibility of these relatively new compounds with *Trichogramma* wasps, such as the detailed study involving *T. pretiosum* and tebufenozide (Cônsoli et al. 1998) with Neem (El-Wakeil et al. 2006) and with other biocontrol agent *Chrysoperla carnea* (El-Wakeil & Vidal 2005).

Example: Side effect on parasitism rates of *T. pretiosum* and *T. minutum* on *Helicoverpa* eggs

El-Wakeil et al. (2006) reported that their results indicated that NeemAzal-T/S reduced the parasitism rates to 50, 48.9, 71.1 and 73.3 % at 2, 1, 0.5, 0.25% cons, respectively (Fig. 2A), compared to 96.6% on control plants. NeemAzal PC 05 reduced the parasitism rates to 70, 67.8, 70 and 80% on succeeding concentrations; 2, 1, 0.5 and 0.25%. Neem blanks achieved a less side effect on *T. pretiosum*. NeemAzal Blank reduced the parasitism rates to 81.1%. NeemAzal PC05 Blank reduced the parasitism rates to 91.3% compared to 98.7% on control plants (Fig. 2A). El-Wakeil et al. (2006) mentioned further that NeemAzal-T/S had reduced the parasitism rates, to 40, 55.4, 77.8 and 81.3 % (at 2, 1, 0.5 and 0.25% cons.), respectively, compared to 93.3% on control plants. NeemAzal PC 05 reduced the parasitism rates to 82.2, 82.2, 74.4 and 83.3% on succeeding concentrations; 2, 1, 0.5 and 0.25% (Fig. 2B). Neem blanks achieved a less side effect on *T. minutum*. Parasitism rates reached to 74.4% in neem blanks. Parasitism rates were reduced by NeemAzal PC05 Blank to 86.7% compared to 93.3% on control plants (Fig. 2B).

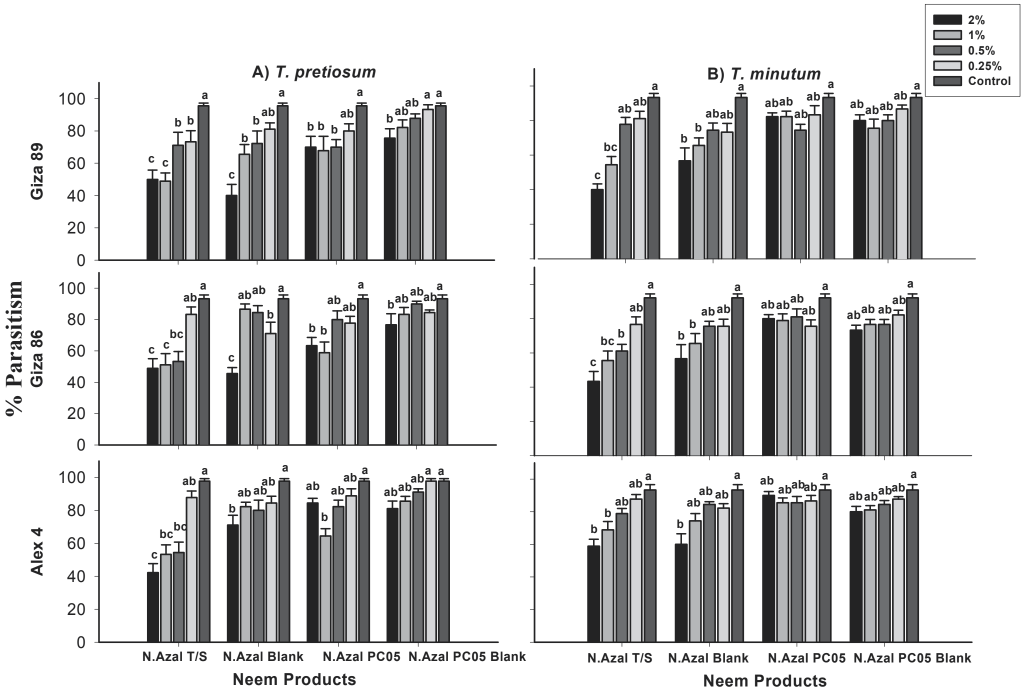

Figure 2. Effect of neem products on parasitism rates of *Trichogrammapretiosum* (A) and *T. minutum* (B) on *Helicoverpa armigera* eggs in the greenhouse. Different letters indicate significant differences.

Li et al. (1986) tested 29 insecticides including Bt & Non Bt in order to study their side-effects on *Trichogramma japonicum* in the laboratory. The authors concluded from the results that Bt & Non Bt were the safest pesticides for the parasitoid. Klemm & Schmutterer (1993) applied NSKE (2.5% and 3%) against *Trichogramma* spp., egg-parasitoids of the diamondback moth, *Plutella xylostella. T. principium* accepted neem- treated eggs in the laboratory and *T. pretiosum* in the field but two treatments prevented the eclosion of adult parasitoids from treated *P. xylostella* eggs completely. Eggs treatment with 2% neem oil (NO) reduced the number of eggs parasitized per female wasp by 13.3. As a further side-effect, Non Bt reduced the emergence of *T. principium* from treated eggs by 45.1%. Lyons et al. (1996, 2003) offered neem-treated eggs of *Ephestia kuehniellu* in shell vials to single females of *Trichogramma minutum* for parasitation. The eggs were fixed with adhesive to strips and held until all parasitoids had emerged from them. Azatin, Neem EC (experim. formul. 4.6% aza) and pure aza were tested at concns. of 50 g and 500 g/ha. At 50 g/ha no significant effect was observed, at 500 g/ha Azatin and Neem EC reduced the female survival by 64% and 40% respectively whereas pure aza showed no effect. Likewise, at 500 g/ha the number of parasitized eggs was reduced by 89% by Azatin, 29% by Neem EC but not reduced by aza. The parasitoid's development success was reduced by all treatments.

Cano & Gladstone (1994) studied the influence of the NSK-based extract NIM-20 on parasitization of eggs of *Helicoverpa zea* in a melon field in Nicaragua. Mass-reared *T. pretiosum* were released at six weekly intervals 1, 2, 6 and 24h after application of NIM-20 at 2.5g/l. No negative effect was observed as up to 84% of the eggs of the pest were parasitized.

Srinivasa Babu et al. (1996) studied the effects of neem-based commercial insecticides such as Repelin and Neemguard on *T. australicum* in laboratory and field conditions. They reported that both the insecticides were relatively safe at lower concentrations but higher concentrations adversely affected the parasitoids both in laboratory and in field. Effects of insecticides on the emergence of *T. japonicum* from eggs of *Corcyra cephalonica* on the third or sixth day after parasitization using chlorpyrifos, quinalphos, monocrotophos, cypermethrin, dimethoate, phosphamidon, fenvalerate, Biolep and Bioasp (both Btk products) and NeemAzal-F and Fortune Aza (both neem-based products) clearly indicate that Bt and neem products had the least effect on the emergence of parasitoids, similar results were stated by Koul & Wahab (2004). On the other hand, fenvalerate and monocrotophos had the least effect while quinalphos had the most. Adult emergence was relatively less when eggs were sprayed on the sixth day after parasitization compared to third day after parasitization (Borah & Basit 1996). Similar results were obtained against *T. japonicum* using Econeem and NeemAzal-T/S (0.1-1.0 %) (Lakshmi et al. 1998). On the whole it has been assessed that neem products were fairly safe to *Trichogramma* spp. (Sreenivasa & Patil 1998; Sarode & Sonalkar 1999a; Koul & Wahab 2004).

However, some neem formulations such as Nimbecidine (0.25-4.0%), Neemgold (2.0-4.0%) and Rakshak (1.0%) are reported to possess adverse effects on parasitism (Lakshmi et al. 1998; Koul & Wahab 2004). Raguraman and Singh (1999) tested in detail the neem seed oil at concentrations of 5.0, 2.5, 1.2, 0.6 and 0.3% for oviposition deterrence, feeding deterrence, toxicity, sterility and insect growth regulator effects against *Trichogramma chilonis*. Neem seed oil at 0.3% deterred oviposition (parasitization) by the parasitoid but the sensitivity varied considerably both under choice and no-choice conditions. Neem seed oil also deterred feeding at or above 1.2% concentration both in choice and no-choice tests. In feeding toxicity tests, neem seed oil at 5% concentration caused < 50% mortality to both males and females but in contact toxicity tests, females were affected sparing males. No sterility effect was observed when the parasitoid was fed with neem seed oil treated honey. Both pre-and post-treatment of host eggs revealed no adverse effects on the development of the parasitoid, the same trend of results was obtained by Saikia & Parameswaran (2001). Thakur & Pawar (2000) tested two neem-based insecticides (3g Achook/litre and 2 ml Neemactin/litre), two biopesticides [1 g Halt (cypermethrin)/litre] and 1 ml Dipel (Btk)/litre], and endosulfan (1.5 ml/litre) in the laboratory for their relative toxicity to newly emerged adults of *T. chilonis*. Results revealed that neem-based pesticides and biopesticides were harmless while endosulfan was slightly toxic to egg parasitoid. These observations also get support from the studies on different groups of moult inhibitors and biopesticides against rice leaf folder, *C. medinalis* and its parasitoid *T. chilonis* (Koul & Wahab 2004).

2.2. Larval and larval/ pupal parasitoids

Schneider & Madel (1991) reported that there was no adverse effect on adults of the braconid *Diadegma semiclausum* after exposure for 3 days or during their lifetime in cages to residues of an aqueous NSKE (0.1- 5%). The longevity of the wasps exposed to neem residues was even prolonged but the difference between treated and untreated individuals was statistically not significant. Females of the braconid, derived from larvae developed in neem-treated larvae of *P. xylostella,* showed no reduced fecundity or activity as compared with controls. Fresh extracts showed no repellent effect. The influence of aza on *Diadegma terebrans,* parasitoid of *Ostrinia nubilalis,* was investigated in the laboratory by Mccloskey et al. (1993). These authors added sublethal doses (0.1 ppm and 0.3 ppm) of aza or ethanol (carrier solvent) to diets of 2nd instar larvae of the pyralid. Both aza concns caused no significant difference of the parasitation percentage; host acceptance by the parasitoids was also not influenced. However, significantly higher mortality of parasitoids was observed in aza-treated groups compared with untreated groups, especially after emergence from the hosts. The duration of the larval instars in the hosts was prolonged and pupae weight and adults from treated groups was reduced.

Schmutterer (1992, 1995, 2002) studied the side-effects of 10 ppm and 20 ppm of an aza-containing and an aza-free fraction of an aqueous NSKE, of AZT-VR-K and MTB/H,O-K-NR on *Cotesia glomerata,* a gregarious endoparasitoid of the larvae of the large cabbage white, *Pieris brassicae,* in Europe. When heavily parasitized 5th-instar larvae of the white were fed neem-treated cabbage leaves, numerous parasitoids could leave their moribund hosts, pupate and emerge as apparently normal wasps. On the other hand, high mortality was also recorded as many larvae could not spin a cocoon and adults were not able to emerge from normally looking cocoons. Intraspecific competition for food among larvae of *C. glomerata* in treated and untreated hosts could have been the main reason for high mortality, which was also observed in controls. In contrast, Osman & Bradley (1993) explained high mortality of C. *glomeraca* larvae and morphogenetic defects of adults derived troni larvae developed in neem-treated hosts mainly as effects of aza on the metamorphosis of the parasitoids. Spraying of high concns of AZT-VR-K on adult braconids and their contact with sprayed cabbage leaves for 2 days had no obvious effect on the wasps (Schmutterer 1992). Beckage et al. (1988) recorded that the development of *Cotesia congregata* was interrupted by aza in larvae of the tobacco hornworm.

According to Jakob & Dickler (1996) adults of the ectoparasitic, gregarious eulophid *Colporljpcus floriis,* an important parasitoid of the tortricid *Adoxophyes orana,* were not adversely affected by application of NeemAzal-S (25 ppm and 100 ppm) in the laboratory and in the field, but 100% of the larvae died, apparently due to lack of appropriate food on the neem-treated decaying larvae of the host.

Hoelmer et al. (1990) evaluated the side effects of Margosan-O on parasitoids of the whitefly *Bemisia tabaci* and the aphid *Aphis gossypii* in the laboratory. The survival of the aphelinid *Eretmocerus calijornicus* was identical on treated and untreated hibiscus leaves, whereas the aphid parasitoids *Lysiphlebus testaceipes* (Aphidiidae) and *Aphelinus asychis* (Aphelinidae) showed more sensitivity to neem-treated leaf surfaces. *E. californicus* pairs in sealed Petri dishes with treated and untreated leaves survived for 5 days. Dipping of aphid mummies parasitized by *L. testaceipes* in Margosan-0 solution did not prevent the eclosion of the wasps. The same applied to the emergence of *Encarsia formosa* and *E. transversa* after dipping of parasitized puparia of *B. tabaci.* Only in the case of *E. calfornicus* was the emergence from treated whitefly puparia reduced by 50% as compared with untreated. Other researches had studied the toxicity of abamectin and spinosad on the parasitic wasp *Encarsia formosa* (van de Veire & Tirry 2003; van de Veire et al. 2004).

Schauer (1985) reported that the aphid parasitoids *Diaeretiella rapae* and *Ephedrus cerasicola* developed normally after spraying of parasitized nymphs or mummies of *Myzus persicae,* using the neem products MeOH-NR (0.1%), AZT (0.05%) and MTB (0.01%) plus sesame oil. NO at concns of 0.5%, 1% and 2% did not reduce the rate of parasitism of *M. persicae* by *D. rapae,* but the emergence of adult wasps from aphid mummies collected from treated plants in the laboratory was reduced to 35, 24 and 0%, respectively, of the controls; similar results were obtained by Jenkins & Isaacs (2007) during their study about reducing the risk of insecticides for control of grape berry moth (Tortricidae) and conservation of its natural enemies, the same vision was recorded by Desneux et al. (2007).

In laboratory trials of Feldhege & Schmutterer (1993), using Margosan-0 as pesticide and *E. formosa,* parasitoid of *Trialeurodes vaporariorum,* as target insect, parasitized puparia of the whitefly were dipped in Margosan-0 solution containing 10 or 20 ppm aza. The lower concn showed little effect on the parasitoid emergence from the puparia and on longevity, but the higher concn caused a slight reduction of the walking activity of the wasps. Stark et al. (1992) studied under laboratory conditions the influence of aza on survival, longevity and reproduction of parasitoids of tephritid flies. The braconids *Psytallia incisi* and *Biosteres longicaudatus* developed in and eclosed from the tephritid *Bactrorera dorsalis* exposed in a diet to aza concns that inhibited adult eclosion. *Diachismomorpha tryoni* also eclosed from *Ceratitis capitata,* exposed to concns of aza that prevented eclosion of adult fruitflies. The longevity of parasitoids emerged from treated flies did not differ significantly from that of controls but reproduction of *P. incisi,* developed in flies exposed to 20 ppm aza, was reduced by 63-88%. The reproduction of other braconid species was not adversely affected.

Stansly & Liu (1997) found that neem extract, insecticidal soap and sugar esters had little or no effect on *Encarsia pergandiella* the most abundant parasitoid of *Bemisia argentifolii* in south Florida vegetable fields and can contribute significantly to natural biological control of this and other whitefly species. Of the 10 species of leaf-mining Lepidoptera collected in apple orchards in south-western Germany in 1996, the most abundant were *Phyllonorycter blancardella, Lyonetia clerkella* and *Stigmella malella* and a mining curculionid, *Rhamphus oxyacanthae,* the same trend of results was confirmed during studying effects of insecticides on two parasitoids attacking *Bemisia argentifolii* by Jones et al. (1998).

Total parasitism by Chalcidoidea and Ichneumonoidea ranged from 10 to 29%. Use of a neem preparation for pest control had no effect on the rate of parasitism (Olivella & Vogt 1997). Sharma et al. (1999) also reported that the extracts from neem and custard apple kernels were effective against the spotted stem borer, *Chilo partellus,* Oriental armyworm, *Mythimna separata,* head bugs, *Calocoris angustatus,* and the yellow sugarcane aphid, *Melanaphis sacchari* in sorghum, but neem extract was non-toxic to the parasitoids and predators of the sorghum midge; as well other parasitoids as stated by Raguraman & Singh (1998, 1999). Sharma et al. (1984) reported that an active neem fraction of NSK had adverse effect on larval parasitoid, *Apanteles ruficrus* of Oriental armyworm, *M. separata*. Injection of 2.5 to 10μg of azadirachtin to newly ecdysed fourth and fifth instar larvae of host either partially inhibited or totally suppressed the first larval ecdysis of braconid, *Cotesia congregata* an internal larval parasitoid of tobacco hornworm, *Manduca* *sexta* (Feng & Wang 1984; Mani & Krishnamoorthy 1984; Peter & David 1988; Beckage et al. 1988). They also reported that the parasitoid growth was arrested, while the host larvae survived for two weeks or longer, following injection of azadirachtin but their parasitoids never recovered and died encased within exuvial cuticle.

Stark et al. (1992) studied the survival, longevity and reproduction of the three braconid parasitoids namely *Psystallia incisi* and *Diachasmimorpha longicaudata* from *Bactrocera dorsalis* and *Diachasmimorpha tryoni* from *Ceratitis capitata*. They also studied the effect of azadirachtin concentration on these three parasitoids. Results of the first test were in conformity with Stark et al. (1990). All larvae that were exposed to sand treated with azadirachtin, pupated. Adult eclosion was concentration-dependent in both fly species, with little or no fly eclosion at 10 ppm. However, *P. incisi* and *D. longicaudata* successfully eclosed from pupae treated with < 10ppm azadirachtin. In all the cases after the exposure of azadirachtin, the adult eclosion was inhibited.

Facknath (1999) and Reddy & Guerrero (2000) evaluated biorational and regular insecticide applications for management of the diamondback moth *P. xylostella* in cabbage and side effects on aphid parasitoids and other beneficial insects; they reported that the these biocontrol agents were not affected by neem treatments, whereas Pirimor R treatments reduced beneficial insect numbers. Although Pirimor R would be the preferred choice for immediate aphid control through contact action in commercial crop production, neem still has a place in the control of aphids in situations such as organic crop production, or in crops where resistance to other chemicals by aphids or their natural enemies has resulted (Stark & Wennergren 1995; Holmes et al. 1999; Hoelmer et al 1999).

Perera et al. (2000) studied the effect of three feeding deterrents: denatonium benzoate, azadirachtin and Pestistat on 4th instar larvae of *Chrysodeixis eriosoma* and *P. xylostella* and on the parasitoid, *Cotesia plutellae*. Their results suggested that the three antifeedants were effective in managing cabbage pests, *C. eriosoma* and *P. xylostella* and could be used in integrated pest management programmes. Denatonium benzoate was comparatively safer to the parasitoids *C. plutellae*.

Bruhnke et al. (2003) evaluated effects of pesticides on the wasp *Aphidius rhopalosiphi*. They emphasize that whole-plant test designs seemed to be more attractive to the wasps than single leaves and there were no harmful side effects. Similar results were mentioned by Mead-Briggs (2008) and Dantinne & Jansen (2008).

3. Side effects of insecticides on coccinellids

Many research studies show that integration of chemical, cultural and biological control measures are getting popular as integrated pest management (IPM), components, throughout the world. In this regard, biological control occupies a central position in Integrated Pest Management (IPM) Programmes. Because biological control agents for pests and weeds have enormous and unique advantages, it is safe, permanent, and economical (Kilgore & Doutt, 1967).

Augmentative releases of several coccinellid species are well documented and effective; however, ineffective species continue to be used because of ease of collect ion (Obrycki & Kring 1998). About 90% of approximately 4,200 coccinellid species are considered beneficial because of their predatory activity, mainly against homopterous insects and mites.

Pesticides are highly effective, rapid in action, convenient to apply, usually economical and most powerful tools in pest management. However, indiscriminate, inadequate and improper use of pesticides has led to severe problems such as development of pest resistance, resurgence of target species, outbreak of secondary pests, destruction of beneficial insects, as well as health hazards and environmental pollution. It is therefore, a high time to evaluate the suitable products to be used in plant protection strategy. In an integrated control programme, it was necessary to utilize some insecticides with minimal toxicity to natural enemies of pests. Such practice might help to alleviate the problems of pest resurgence, which is frequently associated with insecticide up use in plant protection (Yadav, 1989; Meena et al. 2002).

Coccinella undecimpunctata L. (Coleoptera: Coccinellidae) is a euryphagous predator that feeds especially on aphids (Hodek & Honěk 1996). Given its voracity toward these pests, *C. undecimpunctata* offers interesting potential as a control agent in the context of Integrated Pest Management (IPM) (ElHag 1992; Zaki et al. 1999a; Moura et al. 2006; Cabral et al. 2006, 2008, 2009). The success of IPM programs depends, in part, on the optimal use of selective insecticides that are less harmful to natural enemies (Tillman & Mulrooney 2000; Stark et al. 2007), which requires knowledge of their side-effects on the biological and behavioural traits of these organisms (Tillman & Mulrooney 2000; Sechser et al. 2003; Youn et al. 2003; Bozski 2006; Stark et al. 2007). Some studies have been done to assess the susceptibility of *C. undecimpunctata* to different insecticides but all, in some way, adversely affected this species (Salman & Abd-el-Raof 1979; Lowery & Isman 1995; Omar et al. 2002). Recent studies showed that, in general, pirimicarb and pymetrozine had no adverse effects on the biological traits (i.e. developmental time, fecundity, fertility, percentage of egg hatch) of immature or adult stages of *C. undecimpunctata* when sprayed on the insects, which makes these chemicals potentially suitable to use in combination with *C. undecimpunctata* for integrated control of sucking pests (Cabral et al. 2008, 2011).

The coccinellids predatory activity usually starts at medium high level of pest density, so the natural control is not quick, but is often effective. Untreated areas (such as edge rows) close to the orchards serve as refugia and play a strategic role in increasing biological control by coccinellids. The side effects (short term/ microscale) of several organophosphate and carbamate derived insecticides (commonly used to control tortricids, leafminers or scale pests in differnt orchards) against aphid-feeding coccinellid species were evaluated in fields tests in apple, pear and peach orchards according to the method described by Stäubli et al. (1985). The main species of aphid feeding coccinellids found were *Adalia bipunctata*, *C. septempunctata* & *Oenopia conglobata*, in order of population density observed (Pasqualini 1980; Brown 1989).

The influence of 7 pesticides (6 insecticides & 1 acaricide) on different stages (adults, larvae, eggs) of *C. septempunctata* and adults of *A. bipunctata* was evaluated under laboratory conditions by Olszak et al. (2004). It was found that food (aphids) contaminated with such chemicals as pirimicarb, novaluron, pyriproxyfen and fenpyroximate did not decrease neither the longevity nor the fecundity of females of both tested species.

Olszak et al. (1994) investigated influencing of some insect growth regulators (IRGs) on different developmental stages of *Adalia bipunctata* and *C. septempunctata* (on eggs, larvae and adults); who stated generally that the tested IGRs affected all developmental stages of both coccinellid species but the results varied according to stage. Some of the insecticides elicited a drastical reduction of the fecundity, especially in ladybirds (e.g. with teflubenzuron, fenoxycarb and flufenoxuron). Moreover, chlorfluazuron was the most dangerous one for almost all larval stages. From the other hand IGRs exerted a relatively low influence on adult coccinellids, the same trend of results obtained by Olszak (1999) and Olszak & Sekrecka (2008).

Pasqualini & Civolani (2003) examined six insecticides on adults of the aphidophagous coccinellids *Adalia bipunctata* (L.), *C. septempunctata* (L.) and *Oenopia conglobata* (L.) in apple, pear and peach orchards. The insecticides evaluated were the organophosphates (OP) chlorpyrifos, chlorpyrifos-methyl, azinphos-methyl and malathion, the carbamate derived Methomyl and the Nereistoxin analogues Cartap. Azinphos-methyl was consistently toxic to coccinellids with between 76% and 90.5% mortality occurring in four studies. Chlorpyrifos EC resulted in mortality ranging from 40.2% (apples, 1999) to 63% (peach, 2001) over five studies. Chlorpyrifos WDG mortality ranged from 50.8% to 70% over three studies. Chlorpyrifos-methyl resulted in 31% mortality in apples in 1999 and 86.1% mortality in pears in 1998. Methomyl and cartap were evaluated in a single study in apples and resulted in 66.7 and 10% mortality respectively. Malathion was evaluated in a separate study and caused 43.5% mortality.

To further develop IPM against aphids, it is important to evaluate the effects that these insecticides might have on *C. undecimpunctata* predatory capacity, since it is considered relevant to evaluate the predator's potential as a biological control agent (ElHag & Zaitonn 1996; Omkar 2004; Tsaganou et al. 2004). Previous studies indicated that sublethal effects of insecticides may result in an immediate disruption of predatory behaviour and a potential reduction in the efficiency of coccinellids to locate and capture their prey, since chemicals may interfere with the feeding behaviour by repellent, antifeedant or reduced olfactory capacity effects (Singh et al. 2001, 2004; Stark et al. 2004, 2007). The behavioural responses may also alter the predator's search pattern (Thornham et al. 2007, 2008) by avoidance of treated surfaces or ingestion of treated prey, to minimize their contact with insecticides (Wiles & Jepson 1994; Singh et al. 2001, 2004). On the other hand, insecticides can indirectly induce modifications on the dynamic predator/prey, through changes in the state and behaviour of the aphid colony that will influence relative prey value and consequently the predator's active choice. In addition, reductions (or absence) in the mobility and of defensive responses by the aphids can influence the predator's choice, as shown by several authors (Eubanks & Denno 2000; Provost et al. 2005, 2006; Cabral et al. 2011).

In the field, beneficial arthropods can be exposed to insecticides in several ways: by direct contact with spray droplets; by uptake of residues when contacting with contaminated plant surfaces; by ingestion of insecticide contaminated prey, nectar or honeydew (i.e. uptake of insecticide-contaminated food sources) (Longley & Stark 1996; Obrycki & Kring 1998; Lewis et al. 1998; Youn et al. 2003). Since it is known that the susceptibility of natural enemies to insecticides varies with the route of pesticide exposure (Longley & Stark 1996; Banken & Stark 1998; Naranjo 2001; Grafton-Cardwell & Gu 2003), it is important to perform both topical and residual tests as they can provide valuable information about the expected and observed impacts of insecticides on natural enemies in the field (Tillman & Mulrooney 2000). On the other hand, in the field predator/ prey interactions generally occur in structurally complex patches (i.e. plant architecture and surface features), which thereby influences the predator's foraging efficacy (Dixon 2000). Thus, studies regarding insecticide effects on predator's voracity should also reflect such scenarios (i.e. the tri-trophic system predator/prey/plant), particularly when testing systemic insecticides where the presence of the plant allows prey contamination not only by contact, but also through the food source.

Some studies have addressed the susceptibility of immature and adult coccinellids to pirimicarb and pymetrozine, when directly sprayed on prey and/or predators (e.g. James 2003) but nothing is known about the side effects of these chemicals on prey/predator interactions within tri-trophic systems. Thus, Cabral et al. (2011) evaluated effects of pirimicarb and pymetrozine on the voracity of 4th instar larvae and adults of *C. undecimpunctata,* under distinct scenarios of exposure to chemicals within a prey/plant system. Voracity of *C. undecimpunctata* was not significantly affected by pirimicarb or pymetrozine when treatments were directly sprayed on the predator; however, when insecticides were sprayed on the prey/plant system, the predator's voracity was significantly increased. Results suggest that *C. undecimpunctata* does not detect the insecticide on the aphids and indicate that the increase in voracity may be due to a decrease in the mobility of insecticide-treated aphids, since their capture should be easier than highly mobile non-treated prey as reported by Cabral et al. (2011). The consequences of such increase in the voracity for IPM programs are vital and required in aphid control programs.

Other studies suggested that the predatory efficiency of both adult and fourth instar larvae of *C. septempunctata* was significantly reduced, due to the sub-lethal effects of dimethoate residues and treated prey. Prey-choice experiments revealed that adult coccinellids consumed significantly fewer treated than untreated aphids over the 5-h experimental period. Fourth instar larvae preferentially consumed untreated aphids when given the choice of full rate dimethoate treated aphids or untreated aphids. The implications for post-treatment coccinellid survival and integrated pest management are considerable (Swaran 1999; Singh et al. 2004; Solangi et al. 2007)

The cultural practice that has the greatest effect on local populations of coccinellids is the application of insecticides. Accordingly, the greatest gains may be attained through reduction of toxic pesticides in coccinellid habitats. Insecticides and fungicides can reduce coccinellid populations. They may have direct or indirect toxic effect s (DeBach & Rosen 1991). Surviving coccinellids may also be directly affected, *e. g.* reductions in fecundity or longevity, or indirectly affected by decimation of their food source(s). Adults may disperse from treated areas in response to severe prey reductions or because of insecticide repellence (Newsom 1974). Pesticides vary widely in their effect on coccinellids, and similarly, coccinellids vary greatly in their susceptibility to pesticides (Polonsky et al., 1989; Lewis et al. 1998; Decourtye & Pham-Delegue 2002). Botanic insecticides are safer on natural enemies as well insect pathogens as confirmed by many studies (i.e. Ofuya 1997; Schmutterer 1997; Simmonds et al. 2000; Smitha et al 2006). Swaminathan et al. (2010) evaluated side effects of botanicals *viz.*, neem (*Azadirachta indica* A. Juss) leaves (NL), neem seed kernel extract (NSKE), eucalyptus oil (EO) and neem oil (NO) against aphidophagous coccinellids, *Adonia variegata* (Goeze). The side effects of neem seed kernal botanicals on the coccinellid recorded the highest mortality (73.33%) due to NSKE (10%) followed by (65.0% mortality) for neem oil (5.0%); and the post treatment effect (one day after) evinced maximum reduction in feeding (72.0 %) for NSKE (10%) followed by that recorded as 68% for *neem* oil (5%).

Vostrel (1998) stated that most of times tested acaricides, insecticides (carbamates & synthetic pyrethroids), exerted negative effects to varying degrees on all stages of *C. septempunctata*. Average mortality was lowest for acaricides, while fungicides were slightly more toxic. Insecticides nearly always caused comparatively higher mortality of all development stages, but adults were more resistant in many cases.

Based on many years of research, it is stated that bacterial and fungal biological preparations at rates recommended for use in agriculture show low toxicity to the predators *C. septempunctata* and *Chrysoperla carnea*, and to the parasitoids *Encarsia formosa* and *Trichogramma pintoi* (Mikul'skaya, 2000). There is a great importance of biological control in integrated pest management strategy.

4. Side effects on lacewings (*Chrysoperla* spp.)

The common green lacewing, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) is one of the most common arthropod predators (Tauber et al. 2000; McEwen et al. 2001) with a wide prey range including aphids, eggs and neonates of lepidopteran insects, scales, whiteflies, mites, and other soft bodied insects (New 1975; McEwen et al. 2001). It has long been considered as a promising candidate for pest management programs worldwide (Tauber et al. 2000; McEwen et al. 2001) due to its wide prey range and geographical distribution, resistance/ tolerance to pesticides, voracious larval feeding capacity as well as commercial availability (Medina et al. 2003a). Inundative releases of *C. carnea* were effective in controlling populations of pest complexes in various crops (Ridgway & Murphy 1984).

Insecticides, earlier considered as the backbone in crop protection, have become subordinate to other control methods, such as biocontrol which has gained more credibility in the last decades (Zaki et al. 1999b; Sarode & Sonalkar 1999b; Senior & McEwen 2001). But, the effectiveness of bioagents has been jeopardized by these insecticides. The sensitivity of *C. carnea* to insecticides differs from compound to compound. Medina et al. (2001) demonstrated that spinosad had little effect on *C. carnea* adult longevity and fecundity with no impact on eggs and pupae. Also, pyriproxyfen and tebufenozide were harmless at recommended field rates, whereas azadirachtin and diflubenzeuron were toxic to *C. carnea* third instar larvae (Medina et al. 2003 a, b; Güven & Göven 2003). In greenhouses, where organic farming system was applied, spinosad was used to control *Spodoptera littoralis* (Boisd.) on pepper and *Plutella xylostella* (L.) on cabbage, whereas *Chrysoperla carnea* and *Coccinella undecimpunctata* (L.) were released to control aphid populations on pepper and cabbage (Mandour 2009).

Saleem & Matter (1991) observed that the neem oil acted as temporary repellent against the predatory staphylinid beetle, *Paederus alfierii*, the coccinellid, *C. undecimpunctata* and the lacewing, *Chrysoperla carnea* in cotton but otherwise neem oil had no adverse effect on these predators of *Spodoptera littoralis*. That neem oil had no adverse effect on predators is also obvious from the studies of Kaethner (1991), as it was found harmless to the eggs, larvae or adults of *C. carnea* and also *C. septempunctata* (Lowery & Isman 1996)

Joshi et al. (1982) noted that 2 percent neem seed kernel suspension, when sprayed on tobacco plants, conserved the *Chrysopa scelestes*, an egg and larval predator of *S. litura*. The adults of the lacewing, *C. scelestes* were repelled from egg laying on cotton plants after they were sprayed with various commercial neem products of Indian origin and aqueous NSKE (Yadav & Patel 1992). First instar larvae of the predator emerged normally from treated eggs. Polyphagous predator, *C. carnea* treated in laboratory and semi-field trials with AZT-VR-K (1000 ppm) and with a mixture of this product with NO (25030000 ppm) induced no toxicity on eggs or adults; the fecundity of the latter was also not significantly affected (Kaethner 1991). The number of eggs (fecundity) laid by adult females developed from treated larvae was normal. The mortality of larvae fed with neem-treated aphids did not differ from that of controls. In laboratory experiments of Hermann et al. (1998) high mortality of larvae and pupae of *C. carnea* occurred if larvae were kept on NeemAzal-T/S (0.3% and 0.6%) contaminated glass plates, but practically no mortality was found in semi-field trials. Vogt et al. (1997) also studied the effectiveness of NeemAzal-T/S at 0.3 percent against *Dysaphis plantaginea* on apple and on its side-effects on *C. carnea*. A single application of NeemAzal-T/S in April gave very good control of *D. plantaginea* for about 5-6 weeks. After this period *D. plantaginea* builtup new colonies and *Aphis pomi*, too, increased in abundance. The side-effect test revealed that in the field NeemAzal-T/S was harmless to larvae of *C. carnea*. Neem seed extract was also found safe to *C. carnea* in comparison to nine insecticidal products (Sarode & Sonalka 1999a) where chlorpyrifos, deltamethrin and cypermethrin were found highly toxic to *Chrysoperla*. There was no mortality of *C. carnea* due to neem-based pesticides like NSE 5 per cent, Neemark, Achook, and Nimbecidine each at 0.003 per cent and neem oil at 1 per cent (Deole et al. 2000; Viñuela et al. 2000).

Spinosad is registered in many countries including Egypt for controlling lepidopteran and dipteran pests in fruit trees, ornamental plants, field- and vegetable crops. Medina et al. (2001, 2003b) studied the effect of spinosad on *C. carnea* eggs, pupae and adults using direct contact and ingestion treatments. As most of *C. carnea* immature stages do not die when exposed to sublethal doses, sublethal effects may exist that reduce the effectiveness of *C. carnea* progeny in controlling aphid control (Desneux et al. 2007). Mandour (2009) studied toxicity of spinosad to immature stages of *C. carnea* and its effect on the reproduction and survival of adult stages after direct spray and ingestion treatments. Spinosad was harmless to *C. carnea* eggs and pupae irrespective of concentrations or method of treatments. Mandour (2009) stated that oral ingestion of spinosad in artificial diet resulted in rapid death in *C. carnea* adults. After 7 days of ingestion, all tested adults in the three highest concentrations were dead compared to 100% of adult survival in control (Fig. 3). He mentioned also that spinosad ingestion had a profound effect on fecundity of *C. carnea*. In the three highest concentrations, almost all eggs were laid on the first two days after spinosad ingestion, and then surviving adults stopped laying eggs until death (Fig. 4).

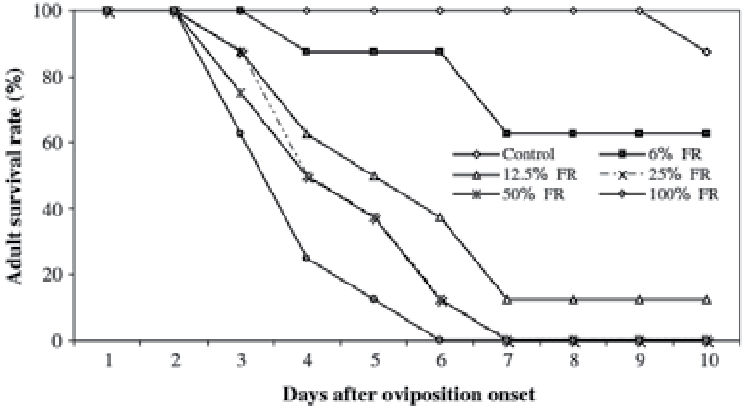

Figure 3. Rate of *C. carnea* adult survival after feeding on spinosad treated artificial diet from the onset of oviposition, FR = field rate (*n*=8) (after Mandour 2009).

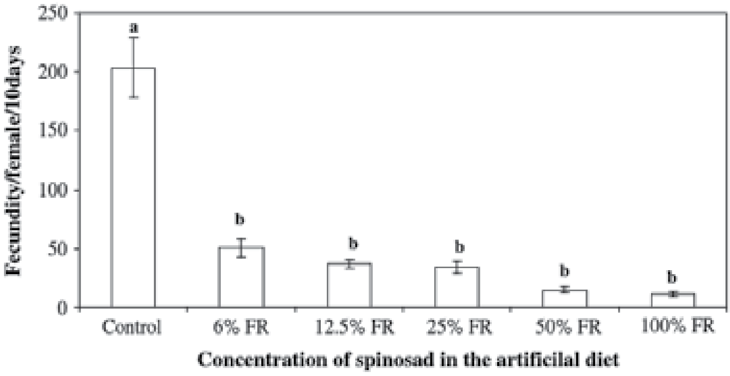

Figure 4. Influence of spinosad concentration on fecundity of *C. carnea* adults when fed with treated artificial diet from the onset of oviposition FR = field rate (*n*=8) (after Mandour 2009).

5. Side effects on predatory spiders and mites

There is an increasing interest in the ecology of polyphagous predators (e.g. Araneae) in agriculture. Spiders are important natural enemies of many insect pests, as they are generalist predators and comprise a large part of the beneficial arthropod community in agricultural fields (Nyffeler 1982; Riechert & Lockley 1984; Sunderland et al. 1986; Young & Lockley 1985; Everts 1990), and a number of case studies in different crops (e.g. Mansour et al. 1981; Nyffeler & Benz 1987, 1988) show that spiders can indeed be effective pest control agents in many situations. However spiders are also easily affected by pesticides (Boller et al. 1989; Everts et al. 1989; Aukema et al. 1990; Volkmar 1995, 1996; Volkmar & Wetzel 1993; Volkmar & Schier 2005; Volkmar et al. 1992, 1996 a, b, 2003, 2004).

Agricultural entomologists recorded the importance of spiders as a major factor in regulating pest and they have been considered as important predators of insect pests and serve as a buffer to limits the initial exponential growth of prey population (Volkmar 1996; Snyder & Wise 1999; Nyffeler 2000; Sigsgaard 2000; Maloney et al. 2003; Venturino et al. 2008; Chatterjee et al. 2009; Jayakumar & Sankari 2010). However researchers have exposed those spiders in rice field can play an important role as predators in reducing plant hoppers and leafhoppers (Visarto et al. 2001; Lu Zhong- Xian 2006, 2007). Several workers reported the predatory potency of spiders in rice ecosystem (Samiyyan 1996; Sahu et al. 1996; Pathak & Saha 1999; Sigsgaard 2000; Vanitha 2000; Mathirajan 2001; Sunil Jose et al. 2002; Satpathi 2004; Sudhikumar et al. 2005; Sebastian et al. 2005; Motobayashi et al. 2006). According to Peter (1988), the crop having more insects or insect visitors always had more spiders.

Many studies have demonstrated that spiders can significantly reduce prey densities. Lang et al. (1999) found that spiders in a maize crop depressed populations of leafhoppers (Cicadellidae), thrips (Thysanoptera), and aphids (Aphididae). The three most abundant spiders in winter wheat, *Pardosa agrestis* (Westring) and two species of Linyphiidae, reduced aphid populations by 34% to 58% in laboratory studies (Volkmar et al. 1992, 1996 a, b; Feber et al. 1998; Yardim & Edwards 1998; Marc et al. 1999; Nyffeler 1999; Holland et al. 2000). Both web-weaving and hunting spiders limited populations of phytophagous Homoptera, Coleoptera, and Diptera in an old field in Tennessee (Riechert & Lawrence 1997). Spiders have also proven to be effective predators of herbivorous insects in apple orchards, including the beetle *Anthonomus pomorum* Linnaeus, and Lepidoptera larvae in the family Tortricidae (Marc & Canard 1997; Buchholz & Kreuels 2009). In no-till corn, wolf spiders (Lycosidae) reduce larval densities of armyworm (Laub & Luna 1992). Wolf spiders also reduced densities of sucking herbivores (Delphacidae & Cicadellidae) in tropical rice paddies (Fagan et al. 1998). Spiders are capable of reducing populations of herbivores that may not be limited by competition and food availability in some agroecosystems (Buchsbaum 1996; Sunderland 1999; Lemke 1999).

Among the identified species, *Lycosa pseudoannulata* (Boes & Stand) was the most prevalent followed by *Atypena formosana* (Oi), *Argiope catenulate* (Doleschalland) *Clubiona japonicola* (Boesenberg and Strand) (Sahu et al. 1996). The population of these four species also varied at different growth stages of rice (Heong et al. 1992). In the first 35 DAT of rice, *Pardosa pseudoannulata* and *Atypena formosana* are considered as the important predators of Green leafhopper (Sahu et al.

1996; Mathirajan, 2001). Moreover *P. pseudoannulata* is the vital predator against brown plant hopper and can also effectively regulate the pest population of Leafhoppers Plant hoppers, Whorl maggot flies, leaf folders, Case worms and Stem borers (Kenmore et al. 1984; Barrion & Litsinger, 1984; Rubia et al. 1990; Ooi & Shepard 1994; Visarto et al. 2001; Drechsler & Settele 2001; Lu Zhong-xian et al. 2006).

Samiyyan & Chandrasekaran (1998) reported spiders were effective against leaf folders, Cut worms and Stem borers. *Atypena formosana* has been observed to hunt the nymphs of plant hoppers and Leafhoppers small dipterans, such as whorl maggot flies (Barrion & Litsiger 1984; Sigsgaard et al. 1999). According to Mathirajan (2001) *Tetragnatha javanas,* is one of the common spider found in rice ecosystem and they effectively reduce the population of Green leafhopper s and brown plant hoppers. The feeding efficiency of four spiders, namely *Lycosa pseudoannulata, Clubiona japonicola, Argiope catenulate* and *Callitrichia formosana* were also studied.

Integrated Pest Management (IPM) aims to avoid harming natural crop spiders. For this, IPM, attempts to synchronize the timing of spraying of pesticides with the life cycle of the pests, their natural enemies (predatory spiders and mites) (Bostanian et al. 1984; Volkmar 1989; Volkmar & Wetzel 1992). IPM also endeavours to use chemicals that act selectively against pests but not against their enemies. Few studies actually investigate effects of insecticides other than their direct toxicity (usually LD_{50}) on non-target animals. However, living organisms are finely tuned systems; a chemical does not have to be lethal in order to threaten the fitness (physical as well as reproductive) of the animal, with un-predictable results on the structure of the biological community (Culin & Yeargan 1983; Volkmar & Schützel 1997; Volkmar & Schier 2005). Pesticides may affect the predatory and reproductive behaviour of beneficial arthropods short of having direct effects on their survival. Thus to show that a pesticide is relatively harmless, or indeed has no measurable effect at all, behavioural studies on the effects of sublethal dosages are necessary. Such studies are not often done, presumably because of their costs in methodological difficulties (Vollrath et al. 1990; Volkmar et al. 1998, 2002, 2004).

5.1. Side effects on predatory spiders

Agricultural fields that are frequently sprayed with pesticides often also have lower spider populations in winter wheat (Feber et al. 1998; Yardim & Edwards 1998; Holland et al. 2000; Amalin et al. 2001). In general, spiders are more sensitive than many pests to some pesticides, such as the synthetic pyrethroids, (cypermethrin and deltamethrin); the organophosphates, (dimethoate and malathion) and the carbamate, (carbaryl). A decrease in spider populations as a result of pesticide use can result in an outbreak of pest populations (Marc et al. 1999; Holland et al. 2000; Maloney et al. 2003).

Spiders can lower insect densities, as well as stabilize populations, by virtue of their top-down effects, microhabitat use, prey selection, polyphagy, functional responses, numerical responses, and obligate predatory feeding strategies and we aim to review the literature on these topics in the following discussion. Nevertheless, as biological control agents, spiders must be present in crop fields and prey upon specific agricultural pests. Indeed, they are present and do eat pest insects. Spiders of several families are commonly found in agroecosystems in winter wheat and many have been documented as predators of major crop pest species and families (Roach 1987; Nyffeler & Benz 1988; Riechert & Bishop 1990; Young & Edwards 1990; Fagan & Hurd 1991; Nyffeler et al. 1992; Marc & Canard 1997; Wisniewska & Prokopy 1997; Fagan et al. 1998; Lang et al. 1999; Marc et al. 1999). Spiders may be important mortality agents of crop pests such as aphids, leafhoppers, planthoppers, fleahoppers, and Lepidoptera larvae (Rypstra et al. 1999; Maloney et al. 2003).

Many farmers use chemical pesticides to help control pests. An ideal biological control agent, therefore, would be one that is tolerant to synthetic insecticides. Although spiders may be more sensitive to insecticides than insects due in part to their relatively long life spans, some spiders show tolerance, perhaps even resistance, to some pesticides. Spiders are less affected by fungicides and herbicides than by insecticides (Yardim & Edwards 1998; Maloney et al. 2003). Spiders such as the wolf spider *Pardosa pseudoannulata* are highly tolerant of botanical insecticides such as Neem-based chemicals (Theiling & Croft 1988; Markandeya & Divakar 1999).

Saxena et al. (1984) reported that the wolf spider, *Lycosa* (=*Pardosa*) *pseudoannulata*, an important predator of leafhoppers in rice fields in Asia, was not harmed by neem oil (NO) and alcoholic or aqueous NSKE. In fact, NO (3%) and aqueous NSKE (5%) were quite safe for the spiders, though endosulfan induced 100 per cent mortality of the predators (Fernandez et al. 1992). NSKE, NO or NCE (10%) treated rice plots had better recolonization of spider *L. pseudoannulata* than in monocrotophos (0.07%) treated plots after seven days of treatment (Raguraman 1987; Raguraman & Rajasekaran 1996). The same neem products also spared the predatory mirid bug, *C. lividipennis* (Mohan 1989). The population of *L. pseudoannulata* and *C. lividipennis* were reported to be unaffected by different neem seed kernel extracts in paddy crop (Saxena 1987, 1989; Jayaraj et al. 1993). Similar observation on rice crop was made by Nirmala & Balasubramanian (1999) who studied the effects of insecticides and neem based formulations on the predatory spiders of riceecosystem.

Samu & Vollrath (1992) assessed a bioassay to test (ultimately in the field) such hidden effects of agrochemicals in their application concentrations. As a paradigm we chose the web- building behaviour of the cross spider *Araneus diadematus* Clerck (Araneidea) and we selected four commonly used pesticides: Oleo Rustica 11E (mild insecticide), Fastac (pyrethroid insecticide), Bayfidan and Sportak (fungicides). Neither fungicides nor the mild insecticide seem to affect web-building behaviour significantly, whereas the pyrethroid insecticide suppressed web-building frequency and severely affected web size and building accuracy.

There are also some studies that prove the neem's lack of toxicity against spiders and mites. Like *Cheiracanthium mildei* (predator of citrus fruit) with its prey *Tetranychus cinnabarinus* that is highly susceptible to neem (Mansour et al. 1986). *Phytoseiulus persimilis* is also not harmed by NSE, specially its fecundity while *T. cinnabarinnus* is up to 58 times more toxic than it (Mansour et al. 1987); the same trend of results was stated by Schmutterer (1997, 1999). Mansour et al. (1993, 1997) reported that the commercial products namely Margosan-O, Azatin and RD9 Repelin showed no toxicity to the spider. Serra (1992) observed that the neem products were not at all toxic to spider predators. Nandakumar & Saradamma (1996) observed the activity of natural enemies in cucurbit fields, where neem-based pesticides were applied for the control of *Henosepilachna vigintioctopunctata*. Natural enemies observed in considerable numbers were *Tetrastichus* sp., *Chrysocoris johnsoni, Tetragnatha* sp., *Oxyopes* sp. and orb-web spiders, and neem product did not inflict any harm to them. Lynx spider, *Oxyopes javanus* was less sensitive To neem oil (NO) (50% EC) than *L. pseudoannulata* (LC_{50} values = 9.73 and 1.18%, respectively) (Kareem et al. 1988; Karim et al. 1992), thereby confirming that NO was the safest pesticide for spiders. In cornfields (Breithaupt et al. 1999) and cabbage fields (Saucke 1995) in Papua New Guinea no significant effect was observed against *Oxyopes papuanus* from aqueous NSKEs (2%) or NeemAzal-S treatments. Serra (1992) did not observe adverse effects from NSKE 4 per cent applied on unidentified spiders in tomato fields in the Caribbean.

Babu et al. (1998) reported that a combination of seedling root dip in 1 percent neem oil emulsion for 12h + soil application of neem cake at 500 kg/ha + 1 per cent neem oil spray emulsion at weekly intervals gave an effective level of control of green leafhopper (*Nephotettix virescens*) infesting rice (var. Swarna). A combination of neem oil+urea at a ratio of 1:10 when applied three times at the basal, tillering and panicle initiation stages gave a superior level of control of brown planthopper (*Nilaparvata lugens*). The treatments, urea+nimin [neem seed extract] and a seedling root dip with 1 per cent neem oil emulsion+neem cake at 500 kg/ha+1 per cent neem oil spray emulsion at weekly intervals was equally effective against *N. lugens*. All neem products had little effect on predators, *C. lividipennis* and *L. pseudoannulata* (Sontakke 1993; Babu et al. 1998). NSKE sprays at 5, 10 and 20 per cent were also substantially safe for spiders and ants in cowpea ecosystems (Sithanantham et al. 1997).

Nanda et al. (1996) tested the bioefficacy of neem derivatives against the predatory spiders, wolf spiders (*L. pseudoannulata*), jumping spider (*Phidippus* sp), lynx spider (*Oxyopes* sp.), dwarf spider (*Callitrichia formosana*), orb spider (*Argiope* sp.), damselflies (*Agriocnemis* sp.) and mirid bug (*C. lividipennis*). It was observed that the neem kernel extract and oil were relatively safer than the insecticides to *L. pseudoannulata, Phidippus* sp. and *C. lividipennis* in field conditions. Markandeya & Divakar (1999) evaluated the effect of a commercial neem formulation (Margosan 1500 ppm) in the laboratory against two parasitoids and two predators. The formulation was tested at the field recommended dose of 10 ml/l. The neem formulation Margosan 1500 ppm was safe to all the four bioagents studied viz., *T. chilonis, B. brevicornis, L. pseudoannulata* and *C. sexmaculata*. Spider population in rice ecosystem was the lowest in carbofuran treatment and highest in neem cake treatments. The mean predator population of *Ophionea indica, Paederus fuscipes, Lycosa* sp. and coccinellid beetles was significantly higher in plots with *Azolla* at 5 t/ha, with or without neem cake at 1.5 t/ha, in field trials conducted in southern Tamil Nadu, India under lowland rice irrigated conditions (Baitha et al. 2000).

5.2. Side effects on predatory mites

Members of the family Phytoseiidae show a remarkable ability to reduce red spider mite infestations. There are many behavioural aspects that need to be considered in the phytophagous and predacious mites. Recognizing these behaviours and the side effects of pesticides on predatory mites can increase the success of biological control. Therefore, successful utilization of biological control could depend on the compatibility of the natural predators with pesticides. Studies on the side effects of pesticides on phytoseiid mites in Portugal have begun in 1995 (Rodrigues et al. 2002; Cavaco et al. 2003). Further research to evaluate these side effects of pesticides on all sensitive stages of the phytoseiid mites were conducted (Blümel et al. 2000; Broufas et al. 2008; Olszak & Sekrecka 2008).

The predatory mite *Phytoseiulus persimilis* (Athias-Henriot) is an economically important species in integrated mite pest management and biological control of spider mites in many countries throughout the world. Mass rearing and releasing natural enemies mainly phytoseiid mites are one of the goals of biological control of these pests in indoor and outdoor conditions (McMurtry & Croft 1997); additional food should be found for predatory mites (Pozzebon et al. 2005; Pozzebon & Duso (2008) in case of rareness of preys. For optimal biological mite management, it is important to know if acaricides have adverse undesirable effects on the predatory mites (Arbabi 2007). Nadimi et al. (2008) evaluated the toxic effects of hexythiazox (Nisorun®, EC 10%), fenpyroximate (Ortus®, SC 5%) and abamectin (Vertimec®, EC 1.8%) on *P. persimilis*. The results showed that the total effect values of all concentrations of hexythiazox were below the lower threshold thus it could be considered a harmless acaricide to this predatory mite. In contrast, the total effect of all concentrations of fenpyroximate, and field, as well as, one half the field concentration of abamectin were found toxic to predatory mite and above upper threshold. The overall results confirmed that *P. persimilis* is promise and crucial to develop IPM programs in agricultural crops; similar results were obtained by (Cloyd et al. 2006, Pozzebon & Duso 2010).

There are many spider mites such as *Tetranycus urticae* (Koch), which is considered one of the most important mite pest species with a wide range of host plants (Herron & Rophail 1993; Bolland et al. 1998). Many efforts have been undertaken to manage *T. urticae* problems in agricultural crops such as the application of new acaricides with the lower concentrations and release of predacious mites such as *Phytoseiulus persimlis* in glasshouses on cucumbers (Arbabi 2007) and in fields of beans, cotton as well as soybeans (Daneshvar & Abaii 1994). It has gained increasing attention by research scientists in many parts of the world. Selective pesticides that can be used to control pests without adversely affecting important natural enemies are urgently needed. Testing programme represented by IOBC (International Organization for Biological Control), is not only meant to provide valuable information on the side effects of pesticides on beneficial organisms but it also gives the testing members an opportunity to improve testing techniques, compare results and exchange experience with colleagues in the Working Group (Hassan et al. 1991).

Biological control of these pests is increasing because of the pressure on growers to find alternatives to chemical pesticides (van Lenteren 2000). In the presence of chemical applications, biological control of spider mites may be achieved by the selective use of pesticides that are less toxic to natural enemies than to pest species (Zhang & Sanderson 1990). Ruberson et al. (1998) suggested that selective pesticide were the most useful tool of integration of biological control agents into pest control programs. A strain of *P. persimilis* was introduced into Iran from the Netherlands (Department of Entomology, Wageningen Agricultural University) in 1988 (Daneshvar 1989) and it was effective in controlling spider mites under greenhouses and outdoor conditions (Daneshvar & Abaii 1994). However, Biological control of spider mites using this predaceous mite is effective only against low population densities of the pest (Pralavorio et al. 1985). When the population densities are high an acaricide treatment is needed to reduce the pest population before release of beneficial mites (Malezieux et al. 1992; Bakker et al. 1992; Hassan et al. 1994). Although various aspect of pesticide effects on *P. persimilis* have been studied by many workers in the past (Samsøe-Petersen 1983; Zhang & Sanderson 1990; Oomen et al. 1991; Blümel et al. 1993, 2000; Blümel & Gross 2001; Blümel & Hausdorf 2002; Cloyd et al. 2006). Only Kavousi & Talebi (2003) investigated side-effects of heptenophos, malathion and pirimiphosmethyl on *P. persimilis*. Moreover, there is no adequate information on the susceptibility of many strains and species to other pesticides, especially acaricides (Zhang 2003).

Bostanian et al. (2004) studied the toxicity of Indoxacarb to two predacious mites: *Amblyseius fallacis* (Garman) (Phytoseiidae) and *Agistemus fleschneri* (Summers) (Stigmaeidae). They reported that Indoxacarb had no adverse effects on *A. fallacis* and *A. fleschneri* adults, number of eggs laid by treated adults of both species and percent hatch of treated eggs of these two species, as stated also by Kim et al. (2000, 2005).

Rodrigues et al (2004) evaluated the toxicity of five insecticides (*Bacillus thuringiensis,* tebufenozide, flufenoxuron, phosalon and deltamethrin) on predatory mites (Acari: Phytoseiidae). The results were similar in both trials: phosalon and deltamethrin had a poor selectivity (harmful) on the phytoseiid mites, *Bacillus thuringiensis,* tebufenozide and flufenoxuron showed a good selectivity to these predators. The most abundant Phytoseiid species identified were *Phytoseius plumifer* (Canest & Fanzag) (91.8%) in Minho region and *Typhlodromus phialatus* Athias-Henriot (96.7%) in Castelo Branco region.

Cavaco et al (2003) studied evaluating the field toxicity of five insecticides on predatory mites (Acari: Phytoseiidae). The dominant species of phytoseiid in the region of Guarda was *Typhlodromus pyri* Scheuten (99.9%) and the dominant species in the region of Castelo Branco was *Typhlodromus phialatus* Athias-Henriot (96.4%). The results of imidacloprid showed good selectivity for phytoseiids while dimethoate was harmful. It was found that *T. pyri* was more tolerant to the other insecticides tested than *T. phialatus*. These results are of interest for the enhancement of integrated pest management programs. They suggest differences in susceptibility of *T. pyri* and *T. phialatus* to the tested insecticides, mainly to vamidothion.

Spinosad controls many caterpillar pests in vines, pome fruit and vegetables (including tomatoes and peppers), thrips in tomatoes, peppers and ornamental cultivation and dipterous leafminers in vegetables and ornamentals (Bylemans & Schoonejans 2000). Spinosad can be used to control pests in crops where the conservation of predatory mites is an important component of Integrated Pest Management (IPM) (Thompson et al. 1997). Additionally, there are governmental and environmental pressures to develop and use products safely with minimum impact on non-target arthropods. Predatory mite species are recognised as both important antagonists of pest species and sensitive indicators of ecologically significant effects (Overmeer 1988; Sterk & Vanwetswinkel 1988).

Miles & Dutton (2003) conducted extended laboratory experiments, semi-field and field tests to examine effects of spinosad on predatory mites. Under extended laboratory conditions (exposure on natural substrates) no effects were seen on *Amblyseius cucumeris*, *Hypoaspis aculeifer* or *Hypoaspis miles* at rates up to 540 g a.i./ha. When *Phytoseiulus persimilis* was tested under semi-field conditions, spinosad was harmless at rates of 9.6, 19.2 and 36 g a.i./hL. No effects were noted to *Amblyseius californicus* at 19.2 g a.i./hL under semi-field conditions. In the field, single applications of spinosad at 48 or 96 g a.i./ha in vines caused no unacceptable effects to populations of *T. pyri* or *Kampimodromus aberrans*. It was concluded that spinosad was highly selective to most predatory mite species and that effects noted in tier I laboratory studies did not translate to higher tiers of testing or use in the field. The reason for this is not clear but could be due to agronomic practice, difference in species sensitivity, sublethal or behavioural effects or even effects on prey. However use patterns safe to predatory mites and compatible with IPM have been developed for a wide range of crops.

Papaioannou et al. (2000) studied the effects of a NSKE (Neemark) and Bioryl(R) vegetable oils against phytophagous and predatory mites using bean leaves treated with different concentrations. Neemark (3 and 5%) was moderately toxic to *T. urticae*, and highly toxic to *P. persimilis*. Other studies investigated the toxicological tests (acute and sublethal effects) of fungicides on predatory mites (Blümel et al. 2000; Auger et al. 2004; Bernard et al. 2004).

6. Conservation and enhancement of natural enemy assemblages

Conservation of predators in the field can be accomplished by reducing both chemical and physical disturbance of the habitat. Natural enemy densities and diversities are significantly higher in orchards and fields where no pesticides have been sprayed (Yardim and Edwards 1998; Marc et al. 1999; Holland et al. 2000; Amalin et al. 2001). Restricting insecticide treatment to crucial periods in the pest life cycle or limiting spraying to midday when many wandering natural enemies are inactive and in sheltered locations can help conserve spider numbers (Riechert & Lockley 1984). Natural enemies can recolonize if the interval between chemical applications is long enough, but several applications per season can destroy natural enemy communities. Some pesticides are also retained in the natural enemies and can be detrimental to those spiders that ingest their webs daily (Marc et al. 1999).

Besides pesticides, other human practices that can disrupt natural enemy populations are mowing, plowing, harvesting, and crop rotation (Nyffeler et al 1994; Marc et al. 1999). Soil disturbance by plowing destroys overwintering sites and can kill any agent already present in the soil (Marshall & Rypstra 1999; Maloney et al. 2003). The movement of farm equipment through a crop field damages spider webs and may destroy web attachment sites (Young & Edwards 1990). Consequently, density and diversity of natural enemies are higher in organic fields than in conventional ones. For example, in cereal fields, Lycosidae made up only 2% of the community in conventional fields, but 11% in organic fields. Most lycosids were found in field edges (Marc et al. 1999). Clearly, human input is harmful to natural enemies, and the best spider conservation strategy may be non-intervention (Young & Edwards 1990; Maloney et al. 2003).

Traditional biological control efforts have focused on using specialist predators to control pest outbreaks, which Riechert & Lockley (1984) liken to "putting out fires rather than preventing their conception". Encouraging natural enemy populations may have the effect of keeping pest levels low and not letting them get out of control. Spiders may be potential the helpful biocontrol agents because they are relatively long lived and are resistant to starvation and desiccation. Additionally, spiders become active as soon as conditions are favourable and are among the first predators able to limit pests. The risks associated with using natural enemies to control pests are minimal. Since diverse species of natural enemies are naturally present in an agricultural system (thus avoiding the problems associated with introductions) and predaceous at all stages of their development, they fill many niches, attacking many pest species at one time (Agnew & Smith 1989; Marc et al. 1999). Because they are sensitive to disturbance, natural enemies may best be used in perennial agroecosystems, such as orchards, that suffer the least disruption and human intervention (Riechert & Lockley 1984; Marc et al. 1999). Natural enemies do have the potential to be highly effective pest management agents, but the overall level of control is specific to each combination of crop and management style (Maloney et al. 2003).

7. Conclusions

Neem products are now widely acclaimed as broad-spectrum pesticides. Schmutterer & Singh (1995) listed 417 insect species as sensitive to neem. In the present era of biocontrol, safety concerns predominate the agro-ecosystem besides pest control. Since neem products are now on large-scale use, their safety to natural enemies has also become a debatable issue. In the case of microbial agents, NPV and Bt are the most successful commercial products. Neem products either pure, crude or commercial so far did not show any adverse effects when combined with NPV or Bt. Though combining neem products with antifeedant property and microbials with stomach poison activity is disputed, the vast volume of research work carried out reveals that the antifeedant principles of neem do not influence in any way the activity of the microbials inside the insect gut. The growth disrupting principles of neem were found to add to the activity inside the insect system along with microbial principles leading to quicker mortality to give a cumulative effect.

In the case of parasitoids, certain guiding principles are suggested in accordance with multi-array activities of neem products in insects. Parasitoids are also susceptible, when they come in direct contact with neem products. In such circumstances blanket application of neem products without understanding the behaviour of the parasitoid may adversely affect the beneficial capacity of the parasitoid. For example, the inundative release of the egg parasitoid *T. chilonis*, should be resorted 3-4 days before/ after neem products application. The external larval parasitoids are no exception to the ill effects if they are in direct contact with neem products. To avoid this, for inundative releases, application of neem products may be followed by the release of the parasitoids and spraying may be avoided if the parasitoids are in larval stages in the field. Hence presampling is suggested to know the stage of the parasitoid, be it internal or external, for timing the application of neem products.

In the case of predatory insects, mites and spiders, certain degree of selectivity is nevertheless appararent, as adult insects show, no or relatively low sensitivity as in the case of earwigs, crickets, true bugs, beetles, lacewings and wasps. This can be explained by the fact that growth-disrupting compounds affect the first line juvenile instars of insects. The fecundity of neem-treated adult, predaceous parasitic insects and the fertility of their eggs are also not or only slightly affected by neem, in contrast to some phytophagous species. In some cases the predation efficiency may be reduced Nymphal/larval instars of beneficial insects are sensitive to neem products. When topically treated, reduction in food ingestion, delayed growth, difficulties in moulting, teretological and morphogenetic defects, reduced activity and increased mortality are normally observed in the laboratory. But, far less drastic or even no effects are observed under semi-field or field conditions. This is partly due to the fast break-down of the active principles underfield conditions.

A desirable biological control agent is a predator that not only reduces pest densities, but also stabilizes them at low levels, while maintaining stable populations itself (Pedigo 2001). Stability in predator-prey systems is achieved by density-dependent responses of the predator to the prey. As prey populations increase, predation pressure should increase, and predation pressure should lessen as prey population decrease. Usually, the greater the importance of a given prey in the diet of a predator, the lower the population size the predator effectively controls. Density-dependent control is thereby affected by the functional response and the numerical response of the predator (Riechert & Lockley 1984; Morin 1999).

The reproductive response of spiders is less studied. Some spiders, especially web-weavers, do show an increase in fecundity with increasing amounts of prey ingested. Such spiders include *Neriene radiate* (Linyphiidae), *Mecynogea lemniscata*, *Metepiera labyrinthea* (Araneidae) and *Agelenopsis aperta* (Agelenidae) (Riechert & Lockley 1984). The extent to which this increase in fecundity can permit tracking of prey populations is limited by long generation times compared to those of pest insect species. Spiders are usually univoltine while generation times for many insect pests are a few weeks (Maloney et al. 2003).

Competition, intraguild predation, and cannibalism can limit the aggregation response of spiders. Spiders are usually territorial and will compete for space and prey at high spider densities, limiting the number of spiders that can coexist in the same area. The result may be migration from a patch of high prey densities and, therefore, less pest control (Marc et al 1999; Marshall & Rypstra 1999). Intraguild predation predation upon members of the same trophic level is a major factor limiting aggregation and spiders' pest control abilities (Fagan et al. 1998; Wise & Chen 1999).

The evidence to date suggests that insecticides derived from the neem tree are unlikely to cause substantial environmental damage and these products appear to be safer than synthetic neurotoxins. However, pesticides derived from neem are poisons and thus should be treated as such. Certain organisms are particularly sensitive to neem and this should be taken into consideration when contemplating their use (Maloney et al. 2003). Currently the development of new means for plant protection has different motivations. Three major groups are apparent: synthetic chemicals, genetically modified products and biological products. The present scenario of regulatory situation in different countries is not very clear and comprehensively laid down; therefore, NeemAzal has been taken as a specific example. An extract "NeemAzal" obtained from seed kernels of the Neem tree *Azadirachta indica* A. Juss and its formulation contains about 54 per cent azadirachtins. NeemAzal-T/S is a formulation of NeemAzal containing 1 percent w/w of azadirachtin A.

The factors that influence effects of either neem products or pesticides on natural enemies (insects, mites & spiders) are type of solvent, soil type, moisture, percent organic matter, temperature, and time of day of spraying. Further, the microhabitat, hunting style, prey preference, and behavior of biocontrol agent also influence their response to pesticide application (Schweer 1988; Volkmar & Wetzel 1993; Krause et al. 1993; Marc et al. 1999). Wisniewska & Prokopy (1997) reported that if pesticides were only used early in the growing season, natural enemy populations increased. Presumably, spiders have a chance to recolonize the field if pesticide use ceases after early June. Spatial limitation of pesticides (such as only applying the pesticides to certain plants or certain plots) also results in higher natural enemy numbers, since they can move out of the treated areas and return when the chemicals dissipate (Riechert & Lockley 1984; Dinter 1986, 1995; Maloney et al. 2003). Comparative studies have been carried out on various beneficial organisms such predatory spiders and mites, providing important data on the impact of pesticides on agro-ecosystems (Sterk et al. 1999; Holland et al. 2000; Amalin et al. 2001; Olszak & Sekrecka 2008).

After the treatment with NeemAzal-T/S larvae suffer feeding and moulting inhibition and mortality; adults show feeding inhibition, infertility and to a lesser degree, the mortality. This specific mode of action is called "insectistatic". These studies with NeemAzal definitely imply that this and several other developments in neem-bsed pesticides have convinced registration authorities not only in Europe and Asia but in USA and Canada as well and Neem has been included among reduced-risk pesticides. That is why main opportunities are seen as arising from the discovery of new leads from high-throughput screening of plant extracts. It is hoped that international harmonized approach will come into force with a uniform set of rules to encourage the development of plant-based products for rational and sustainable agriculture. Of course, the lead from neem-based products now already exists and should be followed globally in order to develop safe and standardized products. NP virus and Bt are highly compatible with neem products. Parasitoids/predators, pre-sampling and timing of application are necessary to avoid the ill effects of neem products, if any, on them. It is obvious that next years will look forward to IPM that will include natural enemies vis-à-vis other biopesticides synchronizing with ecological and behavioural aspects of pests (Landis et al. 2000).

El-Wakeil et al. (2012 unpublished data) studied effects of some insecticides on wheat insect pests (thrips, aphids,creal leaf beetle, click beetles, cicadas, bugs leafhopper and frit fly) and the associated natural enemies (dance flies, coccinellids, hover flies, lacewings, Staphylindis, predatory spider and wasp parasitoids) in winter wheat 2012 in central Germany. The sequential sampling plans (direct count, sweep net, sticky traps and water traps) were used and described in this research to provide an integrated method for less wheat insects. The results showed that both chemical insecticides (Karate and Biskaya) caused more mortality to wheat insects and their side effects were harmful to the natural enemies. On the other hand, neem treatments caused adequate mortality of insects and were safer to the natural enemies (Figs. 5 & 6).

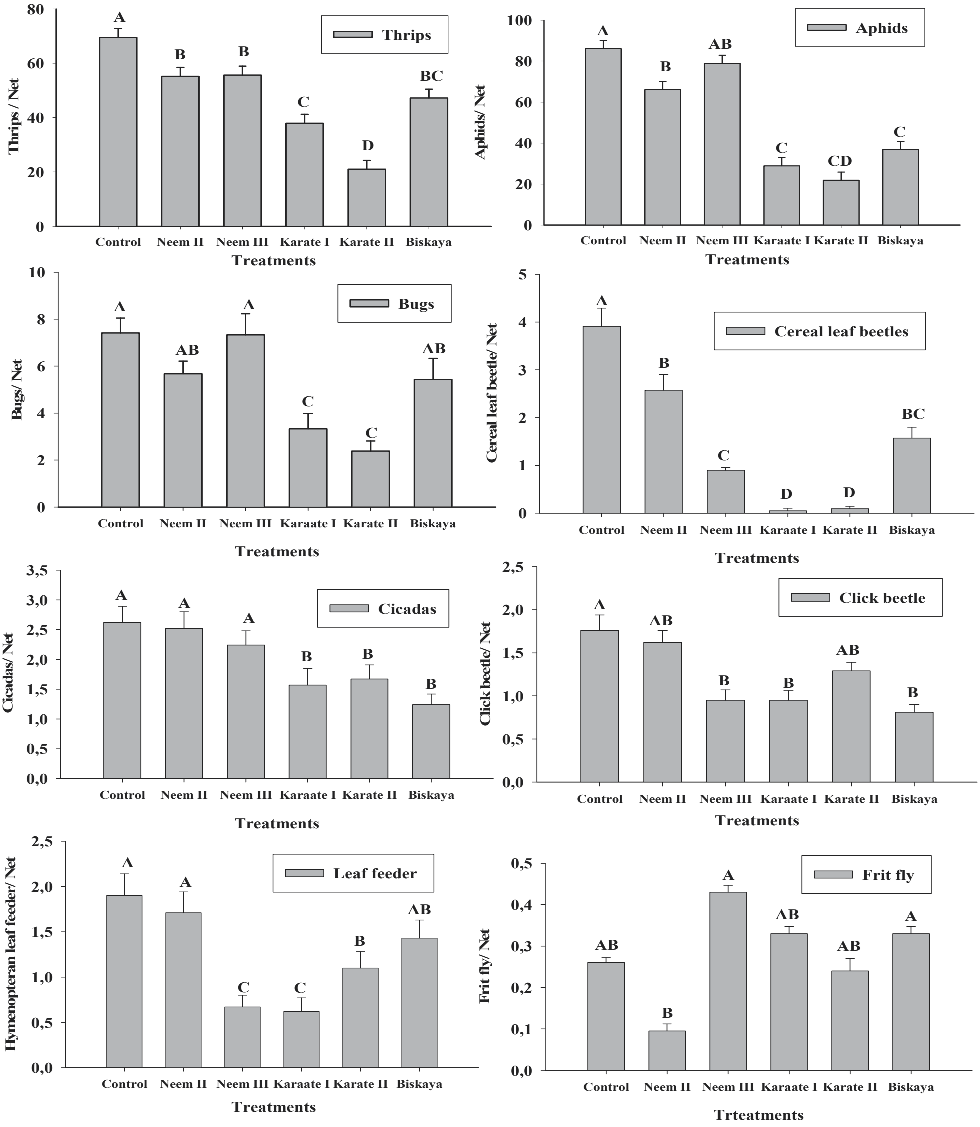

Figure 5. Mean of population ± SE of some wheat insects treated with different treatments and surveyed by sweep net in winter wheat 2012. Different letters indicate significant differences.

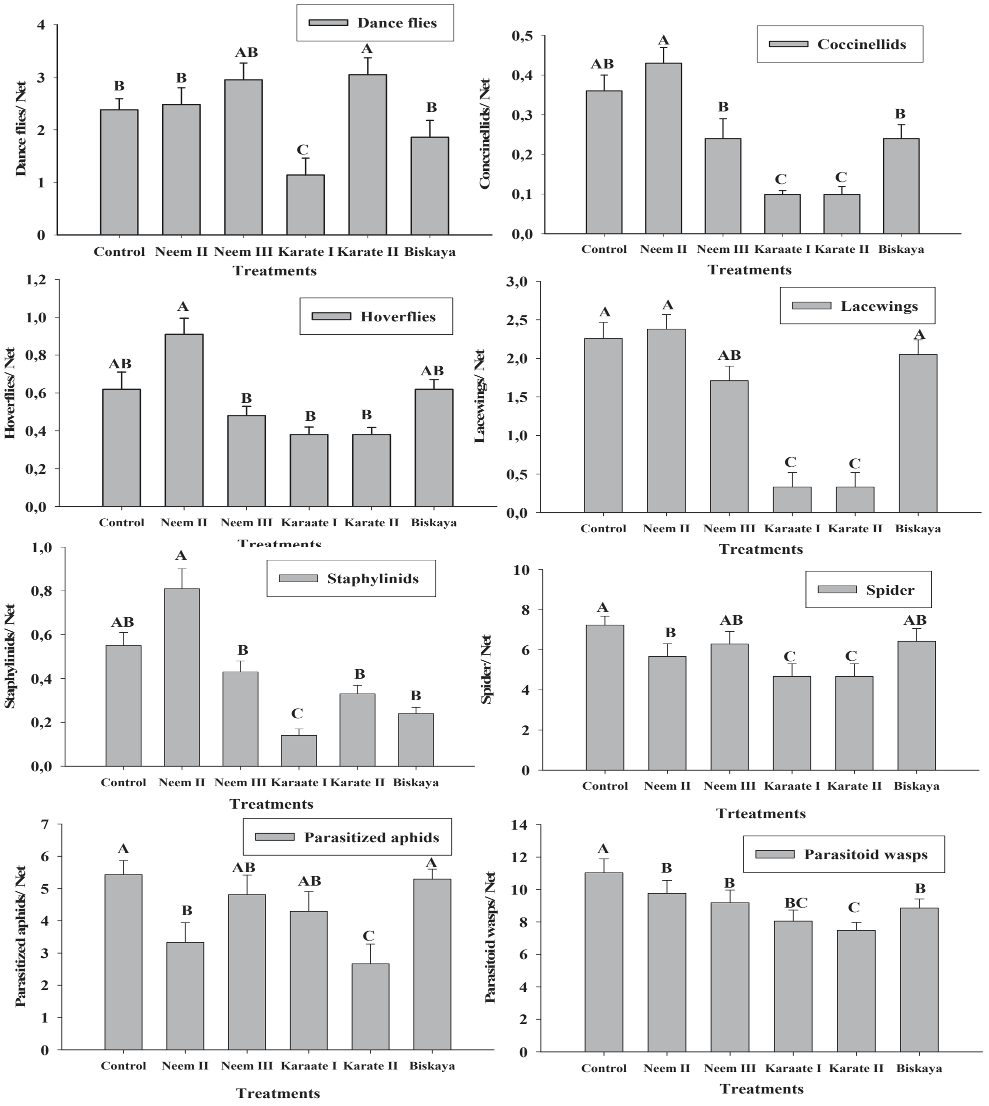

Figure 6. Mean of population ± SE of some natural enemies treated with different treatments and surveyed by sweep net in winter wheat 2012. Different letters indicate significant differences.

Agricultural sustainability requires a focus on the long run, on intergenerational equity. It must be capable of meeting the needs of the present while leaving equal or better opportunities for the future. It must be ecologically sound and socially responsible as well as economically viable. It must also include, as much as possible, the element of local or regional production, and aim for a reasonable level of regional food security. It encourages a shortening of the distance between producers and consumers, to the benefit of both. In a local economy consumers have influence over the kind and quality of their food; they contribute to the preservation and enhancement of the local landscape. It gives everybody in the local community a direct, long-term interest in the prosperity, health, and beauty of their homeland (Buchholz & Kreuels (2009); Shoeb 2010; Cabral et al. 2011).

Organic farming falls under this broader classification of "sustainable agriculture." It is commonly thought of as farming without chemicals, and that is usually the case, but it is much more than that. Organic farmers try to farm holistically - that is, they design production systems that capitalize on the positive synergies among crops, soils, seeds, and animals, in such away that each element of the system promotes the productivity and health of other elements. The rapid growth of organic and sustainable agriculture in Canada is occurring with almost no support from the federal government, whose policies are almost entirely devoted to encouragement of industrial agriculture (El-Wakeil 2003). Other countries are heading in the opposite direction. The cornerstone of Egypt as well Germany's new agricultural policies will be sustainability.

Author details

Nabil El-Wakeil[1,2], Nawal Gaafar[1,2], Ahmed Sallam[3] and Christa Volkmar[2]

*Address all correspondence to: nabil.el-wakeil@landw.uni-halle.de

1 Pests & Plant Protection Dept. National Research Centre, Dokki, Cairo, Egypt

2 Institute of Agric. & Nutritional Sciences, Martin Luther-University Halle-Wittenberg, Germany

3 Plant Protection Dept. Faculty of Agriculture, Sohag University, Sohag, Egypt

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Chapter 2

Pesticide-Residue Relationship and Its Adverse Effects on Occupational Workers

Nabil El-Wakeil, Shehata Shalaby,
Gehan Abdou and Ahmed Sallam

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/54338

1. Introduction

Pesticides use in agriculture is the most economical approach to control various pests, though they are considered major contaminant of our environment. The World Health Organization (WHO, 2005) and United Nations Environmental Program have estimated one to five million cases of pesticide poisoning among agricultural workers each year with about 20,000 fatalities, mostly reported from developing countries (Pimental et al. 1992). There are several definitions of pesticide; the Food and Agriculture Organization (FAO) defines pesticide as any substance or mixture of substances intended for preventing, destroying or controlling any pest during the production, processing, storage or marketing of food in all agricultural commodities for controlling the pests (FAO 1986). Pesticides are playing a pivotal role in meeting the food, cotton fibre and tobacco demand of escalating population and control of vector-borne diseases. However, most of the applied pesticides get dispersed in the environment and affects the health of un-protected agricultural and industrial workers. Pesticides are used extensively throughout the world. The three major routes of entry for pesticides include contamination of the skin, mouth and the nose. Although pesticides furnish some benefits for crop, they entail a number of risks and problems. The public health issue of pesticide exposure is further complicated by the presence of impurities in so-called, inert-ingredients such as solvents, wetting agents and emulsifiers (Hashmi&Dilshad 2011).

The main similarity between pesticide exposure of farm workers' children and lead exposure of children living in poverty is that the substances are present in the home, are difficult for the family to control, and are inequitably distributed across ethnic and socioeconomic groups. Unlike lead, the potential developmental effects of childhood exposure to many types of pesticides are greatly understudied. Some pesticides have been shown to cause behavioural effects in rodents such as hyperactivity, learning and memory problems, and altered habituation (Icenogle et al. 2004). In spite of the paucity of research on the effects of pesticides on human neurobehavioral development, there are reasons to be concerned about children's exposure. First, two widely used classes of insecticides, organophosphates and carbamates, inhibit cholinesterase. Cholinesterase inhibition leads to excess acetylcholine at the synapse, which in turn causes over activation of cholinergic neural pathways. There is evidence that organophosphate and carbamate pesticides can negatively affect early life rodent brain development by interfering with gene signalling by cholinesterases as well as by inducing faulty wiring of the brain via other mechanisms (Slotkin 1999; Aldridge et al. 2005; Sallam et al. 2006, 2009a,b).

These chemicals are suspected of producing adverse health effects based on their structural similarity to proven toxicants. Exposure to pesticides is one of the most important occupational risks among farmers in developing countries (Wesseling et al. 2001; Konradsen et al. 2003; Coronado et al. 2004; Shalaby et al. 2012). Occupational exposure to pesticides is of great interest in order to identify the hazards of pesticide use and the establishment of safe methods of pesticide handling. This is because pesticide misuse in various sectors of the agriculture often has been associated with health problems and environmental contamination worldwide (Soares et al. 2003; Mancini et al. 2005; Remor et al. 2009). Misuse of highly toxic pesticides, coupled with a weak or a totally absent legislative framework in the use of pesticides, is one of the major reasons for the high incidence of pesticide poisoning in developing countries (Konradsen et al. 2003; Hurtig et al. 2003; Atreya 2008). In general, knowledge of the main determinants of pesticide exposure in developing countries is often poor and also exposure situations may differ among countries (Hashmi&Dilshad 2011; Shalaby et al. 2012).

The spray-workers are directly exposed to pesticides during mixing, handling, spray, and through contaminated soil, air, drinking water, eating food and smoking at work places. The farm workers are, therefore, occupationally exposed to pesticides and may absorb them by inhalation, ingestion and dermal contact (Vega 1994; Mathur et al. 2005). The residue concentrations of these compounds in affected workers may lead to a variety of metabolic and systemic dysfunctions and, in some cases, outright disease states. Therefore, the excessive and repeated pesticide use has promoted toxicological problems in spraying community (Brown et al.1989; Karalliede&Senanayake 1999; Azmi et al. 2006). A major factor of pesticide contamination or poisoning in developing countries is the unsafe use or misuse of pesticides. Elements of unsafe use of pesticides that have been identified by past research include erroneous beliefs of farmers about pesticide toxicity, lack of attention to safety precautions, environmental hazards, and information about first aid and antidotes given by the label, the use of faulty spraying equipment or lack of proper maintenance of spraying equipment, and lack of the use of protective gear and appropriate clothing during handling of pesticides (Hurtig et al. 2003; Damalas et al. 2006a, b; Ajayi&Akinnifesi 2008; Chalermphol&Shivakoti 2009; Plianbangchang et al. 2009; Sosan&Akingbohungbe 2009; Hashmi&Dilshad 2011).

In view of the adverse health effects from the unsafe pesticide use, the latency of the adverse effects, the reported lack of awareness of the adverse health effects of pesticides by some farmers, and the erroneous belief of invincibility by others, it becomes imperative that the potential hazards of unsafe pesticide use should be clearly communicated to the farmers. Research has often emphasized the need to increase the awareness of farmers about the consequences of unsafe pesticide use and the importance of communication and education programs aiming to reduction of risk (Ibitayo 2006; Hashemi et al. 2008; Oluwole&Cheke 2009; Sosan&Akingbohungbe, 2009; Damalas&Hashemi 2010)

Exposure to pesticides at any point in the life cycle has the potential for causing a range of short-term or long-term health problems. Documented health effects include a wide variety of illnesses and diseases, from eye irritation, skin rashes and respiratory problems to neurological damage, birth defects, cancer and death. The risk for and severity of adverse health effects from pesticide exposure varies significantly depending on many factors, including individual characteristics such as age and health status, the specific pesticide, and exposure circumstances. Exposure to pesticides at certain developmental stages of life can result in irreversible damage to organ structure and function. Of particular concern is the effect of exposure at during the reproductive cycle, from preconception to breast feeding, because of the possibility of poor birth outcomes, congenital anomalies, developmental deficits, and possibly childhood cancer (Barthel 1981; Karabay et al. 2004; Hernandez et al. 2004; Sanborn et al. 2004; Strong et al. 2004; Hernandez et al. 2006; Hayes et al. 2006; El-Wakeil et al. 2009).

Farm worker families often live near or on the farms on which they work, and thus spend much of their time in close proximity to areas where pesticides are applied on a regular basis. Twenty-one percent of farm workers are women (Carroll et al. 2005), who may be directed to or inadvertently enter recently treated fields while pregnant. Women in farm worker households who do not work in the fields may still be exposed to pesticide residues brought home by farm worker household members on their shoes, clothes and skin; from nearby applications that drift or are directly sprayed on outdoor play areas; and from chemicals used to control pests in and around the home, especially in poor quality housing.

Agricultural extension is a major channel of communication between farmers and research experts which can improve crop production from many points of view as it provides a good link between farmers and research institutes where several agricultural technologies, including pesticides and the relative technology, are developed, tested, and modified accordingly. Training programs can play a crucial role in pest control decisions, providing farmers with the technical knowledge that is necessary for the selection of appropriate pest management methods and also for safe and effective pesticide use. Despite the appearance of homogeneity, often small farmers have different production practices, needs, and constraints (Carr 1989). A successful agricultural extension program, therefore, should not consider all individuals in a target group based on several variables such as age, gender, income, and types of crops (Sallam 2008; Shalaby et al. 2012).

2. Exposure pathways

Pesticides are used in 85% of homes in the US (Whitmore et al. 1992), but they or their residues can be found even on surfaces that have never been directly or peripherally treated. POPs introduced into the environment years ago are still around today, transported by human activity and through the food chain. Despite being banned in the US (and many other countries) some 30 years ago, traces of these insecticides are still found in the homes and bodies of individuals in the US who were not even alive when these products were used (Weiss et al. 2004; Wolff et al. 2007). Chlorpyrifos (a non persistent OP) has also been found to accumulate on newly-introduced surfaces, such as pillows, carpet and soft toys, when brought into a treated area up to two weeks after application, even if applied according to manufacturer's instructions (Gurunathan et al. 1998).

In agricultural settings, work-to-home exposure, or a "take-home pathway," has been identified as a key source of pesticide residues (primarily to OPs) in children's environment ((Fenske et al. 2000; Curl et al. 2002; Thompson et al. 2003; Rao et al. 2006; Coronado et al. 2006). Workers who are exposed on the job on a daily basis, whether as applicators or re-entry workers, are likely to carry home pesticides on their shoes, clothes, skin, and vehicles. Most workers are not provided with adequate washing or changing facilities to remove residues and put on clean clothes before leaving the worksite. If these workers do not take basic precautions (e.g., removing work shoes outside the dwelling, showering before picking up a child); they may transfer residues to the indoor environment or directly to other household members.

The primary routes by which pesticides enter the body are ingestion in food, soil, or water; inhalation, through the skin, and through the eyes (Arcury et al. 2000). OCs are absorbed through the lungs, stomach and skin, and excreted only slowly, sometimes over a period of years (e.g., DDT) (Pohl &Tylenda 2000; Cohn et al. 2007). Dietary ingestion is a significant source of exposure, especially for infants and children (Garry 2004). The residue monitoring program conducted by the FDA in 2003 found measurable levels of pesticides in baby foods, including DDT (6% of samples), captan + THPI (a possible carcinogen) (9%), carbaryl (carbamate) (6%), endosulfan (9%), dimethoate (4%), malathion (3%), and chlorpyrifos (all OPs) (2%) (FDA 2005; Sallam et al. 2006).

Post-natally, infants can be exposed to pesticides via breast feeding. The POPs, despite having mostly been banned, are still found in breast milk because they are stored in body fat (Weiss et al. 2004; Jurewicz et al. 2006). Postpartum weight loss increases the likelihood of the release of OCs into the breast milk (Jurewicz et al. 2006). There is some evidence that the maternal body burden is actually transferred to her children via breast feeding, as the pesticide concentrations decrease with the more times a mother has breastfed (Nickerson 2006). Fortunately, the benefits of breast feeding still far outweigh the possibility of harm from pesticide transfer in breast milk, and should be encouraged for all mothers regardless of exposure history (Nickerson 2006; Eskenazi et al. 2006). Pesticides exposure occurs in different ways: dermal, oral, respiratory and conjunctival routes.

2.1. Dermal exposure

It occurs by not washing hands after handling pesticides or their containers. Splashing or spilling of pesticide on skin by wearing pesticide-contaminated clothing and applying pesticides in the windy weather. Touching treated plants or soil also leads to dermal exposure. Exposures occur by rubbing eyes or forehead with pesticides contaminated gloves or hands, splashing pesticides in eyes, application in windy weather, drift exposure and mixing/loading of dry formulations without wearing goggles.

2.2. Oral exposure

Hands not washed before eating, smoking or chewing, pesticide splashed into mouth. Accidental application of pesticides to food, storing pesticides in drinking containers and drift on lip or in mouth also leads to oral exposure.

2.3. Inhalational exposure

Exposed to drift during or after spraying, mixing/loading, dusts, powders or other dry formulations; use of inadequate or poorly fitted respirators.

2.4. Exposure on respiratory system

The crops, activities, and exposure agents that can lead to respiratory disease are extraordinarily diverse and vary significantly by seasons, geography and type of agriculture. The number of substances affecting respiratory health to which a worker is exposed while working in an agricultural setting is enormous: pesticides, including insecticides, herbicides, and fumigants; other agricultural chemicals, including fertilizers and plant growth regulators; the crops and related allergens, such as pollens, pests, and microorganisms; and the land itself, including organic and inorganic dusts, to name just a few (Schenker et al 1998; Schenker 2005). Further complicating the issue, the likelihood that an individual worker has been exposed to but a single identifiable agent is small. Measuring exposure is also challenging, which makes dose-response relationships difficult to assess, and exposure limits have not been set for most relevant agents. Agricultural respiratory disease often goes untreated and unreported, especially by small operations not regulated by the Occupational Safety and Health Administration (OSHA), making it nearly impossible to determine the true extent of the problem (Kirkhorn& Garry 2000; Ross et al. 2001).

All children are at risk for contact with environmental toxins, but the burden of toxic exposures is disproportionately allocated to poor ethnic minorities (Schell 1997; Moore 2003; Dilworth-Bart & Moore 2006). ''Economic factors not only constrain choices but also inequitably distribute human made stressors.'' and the psychosocial stress and environmental pollutants associated with poverty do not occur independently of one another. Rather, the effects may accumulate through risk focusing, a process by which exposures to toxic or infectious environmental materials are differentially allocated to a specific group partly because of previous exposure to those materials (Schell 1997; Yassin et al. 2002).

3. Pesticides poisonings

Agro-chemical industry has offered thousands of compounds. The climatic condition of Pakistanfavors pest build up that destroys about 20 percent of potential agricultural crop. The health of the pesticides handlers and farmers in particular are at high risks due to irrational use of pesticides. Pesticides cause the acute and chronic health effects; organophosphate and carbamate groups are more important. These insecticides inhibit cholinesterase, an enzyme critical for normal functioning of the nervous system (Travisi&Nijkamp 1998; Gelman& Hill 2007; Soares& Porto 2012).

3.1. Prevalence of pesticides poisoning

In USA, more than 18,000 products are licensed for use and each year more than 2 billion pounds of pesticides are applied to crops, gardens, in homes etc. (U.S EPA 2002). The major economic and environmental losses due to the application of pesticides in public health were 1.1 billion dollars per year in USA (Pimentel 2005). Such wide spread use results in pervasive human exposure. Evidence continues to accumulate that pesticide exposure is associated with impaired health. Occupational exposure is known to result in an annual incidence of 18 cases of pesticides related illness for every 100,000 workers in U.S (Calvert et al. 2004). Pesticide poisoning is a major public health problem in many developing countries (Xue et al. 1987; Jeyaratnam 1990). In developing world, pesticide poisoning causes more deaths than infectious diseases. Pesticide poisoning among farmers and occupational workers in developing countries is alarming (McCauley et al. 2006). WHO estimated approximately 20,000 workers die from exposure every year, the majority in developing countries (Pimentel et al. 1992; Kishi et al. 1995). The number of intoxications with organophosphates is estimated at some 3000,000 per year and the number of deaths and casualties some 300,000 per year (Peter 2003). Ahmed and co workers have reported 64 percent of fatal cases of acute pesticides poisoning in Multan, Pakistan occurred due to Ops pesticide spraying (Ahmad et al. 2002; Ahmed et al. 2006) However another study revealed 21 percent of occupational pesticides poisoning in hospitalized patient (Afzal et al. 2006).

3.2 Acute toxicity

Organophosphorous compound exert acute systemic toxicity by inhibiting the enzymes AChE through a process of phosphorylation. Pesticides bind to cholinesterase and block the hydrolysis of the acetylcholine and acetic acid at the post synaptic junctions without junctioning acetyl cholinesterase; acetylcholine accumulates (Chan &Critchley 1998; Mason 2000). OPs induced neuronal symptoms are a consequence of axonal death. Following OPs exposures inhibition of neuronal enzymes, called neuropathy target esterase, occurs and many of them are irreversible.

4. Occupational health & safety (OHS) (clinical recommendations)

Health care providers are in an ideal position to identify and assess a patient's risk for exposure. The first step is to obtain an environmental history that covers residential and employment histories, types of work activities performed currently and in the relevant past, and possible sources of exposure to biological or chemical agents. For each exposure source identified, additional information needs to be collected, such as frequency, duration, and intensity. Women who are pregnant or planning a pregnancy, especially those currently performing farm work, should be informed of the implications of exposure before, during and after pregnancy, and assisted in making decisions that are appropriate for their individual work and home situations (McDiarmid&Gehle (2006). In addition, providers should encourage mothers to avoid exposure that might contaminate breast milk without unduly alarming them, perhaps by associating it with the importance of not smoking or drinking alcohol during pregnancy and nursing (Pohl &Tylenda 2000; Nickerson 2006). Of course, breast feeding should continue to be strongly encouraged since all evidence indicates that the known benefits far outweigh the potential risks (Eskenazi et al. 2006). Education about pesticide safety is an important measure for preventing exposure. The Migrant Clinicians Network has recently developed a 14-page full-color Spanish language comic book and Wake Forest University School of Medicine has produced patient education handouts and posters in English and Spanish. Women living in farm worker households should be offered additional education on ways they and the farm workers with which they live can reduce take-home exposure:

- remove work clothes and shoes before entering the home
- shower or bath upon returning home and before touching other people
- store and launder dirty work clothes separately from other clothing (Rao et al. 2006)

As the evidence continues to accumulate of the overall hazards that pesticides pose to human health, it is important that health care providers consider the possibility and consequences of occupational, dietary and residential exposure to pesticides for their female patients. Occupational exposure is almost certainly the primary source of exposure for farm workers and their families (McDiarmid&Gehle 2006; WHO 2006). Awareness of the ways in which pesticide exposure occurs and the danger it poses are a crucial component of comprehensive preconception and prenatal care for farm worker women.

Most of the units are seriously concerned with workers health issues. Generally, they have their own OHS plans and policies, which they endeavour to, implement and follow. Following OHS issues, which require more attention are identified because without following these practices a proper assessment of the workers exposure cannot be made:

i. Most of the units are not carrying out the required monitoring of the working air quality, with respect to pesticides and solvents.

ii. Records of accidents and disease are not being properly maintained.

iii. iii. Many of the antidotes are not available readily in the market; this situation is not satisfactory to cope with emergency.

5. Local study

In Egypt the information on the impact of pesticides on health aspects of farm workers and pesticides dealers is lacking and base-line information needs to be generated so that risk exposure of farming community may be minimized. Hence, this book chapter is planned to explain how pesticides are dangerous for humankind, animals and food products; as well to determine pesticide residues in blood and their correlation with biochemical markers for assessment of adverse health effects on farmers, market workers and spray workers as well as to assess the level of knowledge on precautions of pesticides safety.

5.1. Materials and methods

The study was conducted from July 2009 to June 2010 in seven villages (El-Mahmodia, Met Tarif, ElYosifia, Deiarb, El-Daraksa, Hamada and Ali Hendi) located in Dekrnes, Meniate El-Nasr and Baniebad provinces, Dakahlyia governorate, north Egypt.

Basic design and sample size

Seventy healthy male individuals in age group of 30-55 year comprising of 30 farmers, 25 spray workers and 15 market workers were selected for the present study. The individuals selected had history of exposure to different classes of pesticides for 5 to 15 years. They were compared with 25 control individual residents of same area who had no history of pesticide exposure, either as farm worker or as pesticide dealer.

Field survey

All the individuals were provided a questionnaire seeking information on the types of pesticides they mostly used protective equipment or cloths during preparation and application of pesticides, concentrations recommended for pesticides use. In addition, the questionnaire elicited information about the re-entry period (the minimum amount of time that must pass between the times of application of pesticide and the time the farmers could go into the field without wearing personal protective equipment). The individuals selected included those who worked in both field crops and vegetables on the same ground but in different seasons.

5.1.1. Hematological effects

a. Sample collection

Fresh blood samples were collected from the arm vein (10 ml). Each blood sample was divided into three tubes, the 1st tube contained heparin for hematological assays. In the 2 tubes the blood sample was left for a short time to allow the blood to coagulate for biochemical analysis (aminotransferase (AST), Plasma alanine aminotransferase (ALT), acetyl cholinesterase (AChE), urea, creatinine and prothrombin time) and the 3rd tube contained blood sample for determination of pesticide residues.

b. Hematological analysis

The blood in heparinised ampoules was analyzed for white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb) and platelets (PLT) counts as per the method of Schalm (1986).

5.1.2. Biochemical analysis

Plasma was separated by centrifugation at 1500 rpm for 15 min. Serum enzymes and biochemical analysis were carried out by Medical Biochemistry Lab (Faculty of Medicine, MansouraUniversity, Mansoura, Egypt). Plasma alanine aminotransferase (ALT) and aspartate aminotransfersae (AST) activities were determined according to IFCC method Bergmeyer et al. (1998 a, b) while plasma acetyl cholinesterase (AChE) activity was measured as per Ellman's colorimetric method (Ellmann et al. 1961). Urea and creatinine concentrations were determined according to the methods of Sampson & Baird (1979) and Spencer (1986), respectively. Prothrombin time (PT) was measured according to method described by Dacie& Lewis (1984).

5.1.3. Pesticide residue analysis

a. Extraction from blood serum

Extraction of pesticide residues in the serum was as per the method of Rivas et al. (2001). Aliquots of 2.0ml serum samples of each individual and control were spiked separately by adding appropriate volumes of working standard solutions equilibrated for 3.0 h at room temperature in a test tube. Methanol (1 ml) was sequentially added to 2.0 ml sample by mixing in a rotary mixer for 1.0 min, then 2.5 ml n-hexane: diethyl ether (1:1 v /v) was added. The solution was agitated and collected, and the aqueous phase extracted twice with 2.5 ml n-hexane: diethyl ether (1:1 v/v). The combined organic phases were evaporated and concentrated to 1.0 ml in a graduated test-tube under a gentle stream of nitrogen.

b. Clean-up

Clean-up of the extract of pesticide residues in serum was performed according to the method of Mercedes et al. (2004). A florisil column of 200 x 12 mm topped with anhydrous sodium sulfate was prepared and eluated with n-hexane. The extracts of each sample were passed twice through it. Eluate containing pesticides was evaporated and dried completely under a gentle stream of nitrogen. The samples were dissolved in 1.0 ml *n*-hexane and then injected into GLC and HPLC systems.

c. Quantitative determination

The whole cleaned up extracts of organphosphorus and pyrothroid residues were performed by GLC (Hewlett Packard 6890 series) equipped with electron capture detector (ECD) under the following conditions: column: HP-17 (30 m x 0.32 mm x 0.25 μm film thickness), temperatures: column 240ºC; detector 350ºC and injection 320ºC. The quantitative analysis of carbamate pesticide residues was performed by HPLC (Agilent 1100 Series with workstation). UV Diod-array detector set at 220 nm and the analytical column Nucleosil-C18, 5 um (4 x 250 mm) was used. The mobile phase was acetonitrile-water at flow rate 1 ml min^{-1}. All solvents and chemicals used were of analytical grade free of interfering residues as tested by Gas chromatograph. The statistical significance of data was assessed by Duncan and Tukey tests at $p<0.05$ and $p<0.01$ (Snedecor& Cochran 1980).

5.2. Results

Field survey

The most frequently used pesticides by the subjects in this study are shown in Table 1. Inorganic compound, organophosphates, carbamates and pyrothrids are the most pesticides used in Egypt. Zinc phosphide was the most often used insecticide (97.14 %) followed by chlorpyrifos and malathion (94.3%). The study revealed that the majority of study subjects were not taking the necessary precautions to prevent hazards associated with their use (Tables 2 and 3). The results of survey revealed that 60.0, 6.7 and 12.0% of farmers, market workers and spray workers did not wear protective apparels (such as overall, boots, gloves, etc.). While 16.7, 60.0 and 80.0% of farmers, market workers and spray workers, respectively, wear overall only 10.0, 20.0 and 84.0% wear special boots and 6.7, 66.7 and 24.0% farmers, market workers and spray workers, respectively, wear gloves. The farmers did not use mask while 26.7 and 24.0 % of market and spray workers used masks. About 20 and 52 % of farmers and spray workers use hats, but pesticides marketing did not use them.

The survey revealed that the most of subjects were washing themselves after pesticides operation (Table 3). Also 83.3 and 86.7% of farmers and market workers smoke or drink and eat food during mixing or applying pesticides, while about 40.0% of pesticide sprayers practice these habits. About 16.7% farmers and 20.0% pesticide spray workers do have knowledge on re-entry periods. Majority of farmers and pesticide spray workers do not bother to read the pesticide labels and contrarily 80% pesticide market workers read labels. Interestingly 20.0, 13.3 and 40.0% of farmers, market workers and spray workers, respectively, reported that they re–used the pesticide containers, while the majority of farmers and spray workers (80.0 and 60.0 %, respectively) leave it in the field after use.

5.2.1. Hematological effects

No significant differences were observed between RBC counts in pesticides-exposed subjects and control group (Table 4). However, a significant decrease in hemoglobin [Hb] level (-12.1%) and platelet count (-6.6% below control level) was observed in pesticide-sprayer group. On contrary, a significant increase in WBC counts was noticed in pesticides market and spray workers groups (34.6 and 73.9% above the control level, respectively) as compared with control.

5.2.2. Biochemical effects

A slight insignificant increase was observed in AST and ALT activities in all the subjects (Table 5). Higher level of both these enzymes was observed in pesticide sprayers (8.41 and 34.41% above the control level, respectively). A significant decrease in AChE activity was observed in pesticide-exposed groups in comparison to control. High inhibition rate was observed in spray workers followed by market workers (-48.7 and –41.5%, respectively). On the contrary, a significant rise in urea concentration was noticed in spray workers (+50.0%), but no significant differences were observed in creatinine concentration. This study revealed a positive correlation between pesticides exposed with prothrombin time (PT). PT was significantly raised among the farmers, market workers and spray workers (12.0, 23.5 and 44.7% above the normal level, respectively).

Pesticide	Group	WHO category	Type of use	% *
Zinc phosphide	Inorganic compound	IB	Rodenticide	97.14
Chlorpyrifos	Organophosphorus	II	Insecticide	94.3
Malathion	Organophosphorus	III	Insecticide	94.3
Carbofuran	Carbamate	II	Nematicide	90.0
Abamectin	Avermectin	IB	Acricide	80.0
Lufenuron	Benzoylurea	U	IGR	80.0
Copper hydroxide	Inorganic compound	III	Fungicide	78.57
Lambdacyhalothrin	Pyrothrid	II	Insecticide	71.42
Profenofos	Organophosphorus	II	Insecticide	71.42
Mancozeb	Thiocarbamate	U	Fungicide	67.14
Brominal	Hydroxybenzonitrile	II	Herbicide	64.3
Pirimicarb	Carbamate	II	Insecticide	62.86
Fenpyroximate	Fenpyroximate	II	Acricide	61.42
Clodinafop - propargyl	Aryloxyphenoxypropionate	III	Herbicide	61.4
Maneb	Thiocarbamate	U	Fungicide	58.57
Chlorpyrifos-methyl	Organophosphorus	II	Insecticide	57.14
Thiobencarb	Thiocarbamate	II	Herbicide	57.1
Diflubenzuron (IGR)	Benzoylurea	U	IGR	54.29
Dicofol	Organochlorine	III	Acricide	54.29
Dimethoate	Organophosphorus Juvenile	II	Insecticide	50.0
Pyriproxyfen	hormone mimic	U	IGR	44.29
Methomyl	Carbamate	IB	Insecticide	42.86
Fluazifop-ρ-butyl	Aryloxyphenoxypropionate	U	Herbicide	42.9
Glyphosate	Glyphosate-diammonium	U	Herbicide	35.7
Fenvalerate	Pyrothrid	II	Insecticide	32.86
Thiamethoxam	Neonicotinoid	III	Insecticide	28.57
Acetamiprid	Neonicotinoid	II	Insecticide	25.71
Triazophos	Organophosphorus	IB	Insecticide	25.71
Alpha-cypermethrin	Pyrothrid	II	Insecticide	24.29

*Source: WHO (2005) classification: Ib = Highly hazardous, II = Moderately hazardous, III = Slightly hazardous, U = Unlikely to pose acute hazard in normal use. *% = Percent of most frequently by subjects.

Table 1. Pesticides frequently used by the subjects in this study

Precautions equipment	**Farmers (%)**	**Pesticide- market workers (%)**	**Pesticide-spray workers (%)**	Mean (%)
Wear overall	16.7	60.0	80.0	48.57
Special boots	10.0	20.0	84.0	25.70
Gloves	6.7	66.7	24.0	25.70
Mask	0.0	26.7	24.0	14.29
Wide hat	20.0	0.0	52.0	27.14

Table 2. The response of farmers, pesticide marketing and spray workers regarding wearing of protective equipment

Precautions	**Farmers(%)**	**Market workers(%)**	**Spray workers(%)**	**Mean(%)**
Wash after pesticide operation	66.7	93.3	88.0	80.00
Smoke, drink and eating food	83.3	86.7	40.0	60.57
Re-entry period	16.7	0.0	20.0	14.29
Read pesticide labels	36.7	80.0	24.0	41.43
Re-use pesticide containers	20.0	13.3	40.0	25.70
Didn’t follow precautions	16.7	6.7	12.0	14.29

Table 3. The response of farmers, pesticide market and spray workers regarding practices on safety measures during pesticide operations

Treatments	**RBCs (10^6)**	**HGB (mg)**	**Platelets (10^3)**	**WBCs (10^3)**
Control	5.02 a	13.74 a	299.0 a	4.68 c
Farmers	4.83 a (-3.78)*	14.04 a (+2.18)	240.2 ab (-19.67)	4.66 c (-0.43)
Market workers	4.7 a (-6.37)	13.42 a (-2.33)	263.4 ab (-11.91)	6.3 b (+34.6)
Spray workers	4.68 a (-6.77)	12.08 b (-12.08)	159.6 b (-46.62)	8.14 a (+73.93)
LSD 5 %	0.844	1.30	103.89	1.121

The figures superscripted with same alphabets in the same column do not significantly differ from each other as per Duncan test.

*The values in parenthesis are the percent content in comparison to the respective control.

Table 4. Effect of pesticide residues on hematological parameters of farmers, pesticide market and spray workers

Treatments	AST(u / ml^{-1})	ALT(u / ml^{-1})	AchE(u / ml^{-1})	Urea(mg / dl^{-1})	Creatinine(mg / dl^{-1})	Prothrombin Time (second)
Control	21.4 a	18.6 a	1874.6a	28.8 bc	1.25 a	10.96 d
Farmers	21.8 a(+1.8) *	22.4 a(+20.43)	1573.4b(-16.1)	23.4 c(-18.75)	1.12 a(-10.4)	12.28 c(+12.0)
Market - workers	23.2 a(+8.41)	22.6 a(+21.51)	1096.2 c(-41.5)	36.8 ab(+27.8)	1.21 a(-3.2)	13.54 b(+23.54)
Spray - workers	23.2 a(+8.4)	25.6 a(+34.4)	962.0 c(-48.7)	43.2 a(+50.0)	1.33 a(+6.4)	15.86a(+44.7)
LSD$_{0.005}$	11.2	14.76	165.11	12.72	0.298	0.99

The figures superscripted with same alphabets in the same column do not significantly differ from each other as per Duncan test.

*The values in parenthesis are the percent content in comparison to the respective control.

Table 5. Effect of pesticide residues on biochemical parameters of farmers, pesticide market and spray workers

5.2.3. Pesticide residues

The detection limits of pesticides ranged between 0.001 to 0.0025 μg ml$^{-1.}$Percent recoveries in reference samples were 82-93%. Accordingly, the sample analysis data was corrected for these recoveries. About 76.7, 92.5 and 100% of farmers, market workers and spray workers had varied levels of insecticide residues in their blood (Table 6). About 60.0 and 23.3% of farmers had chlorpyrifos and lambada-cyhalothrine (0.022 and 0.014 mg kg^{-1}) residues above the acceptable daily intake (ADI) in their blood. In addition, most of the pesticides market workers were observed to have multiple pesticide residues above ADI. About 80.0% of them had carbofuran residues and 73.3% had chlorpyrifos (0.217 and 0.137 mg kg^{-1}). All pesticides spray workers had high amount of residues detected in their blood; most of them had chlorpyrifos (84.0%), profenofos (72.0%), lambda-cyhalothrine (64.0%), pirimicarb (52.0%), carbofuran (28.0%) and triaziphos (24.0%) residues above the recommended ADI levels because of their extensive use. Further, about 23.3 and 7.5% of farmers and market workers, respectively, had no insecticide residues in their blood.

5.3. Discussion

The present study was carried out in some villages located in Dakahlyia Governorate, Egypt, where infestation level of pests is very high. Ever growing demand for enhancing crop production to meet the requirements of increasing population and the need to enhance farm income tends farmers to use pesticides excessively and irresponsibly. The major pesticides used (58.62%) by the farmers, market workers and spray workers in the study area include zinc phosphide, chlorpyrifos, malathion, carbofuran, abamectin, lufenuron and copper hydroxide. As per WHO (2005) most of pesticides used are classifies either highly or moderately hazardous.

Interviews showed that the majority of farmers and pesticide spray workers do not take the necessary precautionary measures to prevent hazards associated with their use. Also, the low-level education of study groups coupled with lack of good training of pesticides resulted in high exposure hazards (Tijani 2006; Damals et al. 2006). Similar results were obtained by Tchounwou et al. (2002) who reported that in Menia El-Kamh in Sharkia Governorate (Egypt) more than 95% of farm workers do not practice safety precautions during pesticide formulation and application.

Pesticides	**ADI * (mg / kg b.w **)**	**Farmers**			**Pesticides market workers**			**Pesticide spray workers**		
		%	Range mg / kg	Average mg / kg	%	Range mg / kg	Average mg / kg	%	Range mg / kg	Average mg / kg
Chloropyrifos	0.01	60.0	0.07 – 0.34	0.022	73.3	0.1 – 2.08	0.137	84.0	0.31 – 3.76	0.247
Malathion	0.3	33.3	0.009 – 0.26	0.016	53.3	0.09 1.78	0.097	72.0	0.16 – 2.44	0.183
Profenofos	0.01	36.7	0.03 – 0.178	0.008	53.3	0.3 – 1.67	0.088	72.0	0.26 – 2.67	0.208
Pirimicarb	0.02	40.0	0.007 – 0.09	0.004	33.3	0.05 – 0.95	0.034	52.0	0.47 – 1.8	0.117
Carbofuran	0.02	26.7	0.006 – 0.04	0.009	80.0	0.14 – 2.6	0.217	28.0	0.07 – 0.49	0.026
Lambdacyhalothrin	0.005	23.3	0.05 – 0.17	0.014	40.0	0.07 – 0.53	0.046	64.0	1.08 – 2.11	0.136
Methomyl	0.03	0.0	N.D ***	N.D	26.7	0.04 – 0.09	0.007	32.0	0.36 – 0.97	0.067
Triazophos	0.001	3.3	0.001	0.001	0.0	N.D	N.D	24.0	0.0005 – 0.004	0.002
Dimethoate	0.002	0.0	N.D	N.D	6.67	0.001	0.001	12.0	0.0007- 0.0017	0.001
Fenvalerate	0.02	0.0	N.D	N.D	0.0	N.D	N.D	12.0	0.004 – 0.01	0.008
Persons having no residues		**23.3**			**7.5**			**0.0**		

* Acceptable daily intake; ** b.W. = Body weight; ***ND= Not Detected

Table 6. The percent of farm workers, pesticide-market workers and pesticide-spray workers having different pesticide residues and mean concentration of residues (mg kg $^{-1}$ b.w.) in their blood

Our studies revealed significant changes in HGB level and platelets counts in pesticide spray workers and WBC counts in market workers and spray workers. The results are in agreement with Amr et al. 1997; Amr (1999), Abu Mourad (2005) and Al-Sarar et al. (2009) who reported that occupational exposure to pesticides resulted in alterations of hematological parameters. The increase in ALT and AST activities are good indicators of hepatic toxicity (Hall, 2001). The present study showed that insignificant elevation in liver functions (AST and ALT levels) of all study subjects. Similar observations have been made by Al-Sarar et al. (2009). Nevertheless, significant increase in the levels of these enzymes was seen in occupationally exposed workers (Tomei et al. 1998; Khan et al. 2008, 2009, 2010); and in sprayers of grape gardens in India (Patil et al. 2003). Plasma AChE activity has been used for several years as indicator to estimate the risks associated with pesticides induced toxicity in occupationally exposed workers (Dasgupta et al. 2007; Khan et al. 2008; Shalaby&Abd El-Mageed 2010). Present study revealed a negative correlation between pesticide residues with AChE activity. We found significant inhibition in its activity in exposed subjects than that of the control. High inhibition rate was observed in pesticide spray workers followed by market workers thereby indicating that the study subjects had pesticide residues in their blood especially organophosphorus and carbamate compounds. Similar results were noticed among the cotton growers in India (Mancini et al. 2005); in Palestinian farm workers (Abu Mourad 2005) and the tobacco farmers in Pakistan (Khan et al. 2008). In orchard farmers of Kashmir about 31.9% patients (124 out of 389) were orchard-farm workers and the orchard residents and orchard playing children had higher serum cholinesterase (<6334 U/l) level (Bhat et al. 2010).

In present study there was significant increase in urea concentration in pesticide sprayers, with no significant changes in creatinine level in all cases. Some previous studies have shown subtle nephrotoxic changes in workers occupationally exposed to pesticides with higher levels of creatinine and urea (Attia 2006; Shalaby 2006; Khan et al. 2008). Nevertheless, Al-Sarar et al. (2009) observed insignificant elevated levels of serum urea and creatinine among pesticide sprayers of RiyadhMunicipality, Kingdom of Saudi Arabia. In addition, this study revealed significant increase in prothrombin time (PT) in all cases studied. In general, this noticeable effect of pesticide upon blood coagulation could be attributed to the great defect in blood coagulability and to the severe damage of capillaries (Clarke & Clarke 1978; Leck& Park 1981).

Pesticide residues in blood are likely to appear at very low concentrations because as soon as it enters into the body most of the chemical may be metabolized and the metabolites may accumulate to induce toxic effect (Soomro et al. 2008). However, the ultra low quantities of contaminants present in body indicate toxicological impact on exposed population. Present results revealed that all pesticide spray workers had insecticide residues in their blood; however, 23.3 and 7.5% farmers and pesticides marketing workers did not have residues. In addition, the amounts of these residues are high in spray workers than its values in other groups, but these amounts are very low in farmers. The spray workers during spray on crops are directly exposed to pesticides while mixing, handling, spraying as well as through contaminated soil, air, drinking water, eating food and smoking at work places. Also, pesticides market workers are directly exposed to pesticides while handling and opening of pesticide containers in pesticide stores, but farmers are working in the field after pesticides operation. In our study, most of subjects had multiple pesticide residues above the ADI in their blood, which is injurious to health. Most of them had chlorpyrifos, profenofos, carbofuran and lambda-cyhalothrin residues. Similar results were obtained by Coye et al. (1986) and Khan et al. (2008) who reported that the tobacco farmers had multiple pesticide residues above ADI in their blood consisting of 63% methomyl; 56% thiodicarb; 62% cypermethrin; 49% imidaclprid; 32% methamidophos and 27% had endosulfan residues. Sosan et al. (2008) has reported that 42 out of 76 cacao farmers had residues of diazinon, endosulfan, propoxur and lindine in their blood. Similarly Bhat et al.(2010) has reported that 90.04% brain tumour patients (389 out of 432) in Kashmir were orchard farmers exposed to high levels of multiple types of neurotoxic and carcinogenic pesticides for more than 10-20 years.

Generally, controlled studies have shown mixed results about chemical insecticides and chemical fertilizers. We are looking for a solution for the actual problem with intensive insecticide uses. Some support the conclusion that organic production methods lead to increases in nutrients. Other studies show no demonstrable differences. A recent analysis conducted by the LondonSchool of Hygiene & Tropical Medicine provides a comprehensive review of the available literature (Dangour et al. 2009). The authors identified 46 studies with sufficient documentation and quality upon which they performed a systematic review. Eleven nutritional categories were evaluated. The nitrogen content of conventionally-grown plants was higher, and the phosphorus and titratable acidity levels were higher for organically-grown plants. These differences were considered biologically plausible due to differences in fertilizer use (nitrogen and phosphorus) and ripeness at harvest (titratable acidity). There was no difference for the remaining eight categories, including some key ones, including Vitamin C, phenolic compounds, magnesium, calcium, potassium, zinc, total soluble solids, and copper.

6. Conclusion

The present study revealed that most of persons studied did not take necessary precautionary measures to avoid the hazards associated with pesticides marketing, handling and application. Analysis showed that low level of education of users and lack of proper training about safe pesticide usages and handling resulted in high occupational hazards. Most of individuals studied had multiple pesticide residues above ADI in their blood which is injurious to health. The study suggests promotion of awareness among pesticide-users with right practices for safe use and handling of pesticides. Pesticides are absorbed by inhalation, ingestion and/or dermal contact so pesticide users should wear protective clothing. Also, they are highly advised to read pesticide labels before use. The role of government regulations and agriculture experiment services as well as the cooperation of pesticide manufactures of is vital in reducing the risks and hazards of pesticides.

Acknowledgements

Authors are thankful to Dr. Mustafa Neamat Allah, Assistant Professor, Faculty of Medicine, MansouraUniversity, for his immense support during this study. Also, we thank Dr. El-Sayed Ibrahim El-Sayed, Professor of Pesticides, National Research Centre, Egypt, for his assistance in preparation of this book chapter.

Author details

Nabil El-Wakeil[1,3*], Shehata Shalaby[1], Gehan Abdou[1] and Ahmed Sallam[2]

*Address all correspondence to: nabil.el-wakeil@landw.uni-halle.de

1 Pests & Plant Protection Dept. National Research Centre, Dokki, Cairo, Egypt

2 Plant Protection Dept. Faculty of Agriculture, SohagUniversity, Sohag, Egypt

3 Institute of Agric. & Nutritional Sciences, Martin Luther-University Halle-Wittenberg, Germany

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Predicting the Effects of Insecticide Mixtures on Non-Target Aquatic Communities

Alexa C. Alexander and Joseph M. Culp

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53356

1. Introduction

In this study two questions will be posed: firstly, how can single-species, single-compound toxicity test data on non-target aquatic insects predict patterns in stream communities exposed to the same compounds individually and jointly? Secondly, can mixtures of two or three insecticides be treated additively using a concentration addition, Toxic Unit (TU) approach in an aquatic community context? To evaluate these questions, the following studies examined the responses of field-collected benthic (bottom-dwelling) invertebrates exposed to mixtures of organophosphorus insecticides (chlorpyrifos and dimethoate) in detail as well as a preliminary investigation of the effects of adding a third insecticide to the mixture, the neo-nicotinoid (imidacloprid).

Non- target aquatic organisms are routinely exposed to pesticides because these compounds are widely used and are regularly detected during stream biomonitoring [1]. Mixtures of insecticides are particularly worrisome because these compounds can directly alter the abundance and diversity of aquatic insects; consequently, these effects can reshape aquatic food webs. Organophosphorus insecticides are particularly relevant for consideration because they are extensively used in agriculture worldwide and, for example, constitute ~ 40% of the insecticides applied in the United States [2]. In this study, two organophosphorus insecticides were selected, chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) and dimethoate (O,O-dimethyl S-[2-(methylamino)-2-oxoethyl] phosphorodithioate) to examine in detail because both are among the most commonly used in North America. Both are also routinely applied jointly or sequentially for the protection of more than 40 crops globally [2,3].

Chlorpyrifos and dimethoate are also highly toxic to non-target, aquatic species. According to van Wijngaarden *et al.* [4], the 48-h LC_{50} (median lethal concentration to affect 50% of the population) for chlorpyrifos on the non-target mayfly, *Cloeon dipterum* is approximately 1 μg/L and similarly, Baekken and Aanes [5], report that the 96-hr LC_{50} for the mayfly, *Baetis rhodani*, is in the range of 7 μg/L for dimethoate. The third insecticide, imidacloprid (1-((6-Chloro-3-pyridinyl)methyl)-N-nitro-2-imidazolidinimine), is also highly toxic to non-target aquatic species (e.g., the mayfly, *Epeorus longimanus* 24-h LC50 = 2.1 ± 0.5 μg/L, see [6]). Unlike chlorpyrifos and dimethoate however, the primary mode of action of imidacloprid is semi-permanent binding to the acetylcholine receptor rather than the ACh enzyme [7]. This difference may increase toxicity of the ternary mixture because all three insecticides bind the same enzyme and receptor system.

Organophosphorus insecticides are thought to primarily target the acetylcholinesterase (AChE) enzyme, preventing the removal of acetylcholine (ACh) by the enzyme from the post-synaptic gap [8]. Therefore, excessive acetylcholine is bound and continuous nerve signals are sent to cholinergic receptors, which can result in trembling, respiratory duress and ultimately death [8]. Notably, in order for most organophosphorus compounds to become toxic they must first be transformed into their active form, an oxon [9,10]. However, insecticides such as chlorpyrifos and dimethoate are chemically diverse and are able to interact with multiple metabolic pathways and targets. Therefore, indirect biochemical or ecological effects of these compounds may be responsible for observed differences in their toxicity [8,9,10].

In this study, two organophosphorous insecticides (chlorpyrifos and dimethoate) with the same primary mode of action were tested individually and jointly on a natural, macroinvertebrate assemblage using a toxic unit approach. The primary question asked was whether the joint-action of these two insecticides can be reasonably evaluated at a community level using additive assumptions of toxicity. This question was evaluated by determining the appropriate concentrations in toxic units of chlorpyrifos and dimethoate by compiling single-species toxicity test data for orders of insects commonly thought to be sensitive indicators in aquatic biomonitoring of streams and rivers namely, Ephemeroptera, Plecoptera and Trichoptera, or E.P.T. taxa. A 20 day artificial stream experiment was conducted where field-collected benthic (bottom-dwelling) macroinvertebrate assemblages were exposed to four toxic unit (TU) doses of either chlorpyrifos or dimethoate individually (control, 0.2, 0.4 and 0.8 TU) and two, 1:1 mixture doses (0.2 + 0.2 TU and 0.4 + 0.4 TU) of both insecticides applied jointly. Subsequently, responses in the benthos in a community were examined using Principle Components Analysis (PCA). Macroinvertebrate abundance, richness and guild structure was assessed using a factorial ANOVA and a chi-square (χ^2) approach to compare observed responses to control values as well as to predicted responses to treatment across a toxic unit gradient.

2. Methods

This 20-d study was conducted from 12 July to 2 August, 2007 at the Environment Canada mesocosm facility 10-km southeast of Fredericton (New Brunswick, Canada). Aquatic invertebrates were collected in the Nashwaak River (sampling location: 46º14294′N, 66º36722′W). The Nashwaak River is a relatively pristine tributary of the larger Saint John River and runs more than 100 km through forested and rural communities of less than 500 inhabitants in central New Brunswick.

Subsampled invertebrate assemblages were inoculated into 88 outdoor, artificial streams (Figure 1, see also [11,12]). Each partial flow-through stream was circular and had a planar area of 0.065 m^2 and a 10-L volume. Three treatments of organophosphorus insecticides ($n_{\text{replicates per treatment}}$ = 8) were examined in detail: chlorpyrifos (control, 0.2, 0.4 and 0.8 TU), dimethoate (control, 0.2, 0.4 and 0.8 TU) and a 1:1 mixture of both insecticides (0.1 + 0.1, 0.2 + 0.2 and 0.4 + 0.4 TU). An additional ternary 1:1:1 mixture of all three insecticides was also examined as a pilot study and included imidacloprid as well as chlorpyrifos and dimethoate (0.1 + 0.1 + 0.1 TU). Treatment solutions were housed in polyethylene reservoirs and manifolds were used to distribute the treatment solutions at uniform flow rates to each replicate stream. Groundwater from the extensive Saint John River aquifer was used to provide water to the artificial streams. Wastewater from each stream was passed through carbon filters (Culligan Inc.; activated carbon filter cylinder, Moncton, NB, CAN) to remove all contaminants before any water was discharged to the environment.

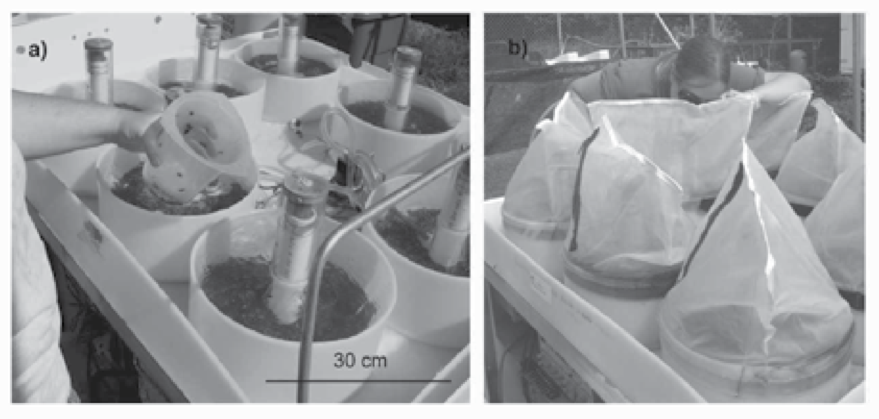

Figure 1. Cylindrical artificial streams. We inoculated 88 outdoor, artificial streams with a field-collected benthic invertebrate assemblage. Each flow-through stream was circular with a planar area of 0.065 m2 and a 10-L volume. In Fig. 1a, 8 streams were inoculated with gravel (coarse and fine) as well as 5 cobbles per stream. Protruding from the centre of each replicate stream is a motorized, rotating paddle that regulated the velocity of water in each stream. In Fig. 1b, streams post inoculation where each stream is covered with mesh to facilitate the collection of adult emergent insects.

2.1. Establishment of the aquatic community

2.1.1. Mimicking in-stream habitats

Prior to initiating the experiment, benthic substrates were introduced into each replicate stream. A realistic benthic substrate was created by inoculating each stream with a mixture of 25% fine gravel (2 - 4 mm) and 75% gravel (4 - 30 mm) that was obtained from gravel beds adjacent to the invertebrate sampling site on the Nashwaak River (Figure 1a). Cobblestones (7-10 cm) were also collected from this site with five stones randomly assigned to each replicate stream. Cobble and gravel were gently washed to remove any attached invertebrates while maintaining the periphyton community. This procedure established a lotic substrate consisting of a 2-3 cm layer of gravel-cobble plus surface stones that were covered with periphyton and was similar to the original habitat of the benthic community examined (Figure 1a).

2.1.2. Field collection

Benthic invertebrates were collected in a single riffle upstream of the gravel collection site on the Nashwaak River with U-nets (area = 0.06 m^2). The subsampling procedure consisted of the collection of twenty-five (25) U-nets collected 8 times by 5 samplers working systematically upstream within the riffle. Twenty-five U-nets were selected to slightly increase (~10%) the ambient density of aquatic invertebrates in the artificial streams, thus offsetting any mortality due to transport from the river to the test site. Each set of 25 U-nets were divided into 16 community subsamples with 5 reference subsamples from each set retained to determine the initial composition of the aquatic community. Streams were systematically inoculated with a subsample from each of the 8 sets of the 25 U-nets collected. Such that each of the 11 treatments levels (low, medium, high or chlorpyrifos, dimethoate, binary mixture, as well as a single comparison of a low ternary mixture and the control) received a portion of the same stream assemblages collected in the field (Figure 2).

2.2. Establishment of treatments

The 96-h LC50s (as 95% C.I.) were estimated for chlorpyrifos (4.68 – 5.69 μg/L) and dimethoate (23.96 – 26.57 μg/L) by curve-fitting single-species, single-compound toxicity test data compiled from public databases (U.S. Environmental Protection Agency Ecotox database [13], Figure 3). Appropriateness of doses was also assessed using tandem laboratory testing of chlorpyrifos and dimethoate on laboratory-reared *Chironomus tentans* and field-collected Heptageniidae mayflies from the Nashwaak River [14]. For imidacloprid (96-h LC50 0.8 – 3.1 μg/L 95% C.I.), where less data was available, appropriate doses were determined in comparison to previous artificial stream studies in our region [15]. Only genera of the orders Ephemeroptera, Plecoptera and Trichoptera (E.P.T. taxa) were included in the estimated riverine community 96-h LC50 (the median lethal concentration that will affect 50% of E.P.T. taxa) because the abundance of these insects is generally thought to be indicative of healthy streams and is widely used in stream biomonitoring [16].

Insecticide solutions were mixed in agricultural grade stock tanks, a 2000-L stock tank for chlorpyrifos, a 520-L stock tank for dimethoate and a 200–L stock tank of each component of the ternary mixture. All solutions were mixed using groundwater from the extensive Saint John River aquifer. Stock solutions of chlorpyrifos (70 μg/L) were made by serial dilution of Lorsban -4E© (NAF-163, Dow AgroSciences, Indianapolis, IN, USA). Stock solutions of dimethoate (200 μg/L) were made by serial dilution of Lagon 480E © (9382, United Agri Products Canada Inc., Dorchester, ON, Canada) and finally, imidacloprid (240 μg/L) by dilution of Admire 240® (Bayer CropScience, Calgary, AB, CAN). The insecticide-treated groundwater was delivered to one of eleven treatment reservoirs by positive displacement pumps (Viking Pumps, Pulsefeeder 25-H duplex pump, Cedar Falls, IA, USA). Secondary pumps then delivered the treatment solutions from each reservoir through a manifold to generate uniform flow rates into the base of each partial flow-through, replicate stream.

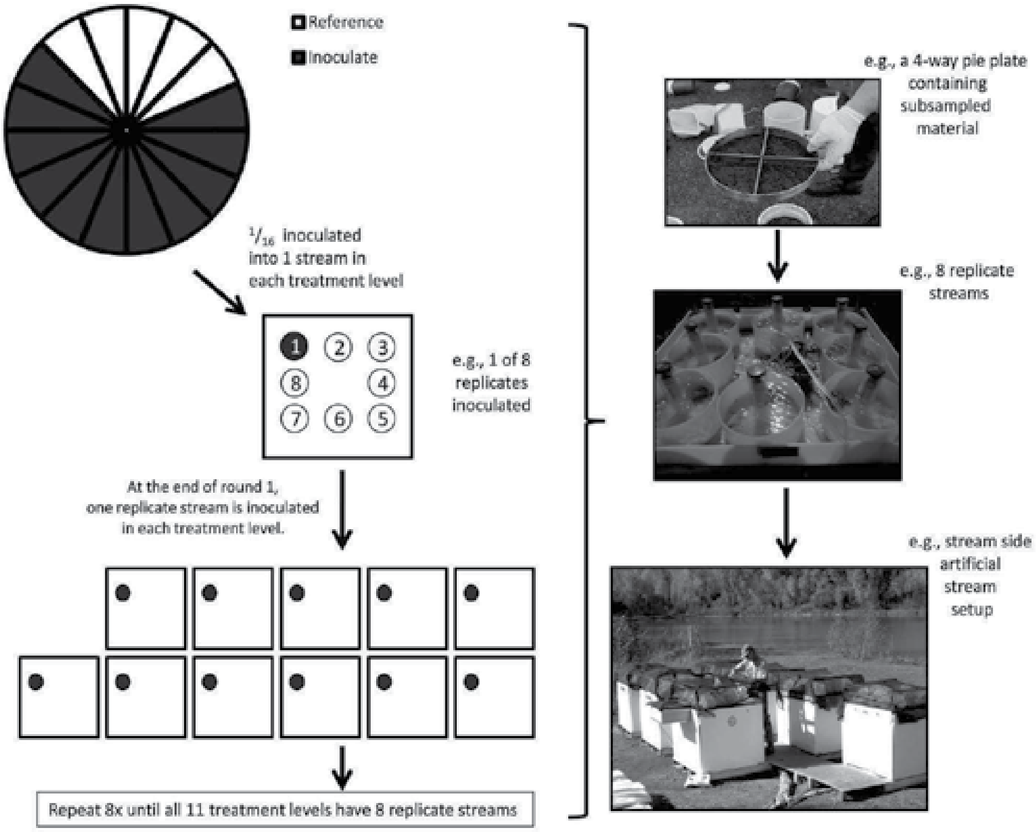

Figure 2. Benthic community subsampling and inoculation procedure for 88 replicate streams (11 treatments each containing 8 replicates). Sets of 25 U-nets (5 samplers collecting 5 U-nets each) were subsampled into 16 equal parts using a pie-plate made from 44 μm mesh. One sixteenth (1/16) of every 20 U-nets collected was inoculated into one replicate stream in every treatment level. This procedure was repeated eight times with each additional set of 25 U-nets systematically inoculated into adjacent replicate streams (one per treatment level). Thus, if the initial stream community had been significantly different in composition differences would have been allocated between treatments. Differences in community composition were not detected between subsamples (*Wilks-L* > 0.86; *P* > 0.99, in both cases).

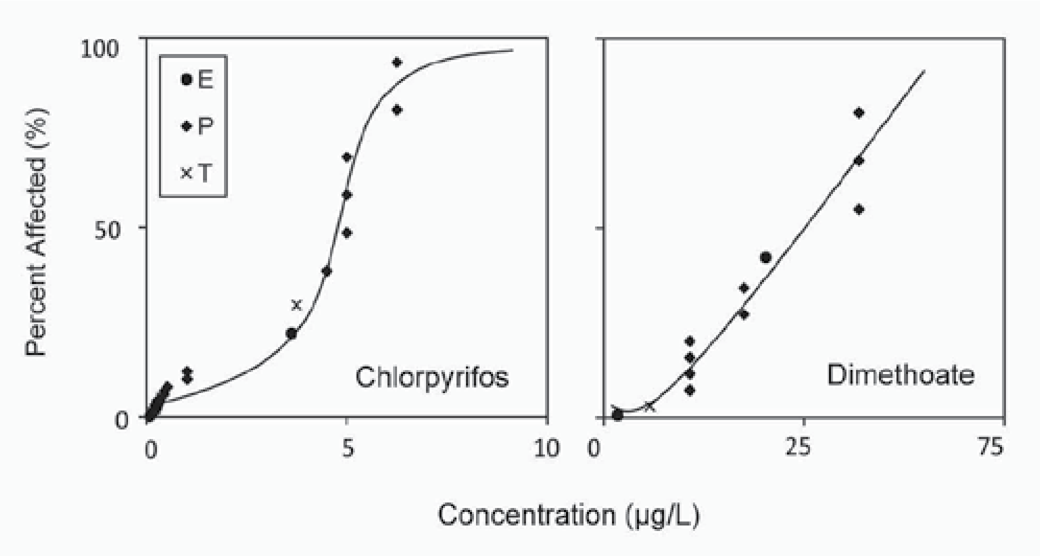

Figure 3. Percent Affected (96-h) of E.P.T. taxa as reported in the literature for the insecticides chlorpyrifos and dimethoate. For imidacloprid (96-h LC50 0.8 – 3.1 µg/L 95% C.I.), where less data was available, appropriate doses were determined in comparison to previous studies in our region [6,15]. Additional, tandem laboratory testing of chlorpyrifos and dimethoate on laboratory-reared *Chironomus tentans* and field-collected Heptageniidae mayflies from the Nashwaak River further corroborated dose selection [14]. Only genera of the E.P.T. Orders (Ephemeroptera, Plecoptera and Trichoptera) were used because the abundance of these insects is thought to be indicative of healthy stream conditions.

Chemical analysis determined the actual concentrations (Table 1) of the three insecticides individually and in mixture. Analyses were conducted at the National Water Research Institute (Environment Canada) in Saskatoon (SK, Canada) using a Waters 2695 Alliance HPLC System interfaced to a Micromass Quattro Ultima triple quadrupole mass spectrometer (LC-MS-MS) equipped with an electrospray ionization interface set to positive ion mode. For chlorpyrifos and dimethoate, chromatography was achieved using a Waters Xtera MS C_{18} (100 mm x 2.1 mm i.d., 3.5-µm particle size, Milford, MA, USA) analytical column and an aqueous acetonitrile mobile phase containing 0.1% formic acid (v/v). For imidacloprid, the mobile phase contained 40% aqueous acetonitrile and 0.2% formic acid (v/v). Water samples were collected in each treatment level on three occasions (July 13, 14, 17 in 2007) during the 96-h insecticide exposure period which began at noon on 13 July. Samples were collected in 500-mL amber vials (EPA vials, Fisher scientific, Fair Lawn, NJ, USA) and stored at 4ºC until shipment to Saskatoon for analysis. The samples were subjected to solid-phase (dimethoate) or liquid-phase (chlorpyrifos) extraction, the extracts taken to dryness, and the extract residue dissolved in deionized water (1.0 mL) prior to analysis by LC-MS-MS. All of the actual concentrations overlapped the target concentrations (Table 1) with an even distribution of under- and over- dosing for each target. Therefore, concentrations were comparable to those determined by laboratory bioassays in the published literature.

Treatment in Toxic Units (TU)	0.2 TU	0.4 TU	0.8 TU	
Target [chlorpyrifos]	0.94 – 1.14	1.87 – 2.28	3.74 – 4.55	
Actual [chlorpyrifos]	0.47 – 1.31	1.64 – 2.70	2.41 – 6.89	
Target [dimethoate]	3.79 – 5.31	9.58 – 10.63	19.17 – 21.26	
Actual [dimethoate]	1.04 – 4.80	9.32 – 12.07	19.93 – 22.96	
Target [imidacloprid]	N/A	N/A	N/A	
Actual [imidacloprid]	N/A	N/A	N/A	
Mixtures in Toxic Units (TU x n)	**0.1 TU x 2**	**0.2 TU x 2**	**0.4 TU x 2**	**0.1 TU x 3**
Target [chlorpyrifos]	0.24 - 0.57	0.94 – 1.14	1.87 – 2.28	0.24 - 0.57
Actual [chlorpyrifos]	0.19 - 0.86	0.78 – 1.61	1.39 – 4.02	0.12 - 0.38
Target [dimethoate]	2.40 - 2.66	4.79 – 5.31	9.58 – 10.63	2.40 - 2.66
Actual [dimethoate]	2.13 – 3.54	2.36 – 5.88	8.18 – 16.43	2.18 - 2.80
Target [imidacloprid]				0.24 - 0.57
Actual [imidacloprid]				0.47 - 0.69

Table 1. Comparison of treatments in toxic units (TU) with respect to the 95% confidence interval (95% CI) of the estimated range of targeted doses and the actual concentrations for chlorpyrifos, dimethoate and the 1:1 binary (x2) mixtures of chlorpyrifos and dimethoate compared to 1:1:1 ternary (x3) insecticide mixtures of chlorpyrifos, dimethoate and imidacloprid. All concentrations are in µg/L. Target concentrations for each insecticide are presented as ranges to reflect the uncertainty in the LC50 estimate.

2.3. Final data collection

At the end of the 20-d experiment, the streams were dismantled and the contents collected. Water samples, periphyton samples and invertebrates were collected from each replicate stream. Benthic macroinvertebrates were collected from each stream and preserved (10% formalin, transferred to 70% ethanol after 1 week) for subsequent laboratory sorting and identification using dissecting microscopes (Leica© Microsystems Ltd., Cambridge, UK). Aquatic specimens were sorted and identified to genus at the end of the experiment according to Environment Canada protocols, with a minimum of 20% of the collected material checked by a certified taxonomist to achieve 95% confidence in the identifications [17]. Some taxa were only identified to Order given time constraints and available expertise (e.g., Oligochaeta, Nematoda, Gastropoda, Collembola and 1st instar Plecoptera). Guilds were inferred from the literature in order to infer the habits of organisms [16,18]. Adult insects were also collected over the course of the 20-d experiment in 2-d intervals and in some cases were used to corroborate the presence of cryptic genera.

2.4. Statistical approaches

Community responses were examined in the factorial portion of the experiment (chlorpyrifos x dimethoate) using Principal Components Analysis (PCA) because the data were continuous with respect to both of the treatment level factors of interest (e.g., actual concentrations of insecticides) as well as the density of in-stream macroinvertebrates [19]. A correlation matrix was used to prevent the different variances in the variables to influence the analysis. Responses in different taxa and guilds were also examined using factorial ANOVA (for chlorpyrifos and dimethoate only) and chi-square (χ^2) approaches. In this study, factorial ANOVA approaches examined response variables with respect to explicit treatment categories: a gradient of toxic units (TU, throughout); different insecticide treatments (I) and the interaction between the dose and the insecticide treatments (TU x I). Post-hoc testing, where applicable, was conducted using 1-tailed Dunnett's tests [20] and compared specific treatments to control levels (ANOVA approach, marked 'a' in corresponding figures). Where necessary (e.g., total and scraper abundance), data were transformed to satisfy assumptions (ln transformation, [21]). Whether the treatments initiated predictable reductions in abundance (of taxa, groups or guilds) was examined by comparing observed differences to those expected (or predicted) using chi-square (χ^2) tests. Expected values were determined by calculating the predicted reduction compared to control values for each invertebrate metric, in abundance from the toxic unit treatment range. Predicted values with respect to control appear throughout and significant deviations from predicted values by the χ^2 approach are marked 'c' in the corresponding figures. Preliminary comparisons of differences between the low binary (0.1 TU x 2) and low ternary (0.1 TU x 3) mixtures (1-way ANOVA) are also made for the six response variables of interest with respect to control, predicted, binary and ternary mixture treatment levels. To simplify, although differences in density per cm^2 were tested for significance, the responses are shown as the percent reduction in response between the ternary and the binary mixtures at 0.1 TU.

3. Results

3.1. Responses to treatment with chlorpyrifos and dimethoate

Principal Components Analysis (PCA) of the 38 genera and 5 orders of benthic macroinvertebrates identified in this experiment were highly responsive to increasing TU treatment and responded differently to treatment with either chlorpyrifos or dimethoate (Figure 4). Factor 1, (Eigenvalue 7.08, 44.3% of variance) was composed of the combined loadings of treatment in toxic units (TU, Pearson's $r = 0.34$) as well as the action of chlorpyrifos (Pearson's $r = 0.58$) or dimethoate (Pearson's $r = -0.15$). Increased insecticide treatment in Toxic Units (TU) reduced the breadth of taxa present in the community assemblage, as indicated by the decreased variation in the distribution of taxa and guilds from left to right along the horizontal axis (Factor 1 in Figure 4). Interestingly, community responses to treatment with either chlorpyrifos or dimethoate were in opposing directions, although both insecticides were important contributors to the distribution of taxa, guilds and treatments in Factor 2 (Eigenvalue 2.36, 14.7%; TU, Pearson's $r = 0.01$; chlorpyrifos, Pearson's $r = -0.31$; dimethoate, Pearson's $r = 0.29$). In particular, chlorpyrifos was an important contributor to the removal of taxa with streams treated with 0.8 TU of chlorpyrifos (C0.8TU) occurring in the PCA quadrant with the fewest taxa (bottom right, Figure 4). By contrast, responses to treatment with dimethoate occurred in the opposite quadrant suggesting firstly, that different members of the benthic macroinvertebrate assemblage were responding to chlorpyrifos versus dimethoate, and that treatment with dimethoate did not decrease density and diversity of taxa as forcefully as treatment with chlorpyrifos (top left, Figure 4). Interestingly, medium dose mixture treatments (M0.4TU) are located in the same quadrant as the equivalent dimethoate treatments (e.g., D0.4TU and D0.8TU) whereas high dose mixtures (M0.8TU) were more closely associated with predictions of additive toxicity in toxic units (Factor 1).

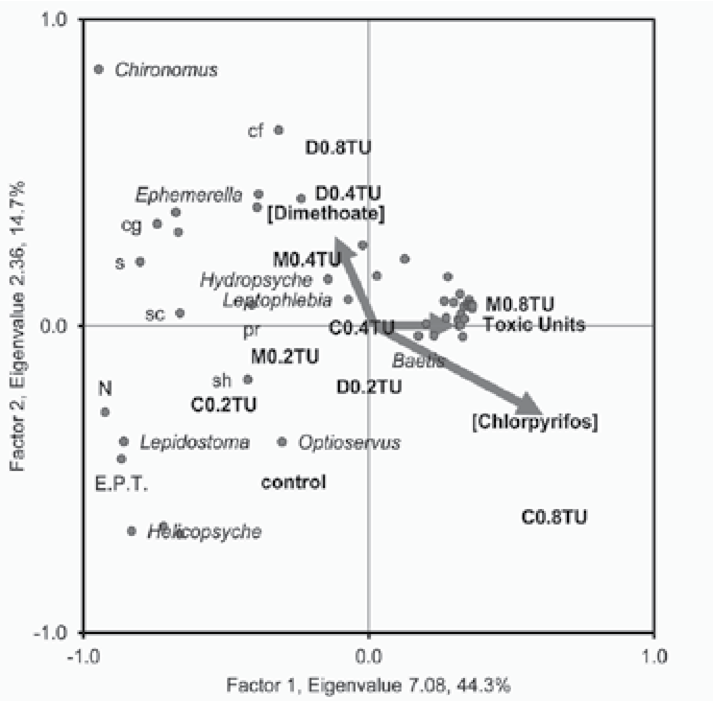

Figure 4. Principal Components Analysis (PCA) of differences in responses of 38 genera and 5 orders of benthic macroinvertebrates (each indicated, •) associated with chlorpyrifos or dimethoate insecticide treatment in Toxic Units (as vectors, above). Each treatment level is indicated (e.g., C0.2 TU, Chlorpyrifos at 0.2 TU). Factor 1 explained 44.3 % of the variance in the assemblages and was primarily driven by increased insecticide treatment in Toxic Units and secondarily by chlorpyrifos treatment. Dimethoate treatment was associated with different assemblages predominantly contributing to pattern in Factor 2 which explained an additional 14.7 % of the variance. Additional notes: guilds are indicated by codes cf = collector-filterers; cg = collector-gatherers; sc = scrapers; sh = shredders; pr = predators; total abundance per cm^2 = N; total richness per cm^2 = s; E.P.T. = sum density of Ephemeroptera, Plecoptera and Trichoptera orders. Remaining labels indicate genera of aquatic insect taxa (e.g., *Chironomus* spp.).

Significant change in measures of average total density per cm^2 and average taxa richness per cm^2 (Figure 5) were only found at the highest dose of chlorpyrifos tested (0.8 TU, abundance or richness, $P < 0.01$). The highly significant interactions (total density, TU x I, $F_{5,\,69} = 68.23$, $P < 0.01$; or richness, TU x I, $F_{5,\,69} = 709.03$, $P < 0.01$) were the result of total density and richness being decreased as predicted under exposure to chlorpyrifos, while dimethoate had no such effect. Throughout this study, dimethoate was non-toxic with respect to total density and richness and no negative effects of insecticides were detected irrespective of dose.

Additionally, mixture treatments were not different than control levels for either total density or richness (e.g., total density in M0.8TU, $P = 0.97$; richness in M0.8TU, $P = 0.75$). Stream communities were significantly more dense than predicted in high dose treatments containing dimethoate including the high mixture (M0.8TU, $\chi^2_{\ 7} = 20.24$, $P < 0.01$) and the high dimethoate treatment (D0.8TU, $\chi^2_{\ 7} = 16.90$, $P < 0.01$). In contrast, taxa richness was not found to be significantly different than predicted.

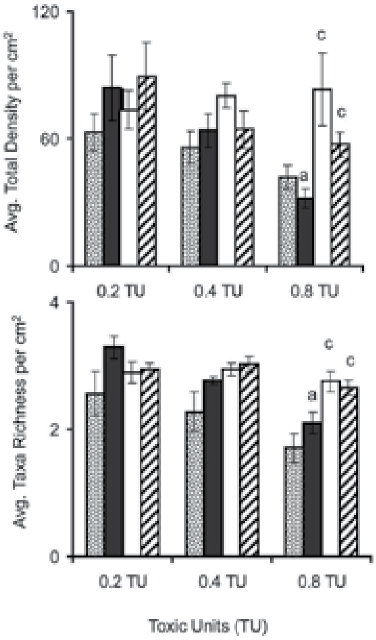

Figure 5. Total abundance and richness per cm^2 (± 1 SE, n = 8) of aquatic macroinvertebrates compared to treatment with the insecticides chlorpyrifos (black bars), dimethoate (white bars) or a 1:1 mixture of both insecticides (patterned bars). Letters indicate: 'a' significant differences compared to control (ANOVA approach), and 'c' differences in specific treatments (χ2 approach).

Responses in the average density of E.P.T. taxa and *Chironomus* spp. per cm^2 (Figure 6) were only found to significantly differ from control values in the highest chlorpyrifos treatment level (0.8 TU, E.P.T. or *Chironomus*, $P < 0.01$). Highly significant interactions were evident (E.P.T., TU x I, $F_{5,\ 69} = 53.91$, $P < 0.01$; or *Chironomus*, TU x I, $F_{5,\ 69} = 50.02$, $P < 0.01$) because density of E.P.T. and *Chironomus* decreased due to chlorpyrifos but not due to dimethoate. However, *Chironomus* midges were highly negatively affected by 0.8 TU of chlorpyrifos and the mean density of larvae in this treatment level was reduced 96% compared to controls (predicted decrease at 0.8 TU = 40%; C0.8TU, $\chi^2_{\ 7} = 31.45$, $P < 0.01$). E.P.T. taxa were highly sensitive to high dose treatment with chlorpyrifos (C0.8TU, $\chi^2_{\ 7} = 12.75$, $P < 0.01$), however, treatments containing dimethoate (e.g., dimethoate and mixture) were much less toxic than predicted (e.g., mean E.P.T. density in 0.8TU mixture, 37 % greater than predicted).

Scraper density was not different than the control, although predators were highly responsive to all high dose insecticide treatments ($P < 0.01$, Figure 6). Once again, significant interactions were found for both guilds (scrapers, TU x I, $F_{5,\,69} = 12.46$, $P < 0.01$; predators, TU x I, $F_{5,\,69} = 26.35$, $P < 0.01$). However, the extent of significant interactions in scraper genera appeared to be largely due to the high variation in the density of the guild in the low dose, chlorpyrifos treatment (0.2 TU). Doses of 0.2 to 0.4 TU of chlorpyrifos and 0.2 TU of dimethoate all contained more scrapers than predicted (e.g., 74 % greater than predicted scraper density in chlorpyrifos 0.2 TU, $\chi^2{}_7 = 50.03$, $P < 0.01$). In contrast, responses in predators were unique in that they responded to high insecticide doses (0.8 TU) by significantly decreasing abundance in these treatments, irrespective of the insecticide applied (e.g., 0.8TU mixture, 46 % less than predicted, $\chi^2{}_7 = 28.38$, $P < 0.01$). Finally, the bell-shaped abundance pattern in predators with increased dimethoate treatment, compared with the linear decrease in abundance of the chlorpyrifos treatment, suggests that responses in predators were more complex than in other groups, potentially as a result of indirect effects due to reduced prey density.

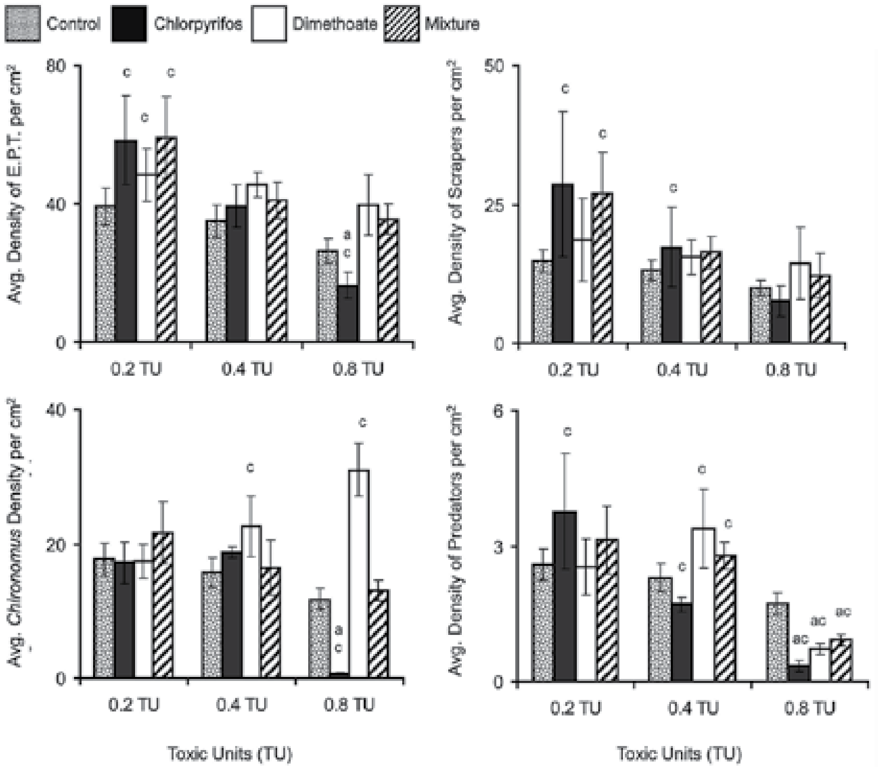

Figure 6. Density of E.P.T., Chironomus spp., scrapers and predators per cm^2 (± 1 SE, n = 8) compared to treatment with the insecticides chlorpyrifos (black bars), dimethoate (white bars) or a 1:1 mixture of both insecticides (patterned bars). Letters indicate: 'a' significant differences compared to control (ANOVA approach), and 'c' differences in specific treatments (χ2 approach).

3.2. Preliminary findings comparing binary and ternary mixtures

Statistical comparisons of the differences in density between binary (0.1 TU x 2) and ternary (0.1 TU x 3) mixtures of insecticides determined that the average total density ($P = 0.02$), taxa richness ($P < 0.01$) and *Chironomus* spp. ($P < 0.01$) were all significantly reduced due to the addition of imidacloprid to the mixture (Figure 7). In contrast, the average density of E.P.T. genera, scrapers and predators were not found to be significantly reduced in the presence of imidacloprid ($P > 0.06$, all cases). On average, the addition of a third insecticide resulted in a 62.9 ± 13.0 % reduction in average density. Density was more greatly reduced in some groups than others with scrapers the most affected (-111.6 ± 16.9 %) and taxa richness the least affected (-18.2 ± 16.5 %).

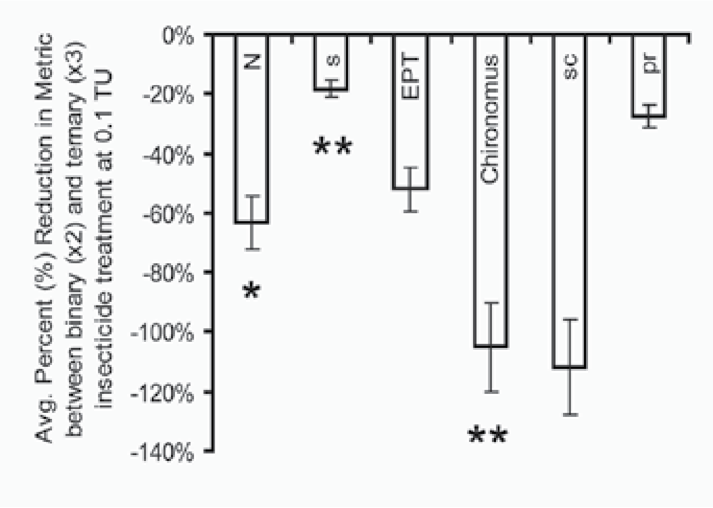

Figure 7. Comparison of % reduction in metrics due to treatment with the ternary mixture of 0.1 TU versus the binary mixture with the same doses. Each 0.1 TU dose should reduce the density of sensitive taxa by 5% because 1 TU = LC50. Therefore, reductions greater than 5% in the density of aquatic taxa is of biological interest even if differences in the density of organisms were not found to be statistically significant.

4. Discussion

4.1. Responses to chlorpyrifos and dimethoate

All of the metrics of benthic invertebrate responses measured also had significant interaction terms (TU x I, $P < 0.1$) suggesting that not all taxa, groups or guilds were equally sensitive to insecticide treatment. Differential toxicity within the organophosphorus insecticides has been reported previously and is predominantly due to the complexity of the biochemical pathway to reach what is considered the primary target, acetylcholinesterase [8,9,10]. Specifically, the toxic potency of organophosphorus insecticides depends on the creation of an oxygen analogue (oxon) via metabolic bioactivation, creating an excretable endproduct which is also potentially toxic [9]. It is the oxon that binds acetylcholinesterase (AChE) and prevents the capture and removal of acetylcholine in the synapses, creating a positive feedback loop whereby uncontrolled neural signalling is initiated. Therefore, increased or decreased toxicity, even from the standpoint of a single mode of action (AChE), is due to the interaction of at least five factors: firstly, in/efficient creation of the oxygen analogue (oxon), i.e., differences in basal metabolism; secondly, insufficient binding of the target esterase(s) and/or binding to alternative targets; thirdly, insufficient accumulation of acetylcholine in the synaptic gap, due to inherent neurochemical differences or deficiencies, e.g., Myasthenia gravis; fourthly, other forms of tolerance and/or resistance, e.g., species, strain or regional differences (e.g., as reported in [22]), and finally, excretion and/or uptake efficiency of the parent toxicant or its metabolites. Furthermore, organophosphates also bind other receptors (e.g., muscarinic and nicotinic receptors), which in themselves can up or down regulate the effectiveness of the insecticide dose [23].

Despite the equivalent toxic unit doses employed in this study, treatment with dimethoate was associated with increased abundance of different taxa and guilds with the exception of predators, which were found to be substantially negatively impacted by all high dose treatments. In mixture treatments, the density of taxa often fell between that of either of the two insecticides individually, or, resembled the relatively non-toxic dimethoate at 0.4 and 0.8 TU. The highly significant declines in abundance of different taxa and guilds due to chlorpyrifos treatment, and the lack of similar findings due to dimethoate treatment are troubling because this study determined the appropriate doses from standard bioassays of the same genera from public databases of the published literature. For instance, according to a Norwegian study by Baekken and Aanes [5], the 96-hr LC_{50} for *Baetis rhodani* exposed to dimethoate was ~ 7 μg/L. In this study *Baetis* not only survived but emerged as adults (37 females and 26 males, not shown) in the 0.8 TU treatment where the dimethoate concentration was in the range of 19.93 – 22.96 μg/L. Disparities such as these invite speculation. If regional differences in sensitivity are as pronounced as the above finding suggests, then modeling may be restricted to more local scales. Alternatively, regional variation in data quality also invites speculation.

This study generally found that the mixture pattern at high doses had intermediate toxicity. Specifically, invertebrate responses to the binary mixtures were between that of dimethoate or chlorpyrifos individually. LeBlanc *et al.* [14] also found mixtures of chlorpyrifos and dimethoate to exhibit dose-level dependency in concurrent laboratory studies using chlorpyrifos and dimethoate in both binary mixtures (i.e., low dose antagonism to high dose synergy). Although high dose exposures are likely less common than sublethal effects (as described in [24]), high dose synergy is a concern because isolated high-dose events (e.g., a rain event) could significantly alter the composition of aquatic communities. Additionally, in more complex mixtures where multiple modes of action may be the norm, the concentration that initiates a synergistic effect may be lower than implied from bioassay results using single-species and single compounds.

4.2. Preliminary findings for responses in binary versus ternary mixtures

In this study, the addition of a third insecticide at 0.1 TU resulted in an average reduction in invertebrate density of approximately 60% (-62.9 ± 13.0%). However, the addition of 0.1TU of imidacloprid should, in theory, only result in a reduction of 5% in the abundance of organisms because 0.1 TU equals the 5% median lethal concentration or the LC5. Therefore, average density was reduced 50% more with the addition of one more insecticide to the mixture despite the addition occurring at what would otherwise be considered a very low dose. The implication of these findings is that the presence of imidacloprid in a mixture, an insecticide with a similar mode of action to chlorpyrifos and dimethoate, may cause significantly greater than additive reductions in invertebrate density in naturally occuring assemblages such as those tested in this study. These findings are similar to those of Leblanc *et al.* [14] where the combined action of imidacloprid resulted in greater than additive toxicity of mixtures of the same insecticides used in this study.

Although we did not detect significant differences when comparing the density of predators in low dose binary versus ternary mixtures, responses in groups such as predators continue to be of interest because of the importance of certain feeding groups in food webs (e.g., see [25]). For predators, the average percent reduction in density was -27.4 ± 9.9% at a dose that in theory will cause a 15% reduction in density (0.3 TU = LC15). However, if the addition of one insecticide can cause (at best) a 30% reduction in density, then what effects are likely for more complex mixtures acting on highly interconnected aquatic communities? Gilliom has previously reported that mixtures of up to 5 insecticides are routinely found in the environment [1]. If the patterns found in this study are true of more complex mixtures, then 5 insecticides at 0.1 TU could remove more than half the invertebrate population (> LC50) at individual doses that are thought to cause a mere 5% reduction in density. Clearly, further study of the effects of mixtures on keystone species, such as predators, will be important for untangling community responses to multiple stressors.

4.3. Implications to additive models: a biological argument

It is questionable whether additive predictions of responses can be made for these insecticides despite having the same (or similar) primary modes of action. Clearly, chlorpyrifos and dimethoate were not sufficiently similar in their actions on organisms in the community assemblage studied here to warrant additive treatment, even though their effects may be similar *in vitro*. In this study, dose-level dependency and genus or guild specific differences were the norm. Therefore, although the use of additivity to predict effects of insecticide mixtures has the appeal of simplicity, pest managers and regulators may be better informed by focused study of common mixtures of multiple compounds on relevant assemblages of organisms. Differences in sensitivity and tolerance may be region or system specific due to the predisposition of different populations to up or down-regulate the production of alternative substrates to which these insecticides can bind [9,26,27].

Thus, arbitrary grouping of two similar insecticides based on their primary mode of action, is inappropriate, particularly in an ecological context. Although grouping organophosphorus insecticides to model responses additively has been demonstrated to be appropriate chemically (as in [28,29]), there appears to be little empirical evidence to support the uniform toxicity, or activity of organophosphorus compounds in biota (see [9]). Rather, non-additive responses appear to be the norm in real systems, perhaps because effects in real systems are mediated by biotic filters such as trait-mediated indirect effects [30,31]. We suggest that grouping these compounds into potency subclasses, as first suggested by Mileson *et al.* [23] will aid modelling efforts to overcome dose dependent effects of similar mixtures with variable potency. This is particularly warranted because dose-dependency appears to be a common mixture pattern [32]. Although concentration addition is widely thought to be a conservative approach to modelling impacts in streams (as in [33]), regional differences in sensitivity, or alternatively data quality, will reduce the usefulness of additive models. Finally, current toxicological models such as concentration addition and independent action, do not consider biological interactions between species. Interactions between species in a community can increase or mask organismal responses to stress and may be more important than isolated laboratory responses for the prediction of community level patterns.

5. Conclusions

In this study, when chlorpyrifos and dimethoate were both applied these mixtures were often intermediately toxic to aquatic invertebrates with the exception of predators that were severely impacted by all elevated insecticide treatments. In contrast, ternary mixtures were generally more toxic than expected and predators were highly affected even at the very low doses tested. Although only an additional 0.1 TU (= LC5) was added of a third insecticide, imidacloprid, responses in the density of different benthic macroinvertebrate metrics were reduced on average by more than 20%. From a community standpoint, it is apparent that different taxa and guilds within the macroinvertebrate community tested were not equally sensitive to treatment with different insecticides despite the use of equivalent toxic unit doses drawn from published bioassays on the same genera of aquatic insects as those examined in this study. As such, additive assumptions of toxicity in a community context are questionable. This is particularly true given that the interactions between species are rarely measured in ecotoxicology and thus, significant biological effects are likely ignored. Pest managers and regulators concerned with the impact of complex mixtures on naturally occurring communities may be better informed by focused study of common mixtures of multiple compounds on locally and regionally relevant assemblages of organisms than predictions derived from laboratory based mode of action models.

Acknowledgements

Many thanks to Kristie Heard (Environment Canada, Fredericton) for her assistance with the taxonomy and subsampling procedure, to Dave Hryn (Environment Canada, Fredericton) for his technical expertise and assistance in conducting the artificial stream experiment, and to Jon Bailey (Environment Canada, Saskatoon) who conducted the chemical analyses.

This work was made possible by the support of Environment Canada and a grant from Health Canada's Pesticide Science Fund. Further support was provided by a National Sciences and Engineering Research Council (NSERC) Discovery Grant to JMC and a NSERC (PGS-D3) to A.C. Alexander.

Author details

Alexa C. Alexander[1,2*] and Joseph M. Culp[2,3]

*Address all correspondence to: alexa.alexander@unb.ca

1 Canada Centre for Inland Waters, Environment Canada, Burlington, ON, Canada

2 Department of Biology at the University of New Brunswick, Fredericton, Canada

3 National Water Research Institute, Environment Canada, Fredericton, NB, Canada

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Chapter 4

Physiological Dysfunction in Fish After Insecticides Exposure

Mahdi Banaee

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/54742

1. Introduction

A fundamental contributor to the Green Revolution has been the development and application of insecticides for the control of a wide variety of insectivorous and herbaceous pests which would otherwise diminish the quantity and quality of food production (Ecobichon, 2001). Unfortunately, in spite of its advantages, chemistry has great disadvantages as well. Insecticides are threatening the long-term survival of major ecosystems by disruption of ecological relationships between organisms and loss of biodiversity. On the other hand, agriculture, as the largest consumer of freshwater and as a major cause of reduction of surface and groundwater resources through erosion and chemical runoff, are close related to the loss of water quality (Wauchope, 1978). The processing industry associated to agriculture is also a significant source of organic pollution in most countries. Conventionally, in most countries, all types of agricultural practices and land use, including animal farming, are treated as non-point sources. The main characteristics of non-point sources are not simply determined or controlled directly and therefore, are difficult to regulate their impacts on ecosystem health (Garcia et al., 2001). Non-point source pollutants are mainly transported overland and through the soil by runoff (Dubus et al., 2000). These pollutants ultimately find their way into groundwater, wetlands, rivers and lakes and, finally, to oceans in the form of sediment and chemical loads carried by rivers (Albanis et al., 1998; EPA, 2003; Aydın and Köprücü, 2005). The major insecticides that are usually applied in agriculture and public healthy sections include organophosphate, organocholorines, pyrethroids and carbamate. Contamination of water by insecticides is mainly due to intensive agriculture combined with surface runoff and subsurface drainage, usually within a few weeks after application (Banaee et al., 2011) Most of insecticides have been known to be highly toxic to non-target organisms that inhabit natural environments close to agricultural fields. Several studies reported that some of surface waters and surrounding environments were contaminated with different insecticides (Arjmandi et al., 2010; Bagheri et al., 2000; Ghassempour et al., 2002; Rahiminezhhad et al., 2009; Tarahi Tabrizi, 2001). The continuous presences of insecticides are the consequence of application (timing, rate, frequency) and the rainfall during the application period (Lydy and Austin, 2004; U.S.A. EPA, 2005; Bouldin et al., 2007; Mast et al., 2007; Echols et al., 2008; Vryzas et al., 2009; Werimo et al., 2009; Ding et al., 2011; Hope, 2012). Although monitoring the presence of insecticides in surface water and ground water are generally poor in much of the world and especially in developing countries, the effect of these pollutants on aquatic animals' health frequently was investigated (Chambers et al., 2002; Richards and Kendall, 2002;Lam and Wu, 2003; Scott and Sloman, 2004; Cengiz, 2006; Box et al., 2007; Sun and Chen, 2008; Banaee et al., 2008; Banaee et al., 2011; Banaee and Ahmadi, 2011).

Since fishes are important sources of proteins and lipids for humans and domestic animals, so health of fishes is very important for human beings. Fish like other aquatic organisms may be exposed to a great range of insecticides during the course of their life cycle. In fish, different insecticides can be absorbed through gills, skin or alimentary ducts (Schlenk, 2005; Banaee et al., 2011; Banaee, 2012). Fishes are particularly sensitive to environmental contamination of water. Hence, pollutants such as insecticides may significantly damage certain physiological and biochemical processes when they enter into the organs of fishes (Banaee et al., 2011). So, the effects of insecticides on fishes are of great concern.

Recently, many studies have been conducted to determine the mechanisms of insecticides' damage in fishes, with the ultimate goal of monitoring, controlling and possibly intervening in xenobiotics exposure and its effects on the aquatic ecosystem. The main mechanism of action of organophosphate and carbamate insecticides is block of enzyme acetylcholinesterase action that results in signs and symptoms of intensive cholinergic stimulation. Organochlorines are neurotoxins which effect on sodium and potassium channels in neurons. Decrease of potassium permeability and inhibition of cadmodulin, Na/K and Ca-ATPase activity occur by organochlorine insecticides. Pyrethroids can block Na channel and effect on the function of GABA-receptors in nerve fiber. Oxidative stress is another mechanism for toxicity of insectisides resulting in cell death includescellular necrosis and apoptosis and dysfunction in cellular physiology include alterations in metabolic and vital functions of the cells.Hence, fish should be able of managing environmental exposure by detoxifying these xenobiotic. In order to do this task, fish like aerobic animals have evolved complex of detoxification system, composed of two main parts including enzymatic and non-enzymatic components. Theroles of enzymatic and non-enzymatic detoxification system of animal's body lessen the potential damages caused by the toxicity of environmental pollutants. Although it is possible that reactive oxygen species (ROS) produced during the insecticides detoxification process in liver tissue may react with vital macromolecules such as lipid, protein, carbohydrate and nucleic acid and result in oxidative damage to aquatic organisms (Üner et al., 2006). ROS derived damage to natural and structure cellular components are generally considered a serious mechanism involved in the physiological and pathological disorders (Sepici-Dinçel et al., 2009).So, much literature suggests an association between impaired detoxification and disease such as oxidative stress, histopathological damages, hormonal dysfunctions, reproductive disorders, immunosuppression syndrome and etc.

Hematological and biochemical studies, along with histopathology, are the major means to learn a toxicants mode of action. Hematological and clinical chemistry parameters can be detected rapidly and hence can be used for prediction and diagnosis of insecticides toxicity. Alterations in these parameters show toxic stress in the treated animals especially on blood and blood-forming organs. Alterations in blood biochemical parameters as important diagnostic tool can be used for the detection of abnormalities in the liver and other tissues (Banaee et al., 2011). This chapter describes some of the important hematological, biochemical and histopathological changes that occur in fish exposed to different insecticides. An initial description is given of the detoxification process, and then the other physiological dysfunction in various biological systems occurring in fish exposed to different insecticides will be discussed.

2. Metabolism of insecticides

In fish, insecticides are readily converted into more polar compounds through biotransformation and stored in the bile until their excretion. In general, detoxification process involves two phase, functionalization (phase I) and conjugation (phase II). The first biotransformation step (phase I) adds oxygen to the insecticides structure and the second step (phase II) conjugates the oxygenated product with and endogenous water-soluble small molecule present in the cell. The final products are highly water-soluble conjugates that are easier to eliminate than the parent compounds.Although, recently, anti-porter activity (p-glycoprotein or multidrug resistance) has been defined as the Phase III detoxification system, there is no exactly information about this detoxification system in fish.Classic detoxification pathways of insecticides in aquatic organisms are presented in Table 1.

3. Phase I

The phase I detoxification system, composed over 10 families of enzymes which played important role in the metabolism of various xenobiotic. The phase I detoxification system, composed mainly of the cytochrome P450 supergene family of enzyme, that are present in all eukaryotes and some prokaryotes and is generally the first enzymatic defense system against xenobiotic.A great diversity of cytochrome P450 enzymes in fish has been recognized (Stegeman and Hahn, 1994), and CYP1A, CYP2B, CYP2E1, CYP2K1 and CYP3A have been recently identified in liver of some freshwater fish (Nabb et al., 2006) which play an important role in the detoxification of organophosphate and carbamate insecticides (Ferrari et al., 2007). The common pathways of biotransformation of different kinds of insecticides include three cytochrome P450 (CYP) mediated reactions: *O*-dealkylation, hydroxylation, and epoxidation of insecticides (Keizer et al., 1995; Kitamura et al., 2000; Straus et al., 2000; Behrens & Segner, 2001; Nebbia, 2001). Most insecticides are metabolized through phase I biotransformation. In general, CYP450 enzymes mediated reactions by using oxygen and NADH, as a cofactor, lead to detoxification and subsequent excretion of xenobiotic. The CYP450 enzymes can also facilitate dealkylation, dearylation, aromatic ring hydroxylation, thioether oxidation, and deamination (Table 1.). However, CYP450 enzymes mediated metabolism can also cause formation of reactive metabolites which is far more dangerous than parental compounds. For example, oxidative group transfer of certain organophosphorous insecticides to the toxicorganophosphate, e.g. conversion of parathion to paraoxon,oxidative dechlorination of chloroform to phosgene,activation of ethyl carbamate to urethan. However, many of these same chemicals are also detoxified by cytochrome P450 by conversion to less toxic metabolites. In some cases, the same enzyme may catalyze activation and detoxification reactions for a given chemical. The resulting toxic effect of a xenobiotic chemical is thus due to a balance between metabolic activation and deactivation (Casarett and Doull, 1996).

4. Phase II

In phase II reactions, metabolites produced in phase I detoxification often conjugate with glutathione, uridyl-diphosphate glucose (UDPG), uridyl-diphosphate-glucuronic acid (UDPGA), amino acid derivatives and sulfate derivatives and can readily excrete from the fish body (Keizer et al., 1995; Kitamura et al., 2000; Straus et al., 2000; Behrens & Segner, 2001; Nebbia, 2001). In fact, this results from enzymatic oxidation and hydrolysis that produce metabolites with OH, COOH and NH2, SH functional groups. These functional groups are then subject to conjugation with carbohydrates, glutathione, sulfate, andamino acids, and then, the final metabolites may also be excreted from the body of fish through the skin, gills, genital products, urine as sulphated and glucuronidated metabolites and stool as glutathione conjugated metabolites (Kitamura et al., 2000; Straus et al., 2000; Behrens & Segner, 2001; McKim & Lein, 2001; Nebbia, 2001).

Metabolism		Category	Reaction pattern
Oxidation	Alkyl oxidation	O1	$R-CH_3 \rightarrow R-CH_2OH \rightarrow R-CHO \rightarrow RCOOH$
	O (N)-dealkylation	O2	$-O\{N\}-CH_3 \rightarrow [-O\{N\}-CH_2OH] \rightarrow -O\{N\}H$
	Ring hydroxylation	O3	$Ar-H \rightarrow Ar-OH$
		O4	$Ar-H \rightarrow Ar-(OH)_2$
		O5	Quinone
	S-oxidation	O6	$-S- \rightarrow -S(O)- \rightarrow -S(O_2)-$
	Desulfuration	O7	$P=S \rightarrow P=O$, $C-SO_3H \rightarrow C-OH$

Metabolism		Category	Reaction pattern
	Others	O8	$C=C \rightarrow$ epoxide, ketone, $NH_2 \rightarrow NHOH$ $\rightarrow NO$, $R-X \rightarrow R-OH\ [X=NO_2]$
Reduction			
	Dehalogenation	R1	$RX \rightarrow R-H + X^{-} [X=\text{halogen}]$
	Dehydrohalogenation	R2	$CH-CX \rightarrow C=C + HX\ [X=\text{halogen}]$
	Nitro group	R3	$-NO_2 \rightarrow [-NO] \rightarrow NHOH \rightarrow NH_2$
	Multiple bond	R4	$-N=N- \rightarrow -NH-NH-$, $-C \equiv C- \rightarrow -C=C- \rightarrow -CH-CH-$
	Sulfone, sulfoxide	R5	$-S(O_2)- \rightarrow -S(O)- \rightarrow -S-$ Hydrolysis
	Carboxyl ester	H1	$-C(=O)OR \rightarrow COOH + R-OH$
	Phosphoryl (sulfonyl) ester	H2	$-P(=X)-YR \rightarrow -P(=X)-OH + RYH$ $[X, Y=O, S]$ $ROS(=O)OR' \rightarrow ROH + R'SO_3H$
	Amide	H3	$-C(=O)NR- \rightarrow -COOH + NHR$
	Carbamate	H4	$-NC(=O)O(\text{or } S)R- \rightarrow -NH + RO(\text{or } S)H$
	Urea	H5	$-NHC(=O)NR- \rightarrow -NH_2 + RNH-$
	Others	H6	$-CN \rightarrow -CONH_2 \rightarrow -COOH$, $R-X \rightarrow R-OH$ [X=halogen]
Conjugation			
	Glucuronidation	C1	$R-XH \rightarrow R-X-Gla\ [X=O,\ COO,\ S,\ NH]$
	Glucosidation	C2	$R-XH \rightarrow R-X-Glu \rightarrow R-X-(6O-R')Glu$ $[X=O,\ COO,\ S,\ NH;\ R'=\text{acetyl, malonyl, pentosyl}]$
	Sulfation	C3	$R-X-H \rightarrow R-X-SO_3H\ [X=O,\ NH]$
	Glutathione conjugation	C4	$R-X + GSH \rightarrow R-SG \rightarrow R-Cys-Gly$ $\rightarrow R-Cys \rightarrow R-(N-\text{acetyl}-Cys)$
	N-acylation	C5	$-NH_2 \rightarrow -NHCHO$, $-NHC(=O)CH_3$
	Amino acid conjugation	C6	$R-COOH \rightarrow RC(=O)NHC(R')COOH$
	Methylation	C7	$R-O(\text{or } NH)H \rightarrow R-O(\text{or } NH)CH_3$
Miscellaneous		M	Isomerization, rearrangement, etc.

Table 1. Classic detoxification pathways of insecticides in aquatic organisms (adapted from Katagi, 2010)

5. Physiological dysfunction in various biological systems of fish by insecticides

5.1. Behavioral response

Behavioral alterations and the change of body's color pattern of fish or darkness of skin and mucosa increase to the skin and gill surface, as well as bleeding around the eyeball and the base of pectoral fins and also, the volume increase of the liver and the gall bladder in fish exposed to insecticides were the main symptoms evidenced in the toxicology studies.

Behavioral changes are the most sensitive indicators of potential toxic effects. Most insecticides affect the behavioral patterns of fish by interfering with the nervous systems and sensory receptors and consequently it can lead to disorders in the fish response to environmental stimuli. The effect of certain insecticides on the activity of acetylcholinestrase may also lead to a decreased mobility in fish (Banaee, 2012). Several studies have demonstrated that insecticides are metabolized in liver to toxic derivate via cytochrome P450 mono oxygenases (Fujii and Asaka, 1982; Hamm et al., 2001; Schlenk, 2005) and finally, these metabolites were hydrolyzed in microsomes (Keizer et al., 1993; Keizer et al., 1995) and excreted from the body. Nevertheless, rainbow trout was very sensitive to organophosphate insecticides toxicity due to a lack of esterase activity and a very sensitive acetylcholinesterase activity to OPs inhibition (Keizer et al., 1995). The phosphorus group of organophosphate insecticides attacks the hydroxyl group of the serine amino acid at the active site of acetylcholinesterase inhibiting the enzyme (Üner et al., 2006; Banaee et al., 2011). Inhibition of AChE in fish was accompanied by an increase in acetylcholine levels (Üner et al., 2006; Banaee, 2012) that can be dangerous since it will impact feeding capability, swimming activity, identification, and spatial orientation of the species (Banaee et al., 2008; Banaee et al., 2011). Thus, AChE inhibition is considered to be a specific biomarker of exposure to organophosphorus and carbamate insecticides like diazinon, chlorpyrifos, propoxur, isoprocarb, (Üner et al., 2006; Cong et al., 2008; 2009; Wang et al., 2009; Banaee et al., 2011;). Similar results have been observed for pyrethroids insecticide toxicity (Koprucu et al., 2006). Disorder in γ-aminobutyrate (GABA) system in brain of rainbow trout exposed to sub-lethal lindane was reported by Aldegunde et al., (1999). GABA receptors inhibit the transmission of nerve impulses; thus disturbances in this receptor would also lead to an over stimulation of the nerves. Researchers have reported the same alterations in *Oryzias latipes*, *Cyprinus carpio*, *Labeo rohita*, *Oncorhynchus tshawytscha*, *O.latipes*, *Cirrhinus mrigala*, *Oreochromis niloticus*, *Clarias gariepinus* treated with chlorpyrifos (Rice et al., 1997; Halappa & David, 2009), malathion (Patil & David, 2008), diazinon (Scholz et al., 2000), endosulfan (Gormley & Teather, 2003), Fenvalerate (Mushigeri & David, 2005), fenitrothion (Benli & Özkul, 2010), dimethoate (Auta et al., 2002), respectively.

6. Oxidative stress

During the detoxification process, ROS are produced (Üner et al., 2006; Isik and Celik, 2008) and they can indiscriminately attack and damage cellular macromolecules -lipids, proteins and DNA- in living cells resulting in serious disturbances in physiological cell processes (Sureda et al., 2006; Tejada et al., 2007). Li et al. (2010c, d; 2011b) believed that cellular antioxidant responses could be used as potential biomarkers for monitoring residual xenobiotic present in aquatic environment. For example, Salvo et al. (2012) found that endosulfan at the sub-lethal concentration in subchronic exposure caused significant changes in liver somatic indices as well as induction of the phase I biotransformation system and oxidative stress in juvenile common carp (*Cyprinus carpio*). Similar results was observed in gar (*Atractosteus tropicus*), Tilapia (*Oreochromis niloticus*), tropical reef fish (*Acanthochromis polyacanthus*) exposed to ethorophos (Mena Torres et al., 2012), lamba-cyhalothrin (Piner and Uner, 2012), Chlorpyrifos, respectively (Botte et al., 2011; Xing et al., 2011; Oruc, 2012).

The antioxidant enzymes that provide the first line of cellular defense to ROS include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), glutathione *S*-transferease (GST) and xanthine oxidase (XOD), etc. However, an imbalance between the activities of cellular antioxidant enzymes and ROS production results in oxidative stress and cellular damage. If the antioxidant system is not able to eliminate or neutralize the excess of ROS, there is an increased risk of oxidative damage (Üneret al., 2006; Oruç and Usta, 2007; Isik and Celik, 2008). It is well established that waterborne pollutants induces oxidative stress and cellular damage in affected aquatic organisms (Sureda et al., 2006; Box et al., 2007).

GR plays a vital role in recycling oxidized glutathione (GSSG) to reduced glutathione (GSH) (Jos et al., 2005; Box et al., 2007; Sureda et al., 2009). GR plays an important role in diazinon detoxification because diazinon can be directly conjugated with GSH facilitating the excretion from the animal body (Banaee et al., 2012). GSH also participates in neutralizing free radicals (Jos et al., 2005; Sureda et al., 2009). This GSH consumption leads to an increase in GR activity in order to recycle GSH. The increase in GR activity observed after seven days of exposure to sub-lethal concentrations of diazinon was followed by a declining trend, which is clearly manifested after 28 days of diazinon contact (Banaee et al., 2012). These results agree with a previous study carried out on fishes that had been exposed to environmental pollutants (Franco et al., 2008). Banaee et al., (2012) found that the decreased activity of GR at the 28th day after an initial antioxidant response may be indicative of a disorder in cell metabolism. GR activity is severely dependent on cellular NADPH levels (Peña-Llopis et al., 2003). It has been reported that the contact with pesticides decreased the synthesis and accelerated the breakdown of GR mediated by a disorder in NADPH synthesis and decreased activity of glucose-6-phosphate dehydrogenase (G6PDH) enzyme (Ozmen et al., 2004; Li et al., 2010c).

Since an increase of GPx activity is necessary to eliminate the excess of H_2O_2 and lipid hydroperoxides produced in hepatocytes of fishes exposed to. The increased activity of GPx accelerates the utilization of GSHto GSSG. This increased GSSG, indicative of a more oxidized state, may explain the decreased levels of total antioxidant capacity in liver cells of fish after exposure to pesticide. A decrease in GPx activity to basal values is probably related to decreasing cellular GSH levels on the days 14 and 28, although it cannot be discarded a direct effect of diazinon on the biosynthesis of the enzyme. Similar alterations in GPx activity were observed in different tissues of *C. carpio* exposed to diazinon (Oruç and Usta, 2007). Decreased

GPx activity in gills, muscle, liver and brain of treated fishes with parathion were also reported, by Monterio et al. (2006).

The SOD enzymes are enzymes that catalyse the dismutation of superoxide into hydrogen peroxide and oxygen whereas CAT catalyzes the decomposition of hydrogen peroxide to water and oxygen. The increased SOD and CAT activities in hepatocytes of fishes exposed to diazinonmight be biochemical responses to over production of superoxide radicals and H_2O_2in hepatocytes, respectively (Banaee et al., 2012). It has been shown that the CAT activity may be related to H_2O_2 production in a xenobiotic detoxification process (Achuba and Osakwe, 2003; Monterio et al., 2006). A previous study by Monterio et al., (2006) reported similar changes in the hepatic CAT activity of freshwater fish, *Brycon cephalus* exposed to methy parathion. Following 2-cholrophenol exposure, alterations in SOD and CAT activities in *Carassius auratus* were reported (Luo et al., 2006). Hai et al, (1997), and Box et al, (2007) showed that organophosphate pesticide and exposure to environmental pollutants caused a significant reduction in CAT activities in different tissues of *Ictalurus nebulosus* and *Mytilu sgalloprovincialis*, respectively. Whereas, Isik and Celik, (2008) reported in rainbow trout exposed to diazinon and methyl parathion a decrease in SOD activities in liver, gills and muscle tissues separately.

Banaee et al., (2012) found that the levels of total antioxidant capacity in hepatocytes of fishes exposed to both concentrations of diazinon were significantly decreased. Similar results were observed in carps exposed to sub-lethal concentrations of cyfluthrin (Sepici-Dinçel et al., 2009). The overproduction of free radicals during pesticide detoxification may be associated with the decrease in the hepatic total antioxidant capacity (Monterio et al., 2006). Impairment in the synthesis of enzymatic and non-enzymatic antioxidant may be the most important factor in reducing levels of cellular total antioxidant. Therefore, the decline in the hepatic total antioxidant levels make the fish cells more vulnerable to oxidative stress damage.

Glutathione S-transferases (GSTs), a family of cytosolic multifunctional enzymes, are detoxifying enzymes that are present in different tissues of fish. They catalyze the conjugation of glutathione with a variety of reactive electrophilic compounds, thereby neutralizing their active electrophilic sites and subsequently making the parent compound more water soluble. For example, the toxicity of diazinon can be decreased by the action of carboxylesterase enzyme which catalyses the hydrolytic degeneration of diazinon and by the action of glutathione *S*-transferase which catalyses the formation of excrete-able conjugate (Keizer et al., 1995). In addition to catalytic functions, the GSTs can also bind covalently/non-covalently to a wide number of hydrophobic compounds, such as insecticides.

Thioltransferase catalyzes the reversible thiol-disulfide interchange reactions. The enzyme has a major role in maintaining intracellular thiol in the reduced state and functions in this capacity by coupling to glutathione and glutathione reductase. Thioltransferase also has a role in the cellular regulation by catalyzing the reversible modification of proteins by thiol-disulfide interchange (Bernstein et al., 1982).

Xanthine oxidase (XOD) is an essential enzyme that converts hypoxanthine to xanthine, subsequent to uric acid. This enzyme contains FAD, molybdenum and Iron are exclusively found in liver, intestine and little amount in other tissues of animals (Sathyanarayana, 2005) also stated XOD played a vital role in transformation of toxic ammonia into nontoxic uric acid. Xanthine oxidase produces hydrogen peroxide which is very dangerous to the animal, and then it converts into HO and O_2. Further, the uric acid may act as an antioxidant and free radical scavenger protects the cells from oxidative damage (Sheehan et al., 2001; Guskovet al., 2002). Naveed and Janaiah (2011) reported that the reduction in XOD activity in liver of fish, *Channa punctatus* exposed to triazophos leads to increase in cellular damage and may be due to non-availability of Iron to the fish during toxic period.

7. Hematological parameters

The main hematological parameters in fish including red blood cell counts (RBC), hematocrit (Ht), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) may be influenced by intrinsic and externalfactors. Toxicology studies show that the disruptive action of different insecticides on the erythropoietic tissue such as kidney and spleen may decrease erythrocyte number and hemoglobin content as an anemic sign, and even lead to death of fish. A low red cell or hemoglobin count indicates anemia, or severe bleeding. Low hemoglobin usually means the animal has anemia. Anemia results from conditions that decrease the number or size of red cells, such as excessive bleeding, a dietary deficiency, destruction of cells because of a transfusion reaction or mechanical heart valve, or abnormality formed hemoglobin (Hisa and Connie, 1998). Decreases in the number or size of red cells also decrease the amount of space they occupy, resulting in a lower hematocrit. A low hematocit, combined with abnormal blood tests, confirms the diagnosis. Decreased erythrocyte count and haemoglobin content in freshwater fish *Channa punctatus*, (Anees, 1978) and *Cyprinus carpio* (Svoboda,et al., 2001; Banaee et al., 2008) after acute and sub-lethal exposure to diazinon.

Another type of hematological response to the effect of organophosphrous compounds was a significant increment of mean corpuscular volume (MCV) associated with increase of hematocrit value and drop of MCHC. MCV is the index most often used. It measures the average volume of red blood cell by dividing the hematocrit by RBC. The MCV categorizes red blood cells by size. Under a microscope, stained red blood cells with a high MCV appear larger than cells with a normal or low MCV. Mean corpuscular hemoglobin (MCH) measures the average amount of hemoglobin within a red cell. A similar measurement, mean corpuscular hemoglobin concentration (MCHC), expresses the average concentration of hemoglobin in the red blood cells. In contrary, values of MCV, MCH and MCHC registered in during exposure to diazinon based pesticide in 60 and 120 μg/L concentrations to common carp were comparable with the control group (Banaee et al., 2008). Alteration in values of MCV, MCH and MCHC in *Cyprinus carpio* was reported (Svoboda et al., 2001).

The white blood cell (WBC) count determines the total number of white cells (leukocytes) in bloodsample. Fewer in number than the red cells, WBC are the body's primary means of fighting infection. There are five main types of white cells (lymphocytes, monocytes, neutrophil, eosinophiland basophiles), each of which plays a different role in responding to presence of foreign organisms in the body. The number of white blood cells may increase or decrease significantly in certain diseases. Banaee et al., (2008) observed significant decrease of leukocyte count of common carp in during exposure to sub-lethal concentration of diazinon. A low white blood cell count may mean dysfunction in hematological tissues (spleen and kidney) or certain infectious diseases. Lower than normal levels of lymphocytes (lymphopenia) can be an indicator of immune system deficiency.

Poisonous substances treatments can also deplete the body's supply of lymphocytes, as can exposure to diazinon. Lymphopenia as a consequence of methy-parathion based pesticide was reported by Nath and Banerjee (1996) in *Heteropneustes fossilis* and also by Siwicki et al., (1990) in common carp after an acute effect of trichlorfon.

Decreased in lymphocyte and monocyte percentage in smear were showed in *Cyprinus carpio* (Svoboda et al., 2001). In contrary, Banaee et al., (2008) observed significant increment of neutrophil percentage in smear of common carp in during exposure to 60 and 120 μg/L concentration of diazinon. The most common and important cause of neutrophilia is infection, and most infections cause neutrophilia. The degree of elevation often indicates the severity of the infection. Tissue damage from other causes raises the neutrophile for similar reasons. Poisonings, and severe disease, like kidney failure all cause neutrophilia (Holland et al., 1997). Ghosh and Banerjee (1993) reported lymphopenia and increased in both neutrophil and eosinophil in *Heteropneustes fossilis*, after an effect of dimethoate in 96h LC_{50} concentration.

8. Blood biochemical parameters

Measurement of blood biochemical parameters are used as important diagnostic tool for the detection of abnormalities in the liver and other tissues (Banaee et al., 2011). Liver serves to protect the body from potentially harmful ingredients absorbed from the intestinal tract, gills, and skin as well as toxic by-products of metabolism by various mechanisms that previously described.

The significant differences in glucose concentrations in plasma between the control and treatment fish, following the action of different insecticides, which may be considered to be the manifestation of stress (Banaee et al., 2011). In agreement with our results, Ceron, et al., (1997) report significant glucose increase in common eel (*Anguilla anguilla*) following a 96 h action of sub-lethal concentrations of diazinon. Bhatia et al., (1972) and Weiss et al., (1984) reported a pronounced increase in blood sugar level which was going parallel to the inhibition of the cholinesterase and the appearance of manifestation of cholinergic stimulation as a result of parathion intoxication.

Glucose increase is a general response of fish to acute pollutant effects, including organophosphates insecticides (Banaee, 2012). Plasma glucose was elevated in treated silver catfish *Rhamdia quelen* after all periods of clomazone exposure (Lazzari, et al., 2006).

Albumin and globulin make up most of the protein within the body and are measured in the total protein of the plasma. Total protein, albumin and globulin tests are used to monitor the course of diseases in immune disorders, liver dysfunction and impaired kidney activity (Banaee et al., 2011). According to the test results, levels of total protein, albumin and globulin were decreased in fish exposed to diazinon. Decreased total protein levels may be due to starvation, malnutrition and chronic liver diseases (Banaee et al., 2011). Other authors also found that the levels of total protein and albumin are decreased in the fish exposed to different pollutants and insecticides (Vijayan et al., 1997; Velisek et al., 2009).

Decreased globulin levels have been reported in immune deficiency. In fact, the effect of diazinon on the immune system of tilapia (*Oreochromis niloticus*) and beluga sturgeon (*Huso huso*) as immunomodulator has been studied by other authors (Khoshbavar-Rostami et al., 2006; Gokcimen et al., 2007). Diazinon-induced tissue destruction and hepatocyte apoptosis might be the most important agent responsible of reducing the synthesis of total protein, albumin, and immunoglobulin by the liver (Gokcimen et al., 2007).

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are found in the liver, heart, skeletal muscle, kidney, pancreas, spleen, erythrocyte, brain and gills (Banaee et al., 2011). When diseases or injuries affect these tissues, the cells are destroyed and these enzymes are released into plasma. Keizer et al (1995) showed that diazinon in fish liver can be metabolized to diazoxon and other metabolites including pyrimidinol by cytochrome P450 monoxygenase and during this process, reactive oxygen species (ROS) are generated. The increase in intracellular levels of ROS may lead to lipid peroxidation resulting in an increased permeability of liver cell membrane. As a result, liver enzymes including AST and ALT are released into plasma. Similarly, ROS produced from the metabolism of diazinon may damage other tissues such as gills, muscle, heart, kidney and spleen causing the leakage of enzymes into plasma. In this sense, if the cellular injury is chronic AST and ALT levels will remain elevated (Banaee et al., 2011). Increased activities of AST and ALT were observed in plasma of *Channa punctatus*(Agrahari et al., 2007) exposed to organophosphorus insecticides. Banaee et al., (2008) have reported increased levels of AST and ALT followed by the exposure of common carp to diazinon.

Lactate dehydrogenase (LDH) is an enzyme found in almost all body tissues, such as heart, kidneys, liver, skeletal muscle, brain, erythrocyte and gills (Banaee et al., 2011). LDH measurement is used to detect tissue disorders and as an aid in the diagnosis of tissue damage (Banaee et al., 2011). Although a significant decrease in LDH content in plasma was observed in fish exposed to diazinon on 7th day, LDH activity was not significantly reduced on 14th and 28th days. Our data on 7 days after initial experiment are in accordance with several reports that revealed decreased LDH activity in tissues under various pesticide toxicity conditions in different fishes such as *O. mossambicus* (Saha and Kaviraj, 2009) and *C. punctatus* (Agrahari et al., 2007). Hernández et al. (2006) reported some insecticides, such as organophosphates are able to cause inhibition of LDH activity. However, the recovery evidenced on 14th and 28th days in LDH activity could indicate that diazinon concentration used in the present study was not enough to produce chronic LDH alterations (Banaee et al., 2011).

Creatine kinase (CK) is an enzyme found in muscle, heart, gills and brain (Banaee et al., 2011). Although, fish exposed to higher dose of diazinon exhibited higher CK activities in plasma on 7th day, plasma CK concentrations resumed to its normal levels or even a reduced activity on

14th and 28th day. However, elevated levels of this enzyme in plasma indicate a transient damage to either muscle fibers or other tissues. Previous results reported that continuous exposure to acute pesticide (bifenthrin) concentrations resulted in significantly increased levels of CK in common carp at 96 h (Velisek et al., 2008).

Creatinine is a breakdown product of CK in muscle. The creatinine test has been usually used to diagnose impaired kidney function and to detect renal damage (Toffaletti and McDonnell, 2008; Banaee et al., 2011) However, the lack of changes in creatinine levels are in accordance with the previous results obtained by Gilbert et al (1989) and Banaee et al. (2011) which reported that plasma creatinine was usually not an accurate biomarker to distinguish dysfunction in kidney tissue. The creatinine test has been usually used to diagnose impaired kidney function and to detect renal damage (Toffaletti and McDonnell, 2008).

The alkaline phosphatase (ALP) plays a significant role in phosphate hydrolysis and in membrane transport as well as is a good bio-indicator of stress in biological systems. The importance of measuring alkaline phosphatase is to check the liver dysfunction (Banaee et al., 2011) and the cellular membrane health. Alkaline phosphatase (ALP) is an enzyme found in different body tissues; in liver, is produced by the cells lining the small bile ducts (Agrahari et al., 2007). Increased activity of ALP in blood plasma on 14th day may be related to hepatic tissue damage and dysfunction due to pesticide toxicity; the elevation in ALP activity on may be due to an increase in transphosphorylation activity (Sharma, 1990). Agrahari et al. (2007) found increased levels of ALP in plasma of *Channa punctatus* exposed to monocrotophos. These changes in ALP activities in plasma were earlier reported in tilapia (Rao, 2006) and in common carp (Banaee et al., 2008). The return of ALP activity to basal values could indicate a fish adaptation to the diazinon treatment at sub-lethal concentrations.

Acid phosphatase plays an important role in carbohydrate metabolism. This enzyme can be found inside the membrane of lysosomes. So, any damage to the membrane of lysosomes can cause the release of this enzyme into muscle and increase its levels.

9. Immune system

The immune system of fish is important for defense against a variety of pathogens. The system is very sensitive to homeostatic adjustments via endocrine regulation and is influenced by the biochemical status of the nervous system. Thus, any impairment in the nervous system and disturbance in the biochemical homeostasis can weaken the immune system of fish.

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

Lysozymes are a family of enzymes with antibacterial activity characterized by the ability to damage the cell wall of bacteria. Ahmadi et al. (2011) found that exposure to diazinon induced a dose-dependent decrease in rainbow trout plasma lysozyme activity. Peroxidases are a large family of enzymes which play important role as natural antibacterial agent in animal immune system, e.g., myeloperoxidase (Clark and Klebanoff 1975). Ahmadi et al. (2011) indicated that diazinon treatment of rainbow trout significantly affected the peroxidase activity.

Globulin is made up of subunit of α1, α2, β, and γ globulins, which are considered as the source of almost all the immunologically active proteins in the blood (Jha et al., 2007; Ahmadi et al., 2012).Decreased globulin levels have been reported in immune deficiency. In fact, the effect of diazinon on the immune system of tilapia (*Oreochromis niloticus*) and beluga sturgeon (*Huso huso*) as immunomodulator has been studied by other authors (Khoshbavar-Rostami et al., 2006). Significant decrease in globulins levels in plasma of fish exposed to diazinon could be due to a disruption in protein biosynthesis (Ahmadi et al., 2011). In the other word, diazinon-induced tissue destruction and hepatocyte apoptosis might be the most important agent responsible of reducing the synthesis of total protein, albumin, and immunoglobulin by the liver (Banaee et al., 2011)

Complement includes over 20 different plasma proteins that are produced by a variety of cells including, hepatocytes, macrophages, and gut epithelial cells. Some complement proteins bind to immunoglobulins or to membrane components of cells. The complement system is an essential and effective part of the innate immune system. It can rapidly distinguish and opsonize bacteria for phagocytosis by specialized phagocytes or destroy them directly by membrane disorder (Rooijakkers and van Strijp 2007; Ahmadi et al., 2012). Ahmadi et al. (2011) believed that significant depression of complement levels clearly indicated the comprehensive effects of diazinon on immune system of rainbow trout. Thus, insecticides may alter the function of the immune system and result in immune-depression, uncontrolled cell proliferation, and alterations of the host defense mechanisms including innate immunity and acquire immunity against pathogens.

10. Metabolism and growth

The change from anabolic to catabolic processes in fish exposed to various insecticides involves important alterations in intermediary metabolism and has important consequences for somatic growth. Changing levels of stress hormones – catecholamine and corticosteroids –, alterations in the ability of digestive enzyme biosynthesis and behavioral changes – hunter and forage behavior – are main factors influencing on the metabolism and growth of fish. For example, histopathological damage to liver, pancreas or hepato-pancreas and intestine can also reduce efficiency of food digestion in fish. Because, these tissues have critical role in regulation biochemical parameters, especially proteins, lipids and carbohydrates, hormones as well as in synthesis and secretion of digestive enzymes, are a target organs for toxicity of insecticides. So, the effect of insecticides on these organs can change metabolism of proteins, lipids and carbohydrates. The purpose of this section of the chapter is to describe briefly the metabolic activity known to be sensitive to various forms of insecticides toxicity. However, it has refused to more descriptions, because of breadth of the topic.

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

11. Carbohydrate metabolism

Most researchers have reported that the increased blood glucose is usually observed in fish under undesirable conditions and it helps the animal by providing energy substrates to vital organs to cope with the increased energy demand (Banaee et al., 2008; Banaee et al., 2011). Elevation of blood glucose levels was widely used as a secondary marker of a stress response (Toal et al., 2004). On the basis of our literature review it is clear that insecticides can be acted as a stressor in fish. Because, hyperglycemia has been reported in many fish exposed to different insecticides. For example, increases in blood glucose levels have been reported in *Heteropneustes fossilis*(Saha and Kaviraj, 2009) and *Cyprinus carpio* (Banaee et al., 2008), *O.mykiss* (Banaee et al., 2011) after exposure to cypermethrin and diazinon, respectively.

Glycogenesis, glycogenolysis, glycolysis and gluconeogenesis are processes that play important roles in regulating blood glucose and carbohydrate metabolism. Glycogenesis is the process of glycogen synthesis from glucose molecules, in which various enzymes such as involved. Hexokinase (HK), glucokinase, and glycogen synthase are key enzyme in the glycogenesis process. Studies show that interfere with activity of these enzymes associated with insecticides exposure of organisms can inhibit glycogen stored in liver (Rezg et al., 2006). Reduced glycogen contents in live and muscle of fish have been reported by some authors.

Glycogenolysis is the catabolism of glycogen which leads to breakdown of glycogen to glucose and catalyzed by enzymes glycogen phosphorylase (GP), phosphoglucomutase (PGM), and glucose 6-phosphotase. Depletion of glycogen content in liver and muscle of fish during exposure to organophosphate insecticides was reported by many researchers (Ghosh, 1987; Nemcsok et al., 1987; Husain and Ansari, 1988).

In fish, catecholamine and corticosteroid hormones have an important role to promote gluconeogenesis. Cortisol is possible through increased levels activity of liver enzyme such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and tyrosine aminotransferase (TAT) have a stimulatory effect on gluconeogenic mechanism.Since in the gluconeogenesis process, glucose is produced from otherorganic molecules like pyruvate, lactate, glycerol, and aminoacids; elevated liver enzyme activity have an important role in the progress of the gluconeogenesis process. Although, pyruvate carboxylase, phosphoenolpyruvatecarboxykinase (PEPCK), fructose 1,6bis-phosphatase, andglucose-6-phosphatase are key enzymes of this process, there are a limit information about their activities in the gluconeogenesis process in fish exposed to insecticides.

Glycolysis is a cascade of biochemical reactions by which a molecule of glucose is oxidized to two molecules of pyruvic acid and two high energy electron carrying molecules of NADH. Glycolysis can occur with or without oxygen. In the presence of oxygen, glycolysis is the first stage of cellular respiration. Without oxygen, glycolysis allows cells to make small amounts of ATP. Glycolysis is catalyzed by enzymes such as HK, phosphor-fructokinase (PFK), and lactate dehydrogenase (LDH).

LDH is an enzyme participated in anaerobic pathway of carbohydrate metabolism. The increase of LDH activity is a diagnostic index widely used to recognize increases of anaerobic metabolism resulting from depletion of energy under anaerobic and environmental stress conditions.The increase of LDH activity can be attributed to the conversion of accumulated pyruvate into lactate which is transported through muscle to hepatopancreas and regenerated glucose and glycogen to supply energy fish exposed to insecticides. In other words, the increase of LDH activity in liver and muscle reflects a possible improvement in tissue glycolytic capacity.

12. Protein metabolism

Proteins are vital ingredient involved in the architecture of the cell, which is the main source of amino acids for building up of new tissues and for the synthesis of biologically important molecules such as enzymes, hormones, etc as well as the source of energy for fish. Alterations in protein content of various tissues of fish exposed to different concentrations of insecticides are linked through a biochemical metabolic pathway. For example, Bose et al. (2011) reported the increase in the protein level in liver of freshwater fish (*Oreochromis niloticus*) was maybe due to check the influence of thiamethoxam and effort to recover from the stress of insecticide at lower doses. They found when the concentration of thiamethoxam was increased; there was decrease in liver protein level. Thus, reduce a significant portion of protein in different tissues, especially the liver, may have been due to their degradation and possible utilization for metabolic purposes. Increases in free amino acid levels were the result of breakdown of protein for energy and impaired incorporation of amino acids in protein synthesis. Since, free amino acids are used in gluconeogentic pathway to glucose production, reduced levels of protein synthesis in fish exposed to insecticides.

Transaminase play an important role in breakdown of protein to free amino acids which may be used an energy source for glyconegenic pathways or used to synthesis new proteins to repair damaged tissues. So, change in plasma free amino acid levels indicates either an increase or a decrease in protein catabolism or biosynthesis.

Aspartate transaminase (AST), or serum glutamic oxaloacetic transaminase (SGOT) is a pyridoxal phosphate (PLP)-dependent transaminase enzyme. AST catalyzes the reversible transfer of α-amino group between aspartate and glutamate and, as such, is an important enzyme in amino acid metabolism. Alanine transaminase or ALT is a transaminaseenzyme. It is also called serum glutamic pyruvic transaminase (SGPT). It catalyzes the transfer of an amino group from alanine to α-ketoglutarate, the products of this reversible transamination reaction being pyruvate and glutamate. Aspartate aminotransferase and alanine aminotransferase are found in the liver, heart, skeletal muscle, kidney, pancreas, spleen, erythrocyte, brain and gills (Banaee et al., 2011). Tyrosine aminotransferase (or tyrosine transaminase) is an enzyme present in the liver and catalyzes the conversion of tyrosine to 4-hydroxyphenylpyruvate. Alteration in deamination and transamination of amino acid are associated with changes in nitrogen metabolism, which can be detected in terms of plasma nitrogenous metabolite levels. Free amino acid is also contributed in the formation of excretory product by the process of conjugation.

The increase in intracellular levels of ROS can lead to lipid peroxidation resulting in an increased permeability of liver cell membrane. As a result, liver enzymes including AST and ALT are released into plasma. In this sense, if the cellular injury is chronic AST and ALT levels will remain elevated (Srivastava et al., 2004; Rao, 2006). Increased activities of AST and ALT were observed in plasma of *Channa punctatus* (Agrahari et al., 2007) exposed to organophosphorus pesticides. Banaee et al., (2008) have reported increased levels of AST and ALT followed by the exposure of common carp to diazinon. Similarly, ROS produced from the metabolism of organophosphate insecticide could be damaged other tissues such as gills, muscle, heart, kidney and spleen causing the leakage of enzymes into plasma (Banaee et al., 2011).

Impact of different insecticides on the hormones involved in the process of protein synthesis can also affect the tissue protein levels. This was in conformity with the effects of dimethoate on *C. punctatus* (Tripathi et al., 2003). Similar changes in protein content was observed in *Labeo rohitha* (Ramani, 2001), *Etroplus maculatus* (Sulekha and Mercy, 2011), *Anabas testudineus*

(Sulekha, 2002; Sulekha and Mercy, 2009) exposed to monocrotophos and phosphamidon. Histological damage to various tissues, especially the liver tissue, caused by exposure to insecticides can also decrease the tissue's ability to synthesis protein. For example, Murray et al., (2003) announced that organophosphate insecticides through methylation and phosphorylation of cellular proteins may lead to a reduction in the reconstruction of necrotic tissues.

In case, due to the presence of unsaturated and sulphuric molecules in the biochemical structure of some amino acids such as phenylalanine, mitonin, cysteine, histidine, and tryptophan that they are sensitive and vulnerable to free radicals and specifically reactive oxygen species (Sureda et al., 2006; Tejada et al., 2007) and it helps breaking the sequence of amino acids, aggregation of amino acid chains and even changing the biochemical structure of amino acids and it leads to proteolytic changes in protein compounds (Stadmann, 1992, 1993; Asada and Barba, 2004).

Albumin and globulin make up most of the protein within the body and are measured in the total protein of the plasma. Total protein, albumin and globulin tests are used to monitor the course of diseases in immune disorders, liver dysfunction and impaired kidney activity (Banaee et al., 2011). Banaee et al., (2011) showed that the levels of total protein, albumin and globulin were decreased in fish exposed to diazinon. Decreased total protein levels may be due to starvation, malnutrition and chronic liver diseases (Kirby et al., 1995; Martin et al., 2010). Other authors also found that the levels of total protein and albumin are decreased in the fish exposed to different pollutants and pesticides (Vijayan et al., 1997; Velisek et al., 2009).

13. Lipid metabolism

Lipids play an important role as source of energy for fish. Since, most insecticides are lipophilic compounds, they can easy pass through biological barriers which content lipids and accumulate in fat tissue.Lipids molecules are highly susceptible to oxidative reactions. Due to cell membrane lipid peroxidation of unsaturated fatty acids, short chain fatty acids with R-COOH, R-OOH, R-CHO, and R-OH bases are created which seriously affect the cellular membrane functions such as the activity of hormone receptors and neural mediators, ion transport channels and the activity of membrane enzymes and the transportation of specific molecules. On the other hand, the formation of malondialdehyde (MAD) during peroxidation process of fatty acids having double bonds can create covalent bonds and polymerize cellular membrane components (Sureda et al., 2006; Tejada et al., 2007). In addition, accumulation of fatty acids in the cytosol increased peroxidation of fatty acids in peroxisomes and the endothelial reticulum, resulting in overproduction of ROS and further damage.

Increased levels of stress hormones such as cortisol in blood of fish exposed to various insecticides, stimulates lipid breakdown in adipose tissue. Both elevated and reduced free fatty acids levels in plasma have been observed in different fish species exposed to insecticides. Changes in cholesterol and triglycerides levels in the blood and other tissues such as muscles and liver of fish treated insecticides indicated that effect of these compounds on lipid metabolism.

14. Growth

From a biochemical point view, ratio of the RNA/DNA can be used as a bio-indicator measure of body growth. Recently, many researchers have focused on the impact of different insecticides on the metabolism of nucleic acid in various tissues of fish (Rathod and Kshirsagar, 2010). Insecticides toxicity indicates change in nucleic acid biosynthesis. Disturbance in the metabolism of nucleic acid can lead to reduction in the RNA content. Also, the deterrent effect of different insecticides such as organophosphate compounds on acid phosphatase and alkaline phosphatase activity in different tissues of fish can also adversely effect on nucleic acid synthesis (Das and Mukherjee, 2000).

Apparently, some insecticides have the potential to inhibit DNA synthesis. For example, the toxicity of dichlorvos has also been related to alterations in DNA replication and chromosome aberration, which causes mutations and cellular hyper-proliferation as a result of local irritation.In fact, insecticides and their metabolites may interfere with the process of DNA synthesis and gene expression by different mechanism. On the other hand, the propagation of malondialdehyde into cells can make the ground for its reaction with nitrogen alkalis of DNA strands (Sureda et al., 2006; Tejada et al., 2007).For example, reduced synthesis of nucleic acids andimpair in the process of proliferation DNA strands as well as inhibition of enzyme activities involved in DNA replication and repair mutations can affect the final product of gene expression. In the other hand, damage to DNA strands caused by oxidative stress and insecticide's metabolites bind to DNA strands (DNA adduct) can lead to impair in the transcription and genes expression. Furthermore, inhibition of DNA synthesis, thus, might affect both protein as well as amino acid levels by decreasing the level of RNA in protein synthesis machinery.

15. Histopathology

Histopathological investigations on different tissues of exposed fish are useful tools for toxicological studies and monitoring water pollutions. Tissue alterations in fish exposed to a different concentration of insecticides are a functional response of organisms which provides information on the nature of the toxicant. In histopathology, we can provide information about the health and functionality of organs. Tissues injuries and damages in organs can result in the reduced survival, growth and fitness, the low reproductive success or increase of susceptibility to pathological agents.

Reactive oxygen species (ROS) produced during the insecticides detoxification process in liver tissue may react with vital macromolecules such as lipid, protein, carbohydrate and nucleic acid and result in oxidative damage to aquatic organisms (Üner et al., 2006). ROS derived damage to natural and structure cellular components are generally considered as a serious mechanism involved in the histological disorders (Sepici-Dinçel et al., 2009). On the other hand, organophosphate insecticides through methylation and phosphorylation of cellular proteins (Murray et al., 2003) may lead to a reduction in the reconstruction of necrotic tissues. The deterrent effect of organophosphate insecticides on acid phosphatase and alkaline phosphatase activity in different tissues of fish can also adversely effect on nucleic acid synthesis (Das and Mukherjee, 2000).Frequency and intensity of tissue lesions depend on the concentrations of insecticides and the length of the period fish are exposed to toxins. Nevertheless, many insecticides cause specific or non-specific histopathological damage (Fanta et al., 2003). For example, histopathological lesions in the liver tissue of freshwater fish (*Cirrhinus mrigala*) (Velmurugan et al., 2009) and common carp carp (*Cyprinus carpio*) (Banaee et al., 2013) were observed after 10 and 30 days exposure to sublethal concentrations of dichlorvos and diazinon insecticides, respectively. Other researchers reported the same histopathological alterations in different tissues of fish treated with diazinon (Duttaa & Meijer, 2003; Banaee et al., 2011), deltamethrin (Cengiz, 2006; Cengiz&Unlu, 2006), fenitrothion (Benli & Özkul, 2010).The pathological changes in the different tissues such as gill, liver, kidney and spleen of fish treated with different insecticides can disturb homeostasis and lead to physiological disorders in these animals.

16. Gill

Fish gills have many important functions including exchange of gases, transport of many mono and divalent ions, excretion of waste nitrogen, and uptake and excretion of various xenobiotics (Zayed and Mohamed, 2004; Evans et al., 2005). Histopathology of gill is the appropriate bioindicator to pollution monitoring. One of the lesions most frequency found on the gills of rainbow trout exposed to 0.1 mg/L diazinon was epithelial hyperplasia of both primary and secondary epithelium, as can be seen in Figure 1. Our results indicated that the major alterations in the gills of rainbow trout exposed to 0.2 mg/L diazinon were edma and epithelial hyperplasia, mucosa cell hyperplasia and fusion of the secondary lamellae. Damage to gill tissue may interfere with gas exchange performance of gill and cause respiratory disorders, ion-regulation and osmoregulation dysfunction and inefficacy of the excretion of waste nitrogen metabolite in exposed fish (Nero et al., 2006; Cengiz and Unlu, 2006; Velmurugan et al., 2007). Gill histopathological damage was also observed after exposure of mosquitofish (*G. affinis*) to deltamethrin (Cengiz and Unlu, 2006), yellow perch and (*P. flavescens*), goldfish (*C. auratus*) to oil sands (Nero et al., 2006), yellow perch (*P. flavescens*) to naphthenic acid (Nero et al., 2006), carp (*C. carpio*) to deltamethrin (Cengiz, 2006), and rainbow trout (*O. mykiss*) to maneb and carbaryl (Boran et al., 2010).

17. Liver

Histopathological analysis reported important alterations in liver, including necrosis, and cytoskeleton disarray, changes in nuclear shape and heterochromatin distribution as well as intense damages in Disse's space between hepatocytes and sinusoid vessels. Increased vacuolization of the endothelial cells, morphological derangement and necrosis in the Disse's space were also evidenced in liver fish exposed to diazinon. These results are in accordance with Cattaneo et al. (2008), who reported disorder in hepatocyte's cords, rupture of the cell membrane and vacuolated cytoplasm in liver tissue of silver catfish, *Rhamdia quelen*, after exposure to 2,4-dichlorophenoxiacetic acid (2,4-D) herbicide. Hypertrophy of hepatocytes, significant increase of kupffer cells, circulatory disturbances, focal necrosis, fatty degeneration, nuclear pycnosis and narrowing of sinusoids has been reported in *G. affinis* and *C. punctatus*, *C. carpio*exposed to deltamethrin and heavy metal, respectively (Cengiz and Unlu, 2006; Mishra and Mohanty, 2008; Vinodhini and Narayanan, 2009). Similar histopathological changes were observed in the liver tissue of *O. niloticus* and *C. carpio* exposed to sub-lethal concentrations of carbaryl and cyfluthrin, respectively (Matos et al., 2007; Sepici-Dinçel et al., 2009). This disturbance in the morphological structure of liver could be associated with a disruption in tissue function, which could be also related to the decreased antioxidant capability in diazinon treated fishes.

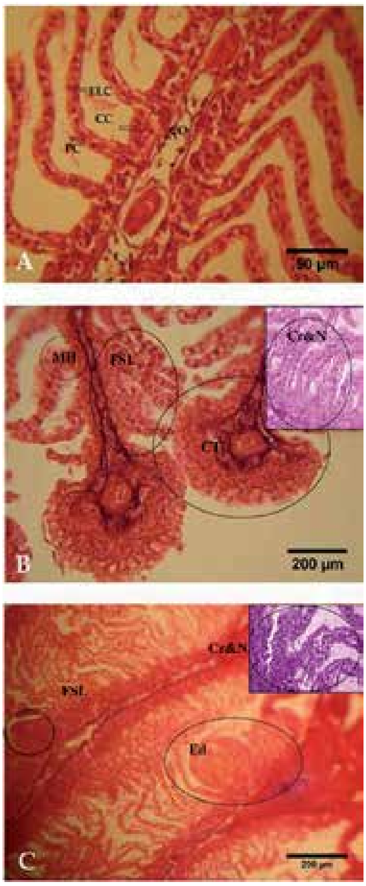

Figure 1. Gills of rainbow trout from the control group (A): Lamella (L), erythrocyte in lamellar capillary (ELC), Chloride cell (CC), Pillar cell (PC), Chondrocytes (CO); Gills of rainbow trout exposed to 0.01 mg/L diazinon (B): crusting and necrosis of secondary lamellae's epithelium (C&N), mucosa cell hyperplasia (MH), clubbing tips of gill filaments (CT); Gills of rainbow trout exposed to 0.02 mg/L diazinon (C): Changes in cartilage tissue of the gill filament, fusion of secondary lamellae (FSL), edema and epithelial hyperplasia (Ed), and the loss of the secondary filaments. Figures are representatives of three replicate experiments (magnification of the sections 400X).

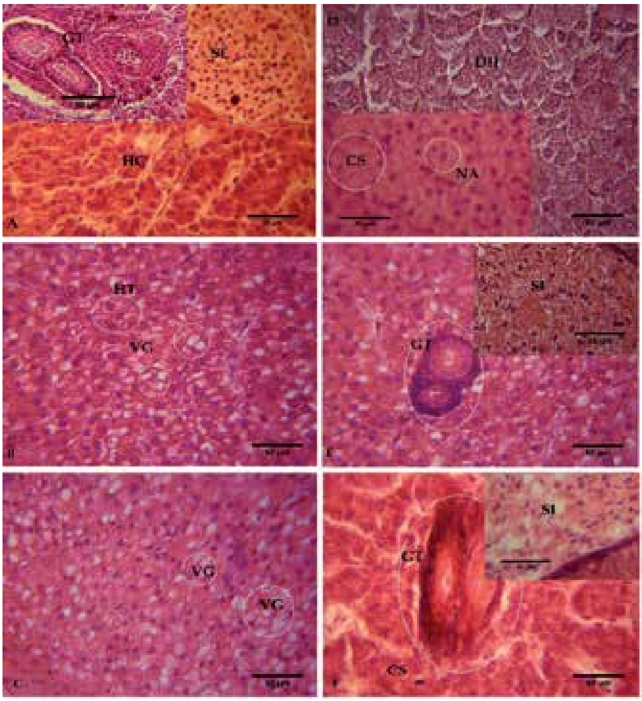

Figure 2. Histopathology of the liver tissue of rainbow trout (*Oncorhynchus mykiss*) is presented after 28 days of exposure. (A) Liver of control fish showing hexagonal hepatic cells (HC) surrounded with the sinusoidal portal blood (SI); this histopathological slide shows cord of hepatocytes separated by sinusoids containing erythrocytes; bile duct (GT). (B & E) Liver of 0.1 mg/L of diazinon-treated fish showing increased hypertrophy of hepatocytes (HC), vacuolization of cell cytoplasm (VG), hepatocyte cloudy swelling (CS); and at the same time hepatocytes lost their normal polygonal structure. (C & D & F) Liver of 0.2 mg/L of diazinon-treated fish showing increased cellular degradation with cytoplasm vacuolization (VG) and nucleus atrophy (NA). The hepatic cells form clusters; hypertrophy (HT) and the disorientation in the hepatocytes and bile duct obstruction (GT), hepatocyte cloudy swelling (CS), are found to be more prominent. Figures are representatives of three replicate experiments (magnification of the sections 400X).

18. Kidney

The head kidney of teleost fish is composed of a variety of cells, including parenchymal cells, lymphoid and hematopoietic tissues (Mela et al., 2007). The functional unit of the kidney is nephron. Morphologically, the nephron of bony fish includes glomerulus, tubules and collecting ducts. In the fish exposed to 0.1 mg/L diazinon, disorientation in the glomerular structure, dilation in the inter space of urinary tubular, cloudy swelling were observed. Histopathological damage in the kidney tissues of fish exposed to 0.2 mg/L diazinon were characterized by degeneration in the epithelial cells of renal tubule, necrosis in the hematopoietic tissue, degeneration of glomerulus, dilation of glomerular capillaries, appearance of vacuoles in cytoplasm epithelial cells of renal tubules with hypertrophied cells and narrowing of the tubular lumen. Increase of ROS production in the diazinon metabolism process may play an important role in lipid peroxidation of components of cellular membrane resulting in reduced nephron number, glomerular lesions, and reduced glomerular filtration rate. Similar studies showed the toxic effect of insecticides on the histology of kidney of Atlantic salmon (*Salmo salar*), rainbow trout, *O. mykiss*, exposed to endosulfan, as pesticide and captan, as the fungicide, respectively (Glover et al., 2007; Boran et al., 2012). These results conform to the deltamethrin and heavy metals effects on kidney tissue of carp that have been reported by Cengiz, (2006) and Vinodhini and Narayanan (2009).

19. Spleen

The spleen has a fibrous capsule, and small trabeculae extend into the parenchyma, which can be divided into a red and white pulp. In rainbow trout, the aggregations of melano-macrophages are less well-defined and lack a capsule, but the association with blood vessels and lymphocytes is maintained (Press and Evensen, 1999). Expansion of red pulp with vascular congestion and a significant deposition of hemosiderin granules in a melano-macrophage center are important histopathological damages observed in spleen of fish exposed to both concentrations of diazinon. One of the typical macroscopic changes associated with diazinon toxicity is enlargement and roughness of the spleen. These results are similar to the 3,4-dichloroaniline and captanin effects that have been described in common goby (*Pomatoschis tusmicrops*) and rainbow trout, respectively (Monteiro et al., 2006; Boran et al., 2012).

20. Digestive tract (intestine)

The intestine of rainbow trout has a mucosa, submucosa, muscularis and serous membrane. The mucosa epithelium has thin and elongated absorptive cells or enterocytes, goblet cells and lymphocytes. Enterocytes are a single layer of columnar cells with apical brush border and basal elongated nucleus with one nucleolus. There are also often lymphocytes at the basal and apical regions of the epithelium. Atrophy and necrosis of mucosal cell, exfoliate of mucosal epithelium, lymphocyte infiltration to lamina propia, reduction in the elastic properties and capillary bleeding in intestine tissue are important histopathological alterations observed in fish exposed to both concentrations of diazinon. Necrosis, degeneration, and accumulation of lymphocyte in lamina propia were observed in the intestine of mosquitofish, *G. affinis*, exposed to Thiodan and deltamethrin (Cengiz et al., 2001;Cengiz and Unlu, 2006) and *Cirrhinus mrigala* treated with lambda-cyhalothrin (Velmurugan et al., 2007). This result is similar to the observations by Glover et al. (2007) in Atlantic salmon (*S. salar*) to dietary endosulfan exposure.

21. Gonads

Some insecticides induced histopathological changes in testes are difficult to study by light microscopy because of the small sizes of the affected cells. Decrease in number and condensation

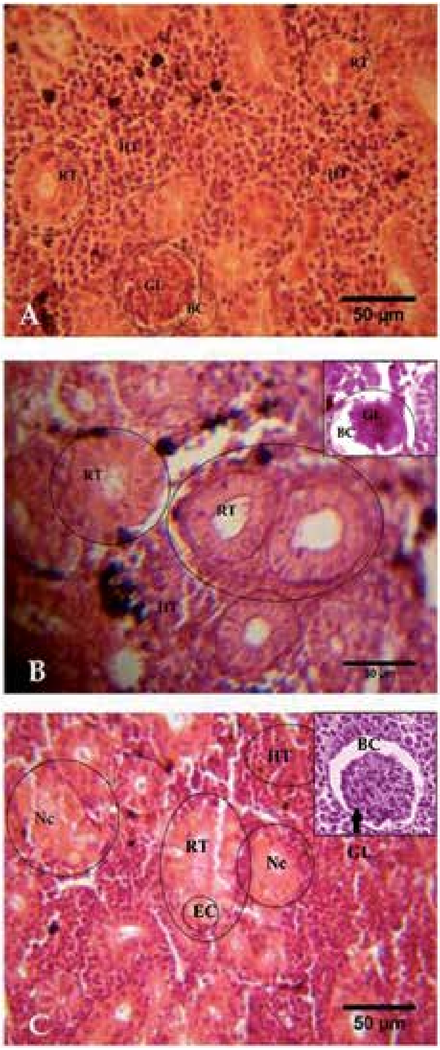

Figure 3. Kidney of rainbow trout from the control group (A): Hematopoietic tissue (HT), renal tube (RT), Glomerulus (GL), Bowman capsule (BC); kidney of rainbow trout exposed to 0.01 mg/L diazinon (B): dilation in inter space of urinary tubular (DUT), cloudy swelling (CS), glomerular destruction; kidney of rainbow trout exposed to 0.02 mg/L diazinon (C): degeneration of tubular epithelial cells (EC), proliferation of epithelial cells in Bowman capsule, cloudy swelling (CS), severe necrosis in nephritic tissue (Nc). Figures are representatives of three replicate experiments (magnification of the sections 400X).

of spermatogonic cells and appearance of a large number of intertubular vacuoles, cloudy swelling and necrosis of seminiferous tubules are important histopathological alterations observed in testis of fish exposed to diazinon (Duttaa & Meijer, 2003; Banaee et al., 2009). This is particularly evident with Sertoli cells, which may undergo major insecticides induced changes in morphology. Thus, Spermatocyte necrosis appears to be a common result of diazinon exposure (Banaee et al., 2009).

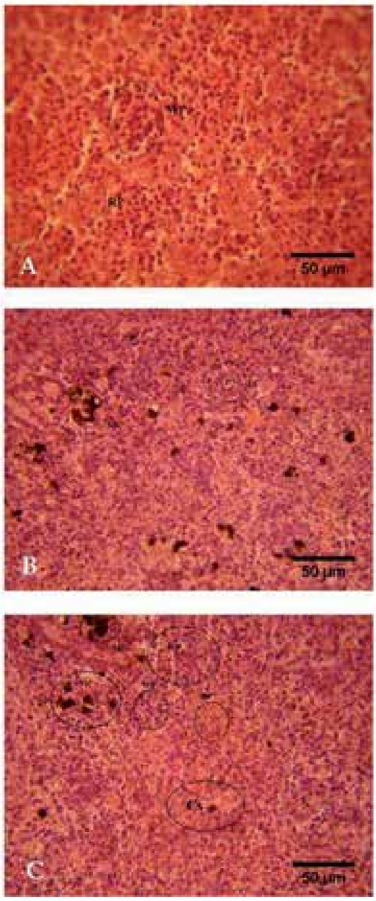

Figure 4. Spleen of rainbow trout from the control group (A): With pulp (WP), Red Pulp (RP), Spleen of rainbow trout exposed to 0.01 mg/L diazinon (B): Melanomacrophage centers (MC), Ellipsoid cells (EC), Penicillus (P), Expansion of splenic red pulp and melano-macrophage centers, disorientation in ellipsoid cells; Spleen of rainbow trout exposed to 0.02 mg/L diazinon (C): Disorder in ellipsoid cells, the increase of number and size of melano-macrophage centers, and cloudy swelling in spleen tissue (CS). Figures are representatives of three replicate experiments (magnification of the sections 400X).

The ovaries of C. *carpio* have shown significant changes on exposure to sub-lethal concentration of diazinon. The oocyte with de-shaped yolk vesicles, vacuolated follicular epithelium and degenerative cytoplasm were reported in fish exposed to diazinon by Banaee et al., 2008.

22. Conclusion

In conclusion, the present literature review shows that exposure to insecticides, firstly, caused an induction of antioxidant enzyme activities indicating the activation of the insecticides detoxification pathways and the antioxidant defenses. However, acute to chronic insecticides treatment disrupted the behavioral response, alterations hematological, biochemical and im-

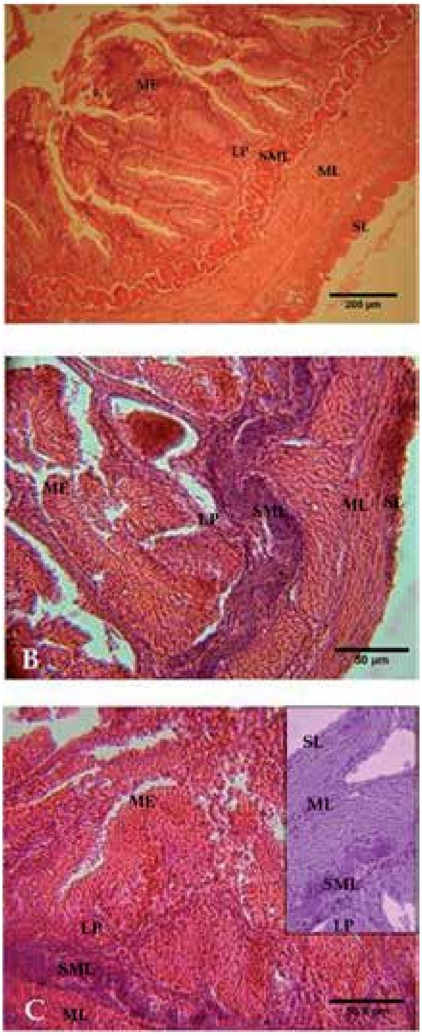

Figure 5. Intestine of rainbow trout from the control group (A): Mucosal epithelium (ME), Lamia's propria (LP), Submucosa layer (SML), Muscularis layer (ML), Serosa layer (SL). Intestine of rainbow trout exposed to 0.01 mg/L diazinon (B): Desquamating of the mucosal epithelium; Intestine of rainbow trout exposed to 0.02 mg/L diazinon (C): Mucosal epithelial hyperplasia, severe necrosis in the mucous layer, loss of the intestine microvilli, disorganization in arrangement of circular and longitudinal muscles in muscularis layer, loss of muscle elasticity and flexibility of the intestine. Figures are representatives of three replicate experiments (magnification of the sections 400X).

munological parameters. In addition, the histopathological analysis performed in gills, liver, spleen, kidney, gonads and intestine of fish exposed to different concentration of insecticides reported significant damage in all tissues indicating that it was a useful methodology for monitoring the effects of insecticides on wild and cultured fish. These changes may be potentially disruptive for the survivability of fish in aquaculture farm and natural resources. This fact should be taken into consideration when this insecticide is used for pest control in agriculture fields surrounding surface water and ground water resources.

Author details

Mahdi Banaee*

Aquaculture Department, Natural Resource and Environmental Faculty, Behbahan University of Technology, Iran

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Section 2

Integrated Methods for Pest Control

Chapter 5

Research on Seasonal Dynamics of 14 Different Insects Pests in Slovenia Using Pheromone Traps

Žiga Laznik and Stanislav Trdan

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53186

1. Introduction

With increasing public concern about the use of toxic pesticides to control insects and other pestiferous organisms, resource managers are turning toward other techniques of integrated pest management. Some of these techniques are common-sense approaches, such as completing sanitation or clean-up activities before the season when the damaging stages of an insect pest are present. Other tools are more "hi-tech", such as the use of odors called semiochemicals, and in particular, pheromones, to manipulate the behavior of insect pests. With these non-toxic and biodegradable chemicals, insects can be lured into traps or foiled into wasting energy that they normally need for locating food and mates. Semiochemicals are chemical signals that are produced by a plant or animal and are detected by a second plant or animal and cause a response in the second organism. Many species depend on these chemical signals for survival.

Pheromones are a class of semiochemicals that insects and other animals release to communicate with other individuals of the same species (Witzgall, 2001). The key to all of these behavioral chemicals is that they leave the body of the first organism, pass through the air (or water) and reach the second organism, where they are detected by the receiver. In insects, these pheromones are detected by the antennae on the head. The signals can be effective in attracting faraway mates, and in some cases, can be very persistent, remaining in place and active for days (Witzgall, 2001). Long-lasting pheromones allow marking of territorial boundaries or food sources. Other signals are very short-lived, and are intended to provide an immediate message, such as a short-term warning of danger or a brief period of reproductive readiness. Pheromones can be of many different chemical types, to serve different functions. As such, pheromones can range from small hydrophobic molecules to water-soluble peptides. Over the last 40 years, scientists have identified pheromones from over 1,500 different species of insects. With insects, though, pheromones have found wide application in the fields of agriculture, forestry, and urban pest management, and there are companies that specialize in the discovery, manufacturing, and sales of pheromone-related products (Thomson, 1997).

There are three main uses of pheromones in the integrated pest management of insects (Witzgall, 2001). The most important application is in monitoring a population of insects to determine if they are present or absent in an area or to determine if enough insects are present to warrant a costly treatment. This monitoring function is the keystone of integrated pest management. Monitoring is used extensively in urban pest control of cockroaches, in the management of stored grain pests in warehouses or distribution centers, and to track the nationwide spread of certain major pests (Thomson, 1997).

A second major use of pheromones is to mass trap insects to remove large numbers of insects from the breeding and feeding population. Massive reductions in the population density of pest insects ultimately help to protect resources such as food or fiber for human use. Mass trapping has been used successfully against the codling moth, a serious pest of apples and pears.

A third major application of pheromones is in the disruption of mating in populations of insects. This has been most effectively used with agriculturally important moth pests (Waldner, 1997). In this scenario, synthetic pheromone is dispersed into crops and the false odor plumes attract males away from females that are waiting to mate. This causes a reduction of mating, and thus reduces the population density of the pests. In some cases, the effect has been so great that the pests have been locally eradicated (Thomson, 1997).

In summary, pheromones are species-specific chemicals that affect insect behavior, but are not toxic to insects. They are active (e.g. attractive) in extremely low doses (one millionth of an ounce) and are used to bait traps or confuse a mating population of insects. Pheromones can play an important role in integrated pest management for structural, landscape, agricultural, or forest pest problems (Witzgall, 2001).

The aim of our research was to study the seasonal dynamics of different insect pests in Slovenia to acquire the informations which are important for improving the control strategy of the pests based on environmentally friendly friendly concepts.

2. Materials and methods

In this chapter we present the results of monitoring of 14 different insect pests in Slovenia (Table 1, Figure 1) with the use of pheromone traps in the period 2004-2010. The majority of the results was performed within the framework of pedagogical work – graduation theses and master thesis – and scientific work at the Dept. of Agronomy, Biotechnical Faculy, University of Ljubljana (Slovenia) under the supervison of Prof. Stanislav Trdan.

Species	**Host plant**	**Monitoring**		
		Location (Coordinates and elevation)	**Year**	**Reference(s)**
Agrotis segetum	maize	Veliki Slatnik (1): 45°47′41″N, 15°13′39″E, 208 m	2007	Srebernjak (2009)
Agrotis segetum	fodder beet	Podobeno (2): 46°8′40″N, 14°11′6″E, 431 m	2010	Dolenec (2012)
Agrotis segetum	maize	Pungert (3): 46°9′23.52″N, 14°20′13.08″E, 340 m	2008	Kalan (2010)
Agrotis segetum	sugarbeet	Cvetkovci (4): 46°24′44″N, 16°3′50″E, 202 m Rakičan (5): 46°39′4″N, 16°11′53″E, 186 m Kranj (6): 46°14′37″N, 14°21′21″E, 388 m	2004	Zalokar (2006)
Anagasta kuehniella	stored cereals and their products	Želimlje (7): 45°55′0″N, 14°34′14″E, 336 m Obrije (8): 46°03′N 14°30′E, 298 m Lipovci (9): 46°37′33″N, 16°13′43″E, 180 m Jable (10): 46°9′45 ″N, 14°34′5″E, 316 m	2004	Selišnik (2007)
Anagasta kuehniella	stored cereals and their products	Želimlje (7)* Obrije (8)* Jable (10)*	2004-2005	Trdan et al. (2010)
Clysia ambiguella	grapevine	Gaberje pri Ajdovščini (11): 45°50′20″N, 13°53′10″E, 191 m	2007	Florijančič (2010)
Contarinia nasturtii	cabbage	Gobovce (12): 46°16′30″N, 14°19′4″E, 374 m	2006	Bohinc (2008)
Contarinia nasturtii	cabbage	Ljubljana (13): 46°03′N 14°30′E, 298 m Rakitnica (14): 45°41′35″N, 14°45′29″E, 490 m Zakl near Braslovče (15): 46°15′45″N, 15°3′14″E, 284 m Škocjan near Koper (16): 45°31′52″N, 13°46′30″E, 48 m	2004	Trdan et al. (2005a)
Contarinia nasturtii	cabbage	Ljubljana (13)* Rakitnica (14)* Zakl near Braslovče (15)*	2004	Walland (2007)
Contarinia nasturtii	cabbage	Ljubljana (13)*	2006	Trdan and Bobnar (2007) Trdan et al. (2008)
Cossus cossus	apricot	Pišece (17): 46°0′12″N, 15°38′53″E, 232 m	2005-2006	Trdan and Jeršič (2008)Jeršič (2009)
Cydia pomonella	apple	Prigorica (18): 45°42′47″N, 14°44′38″E, 485 m	2010	Bartol (2011)
Grapholita funebrana	plum	Dolenja vas (19): 45°42′24″N, 14°45′12″E, 493 m	2007	Pogorelc (2008)
Grapholita funebrana	plum	Gabrnik (20): 46°28′17″N, 15°57′19″E, 221 m Latkova vas (21): 46°14′48″N, 15°5′46″E, 270 m Dol pri Hrastniku (22): 46°8′33″N, 15°7′1″E, 331 m	2004-2005	Humski et al. (2005) Humski (2007)

Species	Host plant	Monitoring		
		Location (Coordinates and elevation)	Year	Reference(s)
		Rakitnica (14)* Želimlje (7)* Dobrovnik (23): 46°39′6″N, 16°20′53″E, 172 m Koper (24): 45°32′47″N, 13°43′46″E, 10 m Kromberk near Nova Gorica (25): 45°57′33″N, 13°40′7″E, 135 m		
Lobesia botrana	grapevine	Gaberje pri Ajdovščini (11)*	2007	Florijančič (2010)
Phyllotreta spp.	cabbage	Ljubljana (13)*	2006	Trdan and Bobnar (2007) Kržišnik (2009)
Plodia interpunctella	stored cereals and their products	Želimlje (7)* Obrije (8)* Lipovci (9)* Jable (10)*	2004	Selišnik (2007)
Plodia interpunctella	stored cereals and their products	Želimlje (7)* Obrije (8)* Jable (10)*	2004-2005	Trdan et al. (2010)
Plutella xylostella	cabbage	Ljubljana (13)*	2006	Trdan and Bobnar (2007) Rešetič (2008)
Scrobipalpa ocellatella	fodder beet	Podobeno (2)*	2010	Dolenec (2012)
Scrobipalpa ocellatella	sugarbeet	Cvetkovci (4)* Rakičan (5)* Gornji Lenart near Brežice (26): 45°55′48″N, 15°34′22″E, 151 m Kranj (6)*	2004	Valič et al. (2005) Čepin (2006)
Sitotroga cerealella	stored cereals and their products	Obrije (8)*	2004-2005	Zalokar (2009)Trdan et al. (2010)
Synanthedon myopaeformis	apple	Bojsno (27): 45°58′13″N, 15°40′15″E, 242 m	2009	Hriberšek (2012)
Synanthedon myopaeformis	apple	Roginska Gorca (28): 46°10′48″N, 15°34′30″E, 208 m	2008	Gradič (2009)

* Coordinates and elevation already mentioned in upper cell

The numbers within parenthesis behind all locations are designed for easier visibility of Figure 1.

Table 1. Insect species monitored with pheromone traps in Slovenia in the period 2004-2010.

Figure 1. Locations of monitoring 14 different insect pests in 26 locations in Slovenia. Each number means specific location where the monitoring took place (seeTable 1).

Studied insect species:

a. Stored product pests

The Indian meal moth (*Plodia interpunctella* [Hübner]; Lepidoptera, Pyralidae)

Mediterranean Flour Moth (*Angusta kuehniella* [Zeller]; Lepidoptera, Pyralidae)

The Angoumois Grain Moth (*Sitotroga cerealella* [Olivier]; Lepidoptera, Gelechiidae)

2. Vegetable pests

Swede midge (*Contarinia nasturtii* [Kieffer]; Diptera, Cecidomyiidae)

The diamondback moth (*Plutella xylostella* [L.]; Lepidoptera, Plutellidae)

Flea beetle (*Phyllotreta* spp.; Coleoptera, Chrysomelidae)

2. Field crop pests

Beet moth (*Scrobipalpa ocellatella* Boyd; Lepidoptera, Gelechiidae)

The turnip moth (*Agrotis segetum* [Denis & Schiffermüller]; Lepidoptera, Noctuidae)

2. Fruit tree and grapevine pests

The Plum Fruit Moth (*Grapholita funebrana* [Treitschke]; Lepidoptera, Tortricidae)

The Codling moth (*Cydia pomonella* [L.]); Lepidoptera, Tortricidae)

The Red-belted Clearwing (*Synanthedon myopaeformis* [Borkhausen]; Lepidoptera, Sesiidae)

The Goat Moth (*Cossus cossus* [L.]; Lepidoptera, Cossidae)

The European grape berry moth (*Clysia ambiguella* [Hübner]; Lepidoptera, Tortricidae)

The European Grapevine Moth (*Lobesia botrana* [Denis & Schiffermüller]; Lepidoptera, Tortricidae)

3. Stored product pests

3.1. The Indian meal moth (*Plodia interpunctella* [Hübner]; Lepidoptera, Pyralidae)

The Indian meal moth is a serious and widespread pest of many stored food commodities (Sedlacek et al., 1996). The larval stage causes the injury. Larvae feed on flour and meal products, dried fruits, nuts, bird food, and dried pet foods. More unusual recorded foods include chocolate and cocoa beans, coffee substitute, cookies, flour, dried mangelwurzel, and even the toxic seeds of Jimsonweed (*Datura stramonium*). As the larva feeds it spins a web, leaving behind a silken thread wherever it crawls. Small particles of food often adhere loosely to the thread, making it conspicuous. Many times an infestation is noticed when moths are seen flying around the home in the evening. They are attracted to lights and often appear in front of the television screen (Sedlacek et al., 1996).

The Indian meal moth has a wingspan of about 18-20 mm. The color of the outer two-thirds of the wings is bronze to reddish brown, while the part of the wings closer to the body is grayish white. The larvae (caterpillars) are about 12-13 mm long when mature. They are a dirty white color, sometimes exhibiting pink or green hues. The pupa (resting stage) is in a loose silken cocoon spun by the larva, and is a light brown color (Hinton, 1943).

A female Indian meal moth can lay from 100 to 300 eggs during her lifetime. Eggs are laid singly or in groups on the food materials. Within a few days the tiny whitish caterpillars emerge. These larvae feed for a few weeks, and when they are mature they often crawl up the walls to where wall and ceiling meet, or crawl to the top of the cupboard, to spin the silken cocoon in which they pupate and from which the adult moth emerges. Mating occurs and the life cycle repeats itself. In warm weather the cycle may take only 6 to 8 weeks (Hinton, 1943).

Female *P. interpunctella* and females of other stored product moths of the subfamiliy Phycitinae produce Z-9, E-12-tetradecandienyl acetate as a component of their sex pheromone blends (Brady et al., 1971). Traps baited with synthetic pheromone are effective and widely used to monitor male stored-product moths (Vick et al., 1986; Chambers, 1990). Pheromone-baited traps have proven successful in detecting low level infestations of these moths (Vick et al., 1986).

In 2004-2005, pheromone traps (VARL+ type, (Csal♀m♂N® Budapest, Hungary) were used to monitor the occurrence of Indianmeal moth in Slovenia. The pheromone traps were set from March to December. Indianmeal moth was monitored during 2004 and 2005 in Želimlje and Jable and during 2004-2006 in Obrije. In Želimlje, the lepidopterian pests were monitored using two traps hung under the ceiling of a corn open air storage (part of a barn). It was used for storage of corn (corncobs) from harvest (the end of September) till the end of July. In the lower part (under this corn open air storage) corn that was ground into flour was kept. In Obrije, an organic farm was monitored using four traps, three in the storage room and one in the mill. In Jable, the traps were placed in the Agricultural Centre, with one trap in the grain storage, a second one in the mill, and two outside the building (in front of the storage). At none of these locations were treatments against stored pests performed. Following the manufacturer's instructions, the pheromone lures were changed monthly. They were checked in 7 day intervals (Želimlje) or 14 day intervals (Obrije and Jable). The trapped males were stored in the lab at room temperature until identification. The determination was carried out using a Olympus SZ30 (manufacturer: Olympus Europa Gmbh, Hamburg, Germany) stereomicroscope (magnification about 10 times). The number of the trapped moths/day was calculated as the intervals were not the same for all the locations (Trdan et al., 2010).

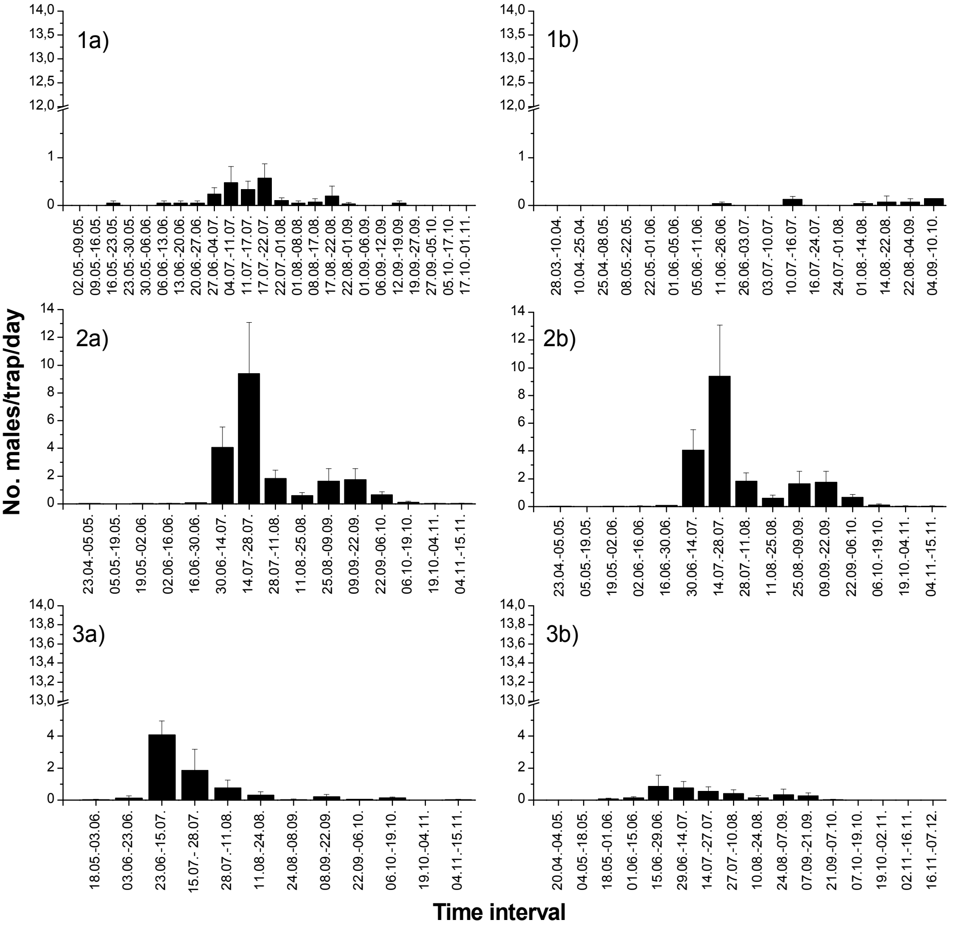

Figure 2. Number of *Plodia interpunctella* males caught by pheromone traps in Želimlje (1), Obrije (2) and Jable (3) in 2004 (a) and 2005 (b).

The males of Indianmeal moth were most numerous in Obrije during the three years of the study. The results suggest two peaks of flight activity, perhaps correlated with generations, of this moth, with the first being rather more numerous (figure 2). The first peak occurred during the second half of July (7-9 males/trap/day). In all three years, the second peak was in the middle of September, with captures of 2 males/trap/day. In Jable, the pest also showed two capture peaks as in Obrije, but captures were less numerous. This can be explained by the fact that two traps were set outside the storage, were only single specimens were captured. The first peak in captures at this location was during the second half of July, and captures were five times more numerous in 2004 (4 males/trap/day) compared to the next year. The second peak in captures was less numerous and appeared at the end of August and in September. In Želimlje captures of Indianmeal moth were the least numerous, probably because monitoring was in a non-protected open air storage, where corncobs were stored. There was never more than 1 male/trap/day caught at this location, and adults were active from the beginning of June till the end of September (both years) (Trdan et al., 2010). Part of this research was published by Selišnik in 2007. These results are opposite to reports of Campbell and Arbogast (2004), who found the greater activity of Indianmeal moth outside wheat flour meal compared to inside of them.

3.2. Mediterranean Flour Moth (*Ephestia kuehniella* [Zeller]; Lepidoptera, Pyralidae)

E. kuehniella is found worlwide but not abundant in the tropic region. The complete life-cycle of this species takes about 50 days. Mediterranean Flour Moth larvae mainly feed on wheat flour but are recorded from a wide range of commodities and from dead insects (Cox and Bell, 1991).

The surface of the newly laid eggs of *E. kuehniella* is white in colour, and shining iridescent when observed by reflected light (Garcia-Barros, 2000). Just before hatching the egg turns light yellow in color due to the development of the embryo which can be seen through the shell of the egg at this time. The egg is 500-550 μm long by 290-325 μm wide. *E. kuehniella* larvae are 0.866 mm long and 0.199 mm wide on average immediately after hatching (Garcia-Barros, 2000). The newly hatched larvae are cream coloured and sparsely covered with long hairs. *E. kuehniella* larvae have six instars (Cox and Bell, 1991). Mature larvae crawl to the surface of the material on which they have fed, and spin silk cocoons intermingled with particles of meal and flour for pupation. Pupae are pale green at the early stage and then turn to reddish brown on the dorsal side of the thoerax. On the last day of development, pupae become dark in color (Garcia-Barros, 2000). Adults are 10-14 mm long when at rest, with wingspan beiing 20-25 mm; forewings are blue-grey with transverse dark wavy bars and a row of dark spots at the tip; hindwing are dirty white with fuscous veins.

In Slovenia the pest was monitored simultaneously with *Plodia interpunctella* males (in the same pheromone lures) since the manufacturer does not offer a specific pheromone for each species. Therefore the same material and methods as it is presented for *Plodia interpunctella* (see the 5th paragraph of the chapter 1.1.) was used (Trdan et al., 2010).

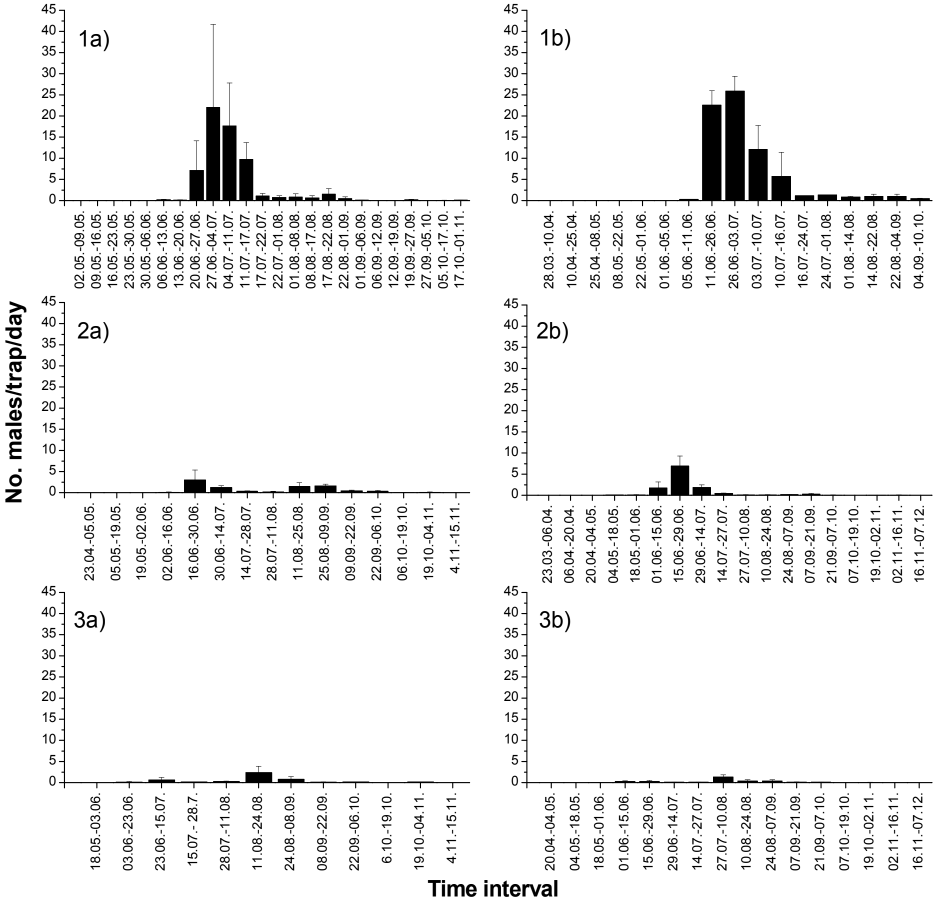

Figure 3. Number of *Ephestia kuehniella* males caught by pheromone traps in Želimlje (1), Obrije (2) and Jable (3) in 2004 (a) and 2005 (b).

Mediterranean flour moth captures in the pheromone traps were more numerous than captures of *Plodia interpunctella*. The captures of this pest, which appeared to have two peaks in capture, was most numerous in Želimlje, where the first peak was in the beginning of June (both years) – 22 to 25 males/trap/day. Later in the year the abundance of this moth decreased significantly, possibly also due to removal of the stored corncobs from the previous season. Single males were found in the traps till the end of the monitoring in October. In closed storage rooms, In Obrije and Jable, captures of Mediterranean flour moth were less numerous, the peak being about 7 males/trap/day in Obrije in the second half of July. The males at these locations were spotted from the beginning of June until October (Figure 3). Part of this research was published by Selišnik in 2007.

3.3. The Angoumois grain moth (*Sitotroga cerealella* [Olivier]; Lepidoptera, Gelechiidae)

Sitotroga cerealella (Olivier) is an important pest of stored grains, whose biology has been well researched (Shazali, 1990). The Angoumois grain moth overwinters as mature larvae that pupate in a silken cocoon in the grain during early spring. Larvae feed inside whole grains on the germ and endosperm, completely destroying the kernel (Arbogast and Mullen, 1987). This insect prefers damp grain to old dry grain, especially barley, corn, oats, wheat, and various seeds. This pest may infest grain, especially wheat and corn, before harvest while it is still standing in the field (Arbogast and Mullen, 1987).

Adults are small, buff or yellowish-white moths with pale yellow forewings and gray, pointed hind wings with a wingspan of 12 mm. The forewings are marked with a few darker lines, and the hind wings are notched at the apical end. The wings also have long fringe around the margins. Mature larvae are pale yellow and about 6 mm long with poorly developed prolegs on the abdomen (Arbogast and Mullen, 1987). Adults emerge in May and June and females begin laying eggs singly or in clusters on or near the grain. Eggs hatch in about a week during the summer, but may require up to four weeks to hatch when temperatures are cooler. Young larvae enter the grain immediately. Larvae mature in two to three weeks, and construct an escape tunnel in the grain through which adults can later emerge. The pupal stage lasts about two weeks (Shazali, 1990). Under favourable conditions, the life cycle may be completed in five to seven weeks. In colder climates, larvae become dormant for four to five months and the life cycle may take up to six months to complete. In unheated storage, there are two generations each year. In heated storage, there are usually four to five generations per year (Arbogast and Mullen, 1987).

The results of investigation in Slovenia (for material and methods see the 5th paragraph of chapter 1.1.), which was performed in Obrije in the period 2005-2006, showing a typical two peaks in flight activity, allow the conclusion, that, on the average, the species under investigation develop two generations under the conditions in Central Slovenia (Figure 4). The results of our research showed that in 2005, the first males of Angoumois grain moth were caught in the first half of June, while more substantial numbers (more than 2 males/trap/day) were observed in Obrije in the first half of July. This period may correspond with the peak of first generation of the year. As the study went on, a second peak was observed in the midst of September (6 males/trap/day), which could indicate a second generation. The adults were active till the first half of November. In 2006, the pest was less numerous and the first specimens were found in the traps during the second half of June. As in 2005, two peaks in trap capture were observed, the peaks being 1 male/trap/day at the beginning of July and in the midst of September (Trdan et al., 2010; Zalokar, 2010).

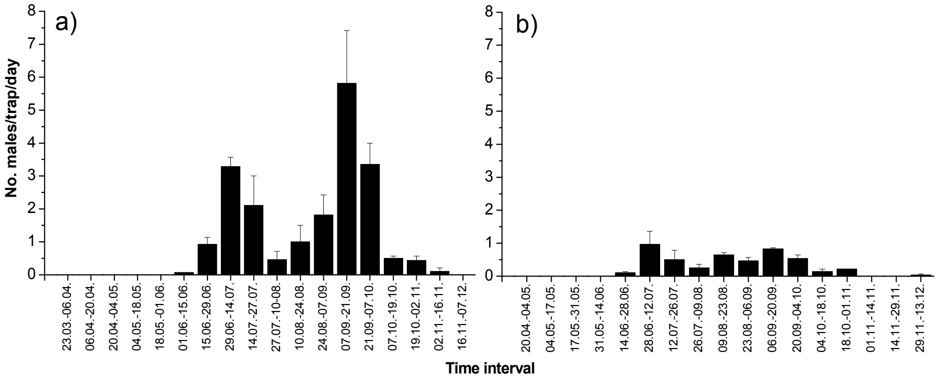

Figure 4. Number of *Sitotroga cerealella* males caught by pheromone traps in Obrije in 2005 (a) and 2006 (b).

4. Vegetable pests

4.1. Swede midge (*Contarinia nasturtii* [Kieffer]; Diptera, Cecidomyiidae)

The Swede midge is a pest of most cultivated Brassicaceae such as broccoli, canola, cauliflower, cabbage, and Brussels sprouts. The species primarily has a Palaearctic distribution and occurs throughout Europe and southwestern Asia to the Caucasus (Olfert *et al.*, 2006). Plant damage is caused by larval feeding; symptoms include misshapen plants and the formation of galls on leaves and flowers (Bardner et al., 1971). The larval stage overwinters in the soil, adults emerge in May and females lay eggs, in clusters of 2–50, on the surface of actively growing plants. Larvae feed on actively growing stems, leaves, and flowers, then drop to the soil to pupate. During the growing season, adults can emerge within 2 weeks or larvae enter diapause in autumn. Depending on temperature and soil moisture, *C. nasturtii* may have 2–5 generations (Hallett and Heal, 2001). Temperature and moisture have been identified to be the two most important factors responsible for population distribution, growth, and control. Population growth was greatest in warm, moist seasons and reduced in cool or dry seasons (Readshaw, 1966).

In 2004, the occurrence of Swede midge on four locations in Slovenia (Ljubljana, Rakitnica, Zakl near Braslovče, Škocjan near Koper) was investigated (Figure 5). Pheromone traps of the Swiss producer (Agroscope FAW, Wädenswill) were set in fields with Brassica plants. The aim of the research was to determine a population dynamics of the pest in vegetation period, to establish the number of generations it develops per year in geographically and climatically different regions. Understanding of the pest bionomics would help to set a strategy for control of Swede midge in Brassica plants. The results of the monitoring indicate that the species has 4 generations per year, also in the continental part of the country. In all four locations a generation was also established in September (Trdan et al., 2005a). Part of this research was published by Walland in 2007.

In similar research, which lasted from the beginning of April to the beginning of November 2006, a seasonal dynamics of Swede midge was investigated at the Laboratory Field of the Biotechnical Faculty in Ljubljana (Trdan and Bobnar, 2007; Trdan *et al.*, 2008). The males of Swede midge were trapped with already mentioned traps of Swiss producer The pheromone capsules were changed in 4-week intervals, while the males were counted on about every 7th day. The first massive occurrence of Swede midge (0.4 males/trap/day) was established in the 2nd decade of May, while the highest number of males (8/trap/day) were caught in the 2nd decade of July. In the 3rd decade of October, the last adults were found in the traps. Based on the results of monitoring we ascertained that in the central Slovenia the Swede midge has 3-4 generations.

In a related research, which was performed in 2006 (Bohinc, 2008), it was confirmed, that the use of synthetic insecticides and fungicides have an influence on a population dynamics of the above mentioned pest.

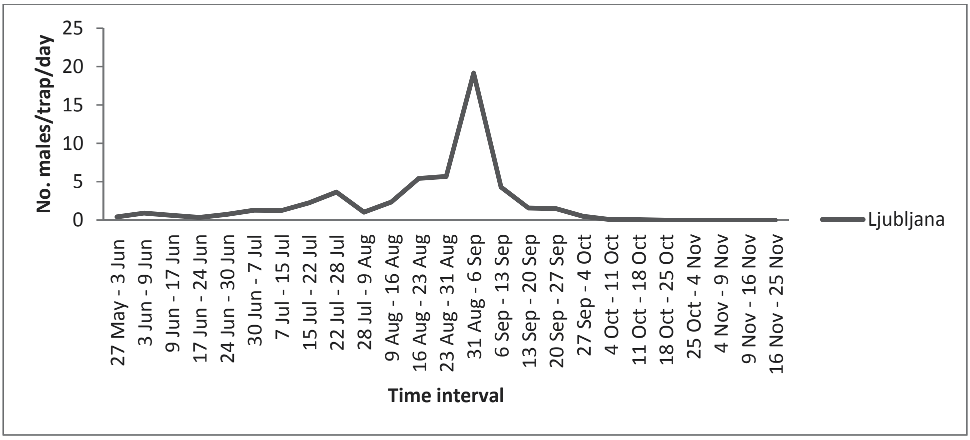

Figure 5. Population dynamics of *Contarinia nasturtii* males in Ljubljana in 2004.

4.2. The diamondback moth (*Plutella xylostella* [L.]; Lepidoptera, Plutellidae)

The diamondback moth is a cosmopolitan species that probably originated in the Mediterranean region (Hardy, 1938). Host plants include both cultivated and wild-growing plants of the family Cruciferae, as well as several ornamentals, such as wallflower, candytuft, stocks, and alyssum. Cultivated crops that are attacked include broccoli, Brussels sprouts, cabbage, cauliflower, Chinese broccoli, Chinese cabbage, flowering white cabbage, head cabbage, mustard cabbage and watercress. Weed hosts, such as mustard and radish, are important reservoir hosts for the species.

The first instars sometimes feed in the spongy plant tissue beneath the leaf surface forming shallow mines that appear as numerous white marks. These mines are usually not longer than the length of the body. The larvae are surface feeders in all subsequent stages. These larvae feed on the lower leaf surface 62-78 % of the time, chewing irregular patches in the leaves (Harcourt, 1957). All the leaf tissues are consumed except the veins. On some leaves, the larvae feed on all but the upper epidermis creating a "windowing" effect. The last stage larva is a voracious feeder; it causes more injury than the first three larval instars. Total development time from the egg to pupal stage averages 25 to 30 days, depending on weather, with a range of about 17 to 51 days. The number of generations varies from four in cold climates to eight to 12 in the south. Overwintering survival is positively correlated with the abundance of snowfall in northern climates (Eigenbrode and Shelton, 1990).

From the beginning of April to the beginning of November 2006, a seasonal dynamics of diamondback moth was investigated at the Laboratory Field of the Biotechnical Faculty in Ljubljana. The males were trapped with the Hungarian traps type RAG (Plant Protection Institute, Hungarian Academy of Sciences). The pheromone capsules were changed in 4-week intervals, while the males were counted on about every 7th day. The first massive occurrence of diamondback moth (1.6 males/trap/day) was established in the 2nd decade of April, and the pest remained active until the 2nd decade of September (figure 6). The adults were the most numerous in the period between the end of May to the middle of June, but even then their number did not exceed three males caught per day. Based on the results of monitoring we ascertained that in the central Slovenia the diamondback moth has 4 generations (Trdan and Bobnar, 2007; Rešetič, 2008).

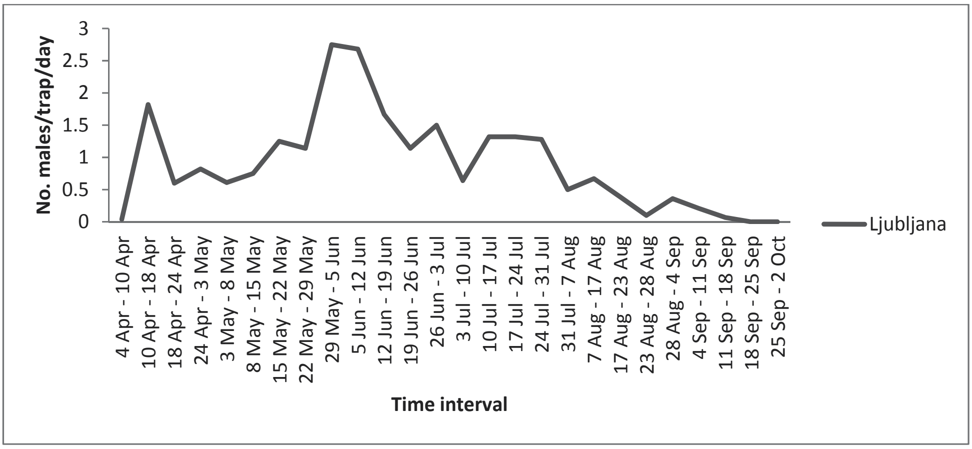

Figure 6. Population dynamics of *Plutella xylostella* males in Ljubljana in 2006.

4.3. Flea beetles (*Phyllotreta* spp.; Coleoptera, Chrysomelidae)

Flea beetles (Coleoptera: Chrysomelidae) inhabit a wide range of environments where cruciferous plants grow, including fields, gardens, and uncultivated areas. Although flea beetles colonize crops every year, their population densities vary widely between years (Andersen et al., 2005). Flea beetles are univoltine. They overwinter as adults, usually outside fields, in margins, in hedgerows, and beneath shrubs, although some find shelter within fields in leaf litter, in stubble, or in grassy areas (Andersen et al., 2005). Physical conditions at their overwintering sites may fluctuate daily, seasonally, and between years. Various species of flea beetle feed on the leaves of Brassica plants throughout the entire growing season (Vig, 2002; Trdan et al., 2005b; Bohinc et al., 2012). The beetles usually cause most problems on young plants, as these are small and hence, can tolerate only small amounts of damage.

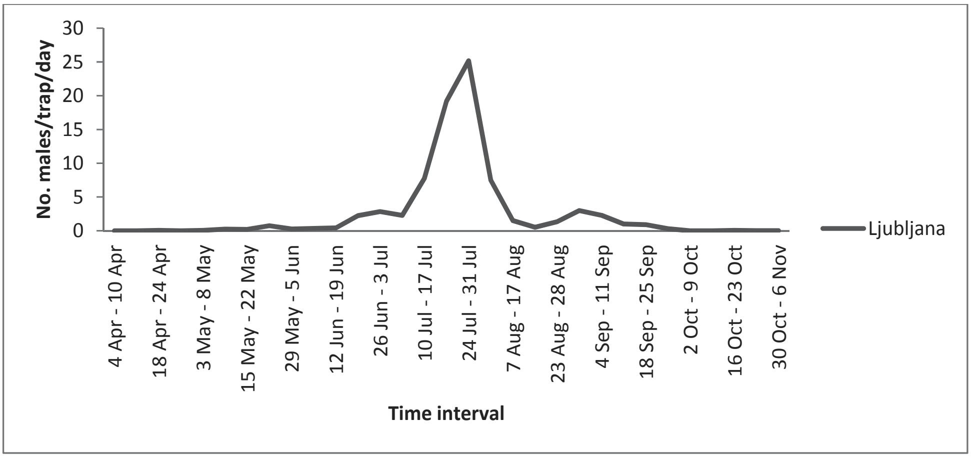

Figure 7. Population dynamics of *Phyllotreta* spp. males in Ljubljana in 2006.

From the beginning of April until the beginning of November 2006, a seasonal dynamics of flea beetles was investigated in Ljubljana (Trdan and Bobnar, 2007; Kržišnik, 2009). The number of males was monitored with pheromone traps (type KLP+) of Hungarian producer. Pheromone baits were changed once per month, and the males which were caught in the traps were counted once per week. The beetles were the most numerous in the second and third ten-days period of July, while the last flea beetle was found in the beginning of November. Weather conditions in the growth period of cabbage, above all the rainfall and air temperature, had important influence on the number of the beetles. Based on the results of flea beetles monitoring we can conclude that in Central Slovenia the pest under our investigation has 1-2 generation (firure 7).

5. Field crop pests

5.1. Beet moth (*Scrobipalpa ocellatella* Boyd; Lepidoptera, Gelechiidae)

Beet moth is a dangerous pest of sugar beet especially in southern Europe (Robert and Blaisinger, 1978). Caterpillars can also feed on other plants of the family Chenopodiaceae (pigweed, seablite, saltwort etc.). The caterpillars skeletonize leaves, braiding them with silk threads. In spring and in the beginning of summer, they mine leaves, usually along main veins, also piercing holes in petioles. The damaged leaves roll and blacken. A black clump of rotten leaves fastened with silk threads is formed instead of the central rosette. In hot and dry years, such damage frequently causes the whole plant to die since the outer leaves die off quickly and new ones are not formed because of the central rosette loss. Caterpillars of the following generations penetrate into roots. In the upper part of the roots they gnaw out narrow, twisting grooves or holes under thin skin, sometimes boring to a depth of 5 cm. These holes under skin also injure lateral parts of roots. The damaged roots become languid and rotten. In parent beet plants, the caterpillars injure flower buds, unripe seeds, and tips of growing floriferous stalks, piercing holes; as a result, the stalks are bent, and yield of seeds sharply falls (Robert and Blaisinger, 1978). The economic damage threshold is exceeded when 4-5 larvae are found on 70% of plants (Valič et al., 2005).

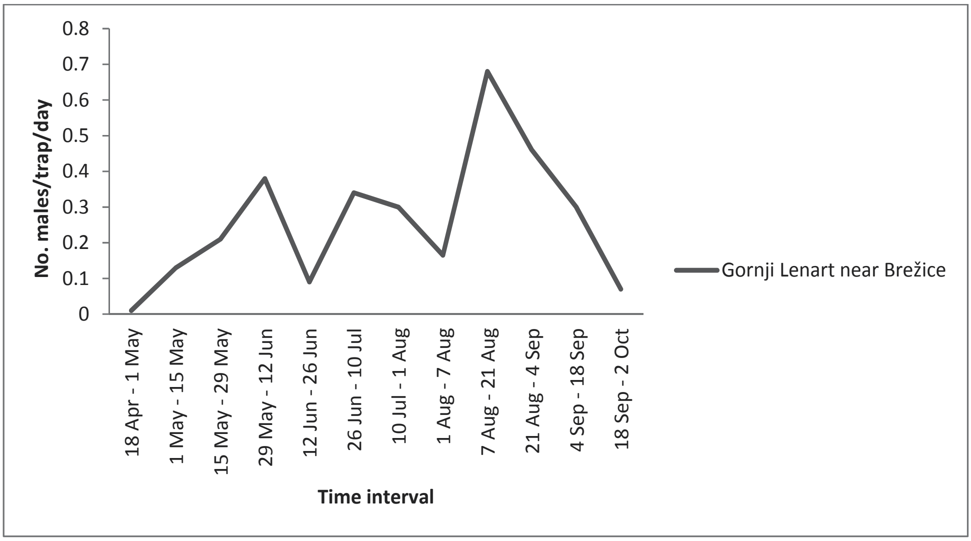

Figure 8. Population dynamics of *Scrobipalpa ocellatella* males in Gornji Lenart near Brežice in 2004.

There are two to five generations a year depending on the climate. Moth flight after wintering begins in April, during shooting of beet in spring. One generation develops 40-65 days. The pest numbers increase in every following generation, reaching maximum by the end of summer. This species overwinters as a pupa or partly-grown larva (Robert and Blaisinger, 1978).

In the beginning of this century, the sugarbeet moth was a new pest of sugarbeet in Slovenia. The first noticeable occurrence of the pest was recorded in 2003, which was distinctively drier and warmer than an average year. Such weather conditions are especially suitable for this species. In 2004, monitoring of the pest was carried out on four locations: Cvetkovci, Rakičan, Gornji Lenart near Brežice and Kranj. The occurrence of the pest was determined by means of setting pheromone traps on the margins of the sugarbeet fields. The greatest number of sugarbeet moths was caught in traps in Gornji Lenart near Brežice, where sugarbeet was grown in close vicinity in 2003. In Kranj, there was no trace of the pest at all. Based on one-year results of the monitoring of the sugarbeet moth we came to a conclusion that in 2004 in Slovenia the pest had 2 generations (figure 8). The sugarbeet moth has caused no severe yield loss in Slovenia so far. Consequently, no insecticides have been registered for its control as yet (Valič et al., 2005; Čepin, 2006). After 2006, the production of sugarbeet has stopped in Slovenia, and that is also one of the reasons that the pest was not recorded in the fodder beet field in the vicinity of Škoflja Loka during monitoring in 2010 (Dolenec, 2012).

5.2. The turnip moth (*Agrotis segetum* [Denis & Schiffermüller]; Lepidoptera, Noctuidae)

The turnip moth is distributed across the European and the Mediterranean regions. *Agrotis segetum* is an important economic insect of turnip, lettuce, Swedes, wild-growing plants, couch grass, bindweed and plantain. Infestation also occurs in neighbouring vegetables including red beet, potato, cereals, tobacco and vine (Wood et al., 2009).

The adult has a 40 mm wingspan; dark brown fore wings with, in the middle, a uniform spot and a clearer circular spot. The hind wings are white in the male and grey in the female. The periphery of the wings bears a thin black border. Larva is 45 to 50 mm, reddish head and a greyish body with two parallel longitudinal lines in the middle region. On each segment, 2 small black spots at the front and two at the back, bearing a small bristle as well.The young caterpillar first nibbles the wild-growing plants and then attacks the neighbouring cultivated species. It feeds at night, gnawing the foliage and cutting the petioles. During the day, it conceals itself by rolling up under a lump of earth or at a slight depth in the ground (Anderson and Löfquist, 1996).

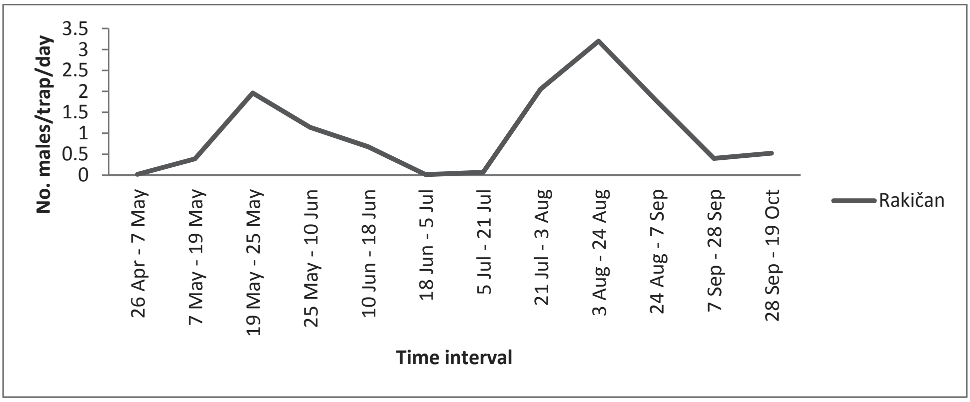

Figure 9. Population dynamics of *Agrotis segetum* males in Rakičan in 2004.

In 2004, the monitoring of turnip moth in sugarbeet (*Beta vulgaris* var. *altissima* Döll.) were performed in two locations in Slovenia: Cvetkovci near Ormož, Rakičan and Kranj (Zalokar, 2006). Pheromone traps (VARL type, Csal♀m♂N® Budapest, Hungary) were used for monitoring the pest under investigation. Four baits were set along the margins of fields (one parcel on each location) where sugarbeet was sowed. Pheromone capsules were replaced once a month and the butterflies captured in them were counted in 2-3 week intervals. We ascertained that, under favourable climatic conditions, the turnip moth are able to overmultiply; that occurs when the air temperature rises above 12°C. The amount of rainfall does not influence the bionomics of the pest. In the neighbouring countries turnip moth develops two generations per year. Based on our observations in 2004 turnip moth appears in two generations also in Slovenia (figure 9). Similar conclusions were gained also in two researches, which took place in the vicinity of Škofja Loka; Kalan (2010), monitored the pest in 2008 on the corn field, and Dolenec (2012) studied the seasonal dynamics of the butterfly in 2010 on the fodder beet field. On the other side Srebernjak (2009) confirmed the occurrence of three generations of the same pest on the corn field in the vicinity of Novo mesto.

6. Fruit tree and grapevine pests

6.1. The Plum Fruit Moth (*Grapholita funebrana* [Treitschke]; Lepidoptera, Tortricidae)

A native of Europe, *Grapholita funebrana* has spread to most other fruit-growing regions of the Palaearctic. It is currently present from Europe and northern Africa across Asia Minor and Central Asia to China, Korea, and Japan (Hrdy et al., 1996). *Grapholita funebrana* is one of the most important lepidopteran pests of fruit in Europe. Larvae can cause significant damage to apricot, cherry, peach, plum, and other *Prunus* species. The Plum Fruit Moth completes 1-3 generations per year; two generations are most common over most of its range. Adults are present from late May to September (Polesny et al., 2000).

First generation females lay eggs singly on fruitlets. Second generation females lay eggs near the base of maturing fruit. Larvae tunnel into the fruit and feed inside. Last instar larvae bore out of the fruit and overwinter in a cocoon spun on tree bark or in the soil. Pupation occurs the following spring. Larvae of the second generation cause the most damage to fruits such as plum that mature in mid- to late summer (Hrdy et al., 1996).

Distribution of plum fruit moth in plum crowns and its apperance in the vicinity of the trees was investigated in 2007 in Dolenja vas near Ribnica (Pogorelc, 2008). Pheromone traps were placed in the first half of February on two trees. Experiment has lasted till the end of September. The purpose of experiment was to find out in which parts of the crowns or how far from the trees the pest appears. It was found out that pest was the most abundant at sunny exposure of crowns, on SE and SW crown parts. Inside of crowns and in northern part of crowns, the pest was less abundant. Before the flowering of plums the abundance of the plum fruit moth in plum crowns and in the vicinity of the trees was almost same numbers. During the flowering and growing season, the number of the moths was higher inside crowns. Plum fruit moth appeared also in the vicinity of the trees. On baits, 10 and 20 m away of them, quite high number of the males has been caught. Air temperature and amount of rain also influenced moth apperance. From the data of our research we can conclude that pest had two generations per year (figure 10), whose the second generation was more abundant from the first generation. In a related research Humski et al. (2005) and

Humski (2007) studied the occurence of the plum fruit moth in 2004 and 2005 on different locations in Slovenia and concluded that the plum fruit moth has 3 generations per year only in the littoral part of Slovenia (Kromberk near Nova Gorica and Koper), while in the continental part of the country it has 2 generations per year.

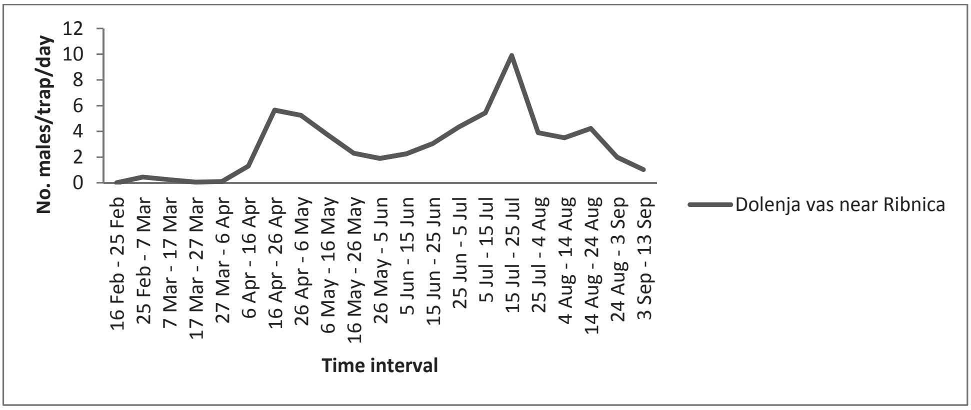

Figure 10. Population dynamics of *Grapholita funebrana* males in Dolenja vas near Ribnica in 2007.

6.2. The codling moth (*Cydia pomonella* [L.]); Lepidoptera, Tortricidae)

The Codling Moth is a cosmopolitan insect pest of deciduous fruits. It has a remarkable ability to adapt to a wide range of climatic conditions. The number of generations gradually increases toward the southern latitude in the Northern Hemisphere and towards the north in the Southern Hemisphere. In, general, the larva of codling moth have five instars (Witzgall et al., 2008). Weitzner and Whalon (1987) describes that the codling moth overwinters as either a fourth or fifth instar diapausing larvae. Codling moth overwinters as a mature larva beneath tree bark scales or at the base of the tree. Adults appear in the spring and eggs are laid singly on or near the fruit. Eggs hatch in 5 to 12 days, depending upon temperature, and the young larvae move to developing fruit within a few hours, chew through the skin, and burrow into the flesh. Subsequently, the larva burrows to the fruit core and feeds on seeds. The potential for crop loss to the codling moth makes it the most important pest of pome fruits. When uncontrolled, the codling moth is capable of annually destroying 80 % or more of an apple crop and 40-60 % of a pear crop. Besides apples, the codling moth can develop on other pome fruits such as pear and quince, on stone fruits such as apricot, plum and peaches as well as on walnuts (Witzgall et al., 2008), apricot, almond, pecan nuts and pomegranates (Weitzner and Whalon, 1987).

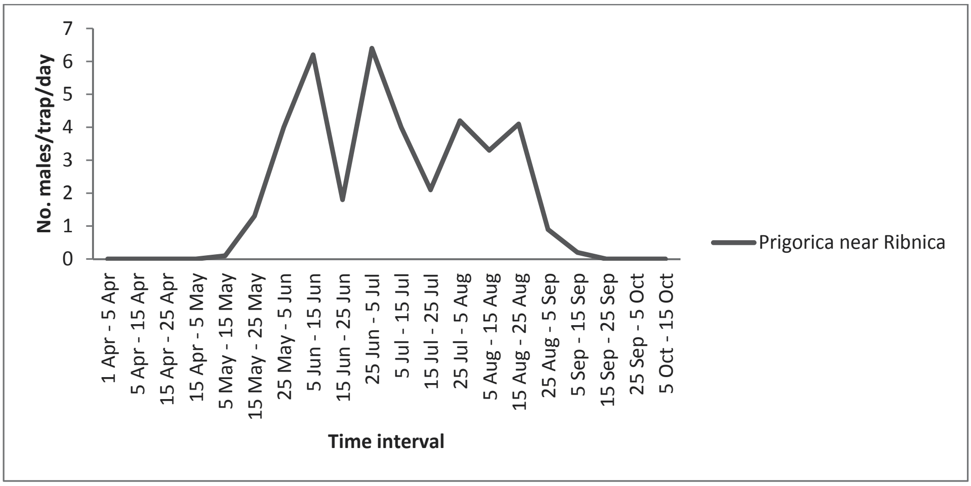

Figure 11. Population dynamics of *Cydia pomonella* males in Prigorica near Ribnica in 2010.

In 2010, a monitoring of codling moth was conducted in an extensive mixed orchard in the village Prigorica near Ribnica (Bartol, 2011). In the orchard, four insect pheromone baits (RAG type, Csal♀m♂N® Budapest, Hungary) were placed and the occurrence of the pest was monitored from early April to mid-October. The purpose of the study was to examine the presence and the numbers in which this pest occurrs, since it was assumed that they differ from that of the intensive orchards. With the research the author obtained useful informations needed to optimize control strategies of codling moth, in which a pheromone baits can also be used for mass trapping of the pest. It was found out that the pest appeared from the first decade of May until the second decade of September and during this time it developed two generations (figure 11). The first generation was larger than the second one. Both, the temperature and the rainfall affect the occurrence and the numbers of codling moth.

6.3. The Red-belted Clearwing (*Synanthedon myopaeformis* [Borkhausen]; Lepidoptera, Sesiidae)

Synanthedon myopaeformis is a xylophagous species that attacks pome and stone fruit trees (Trematerra, 1993). The larval form of this insect lives under the bark of fruit trees, especially apple (*Malus*), but sometimes pear (*Pyrus*), almond (*Prunus amygdalus* Batsch) and a few other closely related plant species (Iren and Bulut, 1981). The larvae located under the bark of tree trunk and thick branches bore deep subcortical galleries 20 to 25 mm long and cut into the phloem (Iren and Bulut 1981). The control of this pest is difficult because the adults have a long emergence period and the larvae develop inside the trunk and thick branches. Failure to prevent injury can lead to reduced tree vigor and yield (Trematerra, 1993).

In 2008 and 2009, a seasonal dynamics of apple clearwing moth (*Synanthedon myopaeformis*) was monitored in two extensive apple orchards (mowed and overgrown) in the village Bojsno in the Bizeljsko region (Hriberšek, 2012) and in Roginska Gorica in the Kozjansko region

(Gradič, 2009). Pheromone traps (RAG type, Csal♀m♂N® Budapest, Hungary), which consist of a triangular plastic casing, a pheromone capsule and a sticky plate were used for monitoring. Four traps were randomly placed in each orchard. Capsules were changed monthly and caught moths were counted in 10 day intervals. The research was conducted from the beginning of April to the beginning of September. The purpose of the research was to examine influence of temperature and quantity of precipitation to the abundance of the pest regarding usage of grassland in the orchards. The results revealed that the usage of grassland has a great influence on the abundance and as well on the appearance of the apple clearwing. The pest was more abundant in the mowed extensive orchard than in the overgrown extensive orchard. First males appeared earlier in the overgrown orchard than in the mowed orchard and they stopped appearing earlier in the overgrown than in the mowed orchard. Captured moths were detected from the end of April to the beginning of August. Moths were the most abundant from June to the beginning of July. Similar conclusions gained also Gradič (2009) in her related research (figure 12). The pest occurred in the mowed extensive orchard when the temperature increased above 15 °C and in the overgrown orchard when the temperature increased above 13, 5 °C. Abundance of the male apple clearwing moths was also increased by the quantity of precipitation from 20 to 40 mm.

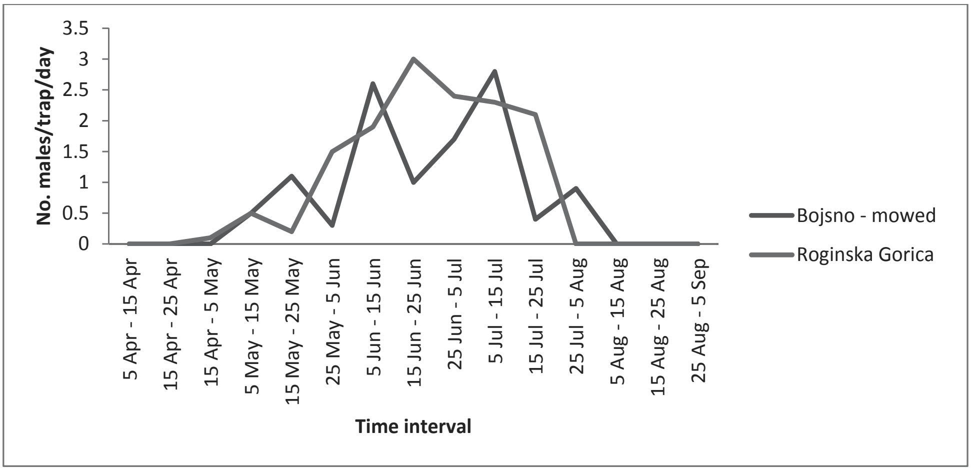

Figure 12. Population dynamics of *Synanthedon myopaeformis* males in Bojsno (2008) and in Roginska Gorica (2009).

6.4. The Goat Moth (*Cossus cossus* [L.]; Lepidoptera, Cossidae)

Goat moth is an important forest insect of Europe. It is distributed in North America, China and Siberia. Larvae cause the main damage. It bores into the heart wood causing extensive damage and finally killing the tree (Oberhauser and Peterson, 2003). In southern Europe this species has been recorded on sugar beet and Artichoke (Pasqualini and Natale, 1999). Caterpillars can also damage wood of pears, apple, plums, cherries, quince, apricot, walnut, persimmon, European olives, wild olives (*Olea oleaster*), mulberries, sea-buckthorn, willow, poplar, aspen, alder, ash-tree, birches, beech, oak, maple, elm (*Ulmus suberosa*), oleaster. Adults fore wings are white and the hind wing grey in colour. Head, thorax and abdomen on the adult has the similar grey colour. Antennal type is pactinate type. Larvae head is black and shiny, body yellowish white with a dorsal band of purplish red. It has special distinctive goat type smell. Larvae bore the trees from bark and take 2-4 years to complete full growth. Pupation takes place in a cocoon made with silk and wood materials (Pasqualini and Natale, 1999).

In the period 2005-2006, the monitoring of male adults of the goat moth (*Cossus cossus* L.) were performed with pheromone traps in plantation of apricots and cherries in the vicinity of Pišece (Jeršič, 2009). In the past years the caterpillars of this insect pest caused languishing state of the trees. In both years the massive occurrence of the pest was established in the period from the end of June until the end of July. In 2005, the highest number of the males (one specimen/trap/2 days) was found in the first decade of July, when the degree-day sum was 595.4 °C. In 2006, the highest number of butterflies (1.4 males/trap/day) were found in the second half of the last decade of June, when the degree-day sum was 519.0 °C (figure 13). The hypothetical lower developmental threshold was 10.0 °C. In Slovenia, no insecticides are registered for controlling the goat moth, therefore other ways of suppressing its damage should be found. One of the most promising methods is the use of controlled-release pheromone dispensers (mating disruption method), which we suggest for implementation in the systems of the fruit production in these areas, where the goat moth is an important biotic factors for limitation of fruit production (Trdan and Jeršič, 2008).

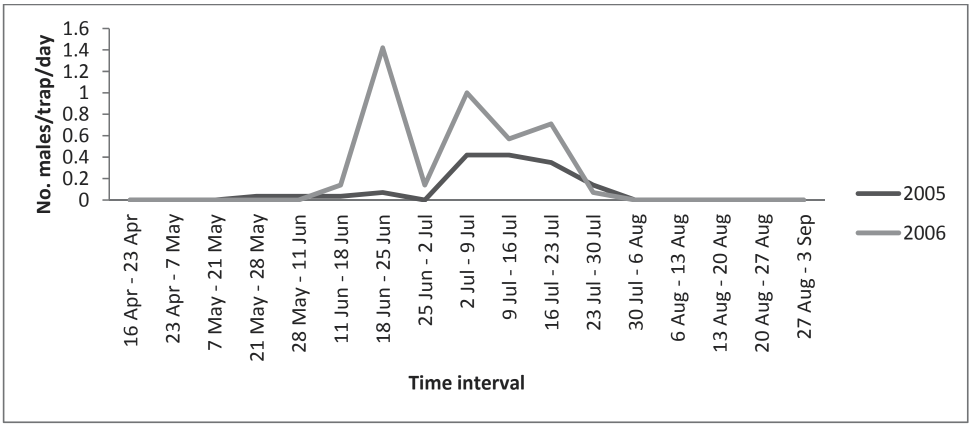

Figure 13. Population dynamics of *Cossus cossus* males in Pišece in the period 2005-2006.

6.5. The European grape berry moth (*Clysia ambiguella* [Hübner]; Lepidoptera, Tortricidae) and the European Grapevine Moth (*Lobesia botrana* [Denis & Schiffermüller]; Lepidoptera, Tortricidae)

The life cycle of *C. ambiguella* is similar to that of *L. botrana*, with the exception of two generations for *C. ambiguella* versus three or more generations for *L. botrana*. Over most of its range, adults are present in May and June for the first generation and again in August and September for the second generation (Gilligan and Epstein, 2011).

Females deposit eggs singly on buds, pedicels, and flowers during the first generation, and on grape berries during the second generation. Early instar larvae burrow into the buds or berries and feed internally; later instars web together buds or berries, and a single larva can feed on up to a dozen berries. Pupation occurs in leaves for the first generation and under bark for the second generation. Overwintering occurs as a second generation pupa. Development time is highly dependent on temperature and humidity. The optimum relative humidity level for development is 70% or higher; eggs will fail to hatch at low relative humidity levels (Gilligan and Epstein, 2011).

Economic losses on grape are caused by direct feeding damage and secondary infections. Feeding damage is similar to that of *L. botrana*. Larvae of the first generation cause minor damage by feeding on flower buds, while those of the second generation cause the most damage by feeding on grape berries. The most significant losses are due to secondary infection of feeding sites on berries and clusters by *Botrytis cinerea.* Economic thresholds vary with the type of grape and cultivar (Gilligan and Epstein, 2011).

During 2007 the occurrence of European grape berry moth (*Clysia ambiguella*) and European grapevine moth (*Lobesia botrana*) was monitored in vineyard at Gaberje village near Ajdovščina (Florijančič, 2010). We observed them on two grapevine varieties: 'Chardonnay' and 'Rebula'. Four pheromone lures (type Pherocon 1C trap, manufacturer Trécé Incorporated, Oklahoma, USA) were placed symmetrically on each variety of grapevine in the vineyard with the size of 0,35 ha. From the end of April to the first days of September, the butterflies developed two generations (figure 14). The peak of the first generation of European grape berry moth was established from 23rd April to 6th May, followed by the second generation, which peaked between 18th June and 1st July. The first generation of European grapevine moth occurred in the period between 7th and 27th of May, followed by the second generation, which established between 11th June and 22nd July. The last three specimens were caught between 27th August and 2nd September. The number of present specimens during the growing season was influenced by weather conditions, namely the air temperature, quantity of precipitations and the relative humidity. In our lures almost twice as many butterflies of European grapevine moth were collected in comparison to European grape berry moth. It is assumed that in Slovenia at least two generations of European grape berry moth and European grapevine moth appear per year. It is assumed also that the variety of grapevine has no influence on the occurrence of both species of butterflies.

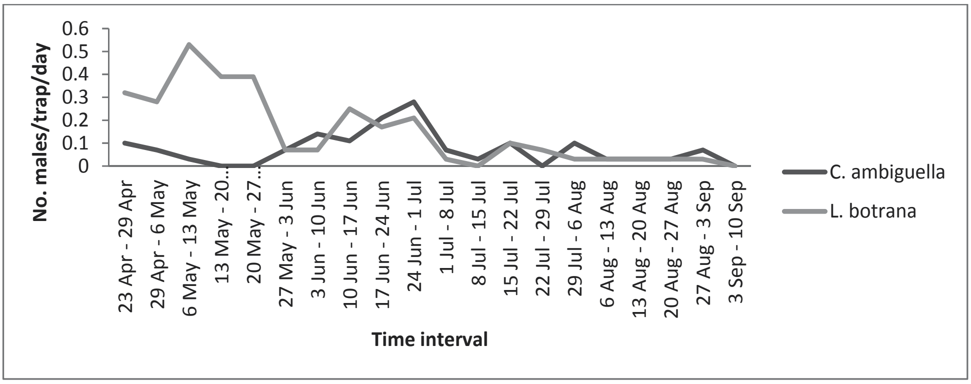

Figure 14. Population dynamics of *Clysia ambiguella* and *Lobesia botrana* males in Gaberje in 2007.

7. Conclusions

Monitoring of harmful insects is one of the basic steps of integrated food production and storing of plant products, since timely detection of the pests prevents their development, spreading and consecutive damage, as well it enables the diminishing of insecticide use. Pheromone traps belong among the most commonly used detection methods of insects, and their advantage over other methods (coloured sticky boards, light traps…) is particularly in their specificity, which is the reason for simple and reliable assessment on the number of harmful insects under investigation. With the use of pheromone traps it is possible – more effective than with other detection methods - to realize one of the fundamental principles of integrated pest management (Milevoj, 2007), i.e. the use of insecticides against insects pests only then, when they reach the damage threshold in agricultural plants and products. In this way the pheromone traps enable the insecticide use in time, which is one of the more important conditions of their satisfactory efficacy. The greater part of investigations, which are present in this chapter, are bounded with the monitoring of insect pests, which bionomics was up to now not studied in Slovenia. Therefore these resuls are important contribution to the field of applied entomology. Results of some researches only confirmed the statements from Slovenian scientific monographies (Vrabl, 1992; Vrabl, 1999), which were in many cases summarized from foreign authors. Other investigations offer less expected results, which can be influenced by climate change, changing of plant varieties, food production techniques etc. In any case this chapter presents the first comprehensive review of monitoring of many insects pests (14 species in 26 different locations) in Slovenia, which can be a good basis for further investigations of complexicity of relations between insects and environment. We hope that with the results of mentioned investigations we will be able to offer the answers for higher present economic impact of some insect pests compared with past decades and to found out if the populations of harmful insect pests can be effectivelly diminished with the use of environmentally friendly methods, in the group of which also pheromone traps have important role.

Acknowledgements

This chapter was performed under the programme Horticulture (P4-0013), funded by the Slovenian Research Agency, and under Professional tasks from the field of plant protection, funded by the Ministry of Agriculture and Environment – Phytosanitary Administration of the Republic of Slovenia.

Author details

Žiga Laznik and Stanislav Trdan

University of Ljubljana, Biotechnical Faculty, Dept. of Agronomy, Chair of Phytomedicine, Agricultural Engineering, Crop Production, Pasture and Grassland Management, Ljubljana, Slovenia

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Chapter 6

The Use of Behavioral Manipulation Techniques On Synthetic Insecticides Optimization

Solange Maria de França, Mariana Oliveira Breda,
Cesar A. Badji and José Vargas de Oliveira

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53354

1. Introduction

The necessity of ever increasing agricultural production has induced farmers to use insecticides to improve yields and profits. The current agricultural mode of production is therefore based on intensive use of insecticides in order to ensure the high productivity by eliminating pests and diseases. Otherwise, others xenobiotics are often used as fertilizer to replace nutrients carried out in agroecosystems. However, in many places, the indiscriminate, prolonged and inappropriate use of these xenobiotics has been affecting the ecosystem and farmers health. Concerning insecticides on agroecosystems, the frequent applications can often cause pest resurgence, environmental pollution and human intoxications, eliminate beneficial insects and enables the emergence of the phenomenon of pest resistance. Under this scenario, the search for other control methods aiming to decrease insecticides use, consequently reducing environmental pollution, the ecological imbalance and human intoxications is required. For this purpose, the understanding of the relationship between the various living organisms in the agroecosystems is essential to provide effective pest control. Living organisms present in the fields of production and its surroundings areas feature between them relationships for survival and preservation. In the specific case of insects, the study of chemical ecology focusing on the intra-and interspecific processes of choice and location of partners, hosts, food sources and shelter have had an important role in the pursuit of a sustainable agriculture. In order to offer more alternatives to control pests, early in the second half of the twentieth century studies were initiated for the development of pest management techniques based on behavioral manipulation of the target organism. When it became clear that insects use their senses to communicate with conspecific and other species of animals or plants, it was possible to

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develop pest behavior research for the benefit of farmers and the environment. What compounds can be used to manipulate the pests and keep them below their level of damage? What behavior can be explored in Integrated Pest Management to reach sustainability in agriculture?

At this current scenario of sustainable development, behavioral control is therefore appropriate by enabling to reduce the use of synthetic insecticides, which usually have broad spectrum and side effects to humans, beneficial insects and environment. Compounds and molecules involved in behavioral pest management such as feeding stimulants and semiochemicals, mostly sex pheromones, can be very useful to reduce synthetic insecticides dosage.

Practical applications of semiochemicals, including the sex pheromones, can lead to modification of pest communication permitting mating disruption, attraction to pint-source lures for monitoring, control by mass trapping, push-pull and attract-and-kill. Attractant lures can also be used for insect population control, in combination with large-capacity traps or a contact insecticide (Witzgall *et al*. 2008). The idea of controlling insect populations through species-specific manipulation of sexual communication, without adversely affecting other organisms, has been a driving force for pheromone research. According to Foster & Harris (1997) manipulation is defined as the use of stimuli that either stimulates or inhibits a behavior and thereby changes its expression. This has been achieved and technological shortcomings have been overcome through a joint effort between researchers, industry, and growers. Adoption of semiochemical-based pest management has increased in the face of dwindling conventional options, such as insecticides, increased government regulations and improved cost-competitiveness.

There are severals main elements of the behavioral manipulation method on which the tactics and strategies targeting the pest management should be based: knowledge about the behavior of the pest, identification of behaviors that should be handled, the ways in which the behavior is manipulated appropriately and the development of methods and tools that are used in the behavioral management of pests. This chapter will discuss pest monitoring and different behavioral manipulation techniques that can be used alone or in combination with other control methods and enable the optimization of synthetic insecticides use in agriculture.

2. Monitoring with attractant-baited traps is an important component of pest-management programs

In the vast majority of farms, pest control is done by spray scheduled. Insecticide applications are made at determined times regardless of the insect population density. When the control by spray scheduled is not used, the presence of a single organism potentially damaging to the culture determines insecticide applications. There is no doubt that those forms of control are not sustainable both environmentally and economically. Therefore, one of the important steps to change those procedures of agricultural production is to quantify the potential damage caused by pests. This determination allows knowing if the insects population density found in the crop can be tolerated. For this reason, one of the major uses of monitoring is the determination of early pest incidence; determine if the pest population has reached levels that will cause economic damage; as well as the detection of infested areas; inspection of quarantine pest presence, among other uses.

The Integrated Pest Management (IPM) which recommends the use of control methods when the pest population reaches the Economic Injury Level (EIL) depends on the monitoring for an effective decision making. Pest monitoring is an extremely important tool, for through it, the proper time to use a control method is defined. If the adopted is chemical control, the insecticides application should be done only when the pest population density reaches control level. Thus, monitoring allows defining the spatial and temporal distribution of the insect. This strategy makes pest control more efficient and economic, reducing costs and time sampling, rationalizing sprayings and preserving biodiversity in the agroecosystems. In pest monitoring, several devices, such as traps, can be used spread out in the fields aiming to quantify specific insects. The number of insects measured is important in the decision making for determine whether the pest population has reached the EIL. At this stage of pest management, assessment is, therefore, qualitative. The important in this case is to obtain a correlation between the number of insects measured and its real population density. For monitoring, a large number of tools can be used as beat cloth, trays or traps. In the case of traps, several attractants can be used, such as pheromones, food and attractive colors.

Pheromones are widely used in pest monitoring (Wyatt 1998) due to their specificity, selectivity and mainly for not affecting the health of workers and environment. They are chemicals used in conspecific communication, acting both in physiology, on individuals development; as in behavior, with a domino effect, prompting immediate action. The domino effect is what is explored in pest monitoring. Among the various existing types of pheromones, the sexual is the most widely studied in insect pest management (Vilela & Della Lucia 2001). The detection and recognition of pheromone molecules by insects are closely related to the olfactory system. The pheromone molecules reach the pores of the antenna sensilla, which are present in the thousands on antenna surface, vertically oriented and capture molecules in the air (Mustaparta 1984). Inside the antenna, the odor molecules collide with the dendritic membrane translating the chemical signal into electrical potential. The nervous impulse is transmitted to the central nervous system to produce a particular behavior in insects. These selective attractants are used in traps spread in the field that are to be monitored. Traps baited with pheromones must be sensitive enough to capture insects selectively, consistently and at low densities. Therefore, the knowledge of the target insect flight behavior is crucial when choosing the type of traps. Trap location and its height in the field, the most appropriate format and the most suitable pheromone mix for the site should be determined from studies of insect reproductive behavior. Additionally, the choice of the trap should be appropriate to the features of the land where the crop is located. Traps which have liquids as surface retention of insects are more difficult to be handled in a land of accentuated incline. In this case, models of traps where the insects are trapped by surfaces impregnated with adhesive glue would be more appropriate. These monitoring tools should be efficient enough to capture the target pest at low population densities, be easy to handle and to carry and also should have low cost, high durability and strength. These parameters, which should be determined, are essential for successful monitoring.

The monitoring has been used on many economic important pest worldwide using traps containing pheromone to estimate population density in important crops such as maize and tomato. The reports of its use to estimate populations of *Spodoptera frugiperda* (Mitchell *et al*. 1985, Tumlinson *et al.* 1986), *Ostrinia nubilalis* (Hudon *et al.* 1989), *Keiferia lycopersicella* (Ridgway *et al*. 1990) and *Tuta absoluta* (Charlton *et al.* 1991) shows that this is not a new tool of pest management. However, given the current need for sustainability of agricultural activities, such monitoring proved to be economically viable and environmentally sustainable, by determining the ideal time of interventions to reduce the population of pests in the field.

Besides pheromone use, monitoring can be performed based on visual stimuli. Insects that use vision to locate hosts can be monitored with the aid of colored traps. The principle of this method is to ensure that the insects are lured into a colored surface impregnated with glue. In reference (Natwick *et al.* 2007) it was recorded the efficacy of colored sticky traps to detect and monitor *Frankliniella occidentalis* in lettuce. In this particular case, the blue color would be more suitable. Studies have indicated that while there are no insects caught in traps, there is no need to carry out chemical control. The adoption of this monitoring by lettuce growers can reduce insecticide applications in the field. It should be noted that when using visual stimuli, based on color as attractant in traps, the preference of the target insect must be followed.

Food attractants can also be used for monitoring pests. In horticulture, the technique is widely used to monitor fruit flies. In this case, food baits are used in traps to attract insects. Several attractants, such as brown sugar, sucrose, hydrolyzed corn protein, fruit juice and wine vinegar in McPhail, Jackson and PET bottles traps are used for monitoring (Nascimento *et al.* 2000). One example is the use of orange and grape juices at a concentration of 25% that are being used in monitoring *Anastrepha fraterculus* in peach orchards (Guerra *et al.* 2007).

3. Mating disruption

The acceptance of the communication-disruption tactic has largely been driven by dissatisfaction with the control levels achieved with conventional insecticides, often because of emerging resistance problems. Furthermore, in contrast to insecticides, the effectiveness of mating disruption increases with long-term use, resulting in a substantial reduction of populations.

Most of the studies on the use of attractants to disrupt a finding behavior have focused on mate location, particularly of moths, in the so-called mating disruption method. This control method has come to prominence in pest control the last decades. Its success has been observed in controlling several pests like the pink bollworrn, *Pectinophora gossypiella* on cotton; the oriental fruit moth, *Grapholita molesta*, on stone fruits; tomato borer fruit *Neoleucinodes elegantalis*, in tomato and codling moth, *Cydia pomonella* in apple, in which codling moth mating disruption enables sustainable and reliable control at low population densities.

This method consists of distributing a large amount of synthetic sex pheromone in the field, aiming to prevent the male to find a female, disrupting mating. Therefore, new pest generations do not occur on treated area. Nevertheless the success of this method is associated with pest migratory ability and biological aspects. According to reference (Cardé & Minks 1995) the use of sexual disruption certainly results in success, due to its ability to reduce the local population of the pest. However, it does not protect the area of immigration from outside populations, when used in an isolated way by a single producer or when there is no geographic isolation (geographic barriers). In order to avoid these potential problems, to ensure that this method will obtains the desirable success, it is required a good knowledge about the ecology of the pest and its immigration ability, aiming the entry capacity of mated females, coming from outside the treated area. The sphere of lures influence must be determinate for obtaining the distance between them in the field. Research will focus on determining the number of lures, their dosages and their spatial arrangement to reduce mating success.

The logistics involved in implementing the method, also deserves attention, as it requires an accurate monitoring program, based on samplings with traps baited with pheromone, to determine or even to ensure that the use of disruption is enough to keep the pest population levels under the economic threshold level, thus justifying the method.

3.1. Mating disruption mechanisms

The sex pheromone may act through different mechanisms in mating disruption, however, the main behavioral mechanisms have been defined as sensory fatigue by diminution of response due to sensory adaptation or habituation, arrestment of upwind flight at high concentrations, shifting the rhythm of response to females, changing the fine structure of or camouflaging a natural plume, outcompeting females, and causing an imbalance of sensory inputs by altering the perceived blend. In spite of the large amount of work on mating disruption of moths, as well as the considerable volume of work on the actual behavioral mechanisms used by male moths in response to pheromone, behavioral control has not been widely used in pest management (Cardé & Minks 1995, Foster & Harris 1997). In this chapter we discuss some of these mechanisms, as follows.

Diminution in responsiveness via either sensory adaptation or habituation: Encounters with formulated pheromone may raise the males response threshold or abolish responsiveness entirely. Either outcome could result from adaptation of peripheral receptors on the antennae or habituation at a central processing level. Continuous release of pheromone formulation, leading to its constant presence in the area, increases habituation.

Shifting the rhythm of response: The continuous presence of pheromone can cause males to respond to formulated pheromone well before females call. Such precocious male activity could contribute to a diminished response when females commence pheromone release.

Camouflage of the plumes from calling females: At some distance downwind of a moth releasing pheromone, the concentration of pheromone emitted from the moth's odor plume should be rendered imperceptible in a background of synthetic pheromone. At those distances the male would be unable to distinguish the natural plume filaments.

Competition between calling females and point sources of synthetic pheromone: Males may remain responsive and orient to pheromone plumes from point-source release devices. The ratio of dispensers to calling females and their comparative attractiveness will set the intensity of competition. The time spent orienting to artificial point sources of pheromone lessens the time available to orient to plumes from calling females.

3.2. Dispenser technology

Practical applications of the mating disruption technique require efficient and economic dispenser materials that release sufficient amounts of pheromone over an extended period. The pheromone dispenser technology determines the efficacy and the economics of mating disruption.

The importance of adequate rate release and consistently for long periods is undisputed in this technique success. That can be observed by several studies testing different release technologies (dispensers), as observed in references (Knight *et al.* 2012) and (Bohnenblust *et al.* 2012). As some successful technologies, we mention the sealed polyethylene vials and the SPLAT®-Specialized Pheromone & Lure Aplplication Technology (Figure 1).

Sealed polyethylene vials with pheromone kept the communication disruption for the millet stem borer, *Coniesta ignefusalus* for up to three months in millet farmers' fields (Youm *et al.* 2012). SPLAT emitters, which is an emulsion paste (wax) and amorphous that controls the release of insecticides and semiochemical was effective to achieve communication disruption by season-long for several Lepidoptera pests like *N. elegantalis* in tomato (França et al. in 2012, unpublished data), *Bonagota salubricola* and *G. molesta* in peach (Härter *et al.* 2010, Stelinski *et al.* 2010) and *Phyllocnistis citrella* in citrus (Stelinski *et al.* 2007).

Not only the pheromone dispenser technology, but the amount of dispensers is an important factor for the efficiency of male mating disruption, since it influences the mechanisms involved in this process. The number of pheromone release sites is related to the initial density of the pest, for when the density is high the number of dispensers has to be raised, in order to obtain the effectiveness of the method. Härter *et al.* (2010) achieved an efficient control of *G. molesta* in peach, using 1000 release sites (with SPLAT® technology) of pheromone, reducing male catch and the damage from this pest. Pastori et al. (2008) testing 1000 release sites (SPLAT® technology) of *B. salubricola* pheromone associated with *G. molesta* pheromone in apple, reduced male catch in both species in the first season. In the following season the catch reduction was only observed for *G. molesta*, however, it did not reflect in damage reduction. For the millet stem borer, *C. ignefusalus* suppression of male catch was obtained when the crops were treated with 400 dispensers / ha (Youm *et al.* 2012).

Figure 1. SPLAT NEO (containing *Neoleucinodes elegantalis* pheromone) on tomato crop in Bezerros, PE. At the top left, the applicator used.

Others technologies have also been used as the microencapsulated formulation. A fortnightly application of the sex pheromone using this technology in peach orchards prevented the chemical communication between the male and female of *G. molesta*, reducing male catch in traps baited with commercial pheromone. Thus, both the SPLAT® and the microencapsulated pheromone was effective to interrupt the chemical communication of *G. molesta* in peach orchards, even when this was associated with *B. solubricola* pheromone (Botton *et al.* 2005).

3.3. Association of mating disruption with synthetic insecticides

An important point is that in several management programs disruptants may need to be used in combination with conventional insecticides, the latter either to reduce initial populations or as an adjuvant to the disruptant. In these cases, a reduction in the use of conventional insecticides is an additional consideration in assessing whether a disruption program is successful (Cardé & Minks 1995). The use of synthetic insecticides associated with pheromone in order to increase the efficiency of control in some situations (Witzgall *et al.* 2008) could increase the adoption of this control technique.

Often the use of this technique reduces the male catch, causing mating disruption, however it does not imply reducing the damage caused by the pest. Probably, this should occur by the entry of mated females originating from untreated areas, so specific applications of insecticides may be indicated to reduce this negative effect. Although, Pastori *et al.* (2008) reported that using SPLAT® dispensers containing *B. salubricola* pheromone associated with *G. molesta* pheromone, with or without cypermethrin in the formulation, found that the presence of the insecticide did not affect the results. Moreover, França et al. (2012) (unpublished data) obtained a greater reduction in both male catch of *N. elegantalis,* oviposition on fruits and damage reduction, when used SPLAT® dispensers containing pheromone associated with cypermethrin compared with SPLAT® containing only pheromone (Figure 2). The mating disruption technique led to the same reduction of damage caused by *G. molesta* in peach orchards, compared with orchards submitted to insecticide spraying, demonstrating the great advantage of using this method (Härter *et al.* 2010). Therefore, using mating disruption, the number of insecticide applications can be reduced or even absence, making the production environmentally sustainable and economically viable, since the money spent on insecticides can be used in obtaining the product to carry out the disruption. Thus, this tactic is quite appropriate for cultures where no residues of pesticides is required or desired, such as fruits for export; and with the consumer profile in transition, becoming increasingly discerning, the search for an alternative control method is essential to the acceptance and retention of the farmer in the current scenario.

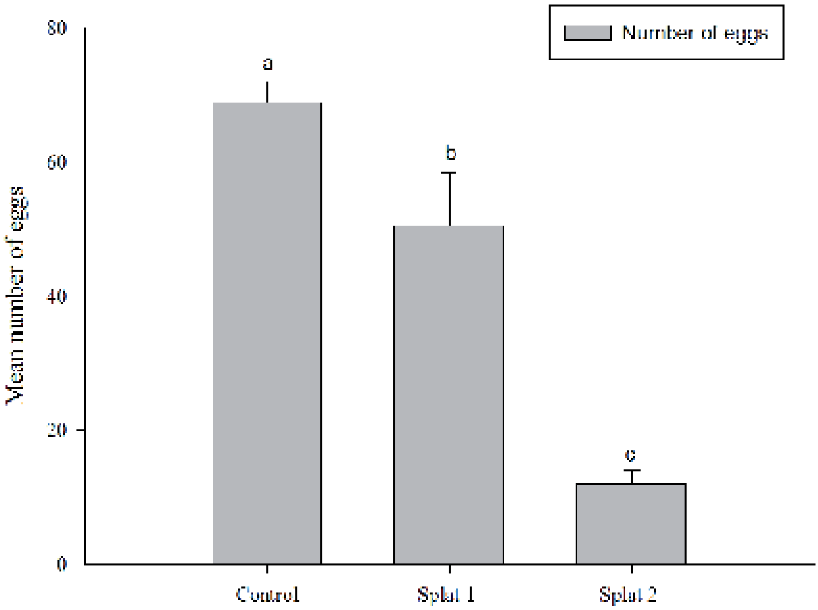

Figure 2. Mean number of *N. elegantalis* eggs throughout tomato crop cycle, variety TY, treated with Splat 1 (with cypermethrin), Splat 2 (without cypermethrin) and control (led by the producer) submitted to treatments 20 and 30 days after transplanting (Camocim de São Félix, Pernambuco, Brazil, 2011-2012). Columns with different letters are significantly different (p>0.05) by Tukey test.

4. Attract-and-kill (A&K) System

A novel approach using sex pheromones is the attract-and-kill system. This strategy is a new pest management technique, an extension of mating disruption, which is characterized by the inclusion of an insecticide (killing agent) in addition to the pheromone active ingredient or a feeding attractant (attracting agent). By doing this, it is possible to achieve the same control methods as mating disruption, with the potential for increased efficacy, resulting from the toxicity of the insecticide (Ebbinghaus *et al.* 2001). Unlike mating disruption, which functions by "confusing" the insect, attract-and-kill system attracts the insect to a pesticide laden gel matrix, distributed as small droplets in the crop, which, upon contact, kills the insect.

With this system, blanket coverage of the crop is not necessary, and so the amount of insecticide can be significantly reduced. Such an approach would permit reductions in the amounts of insecticides used and would minimize contact with the environment, the crop, and beneficial organisms. An additional benefit of an attract-and-kill formulation is that these formulations generally require less pheromone to be effective, since the target species does not need to be overwhelmed by the pheromone; it only needs to be attracted to it.

However, when pheromones are used as the attracting agent, only males are killed. Nevertheless, removal of males from a population may not have a significant effect on reproduction unless a large percentage of the male population is killed, as a small percentage of the male population will serve to mate with most sexually receptive females. Thus, the development of an attract-and-kill system might be enhanced with chemical lures that are effective in bringing females into a target. Although the majority of chemical attractants are female-produced sex attractants that lure males, female may use chemical odorants to locate and select mates, host plants and food.

The use of attract-and-kill suffers from some of the same constraints as mating disruption, including the high degree of pest selectivity, a reduction in efficacy with increasing pest density and risk of immigration of mated females. Besides, the different longevity in the insecticide activity and the attractiveness of the droplets set a time-limit for the application. The attract-and-kill drops often deteriorated quite rapidly under some weather conditions, such as powerful storms, pelting rain, intense heat, and solar radiation. These conditions may affect the stability and longevity of the system.

As with any management tool, the operational use of pheromones must be considered within the context of an integrated pest management system. Commercialization of the attract-and-kill approach has been undertaken by IPM Technologies Inc., who has global rights to a proprietary and patented matrix, combining insecticide and attractant in a UV sensitive carrier material. Marketed as "Sirene ™" and "Last-Call™ " in Europe and the U.S., respectively, this technology was granted US EPA registration in 1998 and California registration in 1999. The robust matrix can accept, protect and release a wide variety of chemicals (acetates, alcohols, aldehydes) so it has the potential to be deployed against many pest species in diverse ecosystems.

Attract-and-kill systems are more powerful than other semiochemical mediated control strategies such as mating disruption in that male moths are incapacitated and removed from the ecosystem. Yet, this approach has the obvious advantage of limiting any potential negative ecological effects of the insecticide, as only those insects coming to the lure will be affected. These systems has been successfully used against several pests, including the boll weevil, *Anthonomus grandis*; codling moth, *C. pomonella* (Charmillot *et al.* 2000) and apple maggot, *Rhagoletis pomonella* (Bostanian & Racette 2001), oriental fruit moth, *G. molesta* (Evenden & Mclaughlin 2004), among others.

In fruit production, the fruit flies control is based on the use of insecticides in total coverage or in the form of toxic lure. The toxic lure is based on the use of food bait associated with an insecticide. In this attract-and-kill system, the insects are killed when in contact or ingest the insecticide. Spinosad baits containing spinosad in different concentrations, water, sugar and attractants were effective in controlling the fruit fly, *Ceratitis capitata* and *Anastrepha fraterculus* (Raga & Sato 2005). The formulation SPLAT ®, afore mentioned at mating disruption, is also used in attract-and-kill system, since its formulation consisting of waxes and oils and allows the inclusion of a wide range of insecticides and attractants with potential to control several species of fruit flies. SPLAT® system has been evaluated as a strategy to attract-and-kill for fruit flies *Bactrocera dorsalis* and *Bactrocera cucurbitae* in the United States with promising results (Vargas *et al.* 2008, Vargas *et al.* 2009). There are some reports in the literature of a SPLAT® formulation containing spinosad 0.10%, which provided control of *C. capitata* adult, even after submitted to simulated rainfall, and showed a smaller effect on the parasitoid *Diachasmimorpha longicaudata* compared with other toxic baits (Zanardi 2011).

Although this method presents the advantage of causing less impact on non-target organisms, some restrictions are observed, for example, the low persistence of toxic lures after rainfall events, as can be seen in the example cited above. These barriers are being solved with the advancement of research on this technology.

Mating disruption and attract-and-kill are similar technologies that have been used to control a wide range of insect pests, typically species in Lepidoptera, Coleoptera, and Diptera (El-Sayed *et al.* 2006). These two technologies may be able to contribute to the eradication of new incursions of invasive species, because like other inversely density-dependent approaches, they have the greatest probability of success against pests at very low density, which is initially the case after an incursion. Making clear the difference between these control systems, the mating disruption relies on the principle of preventing pheromone communication between sexes, but the insects remain alive in this area during the disruption, whereas in attract-and-kill systems they are removed from the population. Besides, attract-and-kill systems for field control typically use insecticides, while in disruption, insecticides may be used but they are not the primary approach of the system.

5. The push-pull strategy

Insects control methods exploiting natural chemical messengers, collectively known as semiochemicals, are becoming increasingly familiar. Semiochemicals are substances that, in their natural context, carry information or chemical cues for a given interaction between organisms, triggering a behavior or a physiological response in the receiving individual. They are subdivided into allelochemicals, related to interspecific communications, and pheromones, in intraspecific communications (Vilela & Della Lucia 2001). One major developments now set to revolutionize the use of semiochemicals is the realization that semiochemicals should not be used alone, but be combined with population-reducing agents such as highly selective pesticides or biological control agents.

Thus, it is rare for a single semiochemical to be very effective when used alone. Instead, the usual approach is a 'push-pull' strategy, also called stimulo-deterrent diversion— which involves 'pushing' the insects away from the harvestable, economic crops, and 'pulling' them onto a trap crop where their population is reduced by a biological control agent or highly specific but slow-acting insecticide (Foster & Harris 1997). Therefore, antifeedants, non-host volatiles, compounds associated with plant defense, visual cues, synthetic repellents, alarm pheromones and oviposition deterrents can be used to achieve the 'push', while the sex pheromone, host volatiles, visual, gustatory and oviposition stimulants can be used to 'pull' the insects onto the trap crop (Figure 3).

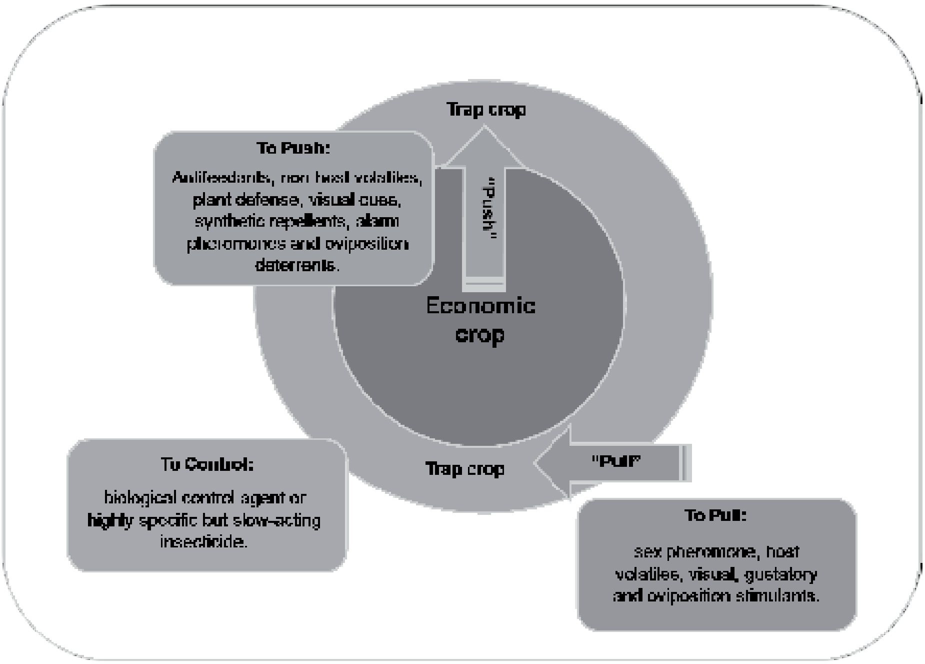

Figure 3. Push-Pull strategies, "pull" and "push" tools and the agents used to reduce pest population.

The olfactory, visual and mechanical stimuli are used by insects to locate and select their hosts. The ones acting at long distance are visual, synthetic repellents, host and non-host plant volatiles, anti-aggregation and alarm pheromones. At short distance, they are called anti-food, oviposition inhibitor and pheromone inhibitor. This technique has been used for a small number of insect pests and needs to be further investigated.

The term push-pull was first conceived as a strategy for insect pest management (IPM) by Pyke et al. in Australia in 1987. The concept was later formalized and refined by Miller & Cowles, who termed the strategy stimulo-deterrent diversion. Most work on push-pull strategies has targeted pest behavior rather than to the manipulation of beneficial organisms. However, it may act to push the beneficial organisms out of the surrounding area and pull them to where they are required for control.

The Push-pull strategies can bring together several pest management tactics, as behavioral manipulation methods, chemical stimuli, habitat diversification strategies (intercropping and trap cropping), biological control and chemical control. According to Cook *et al.* (2007) the principles of the push-pull strategy are to maximize control efficacy, efficiency, sustainability and output, while minimizing negative environmental effects.

5.1. The use of push-pull

The Push-pull strategy successfully controls pests and weeds. The most successful push-pull strategy, indeed the only example currently used in practice, was developed in Africa for subsistence farmers. In Eastern Africa, push-pull works as a novel cropping system developed by the International Centre of Insect Physiology and Ecology (ICIPE) in collaboration with Rothamsted Research (UK), Kenyan Agricultural Research Institute (KARI) and other national partners for integrated pest, weed and soil management in cereal–livestock-based farming systems (Cook *et al.*2007, Hassanali *et al.* 2008, Khan *et al.* 2008).

Millions of rural people in Eastern Africa depend on maize and sorghum for food security and cash income. Despite this, production of these crops is seriously affected by constraints such as stemborers and the parasitic weed *Striga hermonthica* (Nielsen 2001). The push-pull strategy involves the use of behavior-modifying stimuli to manipulate the distribution and abundance of stemborers and beneficial insects in maize or sorghum crops. It is based on in-depth understanding of chemical ecology, agrobiodiversity, plant-plant and insect-plant interactions, and involves intercropping a cereal crop with a repellent intercrop such as Molasses grass (*Melinis minutiflora*) and Desmodium (*Desmodium uncinatum*) (push), with an attractive trap plant such as Napier grass (*Pennisetum purpureum*) and Sudan grass (*Sorghum vulgare var. sudanense*) (pull) planted as a border crop around this intercrop.

Mated stemborer females are repelled from the main crop and are simultaneously attracted to the trap crop. Napier grass produces significantly higher levels of attractive volatile compounds (green leaf volatiles), cues used by stemborer females to locate host plants, than maize or sorghum (Khan *et al.* 2001). However, many of the stemborer larvae, about 80%, do not survive as Napier grass tissues produce sticky sap in response to feeding by the larvae which traps them causing their mortality (Midega *et al.* 2006). Legumes in the Desmodium genus, on the other hand produce repellent volatile chemicals that push away the stemborer moths. *Desmodium sp.* also controls *S. hermonthica* trough an allelopathic effect of the root exudates, produced independently of the presence of the weed, being responsible for a dramatic weed reduction in an intercrop with maize (Khan *et al.* 2002).

Besides this, the push-pull strategy has been studied for controlling several pests, as for example, *Helicoverpa sp.* in cotton, the Colorado potato beetle in potatoes, *Sitona lineatus* in beans, the pollen beetle in oilseed rape, onion maggot on onions, thrips on chrysanthemums, in forestry as the bark beetles on conifers, for veterinary and medical pests as muscid flies, mosquitoes and midges and for controlling urban pests such as cockroaches.

5.2. Push-pull strategy and insecticides

According to Cook *et al.* (2007) the stimuli used to achieve the push-pull strategy generally act by nontoxic mechanisms, thus, integration with population-reducing methods, such insecticides, is also usually needed when the strategies are targeted at pests. The push-pull strategy can be used to displace pests from resources or commodities that are to be protected, and simultaneously lure the pest to an attractant source coupled with an insecticide. In addition, push-pull strategies are beginning to be seriously considered as plausible pest control solutions that help to manage insecticide resistance threats. One study assessing the effects of push-pull strategy with trap crops, neem and Nuclear Ployhedrosis Virus (NPV) in *Helicoverpa armigera* insecticide resistance on cotton, reported that the push-pull strategy was highly effective in reducing the incidence of *H. armigera* and damage (Duraimurugan & Regupathy 2005).

The benefits of a push-pull strategy include a lower requirement for broad spectrum pesticides, saving these valuable materials for a 'fire fighting' role. In addition, there is less risk of producing populations of resistant insects. Because the push-pull components are not individually greatly effective, they do not select for resistance as strongly as conventional toxicant insecticides.

6. Mass trapping

The knowledge of life history traits of the target insect pest is particularly important to determine if this technique can be used. The behavior of the insect in the search of partners or food should be studied to maximize the chances of success in using this strategy. The mass trapping consists of placing a large number of traps with attractants in a crop in order to capture the largest possible number of insects.

The purpose is to reduce the number of individuals of the next generation, removing only males or both insect sexes of the area. As bait, sexual or aggregation pheromones, food attractants or volatile can be used. The density and effectiveness of traps are important factors for the success of population suppression and eradication technique (Steiner 1952).The technique is particularly effective when it aims to control insects with gregarious habits. In these cases, adding a synergist, enhances the capture of both males and females. When using only the sexual pheromones in this technique, generally, only males are caught. The male removal from the area may not have a major impact on the size of the next generation depending on the reproductive biology of the species.

The results achieved with mass collection may not be suitable for pest control as, depending on the mating ability of the species, a single male can fertilize many females, thus preventing the elimination or reduction of the number of individuals of the next generation. To work around this, a combination of pheromone with a food attractant could lead to both males and females catch. The initial size of population should then be estimated by monitoring, before the use of mass collection technique. El-Sayed *et al.* (2006) indicate the technique for controlling populations at low density or sparsely isolated. In the absence of these conditions, it should not be used alone for pest control. When adults of both sexes are considered pests and in the presence of aggregation pheromone, the technique proves to be effective. Several examples can be found using this technique in agricultural pest control, as in the case of *C. pomonella* in Europe and America (Hagley, 1978, Willson & Trammel 1980, Emel'yanov & Bulyginskaya 1999, Beers *et al.* 2003), borer of the genus *Rhynchophorus* in Asia, Middle Eastern and South America; fruit flies in orchards; *A. grandis* in the United States of America and Mexico; *Lymantria dispar* in USA, among others.

The results indicate that the success in suppressing pest population and damage reduction is not always ensured, especially when the pest is found in high population density. Some requirements must be observed according El-Sayed *et al.* (2006) to succeed in using the mass collection. The first is the arrangement of traps in the area, so that a large part of the population has contact with the attractive. The second requirement is that the attractive has to be more effective than the natural source of attraction, as virgin females, foods, among others. This attractive must have an effective release, ranging from emergency to the insect mating season. Traps effectiveness in capture and retention of insects must be observed as well as the cost of the technique.

The mass trapping, when is not enough by itself to control pest population, can be used in combination with other methods of pest control within the philosophy of Integrated Pest Management.

7. Attractive lures and toxic lures

Another aspect of behavioral control is the use of secondary plant substances that play an important role in the feeding of monophagous or oligophagous insects (Cohen 2003). These substances are used in insect behavioral and biology studies, such as attraction / repellence, feeding and oviposition deterrence etc (Martinez & Van Emden 2001). Such chemicals that indicate the presence of food are in many cases, secondary compounds that stimulate chemoreceptor cells located in taste sensilla across tarsi, antennas and mouth parts of insects, inducing feeding and oviposition. When these compounds induce feeding are called phagostimulants or feeding stimulants (Nation 2002).

Attractive food lures containing feeding stimulants make pest management by behavior manipulation an effective tool, helping to reduce the amount and improve pesticides efficiency applied in agricultural production systems. These attractive lures have been used for the following purposes: (i) identification and distribution of insect species, (ii) certification of a region or country for the absence of a particular pest species (free area), (iii) pest eradication programs, (iv) Integrated Management Program, (v) pest monitoring (Nascimento *et al.* 2000).

For a higher persistence of phagostimulants at the lures, the starch can be used as a vehicle of feeding stimulant, for example, in combination with insecticides and dry bran. Several attractive, such as brown sugar, sucrose, hydrolyzed corn protein, fruit juice and wine vinegar in McPhail, Jackson and PET bottles traps are used for monitoring, and when added to insecticides are recommended for pest control, as example the fruit flies (Nascimento *et al.* 2000), contributing to the reduction of pest population in the field. Orange and grape juices at a concentration of 25% have been used in monitoring *A. fraterculus* in peach orchards (Guerra *et al.* 2007).

The attraction of *Mocis latipes* for sweet baits in traps has been studied by Landolt (1995) showing that honey solutions in water (20%) or unrefined sugar in water (5, 10 or 20%) caught a significant number of moths. This author also found that these baits prepared with three days in advance were more effective than baits made with fresh solutions.

Lures prepared with different concentrations of spinosad insecticide have been effective in fruit flies control, *C. capitata* and *A. fraterculus* (Raga & Sato 2005). Toxic lures formulated with hydrolyzed protein, consisting of Biofruit 3% plus malathion (500 Malathion EC) applied on plants located at the orchard edges has been effective in controlling *A. fraterculus* on peach trees. However, this practice is not employed by peaches producers, as it requires changes in the pest management system, replacing scheduled insecticides applications for monitoring implementation (Härter *et al.* 2010).

Toxic lures has also been shown important in the management of lepidopteran pests. According to França *et al.* (2009) the mortality caused by lufenuron and deltamethrin, associated with 10% honey, increased directly with the evaluation periods (0, 0.5, 1, 2, 12 and 24) for males, females and adults of *N. elegantalis* after exposure to 10% honey attractive solution, achieving 100% of mortality in two hours.

One of the advantages of toxic lures is the environmental benefit, since they are not used in the total field area, compared with synthetic insecticides. So, it would provide reduced risk due to the decreased presence of toxic residues in fruits, decreased resurgence of secondary pests, reduction in the amount of insecticides and higher preservation of natural enemies. The use of toxic lure in guava orchards, *Psidium guajava,* had lower influence on adults of Chrysopidae family, compared to synthetic insecticides application (Galli *et al.* 2004).

The great success of behavioral management is the ability to associate technologies to control pests, rationing insecticides use, due to applications based on monitoring. The techniques of behavioral control consort with other control methods such as conventional, and benefit not only the consumers but the ecosystem and all involved in the productive chain of agricultural products.

8. New trends and conclusions

Recent studies have shown that an alternative to improve the pheromone performance on male catch is the use of volatile compounds of plants. Tests at wind-tunnel, demonstrated that the volatile compounds of plants, such as limonene, pear ester, ß-farnesene and linalool increased the attraction of codling moth males, *C. pomonella*, when associated with codlemone pheromone also proving that these volatiles not only increase the attractiveness, but also decrease (shorten) the response time of the males to codlemone (Schmera & Guerin 2012). Thus, based on these observations, it is concluded that admixing limonene, pear ester, β-farnesene or linalool to codlemone aids source location by males, such that host plant volatiles dispensed with codlemone should contribute to better mating disruption of *C. pomonella*. These authors also state that the attractiveness increasing of codlemone to codling moth males can be regarded as a key to increasing the efficiency of mating disruption of codling moth.

The public demand for environmentally correct alternatives to broad-spectrum insecticides or strategies that reduce the use of these pesticides has never been greater. The adoption of behavioral manipulation techniques can help to meet this demand, since the amount of semiochemicals released into the environment is relatively small. In addition, most of the semiochemicals are relatively nontoxic to vertebrates as well as to beneficial insects, having a high selectivity to the target pest species.

Author details

Solange Maria de França[1], Mariana Oliveira Breda[1], Cesar A. Badji[2] and
José Vargas de Oliveira [3]

*Address all correspondence to: solangeufrpe@yahoo.com.br

1 Graduate Program in Agricultural Entomology, Department of Agronomy, Recife, Pernambuco, Brazil, Universidade Federal Rural de Pernambuco, Brazil

2 Universidade Federal Rural de Pernambuco, Academic Unit of Garanhuns, PE, Brazil

3 Universidade Federal Rural de Pernambuco, Department of Agronomy, Recife, Pernambuco, Brazil

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The Performance of Insecticides – A Critical Review

Christian Nansen and Thomas James Ridsdill-Smith

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53987

1. Introduction

As part of minimizing the risk of crop losses due to herbivorous arthropods (here, mainly referring to insects and mites), most of the World's commercial food production systems are subjected to several applications of pesticides before being harvested. Some crops are sprayed 10-20 times, while most field crops are sprayed 1-5 times during the growing season. In the US alone, the average number of insecticide applications ranges from 1-3 for most of the major field crops, and the total annual costs of pesticide applications exceed $1.3 billion ([1], U.S. Department of Agriculture agricultural statistics services: www.nass.usda.gov/). Several large ag-producing countries publish extensive details on insecticide use, including Australia, and the data presented below were collected from a public website (http://usda01.library.cornell.edu/usda/nass/AgriChemUsFruits//2000s/2008/AgriChemUs-Fruits-05-21-2008.pdf) on pesticide applications in the US in 2006. A somewhat extreme but also important example is the fresh sweet maize production in the US, which was grown on about 85,000 ha. Based on data from 14 US states, 20 different active ingredients of herbicides were applied an average 1.04 times to fresh sweet maize and amounted to about 2.6 kg of active ingredients of herbicides per ha. The same data suggested that about 3.5 kg of 23 different active ingredients of insecticides were applied on average 2.10 times per ha. Similarly, tomato fields (grown on about 42,000 ha in the US in 2006) were treated with 12 different active ingredients of herbicides, which were applied, on average, 1.14 times and the equivalent of about 0.7 kg of active ingredients per ha. Regarding insecticides, the same tomato fields were treated with 32 different active ingredients, which were applied an average of 3.6 times and equal to about 4.9 kg of active ingredients per ha. While tomatoes and sweet corn may be close to the top of the list of growing crops receiving pesticide treatments, cauliflower, celery, and many other horticultural crops and fruits are also subjected to intensive pesticide spraying regimes. Thus, farmers acknowledge that weeds and arthropods can potentially cause significant economic losses, and total pesticide application costs are low enough to economically justify a very significant and consistent number of applications in almost all crops. It is beyond the scope of this chapter to address the gradually growing market for organic produce and the possible human health and environmental impacts of intensive pesticide spraying regimes. However, it is worth mentioning that – going back to the fresh sweet maize – applying 47 different active ingredients of pesticides (insecticides = 23, herbicides = 20, and fungicides = 4) at a total dosage of 6,7 kg per ha means that (assuming a maize plant density around 65,000 per ha and that about 50% of the applied pesticide ended up on treated plants) each maize plant received about 0.3 g of active ingredient of agrochemicals. How much active ingredient ends up in the edible portions of crops varies considerably based on exposure of the harvested plant part, the longevity of chemicals (residual effect) and timing of applications in relation to harvest time. Extensive research efforts are being committed to the short and long term effects of pesticide residues in the food products [2, 3]. Despite high levels of pesticides being applied, it is encouraging that publicly available reports, like http://www.ewg.org/foodnews/, suggest that fresh sweet maize does not contain detectable levels of pesticide residues. However according to the same report, other food products (i.e. apples, celery, and bell peppers) quite frequently test positive for pesticide residues.

Several environmental and agronomic/operational factors affect the likelihood of insecticides being applied to a crop. For instance, a comparatively "good growing season", with the right amount and ideal seasonal distribution of rainfall, is equivalent to a high yield potential. A high yield potential means increased risk of potentially high losses incurred by arthropods and weeds, so growers are typically more inclined to apply pesticides to protect a high yield potential. In addition, a "good growing season" may also be conducive to growth of weeds and arthropod pests, which further increases the justifications for applying pesticides, even as a precautionary measure. Among the agronomic factors affecting the likelihood of insecticides being applied, the price of seeds is quite important. A grower may, especially if the prediction is to have a good growing season, decide to plant high-value seeds due to their high yield potential, or because those seeds possess a particular qualitative trait. Similarly, the grower may decide to apply additional (expensive) fertilizer to ensure that the crop grows and yields to its full potential. Planting high-value seeds and "investing" in the crop by applying high levels of fertilizer generally means that growers have lower threshold tolerances for losses incurred. That is, as described in the conventional description of economic injury level and action threshold [4], there is generally a negative relationship between overall value of the crop and the likelihood of pesticides being applied as growers want to protect the growing crop. In other words, investing in high-yielding seeds under favourable conditions may be associated with additional crop protection inputs (such as, pesticide, irrigation, and fertilizer applications), because growers want to take full advantage of the yield potential of the given crop. Another factor increasing the likelihood of insecticide applications is the convenience of "tank mixtures", in which multiple agrochemicals are applied simultaneously. For instance, growers may decide to spray a herbicide just before crop emergence and decide to add a residual insecticide to the formulation to target establishment pests that may or may not be present. That is, growers want to optimize labour and fuel costs, so if they are going to spray fertilizer or herbicides anyway –

they might as well add a low-cost insecticide to the tank mix and possibly get an added benefit. Estimates suggest that insecticide applications constitute 2-10% of the crop yield [5-7], but use of tank mixtures obviously decreases the overall application costs. The three examples provided ("good growing season", value of seeds, and tank mixing) are important, because they are not directly related to actual estimates of the pest population density, but they may still lead to a grower applying insecticides. That is, they are factors that raise the concern about possible pest infestations ("good growing season", value of seeds), or they provide economic justification (tank mix) of a pesticide spray application, irrespectively of whether pest populations have been detected in fields. Finally, it is worthwhile to highlight a psychological dimension to pesticide applications, which is that applying pesticides rather than "doing nothing" may give farmers the feeling of "doing something" (in this case spraying pesticides). This aspect is of particular importance in cropping systems in which sampling/monitoring programs are either not an important part of the operational practices, and/or they are deemed practically unfeasible. Increasing sizes of farms mean that the grower may only get to a certain portion of a field every 2-3 weeks or at even less frequent occasions. Obviously, many things can go wrong in a field that is unattended for long time periods, so growers may feel that they cannot afford NOT to apply insecticides – simply as a preventative measure. Frequent and widespread applications of insecticides are understandable, when very little is known about the actual pest population density, large farming areas are managed by only a few people, and when an insecticide can be easily added at a low cost to an existing spray application. Thus, even though growers are generally considered to be "conservative" in their management style, the brief review above clearly outlines many operational and agronomical factors reasons why most crops are treated with numerous insecticide applications in each growing season. Growers are generally low-risk takers, and are therefore accepting to spend considerable resources on pesticide applications according to a philosophy of rather safe than sorry.

2. Considerations regarding volumes of insecticide formulations

Due to the emphasis and reliance on insecticide applications, it is worthwhile briefly reviewing some of the basic considerations regarding volume of insecticide formulations and other factors affecting spray coverage and canopy penetration, when insecticides are applied to growing agricultural crops [8, 9]. Insecticide labels provide information about required application rates for registered combinations of pests and crops and also about volumes of carrier (most commonly water) to be used. Interestingly, these vary considerably mong countries, so the same pesticide may be applied at a considerable range of dosages among different countries [10]. "Adjuvants" are compounds added to spray applications with the purpose of increasing "stickiness" (adherence to crops), provide UV-light protection (increase the residual effect), increase crop leaf penetration, and/or modify droplet sizes (i.e. reduce drift and increase canopy penetration). Use of adjuvants is therefore a very important aspect of spray application performance. Due to costs and logistics of transporting water, aerial fixed-wing insecticide sprays are applied with much lower spray volumes (rarely applied in formulations above 50 L per ha) than when the same insecticides are applied with ground rigs (50-200 L per ha). Other more specialized insecticide delivery systems include fumigations of post-harvest products or of soils, seed treatments (insecticides coated onto planted seeds) and transgenic insertion of toxin producing genes into growing crop plants (i.e. genes from *Bacillus thuringiensis* in Bt crops). Aerial fixed-wing insecticide applications are often preferred modes of application, when large fields are treated and/or the crop canopy is too high or dense for spray boom applications with a tractor. When insecticides are applied with either aerial or ground rig sprays, the decision on volume of insecticide formulation to be applied to a given field is widely determined by operational considerations, including: 1) size of field, 2) availability and cost of labour, 3) current fuel price, 4) availability of water tanks and/or pumps near the field, 5) height of crop canopy, and 6) whether the insecticide has systemic properties or not (whether it is absorbed through leaf surfaces and translocated within the treated plant – if so, the performance of the insecticide application is perceived to be less influenced by spray coverage and canopy penetration). Other factors more directly linked to the actual pest include: 7) where in the canopy (vertical distribution) the insect pest is most abundant and therefore whether it is important to deliver the insect formulation to a certain portion of the canopy, and 8) the pest's diurnal movement behaviour and therefore whether the spray application has to be completed within a certain time window (for instance, if a pest is highly crepuscular). When applied with modern spraying equipment, the application rate (volume of formulation per ha) is computer controlled mainly through three variables: spray nozzle output (typically delivering mean droplets of 0.05-0.5 μm), boom height above canopy, and speed of vehicle. The practical realities are that aerial insecticide sprays are cheapest but often require contracting of a professional pilot. In Western Australia and many other important agricultural regions, aerial insecticide sprays are frequently about 10 liter per ha, which – assuming that all the spray formulation is deposited on the treated field – amounts to 1 ml per m^2. If that m^2 has crop canopy (not bare ground), then its total surface area is obviously much higher. [11] examined wheat plants planted at a seeding rate of 180 per m^2 and with a specific leaf area of about 30 cm^2 per plant, or the equivalent of 2 (both sides) × 180 plants × 30 cm^2 = 1.08 m^2. Thus, the actual surface area per m^2 was slightly above 2 m^2 when taken the crop surface into account. Furthermore, weather variables are known to greatly impact insecticide spray depositions [1], and issues with pesticide drift are also widely documented [12]. Thus, it seems reasonable to suspect that at least 20% of the sprayed volume is "lost" (not deposited on the target crop but ends up elsewhere). Summarizing these simple calculations, even a fairly conservative estimate of an aerial spray application suggests that not more than 0.4 ml is applied per m^2 to a growing crop. The question raised here is – how likely is it that all insecticide applications deliver enough insecticides to crop leaves so that target pest populations are effectively controlled? In the US, most aerial spray applications are applied at rates around 50 liters per ha, so the spray deposition is likely higher but may still amount to active ingredient leaf coverage in very low concentrations. The calculations presented here may vastly underestimate loss of spray volume due to drift, and they may greatly underestimate leaf areas – especially in dense canopies, so actual spray depositions may be considerably lower. But adhering to the simple calculations presented above, a likely insecticide spray application may consist of applying about 0.4 ml evenly to 1 m^2 – which should convince most about the concern that is being raised. It is important to underscore that the active ingredient (killing agent) is normally just a small proportion of the spray volume, so the amount of killing agent applied to each cm^2 of leaf surface is likely in nanograms. If a contact insecticide is applied – how likely is that target pests acquire a lethal dosage? And if a systemic insecticide is applied – how likely is that the concentration in the vascular tissue is high enough to kill the target pest? Two factors become quite important in the answers to such questions: 1) mobility of the target pest and 2) repellency of the insecticide formulation. Clearly, equivalent to 1-5 ml per m^2 are not evenly distributed within 1 m^2 of crop, so the target pest will only get in direct contact with the active ingredient if distributions of insecticide formulations and of target pests are spatially correlated (i.e. both are most predominant in the same portion of the canopy), and/or the target pest is very mobile. The point is that heavy (almost exclusive) reliance on contact insecticide applications should be accompanied with strong interest and knowledge about the possible performance and constraints. Or put in bold terms, just because a tractor with a boom sprayer travelled through a field or an airplane flew over a field and a certain volume of pesticide formulation was applied does not necessarily mean that target pest control was accomplished.

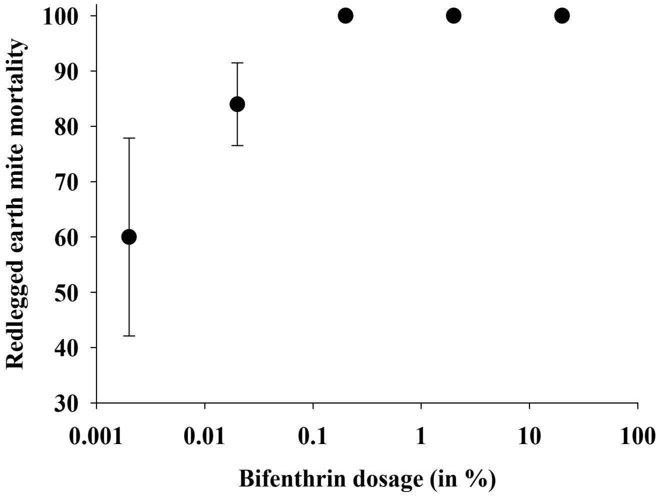


Figure 1. Redlegged earth mite mortality in response to bifenthrin dosage in no-contact bioassay

However, it should be pointed out that insecticides categorized as "contact insecticides" may not always require physical contact in order to cause target pest mortality. Contact insecticides are believed only to kill pests, when insects ingest or get in direct contact with the active ingredient. As an example, bifentrhin is in IRAC (http://www.irac-online.org/eClassification/) class 3A (pyrethroids and pyrethrins), which are sodium channel modulators, and bifenthrin is labelled as a contact insecticide. A simple study was conducted in which formulations of bifenthrin were transferred to a 2 ml Eppendorf tube, which was placed inside a 50 ml plastic container with a lid. We tested the following dosages (% by volume) of bifenthrin in separate trials: 0% (positive control), 0.02, 0.20, 2.00, and 20.00. The recommended application rate for control of redlegged earth mite [*Halotydeus destructor* (Penthaleidae: Acari)] in Australia is 50-100 ml per ha in 50-200 L formulation per ha, and it is normally applied at about 100 ml in 100 L water, which is equivalent to 0.1% (by volume) (www.syngenta.com). Redlegged earth mites collected from a field site near York (Western Australia) were transferred to the 50 ml plastic container and provided a single leaf of a suitable host plant (common vetch, *Vicia sativa*). The rim of the Eppendorf tube containing the bifenthrin formulation was covered with vaseline, so that the redlegged earth mite could not get in direct contact with the insecticide formulation. The lid of the 50 ml plastic container was sealed, so that possible volatilization of the bifenthrin would saturate the air inside the 50 ml plastic container. After 24 hours, the status of the redlegged earth mites was assessed. The results from this bioassay showed a fairly standard log-scaled dosage response in which all redlegged earth mites succumbed when the bifenthrin dosage exceeded 0.2% (Fig. 1). This simple study highlights important characteristics associated with certain "contact" insecticides, like pyrethroids, as they may actually suppress target pests due to volatilization – i.e. creating a scarce cloud within the crop canopy. And returning to the calculations of applications per m^2 described above, volatilization may, at least partially, explain how it is possible that insecticide applications applied at a dosage below 1 ml per m^2 are able to provide successful pest control. However, the simple laboratory experiment was conducted in sealed containers, and it is unknown to what extent the micro-environment inside the sealed containers reflects field conditions. More research is needed to investigate the possible roles of factors like insecticide concentration and droplet size on volatilization as a possible mode of action in dense crop canopies, and it is unknown whether volatilization plays a major role across insecticide classes.

3. Control measures of insecticide applications

Acknowledging the magnitude of resources spent on insecticide applications, and the possible risk of low insecticide performance due to low and inconsistent insecticide applications - it is somewhat noteworthy that there are no widely used quality control measures available. As discussed by [13] and many others, there are numerous factors which can contribute to low performance of a given insecticide application, including: incorrect storage, water pH, wrong concentration of insecticide, nozzles not being turned on, and incorrect application volume. An interesting, but under-utilized resource for assessment of spray coverage, is water sensitive spray cards, which enable growers, consultants, and pesticide applicators to quantify the spray coverage obtained. Water sensitive spray cards are coated with bromoethyl blue, which reacts with water and turn blue-purple depending on dosage of water [14] (Fig. 2b). Although mainly used in applied research projects, they are commercially available through a number of companies and can be used quite effectively to make quantitative assessments of spray applications in response to agronomic variables and weather conditions. [1] used water sensitive spray cards to analyse spray coverage during commercial spray applications in potato fields, of which eight were applied with fixed-wing airplane (spray volume of 48 liter per ha) and six with ground rig (spray volume of 194 liter per ha). During each spray application, 10 water sensitive spray cards were deployed at the top of the canopy in different parts of the field, and both average and range of spray coverages were analysed (N = 140). Canopy penetration data were also obtained from nine of the 14 commercial spray treatments by having additional spray cards placed about 15 cm from the bottom of the potato canopy. In a recent study conducted in Western Australia, we quantified the "potential spray coverage" of commercial spray rigs by placing water sensitive spray cards at the ground level in a bare field (Fig. 2c). Thus, there was no crop, so the obtained spray coverage represented the highest possible under the given conditions.

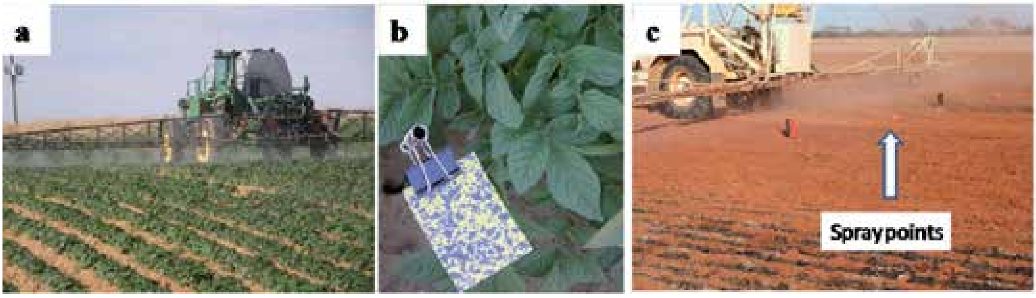


Figure 2. Ground spray rig applications and use of water sensitive spray cards

Weather conditions were recorded, and spray volume (30-130 liter per ha), tractor speed (15-25 km/h) and nozzles type (various types tested) were experimentally manipulated to obtain spray data from a wide range of commercial spray scenarios. Spray data for this study were collected in three combinations of fields and locations, and we obtained data from 77 unique combinations of spray conditions (location, date, spray volume, tractor speed, and nozzle types) and with four replicated spray cards for each combination (N = 308). Fig. 3a shows average spray coverage at the top of the canopy or above bare ground in response to spray volume, and, as expected, there was a highly positive correlation (df = 1,90, adjusted $R^2 = 0.790$, $F = 340.48$, $P < 0.001$). Thus, despite high variability in spraying conditions, spray coverage is clearly driven by volume and reached about 40%, when the equivalent of 200 liter per ha was applied. Average spray coverages for the three data sets (aerial and ground rig applications in Texas and ground rig applications in Western Australia) were examined, and spray coverage was divided by the spray volume applied as a measure of spray performance (Fig. 3b). When applying spray formulations with airplanes, the spray coverage performance was about 0.15 (meaning that for each extra liter per ha, the spray coverage increased, on average, by 0.15%), while it was about 0.17 in experimental studies conducted in Western Australia and about 0.24 in ground rig applications in Texas. Thus in terms of "conversion efficiency" (converting spray volume into spray coverage), the ground rig applications in Texas appeared to be most efficient. In addition to comparison of averages, it is important to examine the range of consistency (difference between minimum and maximum) within a given spray application. This information is important, because it may be used to assess the risk of certain portions of treated fields receiving sub-lethal treatment levels. Of the 91 spray trials, 66 (73%) produced spray coverages, in which the lowest spray coverage on a single spray card was below 10% (Fig. 4). At the same time, the spray range (maximum/minimum) was above 110 in two of the spray applications with airplane and was above 5-fold in 17 (19%) of the spray trials. Low and less uniform spray coverage, especially with airplane applications, is most likely attributed to using smaller spray volumes and nozzles, which deliver smaller spray droplets and therefore increases the risk of spray drift [12]. Among the spray trial data obtained from Western Australia, the highest spray coverage obtained from a single spray card was about 40%, which is an indicator of the "maximum spray potential". That is, bare ground was sprayed with up to 130 liter per ha, and most growers in this region do not apply more than 90 liter per ha. Consequently, the data collected suggest that it will be very difficult to exceed this level of spray coverage of a growing crop.

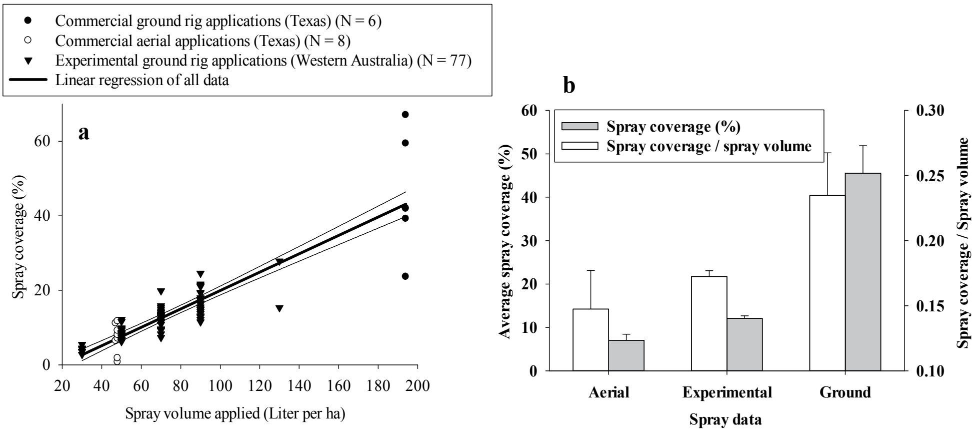


Figure 3. Spray coverage in response to spray volume

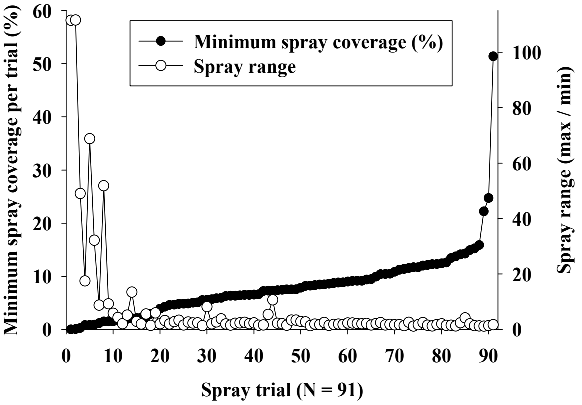


Figure 4. Minimum and range of spray applications

The final aspect of spray applications discussed here is "canopy penetration" – or the level of insecticide being deposited in the lower portion of a given crop canopy. The spray data presented so far were all collected either from the top of the canopy or above bare ground. Based on analysis of nine of the spray trials from Texas, it was possible to compare spray coverages at the top of the canopy with in the lower portion of a potato canopy above 35 cm tall. On average, the bottom portion of the canopy received about half the spray coverage of the top portion, and only one of the nine applications provided over 10% average spray coverage in the bottom portion of the canopy. Published spray coverage studies using water sensitive spray cards have shown that it is not uncommon, especially with aerial spray applications, to obtain spray coverages below 1% [15-17].

These spray results obtained across a wide range of operational conditions clearly highlight that, although spray volume is the most important variable, other variables need to be taken into account if the goal is to predict the obtained spray coverage. Furthermore, these results underscore that most insecticide spray coverages are likely quite low and highly influenced by weather variables and spray application settings. Thus, it is paramount to develop decision support tools to optimize timing of applications in accordance to weather variables, so that farmers are in a position to apply insecticides with highest likelihood of obtaining good coverage and therefore high performance. Otherwise, it is possible that spray applications of low and inconsistent insecticide dosages contribute to resistance development in target pest populations [10, 18].

4. Arthropod pests and insecticide resistance

[13] pointed out that insecticide resistance is among the most significant challenges to food production systems and to public health through management of insect vector born diseases. There are clear indications that many major pests are able to develop physiological and or behavioural insecticide resistance to a large number of insecticides. In this context, physiological insecticide resistance is defined as genotypes being able to tolerate high dosages of neurotoxic ingredients, which are lethal to most individuals of the same species. The most common physiological resistance mechanisms are [19]: 1) catabolic processing of the active ingredient, 2) changes in binding sites that are targeted with a given toxin, 3) decreased uptake rate, and 4) binding of toxin to sites with no toxic effect. Behavioural resistance [20] has been documented for the past 40 years, and it is interpreted as a behavioural adaptation, which reduces the likelihood of target pests acquiring a lethal dosage of insecticide. Behavioural insecticide resistance has mainly been discussed in the context of "bait aversion", in which, for instance glucose based bait for control of cockroaches [21-23] no longer works, because the cockroaches avoid the bait. However as discussed below, it also seems plausible that behavioural insecticide resistance may develop in response to low and incomplete spray coverage. Concerns about behavioural insecticide resistance may be of particular concern when target pests predominantly occur on the abaxial (lower) side of crop leaves and insecticides are not translaminar or systemic. For instance, in a simple study in which either one or both sides of potato leaflets were treated, [24] showed that for some insecticides pota-

to psyllid mortality was much lower when only one side was treated compared to when both sides of the leaflet were treated. These findings were interpreted as potato psyllids [*Bactericera cockerelli* [Sulc] (Homoptera: Psyllidae)] moving away from (avoiding) the treated leaflet side when given a choice between treated and untreated sides.

The first reported incidence of physiological pesticide resistance was of San Jose scale [*Quadrispidiotus perniciosus* (Comstock) (Homoptera: Diaspididae)] to lime sulphur in 1914 [25]. Since then, more than 550 arthropod species have been reported as being resistant to one or more pesticides [13]. However already in 1977, more than 364 species of arthropods were reported to show physiological pesticide resistance [26], 447 species in 1984 [27], and [28] 503 species in 1991. A few examples of documented physiological resistance against active ingredients are presented here and are based on data from the Arthropod Pesticide Resistance Database (APRD, http://www.pesticideresistance.org/): 1) two-spotted spider mite (*Tetranychus urticae* Koch, Acari: Tetranychidae) has developed resistance to 93 active ingredients, 2) diamondback moth (*Plutella xylostella* L, Lepidoptera: Plutellidae) has developed resistance to 82 active ingredients, 3) green peach aphid *Myzus persicae* (Sulzer) (Homoptera: Aphididae) has developed resistance to 74 active ingredients, 4) Colorado potato beetle (*Leptinotarsa decemlineata* (Say), Coleoptera: Chrysomelidae) has developed resistance to 51 active ingredients, 5) silverleaf whitefly (*Bemisia tabaci* Gennadius, Homoptera: Aleyrodidae) has developed resistance to 46 active ingredients, 6) cotton bollworm / corn earworm (*Helicoverpa armigera* Hübner, Lepidoptera: Noctuidae) has developed resistance to 44 active ingredients, and 7) beet armyworm (*Spodoptera exigua* Hübner, Lepidoptera: Noctuidae), has developed resistance to 29 active ingredients. Diamondback moth was the first pest to become resistant to DDT (dichlorodiphenyltrichloroethane) [29, 30]. From the mid-1990s, the use of formulations of toxins derived from strains of the soil borne bacterium, *Bacillus thuringiensis* (denoted Bt toxins) have been promoted to control key lepidopteran and coleopteran pests and at the same time preserve natural enemy populations within crops [31]. Due to its high efficiency, low cost and simple application, Bt-based pesticides rapidly became used for control of many pests, and diamondback moth was the first insect pest to become resistant to Bt toxins [32-34]. Thus, certain characteristics in the diamondback moth genome, its biology, and its interactions with food cropping systems seem to expose an incredible adaptability and responsiveness to imposed pesticide-induced selection pressures. Consequently, [13] made the important point that while there is a steady increase in reported cases of resistance, the number of new species with documented resistance is not increasing nearly as fast. It is therefore important to consider that the most important insect pests will likely continue to develop resistance to the insecticide pressures that are imposed upon them, and that the ability to develop physiological resistance to insecticides may be one of the driving selection pressures for species to become pests. That is, the economically most important arthropod pest species may share certain common denominators, which enable them to be successful under commercial/agricultural conditions with high levels of selection pressure imposed by insecticide treatments. It may be argued that insight into such denominators is critically important for development of future pest management programs, as it may open avenues for management strategies that rely less on insecticides. With less reliance on insecticide applications, pest populations would be under less insecticide-induced selection pressure, which would decrease the risk of pest populations developing insecticide resistance.

When addressing concerns about risk of insect pest populations developing physiological resistance and how management practices can be developed under commercial settings to reduce this risk, it is worthwhile setting the general context. Firstly, we wish to emphasize that there are only two extreme scenarios, which do not potentially lead to development of physiological resistance in target pest populations: 1) always applying an insecticide dosage low enough so that all genotypes survive, 2) applying a high enough dosage to ensure that individuals of all genotypes die. Obviously, the first option is of no interest to growers, as it means zero pest control, and therefore represents waste of resources. As already described in detail based on the analysis of water sensitive spray cards, the second option is in most cases unfeasible from a practical standpoint, and it may also imply very high economical costs. This means that under real-world conditions, applications of insecticides are always imposing a selection pressure on target pest populations.

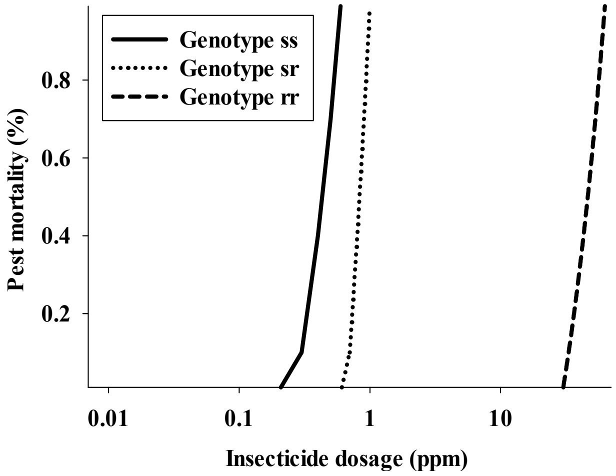
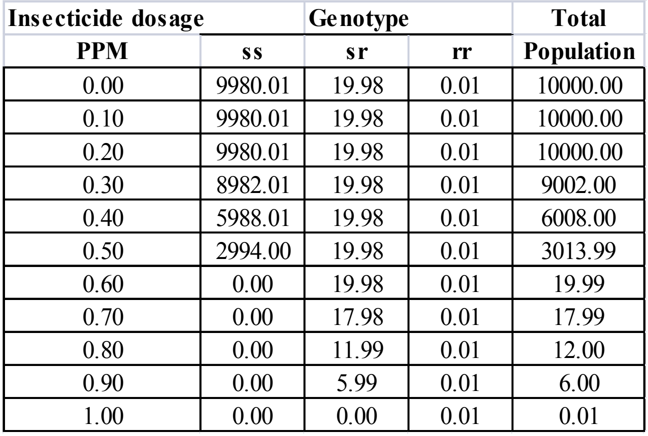


Figure 5. Dosage response

The important point is that the mortality of a given pest individual is NOT random within a pest population: an individual will only succumb to an insecticide application, if the individual is actually susceptible to the pesticide and exposed to a dosage above a certain level (minimum lethal dosage). Moreover, pest individuals within a population vary in their ability to tolerate an insecticide, and – based on their behaviour – vary in likelihood of getting exposed to the insecticide. The intraspecific variation in tolerance to an insecticide is linked to the fact that the mode of action of the vast majority of insecticides is very specific and associated with allelic variation at one (monogenic) or two loci. That is, the insecticide operates by interfering with a very specific metabolic function, but even the slightest change in binding site (induced by mutation at a single locus) may compromise the performance of the



Insecticide dosage		Genotype		Total
PPM	ss	sr	rr	Population
0.00	9980.01	19.98	0.01	10000.00
0.10	9980.01	19.98	0.01	10000.00
0.20	9980.01	19.98	0.01	10000.00
0.30	8982.01	19.98	0.01	9002.00
0.40	5988.01	19.98	0.01	6008.00
0.50	2994.00	19.98	0.01	3013.99
0.60	0.00	19.98	0.01	19.99
0.70	0.00	17.98	0.01	17.99
0.80	0.00	11.99	0.01	12.00
0.90	0.00	5.99	0.01	6.00
1.00	0.00	0.00	0.01	0.01

Table 1. Dosage response

insecticide, so pest individuals possessing such changes will have a higher chance of survival, while individuals without the specific allele will be eliminated. If the insecticide resistance is monogenic, and only two alleles exist (r = resistant and s = susceptible) - dosage response curves for the three genotypes are typically presented with mortality increasing along a logarithmic dosage scale [27]. In a theoretical example of a pest population of 10,000 individuals (Fig. 5), individuals of genotype ss may be expected to succumb when the insecticide dosage ranges between 0.3-0.6 ppm, sr individuals when the insecticide dosage ranges between 0.6-1.2 ppm, and rr individuals when the insecticide dosage is above 30 ppm. If p = 0.001 is the allele frequency of r and q= 0.999 is the allele frequency of s, and the genotypes occur in Hardy-Weinberg proportions, then the demographic composition of the pest population in response to insecticide dosage is as outlined in Table 1. It is seen that subjecting a pest population to a dosage above 0.5 ppm causes a >99% reduction of the overall population, but if it less than 60 ppm it also increases the proportion of resistant individuals in the remaining pest population. And although this fairly simple relationship between survival of genotypes and insecticide dosage has been investigated intensively over the last 3-4 decades and been greatly expended upon – it illustrates the core challenge that insecticide based pest control is faced with: Growers want to suppress as large a proportion of the pest population as possible to minimize the economic loss they incur, and therefore apply high dosages of insecticides. However, they are not able to apply a high enough dosage to completely suppress all pest individuals, so a selection pressure is imposed on the pest populations and the end result may be that the pest population develops physiological resistance because it is practically impossible to kill all the homozygous resistant genotypes.

In this brief and very general discussion of the importance of insecticide dosages, it is important also to mention that the efficiency or performance of an applied insecticide declines over time. The term "residual effect" is used to describe the longevity of the time period in which a given insecticide provides effective pest control, and rarely (continuous expression of Bt toxins in transgenic Bt crops would be an exception) will an insecticide have a residual effect after 7-10 days. In a study of abamectin, [24] conducted experimental sprays of potato leaflets in different vertical portions of a potato canopy under field conditions. During time intervals after spraying, treated and untreated leaflets were collected and used in bioassays with potato psyllids to determine the adult psyllid mortality over time. Based on this study, [24] concluded that the residual effect of abamectin is less than 48 hours. Although most insecticides have longer residual effect than abamectin, the example highlights the challenge that the effective dosage applied will decline over time, so pest individuals that are not affected immediately after application may not be exposed to a lethal dosage. For instance at the time of application, pest individuals may not be present at a vulnerable stage (for example mite eggs are not killed by systematic sprays whereas active adult mites will be killed), or the life stage may not be exposed to contact with chemicals (this is especially important for larvae of insects feeding on roots of plants in the soil). This problem or challenge, with not all life stages being equally susceptible to an insecticides application, becomes an even larger issue, if multiple pest species are present, and these different species occur in different parts of the crop canopy, have different movement patterns within the canopy, show difference in terms of seasonal population dynamics, and also have different migration patterns between the given crop and neighbouring alternative hosts. Immigration by pest populations deserves to be highlighted as a serious constraint: if a pasture or field is treated and all present pest individuals are killed but high immigration means that a new population of pest individuals move into the given field or pasture a shortly after. If so, a grower might think that the insecticide application "didn't work" – but the reality is that the immigration rate of the pest needs to be taken into account when assessing what insecticide to apply and when to apply it. It is not practically feasible to apply insecticide specifically for each pest present separately and so inevitably each application event may effectively control some species or life stage, while other pest individuals will be exposed to sublethal dosages.

In addition to concerns associated with physiological resistance of target pest populations, behavioural resistance may possibly develop in response to incomplete pesticide coverage, as target pests are given a "choice" between treated and untreated surfaces. If the target pest is able to discriminate between treated from untreated surfaces and eventually avoid treated surfaces, the pest will be less exposed to the insecticide. Consequently, the ability to avoid treated surface becomes a strong selection pressure, which can lead to development of behavioural-based resistance, and it has been demonstrated in diamondback moth populations [35, 36], German cockroaches (*Blatella germanica* L. [Blattodea: Blattellidae] [23, 37], and maize weevils (*Sitophilus zeamais* Motschulsky [Coleoptera: Curculionidae]) [38]. [39] demonstrated that spider mites are repelled by the contact miticide, propargite. In a recent study of spider mites on cotton plants, [40] quantified the consequences of behavioural avoidance and based on theoretical modelling showed that behavioural avoidance can have significant impact on population dynamics.

Summarizing this section, the ability to develop physiological resistance to insecticides is one of the key characteristics of the most economically important arthropod pests. There are widespread examples of pests developing behavioural resistance by avoiding treated leaf surfaces or baits containing the active ingredient. With regards to contact insecticides, it is possible that a combination of frequent and low performing pesticide applications creates a selection pressure which favours pest individuals avoiding treated portions of crop leaves, as individuals: 1) have ample opportunity to recover after sub-lethal exposures and therefore "learn" to avoid insecticide treated surfaces, and 2) will be under a directional selection pressure for non-feeding on treated surfaces. However, we are unaware of experimental studies actually addressing the possible relationship between insecticide spray coverage in agricultural field pest populations and behavioural resistance in target pest populations. It is likely that the most important pests will continue to develop resistance to insecticides, as certain traits in their biology and/or ecology appear to enable them to adapt to these severe selection pressures. Thus, continued emphasis on almost exclusive insecticide-based pest control may be a strategy that deserves serious revision, as it seems to play to one of the key "strengths" (their adaptability) of the most important pests. The fundamental challenge is therefore to develop management practices, which minimize the risk of resistance development, and theoretical modelling is critically important in this context, because it can be used as a working tool to examine changes in population genetics over time and under different selection pressures. That is, instead of waiting until growers actually face the severe consequences of insecticide resistance, we can use theoretical modelling to predict its progress and hopefully find ways to slow it down.

5. Sensitivity analysis of physiological resistance development to pesticides

Studying resistance development in controlled populations is, in addition to being highly laborious, associated with some basic concerns. The frequency of resistance alleles in a given pest population is typically extremely low (one in a 1,000 or less) and therefore requires individual analysis of very large numbers (millions or billions) of individuals. And when small laboratory populations are subjected to intensive insecticide based selection pressure, there is a considerable risk of unforeseen recessive genetic defects being expressed and affecting the observed population dynamics. [27] provided an in depth discussion and review of the concerns related to rearing of laboratory cultures for studies of how fast pest populations are able to develop resistance. In brief, they mentioned how the rearing may lead to "genetic bottlenecks" or selection pressures, which are different from those imposed on field populations. Consequently, it is highly likely that a laboratory strain carries major resistance alleles at frequencies that are very different from field pest populations and that studies of resistance development in laboratory strains therefore are unable to mimic actual field conditions. Finally, numerous factors are considered important when assessing the likelihood of a pest population developing physiological resistance, and based on [41], they can be divided into four categories: 1) genetic factors (i.e. frequency, dominance, and expressivity of resistant alleles and their interactions with other alleles, past selection pressures in pest population, and whether the resistance is monogenic or polygenic), 2) biological factors (fecundity, generation and development times, mating behaviour, level of polyphagy, migration/dispersal and mobility, fitness costs of resistance development, and feeding biology), 3) operational (mode of action of insecticide, residual effect of the insecticide, adjuvants added to sprayed formulations, timing of applications in relation to pest population development (which life stages are targeted), dosage applied, crop density at the time of application, type of spray nozzles used, height of spray boom, and 4) weather conditions (which are known to greatly affect spray depositions, see above). With such complexity of factors involved, it is not surprising that much of the current understanding of pesticide resistance development in pest populations is based on genetic population modelling and theoretical sensitivity analyses [10, 42-45]. Such modelling efforts are in many ways constructive and can be used to develop strong justifications for specific research projects and management practices. However at the same time, their validity depends on the assumptions used in their construction [46, 47].

The following section is a sensitivity analysis based on genetic population modelling, which expands on work presented in two theoretical modelling papers [26, 41]. Although published almost 40 years ago, these studies present the basic modelling framework needed to examine fairly simple/basic questions about resistance development. Results presented here are based on a theoretical arthropod pest population "X" with an initial population of 11,000 individuals followed over 20 subsequent generations, and it is assumed that: 1) adults only give offspring in one generation, 2) each generation was exposed to a single insecticide application, 3) resistance development occurs in a single locus with two alleles, r (resistant) and s (susceptible), 4) $p = 0.0001$ is the gene frequency of r and $q= 0.9999$ is the gene frequency of s, 5) genotypes occur in Hardy-Weinberg proportions, 6) dominance is assumed to be intermediate, so that, under insecticide based selection pressure, the survival of genotypes is rr > rs > ss, and 7) resistance was associated with a "fitness cost", which is defined as resistant genotypes having lower fitness than susceptible genotypes in the absence of the particular insecticide [45]. Based on a review by [45] of 77 studies of Bt resistance, it was assumed that physiological insecticide resistance was associated with a "fitness cost" of 15.5% for each allele. Although the possible importance of "incomplete resistance" [42] and "hybrid vigor" [45] have been highlighted, these factors were not included in this analysis. The following sensitivity analysis of r allele frequency and pest population density is based on 1,000 simulations of different scenarios with random variables. Similar to [26], the population density after each discrete generation, N′, was assumed to be density-dependent and described by the following equation 1:

$$\begin{aligned} N' &= [W_{rr} \times N_{rr} \times exp\left(r_{rr} \times \left(K - N_a / K\right)\right] + \\ &[W_{rs} \times N_{rs} \times exp\left(r_{rs} \times \left(K - N_a / K\right)\right] + \\ &[W_{ss} \times N_{ss} \times exp\left(r_{ss} \times \left(K - N_a / K\right)\right]. \end{aligned} \tag{1}$$

In which W denotes the survival of each genotype, N denotes the number of adults in the previous generation, K denotes the carrying capacity, and N_a denotes the initial population.

In the following, we present modelling results from two scenarios, and the main point is to demonstrate some of the advantages of using modelling as part of demonstrating how physiological insecticide resistance appears to develop across a very wide range of scenarios.

Scenario 1: Effects of reproductive fitness and crop suitability on resistance development. Using Equation 1 to estimate total population and r allele frequency over 20 generations, the following settings for allele frequencies were kept constant: $p = 0.0001$ and $q = 0.9999$, and survival rates of the 3 genotypes (W) were: $W_{rr} = 1$, $W_{rs} = 0.5$, $W_{ss} = 0$. Thus, in this scenario, none of the susceptible individuals (W_{ss}) were expected to survive and did therefore not contribute to the sensitivity analysis. [26, 41] assumed the reproductive fitness of pest population X, "r", to be constant and equal to ln(5) between generations, and many subsequent and more recent studies have also been based this assumption. Here, the reproductive fitness of the resistant genotype was assumed to vary randomly from ln(3)-ln(7) (random numbers with two-decimal points) between generations. With the reproductive fitness of the resistant genotype varying randomly between ln(3)-ln(7) and the fitness cost of resistance being 15.5%, that of the heterozygous genotype, $r_{rs} = r_{rr} \times 1.155$. A constant carrying capacity implies that a certain habitat can sustain the same pest population in all growing seasons and irrespectively of regional differences in environmental conditions. Seasonal variations in growing conditions (i.e. drought stress levels and fertilizer regimes) clearly cause marked variations in number of pest individuals a crop plant can harbour. For instance, nutritional composition of crops can vary considerably in response to drought stress [48-51] and is known to vary considerably between growing seasons [52, 53]. [54] estimated varying carrying capacity of the milkweed-oleander aphid [*Aphis nerii* Boyer de Fonscolombe (Hemiptera: Aphididae) on one of its host plants, milkweed (*Asclepias tuberosa*) in response to nitrogen applications. Based on data collected under controlled conditions, the authors obtained ranges from 29.5-35.1 (19%) aphids. Thus, here we assumed a 20% random variation in carrying capacity between growing seasons (K ranging randomly from 10,000-12,000 between subsequent generations).

In all 1,000 scenarios, the initial pest population was reduced by >99.9% after the initial infestation (N = 11,000) was exposed to the first insecticide application. After the initial knock-down, the pest population remained low for about eight generations, after which the pest population density started to increase steadily (Fig. 6a). Almost complete insecticide failure (back to initial pest population density due to complete physiological resistance) was achieved within 20 generations. Comparison of the average curve of the pest population density under varying reproductive rate and carrying capacity and that of fixed variables [with constant reproductive fitness ($W_{rr} = 5$, $W_{rs} = 5.775$ and carrying capacity ($K = 11{,}000$)] suggested that incorporation of variability into reproductive fitness and carrying capacity had limited effect. That is, the average of the 1,000 simulations was very similar to that of fixed variables except for a few and rare simulations (indicated by the curve of maximum pest population) reduced the time to complete physiological resistance by a few years (shifted the curve to the left). Fig. 6b showed that varying crop carrying capacity and reproductive fitness had almost negligible impact on the r allele frequency in the pest population (as expressed by the range of minimum and maximum curves). Most sensitivity studies of genetic populations are based on large populations (i.e. 10,000 individuals), and it is assumed that individuals mate randomly within these large populations. This is highly unlikely and is the main reason why recent genetic population modelling uses a meta-population modelling approach, in which a large population is considered to be composed of many smaller and somewhat segregated populations. Such meta-population based approaches include assumptions about movement between populations and sizes of sub-populations, and these assumptions were not included in this analysis. Another approach is to use individual-based modelling [10]. Based on the analysis of scenario 1, we have highlighted that the influences of incorporating varying crop carrying capacity and reproductive fitness into modelling predictions of population densities over 20 generations were modest, when the fitness cost was kept constant (15.5%), and they had negligible effect influence on r allele frequency.

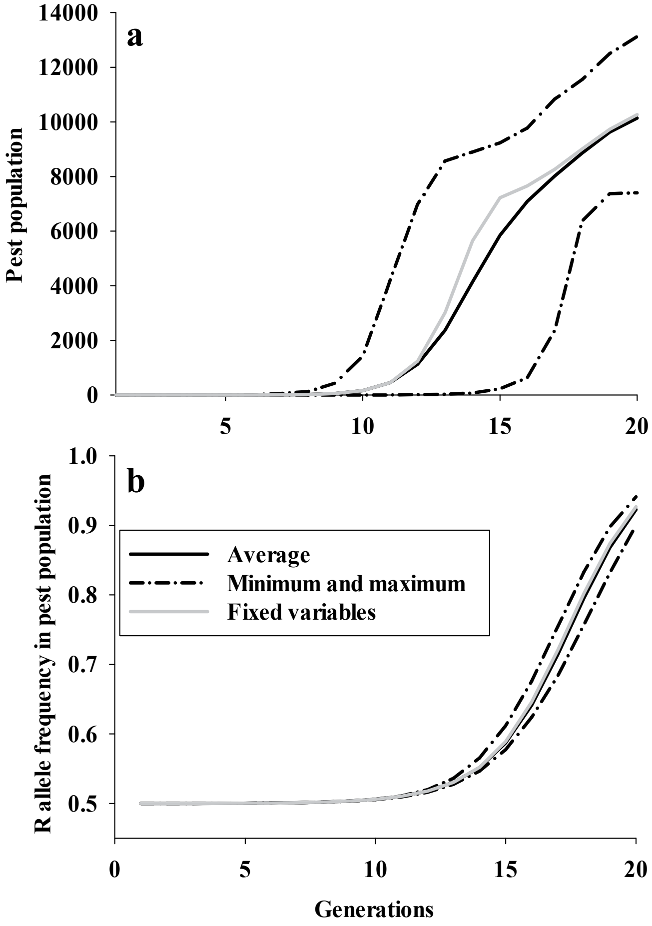


Figure 6. Effects of varying reproductive fitness and carrying capacity

Scenario 2: Effect of varying pesticide spray performance. In this scenario, varying reproductive fitness rates and carrying capacity were maintained as described in scenario 1. The survival rate due to pesticide applications was kept constant for the homozygous resistant (W_{rr} = 1), but this scenario was conducted with varying survival rates of W_{rs} and W_{ss}. That is, it was assumed that low and inconsistent spray coverage would in some generations increase the survival of W_{rs} and W_{ss}. The basis for investigating this scenario with varying survival rates of W_{rs} and W_{ss} was supported by the field spray data presented in Fig. 3: Out of the 91 insecticide spray data sets, several data sets showed spray coverage ranges above 50 times (difference between minimum and maximum). It therefore seems reasonable to assume that there is considerable variation in insecticide dosages and therefore survival rates of subsequent pest population generations. Consequently, a random number function was used to generate survival rates from 0.3 to 0.7 for W_{rs}, and survival rates below 0.5 were considered to be equal to 0.5. In other words, the random function generated one of five outcomes (0.3, 0.4, 0.5, 0.6, or 0.7) with equal probability, and three of these (0.3, 0.4, and 0.5 or 60% of the outcomes) were equal to 0.5, and there was a 50% chance [(0.7-0.5)×100/(0.7-0.3)] of increased survival due to low and inconsistent spray coverage for W_{rs} genotypes. Regarding genotype W_{ss}, the same random function approach was applied to generate random survival rates from -0.2 to 0.2, and all rates equal to or below 0 denoted no survival. In other words, there was about 50% chance of W_{ss} genotypes contributing at least some offspring to the next generation. As in scenario 1, a 15.5% fitness cost was maintained for each resistant allele, which meant that the reproductive fitness of $r_{rs} = r_{rr} \times 1.155$ and that of $r_{ss} = r_{rs} \times 1.155$. In other words, the survival of W_{ss} genotypes had the potential of contributing substantially to subsequent generations in simulations with $W_{ss} > 0$.

In this scenario with varying survival rates of W_{rs} and W_{ss}, the population density after the initial knock-down was, on average, 95%, but there were simulations in which it was below 80%. It should be expected, that increased survival due to low and inconsistent pesticide applications increased the pest population growth during 20 generations, but, in comparison with scenario 1, the effect on pest population was actually quite modest (Fig. 7a). As an example, in scenario 1 (with no survival of homozygous susceptible individuals) the average population density was about 6,000 individuals after 15 generations, while it was about 7,000 individuals in scenario 2. Thus, with half of the simulations allowing 1-20% survival of homozygous susceptible individuals there was only a modest increase in average pest population density. However as indicated by the maximum curve, there were indeed scenarios in which high pest populations were achieved within about 11 generations. With fixed variables and assumption about Hardy-Weinberg allele frequencies, the r allele frequency obviously stayed above 50% and increased as the homozygous resistant genotype increased in relative proportion. Fig. 7b showed, as expected, that the varying survival of homozygous susceptible individuals (when $W_{ss} > 0$) led to a decrease in r allele frequency. In fact after 20 generations, none of the 1,000 simulations led to a higher r allele frequency than 93%, while with fixed variables it was >96%. In other words, this simple exercise suggested that by allowing susceptible genotypes some level of survival, low and inconsistent pesticide applications appear to postpone development of complete resistance. However, low and inconsistent pesticide applications also lead to higher risk of high pest population densities (comparing Fig. 6 a and 7 a) and therefore crop damage and corresponding yield losses. Thus, here it is highlighted that with a consistent selection pressure (all generations of pest individuals subjected to a pesticide applications) the performance of pesticides and negligible development of resistance are antagonistic. In other words, growers should not expect to accomplish both: a high-performing pesticide application will create a strong selection pressure and therefore lead to resistance development. On the other hand, low and inconsistent pesticide applications appear to reduce the likelihood of pest populations developing resistance, but it will also reduce the performance of pesticide applications. However, it is likely that low and inconsistent insecticide applications increase the risk of target pest populations developing behavioural resistance, but that is not incorporated into the modelling presented here. A recent modelling based study of herbicide resistance in weeds addressed this specific question about the effect of dosage [10]. The authors concluded that in cases of monogenic resistance, pesticide dosage had negligible effect on the number of generations before complete failure. However, they also pointed out that in cases of resistance being "non-target specific" (i.e. metabolic and/or polygenic resistance), there is growing evidence of herbicide resistance developing faster under low-dosage selection pressure. There are important differences in factors leading to resistance in weed and insect pest populations (i.e. reproduction/mating biology, generation time, and dispersal strategies), so it may not be accurate to assume the exact same responses by insects and weeds. However, it is clear that reliable and accurate sensitivity analysis of how certain variables affect the likelihood of a pest population developing resistance requires that the underlying genetics are sufficiently understood (especially whether resistance in mono- or polygenic).

6. Some realities associated with rotation of insecticides

It may be argued that the scenarios outlined above are far too simplistic and do not take into account that growers are rotating insecticides as part of resistance management practices. The core of resistance management programs is to rotate between active ingredients, as cross-resistance to multiple insecticides is much less likely to develop. Regarding transgenic crops expressing Bt toxins, incorporation of non-treated refuges in cropping systems is also being advocated [see [45] for review). We are unaware of recommendations about non-treated refuges for any other insecticide treatments. Consequently rotation of classes of active ingredients is the only widespread resistance management strategy, but there are crop-pest systems in which only a few active ingredients are registered for use. For instance in Western Australia, there are three species of aphids [The cabbage aphid, *Brevicoryne brassicae* (L.), the turnip aphid, *Lipaphis erysimi* Kalt, and the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphidae)] attacking canola during the flowering/podding period – yet only ONE insecticide (Pirimicarb 500) is registered for use against these pests! In addition, active ingredients are increasingly being faced out (banned) - so growers are left with only a few options. And if one particular pest is under a single insecticide selection pressure in one cropping system, then this may be the source for a resistant pest population to emerge. In addition, rotation of insecticides is only an effective option as long as cross-resistance is close to negligible, although there are ample examples of arthropod pests developing resistance to a many

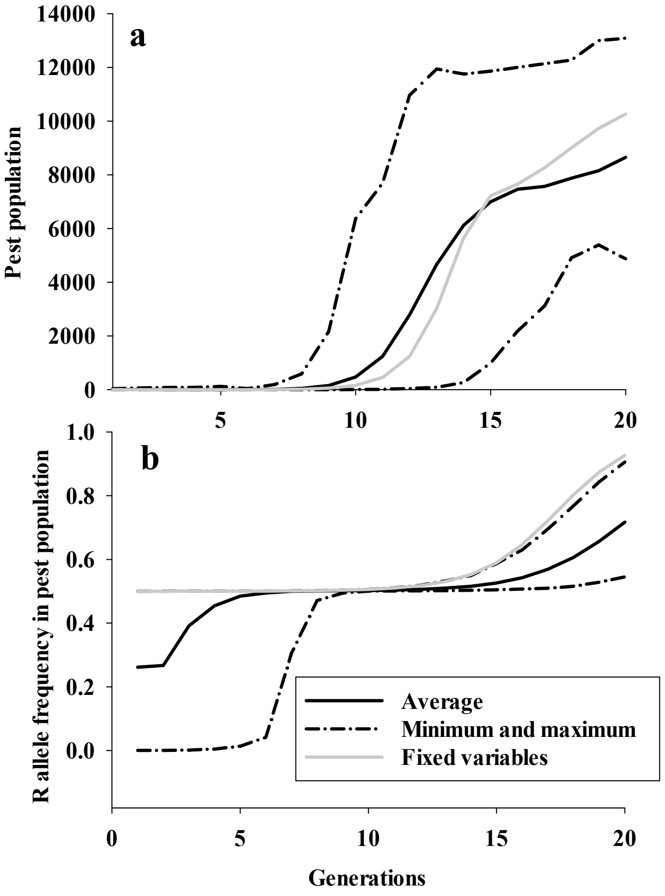


Figure 7. Effects of varying spray application performance

insecticides (examples listed above). Another important aspect of insecticide rotation is that during the last 50 years, it has been a successful but short-term strategy to rely on a continuous development of new pesticides, so the steady increase in insecticides losing their performance has been less of an issue. However, there seem to be a trend of chemical companies registering fewer new insecticides, and at the same time older chemistries are being faced out. So the total number and the diversity of commercially available insecticides are decreasing. And with less available options to choose from, there is obviously an increased overall risk of resistance development. The declining number of new insecticide registrations is very interesting and likely explained by a complex of factors. But it is clear that, in recent years, regulatory bodies have increased the amount of risk assessment studies required for a successful registration, and many of these quite expensive. Thus, chemical companies are less inclined to register new insecticides unless they target very large commercial markets. So risks of insecticide resistance, due to few insecticide alternatives to choose from, may be of particular concern to comparatively smaller markets (cropping systems). In addition to chemical companies being less inclined to register new insecticides due to registration costs, it also means that newly registered insecticides are often considerably more expensive than older insecticides, because the registration costs are passed on to end-users. And a stark difference in price between old and new insecticide obviously creates an economic incentive for continuing insecticide treatment programs based almost exclusively on old/less-expensive insecticides. Thus, resistance in target pest populations may continue to develop due to lack of rotation of insecticides, because growers are unwilling to incorporate newer and more expensive insecticides into their insecticide application regime even though alternative products are commercially available. In short, development of resistance to one active ingredient is a serious concern, because it may initiate a "snowball effect", as loss of one active ingredient, and effectively an entire insecticide class, means that growers can only rotate among pesticides with a few other modes of action, and that increases the risk of resistance development to those alternative pesticides. Thus, for a range of economical, biological/ genetic reasons - growers and other stakeholders associated with the food industry should be profoundly concerned about the long-term sustainability of pest management programs relying almost exclusively on pesticide applications. There needs to be far greater awareness of the risk of resistance developing and its likely long-term cost, so that better decisions can be made on the benefits to rotating with more expensive compounds.

7. Appreciation of the seasonal variability of pest population dynamics

Integrated pest management (IPM) has been an applied research discipline since it was first defined by [55]. One of the initial drivers for development of IPM was the recognition of pest populations developing resistance to pesticides [56]. Many definitions and in-depth descriptions of IPM have been provided [4, 57-59]. Broadly speaking, IPM involves integration of different tactics such as pesticides, biological control, measures to prevent initial pest establishment, use of plant resistance and cultural control. Consequently, IPM requires in-depth understanding of a given target pest's biology and ecology so that cropping systems can be established and managed in ways that minimize risk of pest infestations and subsequent yield losses. IPM is expected to reduce dependence on pesticides, and [60] argued that in several respects IPM may be viewed as "IIM", or integrated insecticide management. However, the most important difference between IPM and other crop management systems is that IPM is based on two fundamental assumptions about yield loss: 1) that it is correlated with pest density and 2) predictable and therefore can be modelled and/or forecasted. Thus, an IPM approach implies that if the pest population density can be accurately estimated, it is possible to determine when and where deployment of responsive management options (such as pesticide applications and/or releases of natural enemies) are needed. Reliable and practically feasible sampling or monitoring plans are therefore needed to estimate the pest population density. The pest density estimate is converted into a decision based on an "economic threshold" (ET), which represents the pest density at which the value of estimated yield loss equals the cost of responsive intervention. If it is assumed that yield loss can be predicted or forecasted based on a given pest population density, then the economic injury level (EIL) can be used as benchmark for when to take action. Consequently, responsive intervention, such as applying an insecticide, should only be deployed, when/if the pest density estimate is expected to exceed the EIL or the ET. The "textbook" concept of EIL (i.e. [4] includes the following variables (Equation 2):

$$\mathrm{EIL} = \mathrm{C} / \left(\mathrm{V} \times \mathrm{D} \times \mathrm{K}\right) \tag{2}$$

In which "C" denotes the cost of action (i.e. application of an insecticide), "V" denotes the market value of the crop, "D" denotes the relationships between pest density and damage, and "K" denotes a coefficient of unavoidable loss (between 0-1). With IPM being an approach based on knowledge about the pest density and the relationship between pest density and economic loss, we argue that costs associated with sampling and the willingness to accept risk should also be included in the calculation of EIL. In development of sequential sampling plans, it is inherently assumed that there is a positive correlation between the precision of the population density estimate and the required sampling effort. In most cases, this relationship is probably asymptotic – so the question becomes how much is gained by collecting, for instance, 25 leaf samples compared to 20, or 47 instead of 42? The answer to this question is not necessarily straight forward, because the "cost" or effort associated with sampling should be taken into account, and the relative cost or effort per data point is not necessarily linear. In other words, most of the sampling costs may be associated with actually driving to the field, and once you are there, it may cost almost the same to take 20 or 25 samples. However for simplicity, we have added two variables to the calculation of the EIL (Equation 3)

$$\mathrm{EIL} = \left[\mathrm{C} \times \left(\mathrm{S} / \left(1 - \mathrm{P}\right)\right)\right] / \left(\mathrm{V} \times \mathrm{D} \times \mathrm{K}\right) \tag{3}$$

With "S" denoting the cost of collecting one sample (i.e. counting number of nymphs on a crop leaf) and "P" denotes the required precision of the sampling effort ($0 < P < 1$). Thus with these additions to Equation 2, it is acknowledged that "expensive" or labour intensive sampling will increase the pest population density which triggers action, and that requirements of high precision of pest population estimates will increase the needed sampling effort and therefore the the EIL. The concept of adding precision or tolerance of error to sampling methods is expanded further in sequential sampling [4, 61].

The concept of IPM – or only taking action on a when-needed basis- is supported by the fact that in most cropping systems, densities of pest species and their economic importance (expressed in crop damage or yield loss) are markedly influenced by weather and agronomic factors and therefore not constant across growing seasons or regions. Thus, individual growers face “low risk” and “high risk” growing seasons, and this is tightly linked to the often opportunistic character of arthropod pest populations – that they are able to take advantage of certain combinations of environmental and agronomic conditions; but they also suffer under other combinations, and in those years insecticide applications may not be warranted. As an example, the diamondback moth is among the most important pests on growing canola in Australia (winter crop). [62] summarized the widely accepted hypothesis regarding the ecological mechanisms driving diamondback moths outbreaks in winter rainfall regions of Australia: in years with good summer rainfall, supporting various cruciferous plants, including wild radish, turnip weeds and volunteer canola before the growing season. These host plants provide a “green bridge” during the summer months and enable early establishment of diamondback moth populations. At the same time, good summer and autumn rainfall means that canola is planted comparatively early and therefore establishes well under those growing conditions. Canola is a highly preferred host by diamondback moth [63], so populations developing in weeds and non-agricultural habitats may migrate into canola and cause economic damage. The risk of severe diamondback moth infestations seem to be further increased if, after early rains, the canola becomes slightly drought stressed. During the last 10 years, seasonal weather patterns characterized by good summer and autumn rainfall seem to explain a couple of growing seasons with high losses in large canola growing regions due to diamondback moth infestations. However in most years, diamondback moth is not considered a major pest on a wide geographical scale. As already mentioned, diamondback moth is one of the most adaptable arthropod pests regarding insecticide treatments, as it was the first pest to develop resistance to DDT and Bt, and, as a species, it is considered resistant to at least 82 active ingredients (may vary among local populations). Thus, for long-term sustainable management of diamondback moth, it is highly important that its somewhat sporadic pest status is taken into account and that insecticides are only applied when and where they are deemed necessary. The important aspect of arthropod pest densities only occasionally leading to significant economic losses is that it provides justification for some times (in some growing seasons and/or in some cropping systems) NOT to apply insecticides, when pest populations are below a given threshold. However, diamondback moth being a sporadic pest in canola in Western Australia is by no means a unique pest–crop system, as most insect pests vary economic importance across seasons. For a wide range of orchards pests [including a moth pest complex of peach [64] and *Acrobasis nuxvorella* Nuenzig (Lepidoptera: Pyralidae) in pecan [65], and field pests [including *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) in alfalfa [66], and *Cylindrocopturus adspersus* (LeConte) (Coleoptera: Curculionidae) in sunflower [67], *Diabrotica virgifera* virgifera LeConte (Coleoptera: Chrysomelidae) adults in maize [68], and *Sitodiplosis mosellana* (Géhin) (Diptera: Cecidomyiidae) in wheat [69], there are well-established degree-day models to predict “low risk” and “high risk” growing seasons. Such degree day models represent two important notions: 1) that the economic importance of a given pest shows considerable regional and seasonal variation, and 2) that the considerable spatio-temporal variation in economic importance can be predicted/forecasted based on quantitative models. Such models can be used very effectively to estimate whether a particular arthropod pest in a given growing season poses a threat to a certain crop system and provide strong foundation for only using insecticides on a when-needed basis. Only applying insecticides when needed may save growers money, and it will undoubtedly reduce the risk of insecticide resistance.

8. Applying insecticides on a when-needed basis

Based on the description of EIL in IPM based approaches to pest management, Fig. 8a shows average pest population densities for the Scenario 2 simulations (see above) after including a pesticide spray application threshold ranging from 0-10% of the carrying capacity (11,000 pest individuals) for the particular sampling universe (i.e. a field). In other words, it was assumed that sampling was conducted and that the average number of pests per plant was used as an action threshold for insecticide application. If the action threshold = 0, all pest generations were subjected to an insecticide application, while an action threshold = 2.5% meant that insecticide applications were only deployed if the estimated population density exceeded 275 pest individuals (or 2.5% of 11,000). Quite interestingly, the simulations suggested that using an action threshold led to slightly lower population density after 20 generations compared to a threshold = 0. In addition, the zero threshold showed a gradual increase in population densities from 18 generations and onwards, while those simulations with a threshold showed a population density stabilizing after about 12 generations. More importantly, the increase in r allele frequency was markedly reduced when a threshold was used, and it stabilized at about 50%, while it continued to increase in the scenarios without a threshold = 0 (Fig. 8b). Another interesting aspect of this analysis was that with 1,000 simulations and 20 generations, there was a total of 20,000 combinations of generations and simulations, and: 1) threshold = 0 obviously triggered 20,000 insecticide applications, 2) threshold = 2.5% of carrying capacity triggered 13,890 insecticide applications, 3) threshold = 5.0% of carrying capacity triggered 13,340 insecticide applications, and 4) threshold = 10% of carrying capacity triggered 12,517 insecticide applications. This means that, in addition to postponing complete insecticide failure (development of complete resistance development in the pest population), even a fairly low threshold of 2.5% of the carrying capacity reduced costs associated with insecticide applications by 31% [(20,000 - 13,890) × 100 / 20,000].

This exercise highlights some of the possible benefits of allowing some individuals of the homozygous susceptible genotype (with a higher reproductive fitness) to survive. They will obviously impose some level of crop damage and therefore cause yield losses, but their beneficial “dilution effect” is clearly outlined in the results from this simple exercise. In addition, it seems plausible that only spraying when the population density is above a certain threshold may enable natural enemies to become established and at least partially suppress the target pest populations. Incorporation of a pest density threshold as part of resistance management is analogous to the use of refuges as part of managing risk of arthropod resistance in transgenic crops (see [45] for review). Of course, the potential of taking advantage of benefits from reduced insecticide application is based on the assumption that a combination of detection/monitoring and degree-day modelling can be converted into accurate and reliable decision support tools. Thus, it is paramount to envision the development of arthropod pest population growth models under field conditions as an essential part of optimizing use of insecticides – both in terms of when application is needed and as part of resistance management.

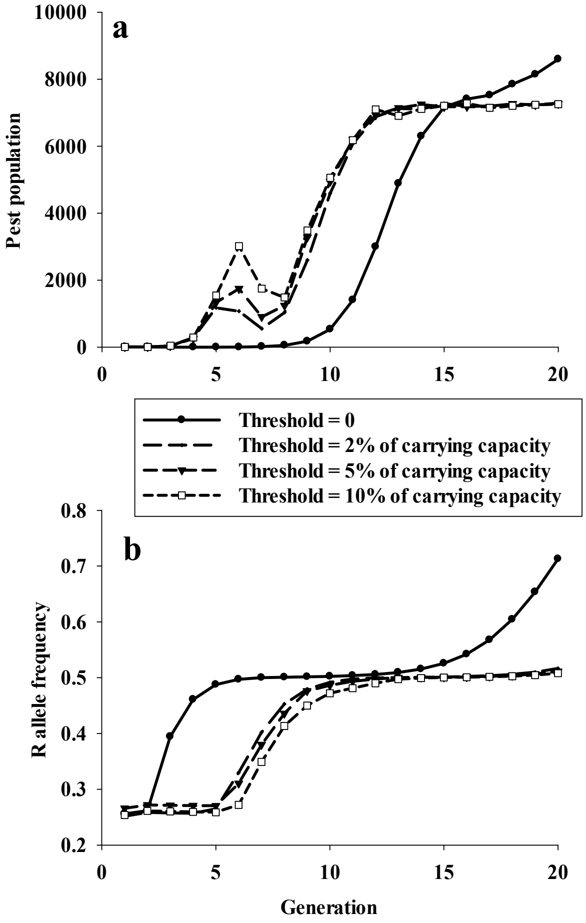


Figure 8. Effects of incorporating an action threshold

9. Pest infestations – symptoms rather than problems

Above, it was established that, mainly as "peace of mind" or because of operational convenience and less as a response to actual emerging pest infestations, insecticides are being sprayed/applied more often than what is economically justifiable and/or necessary to control target pest populations below action thresholds. In addition, we highlighted some practical/ operational circumstances, like tank mix, which may justify insecticide application even without knowing whether emerging pest populations are present or not. It was also shown, based on detailed analysis of water sensitive spray cards deployed under commercial pesticide applications, that expected performance of spray applications may often be below optimal due to quite low spray coverage and canopy penetration. That is, we have outlined the heavy reliance on insecticide applications and also shown that most of the quantitative data available in published reports suggests that insecticide spray coverages are often quite low. In many situations, the benefits of applying insecticides are clear and pest control is being effective, but for other major pests chemical control does not seem to work. Further research into the biology and ecology of these pests can provide valuable clues to how we can reduce our reliance on insecticide based management of important pests. For example, in regions of southern Australia with summer rainfall, root-feeding larvae of scarab beetles at times can destroy all the roots of pasture grasses, so that the pasture can be rolled up like a carpet. During 1970-1975 some 20,000-40,000 ha pasture were estimated to be sprayed annually at a cost of about $AUD 10 per ha. However, research showed the presence of potentially damaging populations was only evident after the damage had occurred and spraying at this time not only failed to prevent damage but also killed valuable natural enemies [70]. Larvae of the root-feeding scarab species selectively feed on living roots of the grasses in the soil [71], which initially causes a reduction in root growth, but a reduction in foliage growth was observed only when the plants were also defoliated (grazed), and these plants are vulnerable to any periods of water stress due to lack of rainfall [72]. At times when younger larvae are feeding actively and plants are growing well damage is not evident, and it is at a later time when older larvae are present that the plants may die due to drought. Spraying at this time does not prevent the appearance of damage because the plants were damaged previously. In addition when roots are growing actively and larvae are feeding strongly it is the lower densities of larvae that cause greatest reduction in the root yield [72]. A model of the interaction between plant growth, sheep grazing and insect feeding indicate that greatest impacts of the insect on pastures are occurring at low grazing pressures and low insect densities when plants are growing well [73]. An adjustment of spraying strategy is needed for these root-feeding scarabs. The redlegged earth mite is a major foliage-feeding pest in regions of southern Australia with winter rainfall, feeding on annual clovers in pastures. Potential economic losses due to redlegged earth mite damage in pastures are estimated at $200 million a year [74]. Pesticides are applied mainly in autumn as bare earth treatment before the mites emerge or in autumn and spring as foliar sprays without any one approach to control being consistently superior. Mites feed on annual crops and pastures during the cooler wet winter but avoid the hot dry summer as diapausing eggs. Emergence of mites in autumn varies from year to year depending on rainfall and temperature, but the onset of diapause in spring in this species remains the same for any one site from year to year [75]. A very accurate prediction for the onset of summer diapause was made for redlegged earth mites based on day length and length of long term growing season everywhere in southern Australia where this species was present [76]. This model was used to give an optimal spring spray date for a single well timed spray, which has resulted in very effective control (over 95%) to the populations emerging the following autumn 8 months after the spray [77]. In both of these examples growers need to make decisions to control the pests long before the actual damage occurs. The mites are easier to see than the root-feeding scarabs which may make it easier to decide on the future risk of damage occurring but the strategy in both cases is to reduce the risk of damage occurring. Growth of plants also affects the plant-insect interaction. Grazing management can affect the populations of some pasture pests, as pastures have a carrying capacity for pests as they do for sheep. Heavy grazing can be used to suppress pest populations in the pasture [78, 79]. Other factors affecting plant growth will also interact with the populations of pests feeding on them. The risk of spider mite (Acari: Tetranychidae) infestations, have been shown to increase in response to crops being grown under drought stressed conditions [for sorghum, *Sorghum bicolor (L.)* Moench [80], cucumber, *Cucumis sativus* L. [81], pepper, *Capsicum spp.* [81] and strawberry, *Fragaria spp.* [81], ornamental plants [82], soybean, *Glycine max* L. [83], cowpea, *Vigna unguiculata* (L.) Walp [84], and maize (*Zea mays* L) [85, 86]. Thus, effective irrigation management could be considered a spider mite management tool. Numerous studies have demonstrated that nitrogen fertilizer tends to increase risk of spider mite infestations [87-90]. Thus application of nitrogen fertilizer will result in improved production but can lead also to the need for further cost inputs (pesticides). [91] reviewed over 2,000 studies regarding effects of potassium on pest and disease incidence in plants. In about 63% of these studies, application of potassium led to a decrease in pest pressure, but opposite and inconsistent effects of potassium applications have also been observed. The exact causes of a positive correlation between potassium deficiency level and susceptibility to pests are not fully understood, but there seems to be considerable evidence of potassium deficiency causing accumulation of soluble sugars and amino acids and interference with constituent host plant defence mechanisms [92].

Pest insects tend to feed mainly on one stage of growth of the plant. For many crop plants it is the seed which is harvested and sold and damage at this stage or post-harvest has a very direct impact on yield. Feeding damage by redlegged earth mite on seedlings can carry through to loss of seed yield by mature plants. In a carefully controlled study on yellow lupins (*Lupinus luteus*), redlegged earth mites at different known densities were allowed to feed for 14 days on seedlings and then removed, and the plants were grown to final seed yield 142 days after mite application [93]. This study revealed a clear negative correlation between redlegged earth mite density and yield with about 56% lower yields at the highest redlegged earth mite density. To avoid this damage the plants would have had to be sprayed with an effective miticide at the seedling stage. This is another example where the decision to spray has to be taken some time before any damage is evident. When the pest is feeding on the seed pod (as with pod borers) some plants are able to compensate. Population-level compensation is observed in cotton following feeding by *Helicoverpa* larvae (Lepidoptera) [94], although obviously very considerable loss of seed yield due to this species can also be seen at other times. The examples above illustrate how insecticide applications should be based on extensive knowledge about the target pest's biology, and that overall management practices can dramatically influence the susceptibility of crops to pest species. It therefore seems possible to both time insecticide applications more accurately and also reduce the number of applications by managing crops so that they are less likely to become infested. This approach will obviously reduce the number of insecticide applications, and therefore also decrease the likelihood of pest populations developing resistance. In this context, it is also important to highlight the adverse effect of insecticides on populations of beneficial insects. The example given here is for adverse effects on dung feeding beetles which provide ecosystem services by dispersing and burying dung and reducing populations of dung breeding nuisance flies. Avermectins are a family of drugs used to control internal parasites of cattle, horses and sheep. Residues from these compounds that are excreted in the dung of cattle can kill both the dung breeding nuisance fly pest, *Musca vetustissima,* and adversely affect the breeding of introduced scarab dung beetles [95]. Several authors have expressed concern that widespread use of avermectins as cattle drenches could adversely affect the populations of recently introduced scarab dung beetles [96]. Research has shown that scarab dung beetles in southern Australia breed mainly for 2-3 months in spring, and if farmers avoid using avermectins to drench cattle in these critical months an impact on dung beetle breeding would be minimised [97]. As can be seen from these examples, it is necessary to have a good biological and ecological understanding of the pest and the crop plant in order to optimise the control of pests and reduce adverse effects of using insecticides.

10. Conclusions

When concerns are raised about efficacy of currently available pest management programs, it is important to remember that humans have battled arthropod pests for as long as we have had agricultural production. There are 4,500-year old records of insecticide-based management practices for control of insect pests in pre- and post-harvest agricultural products. Even biological control has been practiced for over 2,000 years [58, 59]. Yet, we have not been able to develop arthropod pest management Systems based on pesticide applications, which consistently (across many growing seasons and in most growing regions) maintain individual pest species below densities of economic concern. In stored grain, orchards, horticulture, row crops. As a consequence, we are today researching management programs for the same pests as we did 50-100 years ago, or even before that. Despite incredible technological advances and scientific innovations during the development of human civilizations, we are still unable to "outsmart" the insects and mites in our food production, processing, and storage systems. On the other hand, there are several important examples of how classical biological control has provided almost complete control of different pests (i.e. weevils to control water hyacinth infestations in rivers and lakes, parasitoids to control cassava mealybugs in Western Africa, and moths to control prickly pear in Australia). Transgenic Bt technology may be considered an encouraging exception, as it has provided remarkable control of several key coleopteran and lepidopteran pests with high levels of resistance to other insecticides. However, even here there is widely reported documentation of Bt resistance (http://www.pesticideresistance.com/irac.php), and/or examples of how secondary pests, unaffected by Bt toxins, have adapted and taken advantage of the absence of Bt-controlled competitors. Thus for growers, Bt may have solved one pest problem but at the same time created other problems. Even after more than 50 years of IPM, and with growing challenges with target pest populations developing resistance, it is somewhat striking that the number of documented IPM "successes" is fairly low. A simple scientific literature review search provided the following number of hits (based the literature search engine, Agricola, from 1972-2012): 1) "insecticide" = 17,629, 2) "insecticide resistance" = 4,900, 3), "IPM" = 2,243, and 4) "IPM" and "success" = 23. This recognition of our to-date inability to eliminate or completely control pests leads to an important question–what benchmarks should be used to determine whether a pest management program was successful or not? [59] highlighted this aspect as being one of the leading short-comings in current IPM programs.

It is indisputable, that insecticides are very important in our food production systems, and that they will continue to play a very important role far into the future. The purpose of this chapter is by no means to diminish their importance and the benefits associated with their usage in food production – in fact it is closer to the opposite: that exactly because of their importance and value, it is paramount that we understand how to use them effectively and that their performance is not being eroded due to resistance development. Another intended message from this chapter is that, when an insecticide "is not working" it is likely attributed to application failure rather than the insecticide not being effective against the given target pest. In short, we would be in serious trouble if we could not rely on insecticides, and that is precisely why they have to be used as wisely as possible. With the continuously growing list of insecticides becoming ineffective due to resistance, insecticides being faced out due to concerns about their adverse environmental effects, and with chemical companies having to spend increasing amounts of resources on getting new active ingredients registered for commercial use – it seems reasonable to reflect on the long-term sustainability of pest management practices based almost exclusively on insecticide applications.

Author details

Christian Nansen* and Thomas James Ridsdill-Smith

*Address all correspondence to: christian.nansen@uwa.edu.au

The University of Western Australia, School of Animal Biology and the UWA Institute of Agriculture, Perth, Western Australia, Australia

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Chapter 8

Insecticide Use and the Ecology of Invasive *Liriomyza* Leafminer Management

Stuart R. Reitz, Yulin Gao and Zhongren Lei

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53874

1. Introduction

Leafmining flies in the genus *Liriomyza* (Diptera: Agromyzidae) are among the most economically important pests of vegetable and floriculture crops worldwide. Of the more than 300 species in the genus, approximately 24 species are economically important (Spencer, 1973). Among these, three species are of particular importance as crop pests. *Liriomyza huidobrensis* (Blanchard), *Liriomyza sativae* Blanchard, and *Liriomyza trifolii* (Burgess) are highly invasive species that have become established in agricultural areas throughout the world. These three highly polyphagous species cause extensive damage to a wide range of high value vegetable and floriculture crops. Other locally important members of the genus include *L. langei* Frick, which is a pest of ornamental and vegetable crops in coastal areas of California, USA (Parrella, 1982; Reitz et al., 1999); *L. bryoniae* (Kaltenbach), which is primarily a pest of glasshouse tomatoes in Europe (Smith, 1999), and of glasshouse and field crops in east Asia (Abe & Kawahara, 2001); and *L. chinensis* (Kato) which is a pest of *Allium* crops throughout Asia (Andersen et al., 2008; Chen et al., 2003; Spencer, 1990). Because these and other regionally important pest species share many of the same biological attributes and pest management challenges of the major invasive pest species, they too may become invasive pests of concern in the future. The following discussion of lessons learned from the three predominant pest *Liriomyza* species will help to provide information to minimize the threat of problems arising from other species and to avoid past mistakes. Ultimately, successful management of any of these species depends upon on development of comprehensive integrated pest management (IPM) strategies that address management of all pests in a cropping system.

Crop plants are damaged by *Liriomyza* by two means. The first form of damage is caused as females use their ovipositor to puncture the leaf surface to lay eggs and to create feeding holes (Bethke & Parrella, 1985). The stippling patterns left from these punctures degrade the aesthetic value of ornamental plants, and sufficiently high levels of this puncture damage can reduce plant photosynthesis (Trumble et al., 1985). In addition, young seedling plants can be killed by intense puncture damage (Elmore & Ranney, 1954). Nevertheless, damage from female feeding and oviposition is generally minor in comparison with the more pronounced mining activity of larvae as they feed within leaves and stems of plants. Larval feeding not only reduces the marketability of plants because of the aesthetic damage, but it also reduces the photosynthetic capacity of plants, which reduces plant vigor, growth and yield (Al-Khateeb & Al-Jabr, 2006; Trumble et al., 1985). Sufficiently high densities of larvae can lead to defoliation. Leafminer caused defoliation can lead to significant losses in fruiting crops because the fruit becomes exposed to sunscald damage from the loss of the plant canopy (Schuster & Everett, 1983). On a per capita basis, *Liriomyza huidobrensis* has the most significant effect on host plants because it creates large mines in the spongy mesophyll of foliage and in petioles (Parrella et al., 1985). It is also known to mine pods of pea plants (*Pisum sativum* L.) (CABI, 2004). In contrast, *L. trifolii* and *L. sativae* tend to mine only through the upper palisade mesophyll of foliage.

In addition to the direct damage inflicted to crop plants, producers may suffer further economic losses because of quarantine restrictions that constrain international trade (Gitonga et al., 2010). Producers lose export markets when importing countries ban products because of the actual or potential presence of leafminer infestations in the country of origin. Even without complete bans, phytosanitary measures (e.g., fumigation or irradiation (Hallman et al., 2011)) required by importing countries may make exports cost prohibitive for producers in the country of origin.

2. Taxonomy, origins and invasiveness

One of the great challenges in understanding the pest status of *Liriomyza* spp. and effectively managing them has been the uncertainty regarding taxonomy and misidentification of pest species. Agromyzidae species occur throughout the world, and many species are morphologically similar, making distinctions among species difficult. Minkenberg (1988a) suggests that as a consequence invasive populations of *L. trifolii* became well established in some countries because specimens of early colonizers were misidentified as native species, and so no management programs were adopted. More thorough species determinations were not undertaken until widespread crop losses were reported by growers.

Among the three major pest species, there have been considerable historical problems with their taxonomy and identification. *Liriomyza sativae* was originally described from Argentina by Blanchard (1938) and is thought to be endemic to regions of South and North America (Spencer, 1973). It was recorded as a pest of numerous horticultural crops in southern Florida (USA) by the 1940s. Many early records of *Liriomyza* pests in Florida, USA refer to *Liriomyza pusilla* (Meigen) although the actual species of concern was almost certainly *L. sativae* (Spencer, 1973). *Liriomyza sativae* has probably been present in California, USA since the early 20th century, but it is uncertain if the species that Oatman and Michelbarger termed *Liriomyza pictella* (Thomson) in a series of seminal biological studies (Oatman, 1959; 1960; Oatman & Michelbacher, 1958; 1959) was *L. pictella*, *L. sativae* or another undescribed sibling species.

The endemic range of *L. trifolii* is thought to encompass eastern North America, the Caribbean Basin, and parts of South America, although this range must be interpreted cautiously, again because of historical taxonomic uncertainty (Scheffer & Lewis, 2006; Spencer, 1965; 1973). Spencer (1965) noted that *L. trifolii* was widespread throughout Florida but did not consider it to be as significant of a pest as *L. sativae* at that time. The *L. trifolii* discussed by Frick (1959) as occurring in the western USA (California, Oregon, Washington) was later determined to be a new species, *L. fricki* Spencer (1965).

Liriomyza huidobrensis was first described, as *Agromyza huidobrensis,* from specimens reared from *Cineraria* in Argentina (Blanchard, 1926). For many years, *L. huidobrensis* was considered to be endemic to North America and to South America, although it was not recorded from Central America (Parrella, 1982; Spencer, 1973). In North America, this species was considered to be present in the far western states of the United States (California, Hawaii, Oregon, and Washington) (Spencer, 1973), but recent molecular research has confirmed that this North America taxon is a distinct species, *Liriomyza langei* Frick (Scheffer, 2000; Scheffer & Lewis, 2001).

Adding to the taxonomic complexity regarding *Liriomyza* is the recent discovery that *L. sativae* and *L. trifolii* are each composed of biologically distinct cryptic species (Scheffer & Lewis, 2005; 2006). There is evidence that other pest *Liriomyza* species may also be composed of biologically distinct cryptic species (Lonsdale, 2011; Morgan et al., 2000; Reitz & Trumble, 2002b). Genetic and ecological differences among such cryptic species have important implications for understanding the pest status and management of these species (Rosen, 1978; Scheffer & Lewis, 2005).

In addition to our evolving understanding of the taxonomy of *Liriomyza,* the history of *Liriomyza* spp. as pests has changed substantially over time. Although leafminers have been recognized as pests for many years, they remained relatively minor pests in limited geographic areas through the early 20th century (Hills & Taylor, 1951). In Florida, problems with leafminer control began to appear in the 1940s, which coincides with the advent of the use of synthetic insecticides (Hayslip, 1961; Wene, 1953). The initial species to cause these problems was *L. sativae* (Spencer, 1973). From the 1940s through the 1970s, there were repeated failures of insecticides to control leafminers in Florida (Hayslip, 1961; Levins et al., 1975; Wolfenbarger, 1954) and in the Rio Grande Valley of Texas (Wene, 1953), leading to substantial crop damage periodically. By the late 1970s, *L. trifolii* had become the predominant leafminer pest in Florida, and it soon became the most important pest of tomato (*Solanum lycopersicum* L.) in the state (Waddill et al., 1986). This sudden explosion of leafminer problems led growers to intensify insecticide treatments in attempts to manage the problems. Waddill et al. (1986) note that soon after the outbreak of *L. trifolii* growers were making three or more insecticide applications per week against leafminers with little success in managing the problem.

Although *L. sativae* was the predominant leafminer pest in California during the middle of the 20th century, it was not considered to be a major pest (Parrella, 1982; Trumble, 1981). Sporadic outbreaks of the species now recognized as *L. langei* did occur through coastal areas of California during the 1930s - 1950s (Elmore & Ranney, 1954; Frick, 1951; 1957; 1958; Lange, 1949; Lange et al., 1957). These outbreaks tended to be relatively short lived events, with *L. langei* reverting to a minor pest in between outbreaks. However, beginning in the mid 1990s sustained pest problems with *L. langei* emerged in coastal California (Heinz & Chaney, 1995; Reitz et al., 1999).

Liriomyza huidobrensis was not widely discussed as an important pest in South America until the 1970s (Chavez & Raman, 1987). The change in its pest status at that time has been attributed to insecticide induced outbreaks that resulted from intense insecticide treatments made against the primary pest of potato (*Solanum tuberosum* L.) in Peru, the leafmining moth *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae). According to this hypothesis, constant exposure to insecticides for *T. absoluta* led to the evolution of resistance in *L. huidobrensis* populations, but the parasitoids that had contained *L. huidobrensis* populations were eliminated, creating classic secondary pest outbreaks (Luck et al., 1977). As a conclusion, the emergence of *Liriomyza* spp. as consistently important pests can be attributed to the selection for insecticide resistant populations.

In the mid 1970s, as more frequent and severe outbreaks of *Liriomyza* spp. began to be observed (e.g., Chavez & Raman, 1987; Leibee & Capinera, 1995; Oatman & Kennedy, 1976), leafminers began to emerge as globally important invasive pests of a wide range of horticultural crops. At this time, international trade in horticultural products (e.g., fruits, vegetables and cut flowers) began to escalate tremendously (Huang, 2004), which provided the opportunity for *Liriomyza* spp. to spread through the world on infested plant material (Minkenberg, 1988a).

Invasions of these *Liriomyza* spp. has continued unabated from the 1970s through to the present (Abe & Kawahara, 2001; Lei et al., 1997; Scheffer et al., 2006; Weintraub & Horowitz, 1995). All three of the major pest species now occur on all continents, except Antarctica. Even though the three major pest species share the common characteristic of being transported to new geographic areas via exported plant material, they have their own unique invasion histories. In many regions, more than one of the species has been introduced. These sympatric introductions have led to many complex interactions among the species, whereby one species is able to displace another previously established invasive species.

The introduction of *L. trifolii* into California, beginning in the late 1970s from plant material shipped from Florida brought the issue of invasive leafminers to the fore (Parrella, 1987). Soon after its introduction into California, *L. trifolii* displaced the previously established *L. sativae* as the predominant species in the state (Trumble & Nakakihara, 1983). To a large degree, this displacement appears to have resulted from the lower susceptibility of *L. trifolii* to commonly used insecticides (Palumbo et al., 1994; Reitz & Trumble, 2002a). The establishment of *L. trifolii* in California facilitated its spread to other countries, as infested propagation plants are shipped to production facilities in other countries. Then, final products are redistributed from these countries to yet other countries

(Minkenberg, 1988a). Today, *L. trifolii* is widespread throughout Europe, Africa and Asia. In the Americas, invasive populations have probably been introduced into regions where the species is indigenous (Minkenberg, 1988a).

Whereas *L. trifolii* has invaded many European countries, *L. sativae* has a more restricted distribution in Europe. It has been more widespread than *L. trifolii* in Asia and in Oceania, even though its presence was not recorded before the early 1990s. However, this distribution may be changing as *L. trifolii* invades more areas of Asia. For example, in the Chinese province of Hainan, *Liriomyza* spp. have been the predominant pest of cowpea, *Vigna unguiculata* L. Walpers, since 1993 when *L. sativae* first invaded the island and spread to other provinces. Subsequently, *L. trifolii* invaded Hainan in 2006, which has lead to the displacement of *L. sativae* (Gao et al., 2011). In contrast to the displacements of *L. sativae* by *L. trifolii*, *L. sativae* appears to have recently displaced *L. trifolii* as the predominant species in Japanese vegetable crops (Abe & Tokumaru, 2008).

Liriomyza huidobrensis has spread rapidly through the world since the late 1980s when it was first recorded throughout Europe (reviewed in Weintraub & Horowitz, 1995). By the mid 1990s, it was well established throughout Asia, Africa and Central America (He et al., 2002; Scheffer et al., 2001; Shepard et al., 1998). It is not known at this time if *L. huidobrensis* has displaced either *L. sativae* or *L. trifolii* in any geographic region. Where these species co-occur, changes in demographics have been linked to climatic conditions, with *L. huidobrensis* predominating in cooler seasons or at higher elevations with cooler climates (Mujica & Kroschel, 2011; Tantowijoyo & Hoffmann, 2010; Weintraub, 2001a). Although *L. langei* has become a significant pest in California, it has not become an invasive species to date (Scheffer et al., 2001).

3. Biological influences on pest status

The pest status of *Liriomyza* spp. is closely tied to their biology. In part, their pest status results from the ability of populations of these flies to build up rapidly. Although there is considerably variation in the fecundity of *Liriomyza* spp. across studies, it is clear that females have a high reproductive capacity. For example, the mean fecundity for *L. sativae* females observed by Tokumaru and Abe (2003) was over 600 eggs per female. Although this may be an unusual observation, other studies routinely report fecundity in excess of 100 eggs per female. These species also have very rapid developmental rates, with a generation able to be completed in fewer than 20 days at optimal temperatures (Lanzoni et al., 2002; Minkenberg, 1988b; Parkman et al., 1989). Consequently, multiple, overlapping generations can be produced within a single cropping season. *Liriomyza sativae, L. trifolii* and *L. huidobrensis* are among the few members of the genus that are highly polyphagous. The host range of each species encompasses hundreds of species in a wide range of plant families (Spencer, 1990). This polyphagy allows populations of these species to develop on multiple crops, as well as uncultivated hosts, and then disperse into newly planted crops (Jones & Parrella, 1986; Trumble & Nakakihara, 1983; Tryon et al., 1980). Their polyphagy also presents many opportunities for movement on plant material to new regions. As the eggs and larvae of *Liriomyza* spp. are concealed internally within plant foliage, they can be easily moved within shipments from production areas to final markets, and detection is difficult (Parrella, 1987).

4. Response to insecticides

One of the most important factors in leading to *Liriomyza* spp. becoming pests is their ability to evolve resistance to insecticides (Parrella & Keil, 1984). Leibee (1981) compiled a list of insecticides used against *Liriomyza* spp. in Florida and the life spans of their field efficacy in commercial use. The list of ineffective materials includes almost all classes of insecticides developed up to that time. Some insecticides became ineffective in as little as two years. This review confirmed the widespread importance of insecticide resistance in driving the pest status of *Liriomyza* spp. Despite the rapid failures of different insecticides, there has been a general belief, at least through the middle of the 20th century, that new chemistries would become available to replace ineffective ones, and provide a few additional seasons of control. Consequently, there was little emphasis on alternative management techniques until the advent of the worldwide leafminer crisis in the 1970s (Leibee & Capinera, 1995).

Intense insecticide use is the most common strategy used to eradicate newly discovered outbreaks of *Liriomyza* spp. (Bartlett & Powell, 1981). The success of this strategy is dependent on the susceptibility of invasive populations to available insecticides. Because invasive populations are already likely to be resistant to various insecticides (MacDonald, 1991; Parrella & Keil, 1985), eradication programs may not be successful.

Cross resistance to multiple classes of insecticides is also likely in *Liriomyza* spp. Despite a short history of pyrethroid use in Hawaii, high levels of tolerance to fenvalerate and permethrin were detected in field populations of both *L. sativae* and *L. trifolii* (Mason et al., 1987). The authors speculated that the tolerance/resistance arose as a result of cross-resistance to longer used organochlorine insecticides, which have a similar mode of action to pyrethroids. Populations of invasive *L. trifolii* obtained from greenhouses in Canada treated intensively with the organophosphate pyrazophos for less than 1 year showed high levels of resistance to that insecticide and to other types of organophosphates that had not been used previously (Broadbent & Pree, 1989). Fortunately from a pest management perspective, reversion to susceptibility to organophosphates and pyrethroids has been shown to occur within a few generations (within 1 year) (Broadbent & Pree, 1989; Parrella & Trumble, 1989). Interestingly, these Canadian populations showed no susceptibility to carbamates. It is possible that these populations were already resistant to carbamates and that laboratory-reared flies maintained their resistance for 5 years, or that carbamates are not toxic to *L. trifolii.*

At present, two of the most effective insecticides for *Liriomyza* management are abamectin and cyromazine. Both insecticides target the larvae inside the plant foliage. Cyromazine acts as a growth regulator; whereas abamectin is a neurotoxin that acts as a GABA agonist. Both have translaminar properties, allowing them to reach the larvae within the plant. Research by Schuster and Everett (1983) documented the effectiveness of both insecticides under field conditions. Since that time, both have been commercially available. Despite this long history of use, resistance has not been a major problem in their use (Ferguson, 2004). The one recorded case of resistance to cyromazine cited in that study showed that reversion to susceptibility occurred within 8 generations in a laboratory strain and that field efficacy was restored within 2 seasons of reduced exposure.

Another class of insecticide with efficacy against *Liriomyza* spp. is the spinosyn class (spinosad and spinetoram). Spinosyn insecticides have been widely used since their introduction in the US in 1997. Similar to abamectin and cyromazine, spinosyns have translaminar properties, enabling them to target leafminer larvae. Spinosyns are neurotoxins also. However, they have a different mode of action than abamectin, one that disrupts nicotinic acetylcholine receptors (Salgado, 1998). Spinosyns are classified as Group 5 insecticides and abamectin is classified as a Group 6 insecticide by the Insecticide Resistance Action Committee (IRAC International MoA Working Group, 2011). There have been few reports of resistance to spinosyns to date among *Liriomyza* spp. (Ferguson, 2004). The lack of reported cases of spinosyn resistance may be considered surprising, given that spinosyn products are widely used against other key pests that co-occur with leafminers, including thrips and Lepidoptera pests (Demirozera et al., 2012; Reitz & Funderburk, 2012; Reitz et al., 1999). Incorporating the use of a penetrating surfactant improves the efficacy of spinosad against *Liriomyza* larvae (Bueno et al., 2007), allowing growers to improve management with lower rates of insecticide. This approach may also help reduce selection pressures. It is reasonable that increasing penetration of abamectin or cyromazine into plants would, likewise, increase their efficacy.

Selection of appropriate insecticides and rates for use in the field also depends upon proper identification of leafminer species. Parrella and Keil (1985) found that *L. trifolii* was much less susceptible to methamidophos than was *L. sativae* or *L. langei.* Likewise, *L. trifolii* populations in China are significantly less susceptible to abamectin and cyromazine than are populations of *L. sativae* (Gao et al., 2012) In contrast in Japan, *L. sativae* populations were less susceptible to several commonly used insecticides than were local populations of *L. trifolii* (Tokumaru et al., 2005). There is evidence that invasive populations of *L. huidobrensis* are more tolerant to certain commonly used insecticides than are sympatric populations of *L. trifolii* (Weintraub, 2001a).

5. Management trends

The premise that leafminers are secondary pests, which are released from natural control when their enemies are eliminated (Luckmann & Metcalf, 1994), has a long history, even if it has not always been fully appreciated. Studies dating back to the 1940s have shown the importance of parasitoids in maintaining *Liriomyza* spp. populations below economically damaging levels (Hills & Taylor, 1951) and where parasitoid populations are reduced in agroecosystems, there are outbreaks of *Liriomyza* spp. populations (Oatman & Kennedy, 1976; Ohno et al., 1999). Consequently, there has long been interest in identifying insecticides with low toxicity to *Liriomyza* parasitoids (e.g., Wene, 1953).

In every geographic region where *L. huidobrensis, L. sativae* or *L. trifolii* are indigenous, there is a rich complex of hymenopteran parasitoids (Liu et al., 2009). Parasitoid complexes associated with *Liriomyza* spp. generally consist of several species of larval and larval-pupal hymenopteran parasitoids. Many, but not all, of the species are oligophagous so that they may attack the different pest species and native non-pest *Liriomyza* spp. (Nicoli, 1997). It should be noted that there is evidence of differential parasitism across *Liriomyza* spp. Although, many parasitoids of *Liriomyza* are fairly generalized and are able to successfully attack various species, their reproductive success varies with the host (Abe et al., 2005) Still other parasitoids are not able to parasitize all *Liriomyza* species. This differential parasitism ability can have extreme implications for leafminer ecology. Greater levels of parasitism of *L. trifolii* than of *L. sativae* has been cited as one of the key factors in the displacement of *L. trifolii* by *L. sativae* in Japan (Abe et al., 2005; Abe & Tokumaru, 2008).

Parasitoids associated with native non-pest *Liriomyza* spp. have the potential to provide biological control of invasive leafminers because the native hosts serve as reservoirs for parasitoids populations (Chen et al., 2003; Nicoli, 1997; Tran et al., 2006) Often, parasitoids of *Liriomyza* pest species are introduced along with their alien hosts (Bjorksten et al., 2005; Tagami et al., 2006). These relationships may then be exploited as a form of unintended classical biological control.

Whereas parasitoids are valuable control agents, making effective use of them in practice can be challenging. Parasitoid populations, by their nature, will lag behind the development of their host populations (Hofsvang et al., 2005; Trumble & Nakakihara, 1983; Weintraub, 2001a). In these types of situations, growers may need to apply insecticides to keep growing leafminer populations below economically damaging levels. In a similar vein, growers may need to use insecticides to treat other pest problems, which then may have detrimental effects on leafminer management (Getzin, 1960). The outcome of either situation is that leafminer populations are released from their natural control and rapidly increase because many of the insecticides used against leafminers or other pests are highly toxic to their parasitoids. Should such a rapid increase occur growers are likely to believe that further insecticide treatments are warranted. This then becomes the very definition of the pesticide treadmill.

Most broad spectrum synthetic insecticides developed since the 1940s are highly toxic to parasitoids of *Liriomyza* spp. (Hidrayani et al., 2005; Oatman & Kennedy, 1976; Saito et al., 1996; Schuster, 1994). Classes of insecticides that have shown high toxicity to parasitoids include carbamates, organochlorines, organophosphates and pyrethroids, which are also insecticides that show limited efficacy against *Liriomyza* spp. (Hara, 1986; Hidrayani et al., 2005). Therefore, these types of insecticides should be used with great caution in systems where *Liriomyza* spp. are key pests. Several studies have shown that parasitoids are able to evolve resistance to insecticides under routine selection pressures in the field (Rathman et al., 1990; Spollen et al., 1995). Should parasitoids be resistant to a particular insecticide, that insecticide could be integrated into (IPM) programs. This would be especially true if the insecticide were targeting another pest species. However, to be effective, levels of resistance in the parasitoid population must exceed the field rate of the insecticide.

Of the three most effective insecticides for use against *Liriomyza* spp. today (abamectin, cyromazine, spinosyns), there have been variable conclusions regarding their effects on *Liriomyza* spp. parasitoids. Cyromazine is the least detrimental of these insecticides to *Liriomyza* parasitoids. As a growth regulator specific to Diptera, it does not directly affect the development of parasitic Hymenoptera. It does reduce the number of available hosts, and it will kill *Liriomyza* larvae before parasitoids may complete their development. However, these effects should complement the action of parasitoids to enhance overall management of *Liriomyza* pests.

Results of various studies provide conflicting results for the effect of abamectin on leafminer parasitoids (reviewed in Kaspi & Parrella, 2005). In general, field studies have demonstrated that abamectin and spinosyns are not as detrimental to parasitoid populations as carbamates, organophosphate or pyrethroids, but they are more deleterious than cyromazine (Prijono et al., 2004; Schuster, 1994; Trumble, 1985). The greater toxicity of abamectin and spinosyns compared with cyromazine to *Liriomyza* spp. parasitoids have been demonstrated in laboratory studies (Babul Hossain & Poehling, 2006; Bjorksten & Robinson, 2005). In particular, abamectin and spinosyns are lethal to parasitoid adults. Interestingly, parasitoid populations may rebound faster in abamectin treated fields compared with cyromazine treated fields, a result attributed to the longer residual period of cyromazine (Weintraub, 2001b). These results clearly show that insecticide use should be approached cautiously and that growers should be encouraged to consider the costs and benefits of different insecticide uses.

In recognition of the importance that parasitoids play in managing leafminers, Trumble and colleagues initiated development of IPM programs for field grown vegetables in California and Mexico. One of the first aspects addressed in the research program was to establish realistic economic thresholds in these agroecosystems for the key insect pests, *L. trifolii* and the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Reitz et al., 1999; Trumble, 1985; Trumble & Alvarado-Rodriguez, 1993; Trumble et al., 1997). According to program guidelines, when systematic sampling shows pest populations exceeding economic thresholds, growers would select an insecticide to bring the populations under damaging levels. The key to maintaining stable management of *Liriomyza* spp. rests in selecting insecticides that are the least disruptive to the leafminer parasitoid complex.

In commercial scale trials conducted in celery (*Apium graveolens* L.) and tomato, these IPM programs were compared with conventional high input management programs. Insecticides for the IPM programs consisted of *Bacillus thuringiensis,* tebufenozide (an insect growth regulator) and spinosad for management of Lepidoptera, and abamectin and cyromazine for *Liriomyza* management (Reitz et al., 1999; Trumble & Alvarado-Rodriguez, 1993; Trumble et al., 1997). To minimize the risk of insecticide resistance, growers are encouraged to rotate abamectin and cyromazine, should multiple applications be needed in a crop. The conventional, high input management programs reflected standard grower practices of the time and included weekly applications of broad spectrum synthetic insecticides, including methomyl (carbamate), permethrin (pyrethroid) and methamidophos (organophosphate).

These trials consistently showed that the IPM programs had consistently lower populations of *Liriomyza* spp. than the high input conventional programs. These results were seen because the IPM programs were able to conserve the leafminer parasitoids. More importantly for growers, because insecticide applications in the IPM programs were based on scouting results and linked to economic thresholds, fewer insecticide applications were made in the IPM programs than in the conventional programs. By focusing on conservation of leafminer parasitoids, growers can reserve use of the few highly efficacious insecticides for situations where there is a danger of leafminer population outbreaks. Limiting their use to these situations mitigates the risk of resistance developing to these insecticides.

Despite the lower insecticide use, growers were not sacrificing the amount of crop harvested or its quality. Ultimately, these IPM programs based on economic thresholds with the goal of conserving *Liriomyza* spp. parasitoids enable growers to produce high quality crops at lower cost and with typically greater profit than programs with higher insecticide inputs. By including economic comparisons of management programs, these trials provide growers with an economic rationale to alter their management methods (Reitz et al., 1999).

Liriomyza spp. management in protected environments, such as enclosed glasshouse and greenhouse production systems, generally requires greater inputs than for field grown crops. Greenhouses are highly managed environments where growers have extensive control over crop conditions (Shipp et al., 1991). Yet, given the potential value of crops and the high production costs, many growers produce crops year round without periods to sanitize facilities. This continuous production is conducted at optimal temperatures for plant and, consequently, insect development. Therefore, the greenhouse environment is highly conducive to the development of pest populations, but colonization by naturally occurring beneficial organisms is restricted. With the lack of naturally occurring biological control available to most greenhouse systems and the high crop value, growers historically relied on intensive insecticide use for pest management, and this reliance on insecticides has hindered the development of IPM programs for greenhouse production (Parrella & Jones, 1987). Further complicating adoption of IPM programs in greenhouses are the exceedingly low damage threshold for floriculture and vegetable crops that are grown in protected environments (Yano, 2004).

Despite these constraints, there have been successful demonstrations of integrated management of *Liriomyza* spp. and other pests in greenhouse systems. The initial impetus for development of IPM programs has, not surprisingly, been the development of resistance and failure of insecticides to effectively manage pests. IPM programs for greenhouse systems have been widely adopted in northern and western Europe (van Lenteren, 2000). There, natural enemies are commercially available for all major pests, including parasitoids in the genera of *Dacnusa, Diglyphus* and *Opius* for *Liriomyza* management. These parasitoids can be released augmentatively and become established in greenhouses for long term management of leafminers. Because of the high demand for natural enemies to meet the needs of the large European greenhouse industry, mass produced natural enemies are cost effective for European growers to use. However, while augmentative biological control with parasitoids in the United States and other non-European countries has been shown to be effective in managing *Liriomyza* populations, to date, it has not been as economically cost effective as the judicious use of insecticides (Chow & Heinz, 2006; Ozawa et al., 2001). These economic differences make growers less likely to adopt insecticide alternatives

An ideal insecticide for incorporation into a greenhouse IPM program is one that is pest specific and not harmful to biological control agents of that pest, or those of other pests in the system (Kaspi & Parrella, 2005). Although not harmless to parasitoids, the use of abamectin can be successfully integrated with augmentative releases of the parasitoid *Diglyphus isaea* (Walker) for management of *L. trifolii* (Kaspi & Parrella, 2005). They found that the residual period for abamectin was approximately 1 week. By releasing parasitoids after that time, the abamectin would no longer be toxic for the parasitoids. In this manner, an early season application of abamectin could sharply lower *L. trifolii* populations quickly, and released parasitoids could then provide longer term management of *L. trifolii. Diglyphus isaea* larvae paralyze their hosts, and consequently some *D. isaea* larvae would be protected from abamectin sprays because their hosts would no longer be feeding to ingest the toxin. As with the IPM programs for field grown vegetables discussed above, this integrated management program for greenhouse leafminers presents several advantages for growers. Because released parasitoids are self-perpetuating, a single release may substitute for several insecticide applications. Again, this integrated approach reduces the probability of resistance developing. Also, this approach could reduce inputs for growers without sacrificing crop yield and quality. This integrated management program for *L. trifolii* could be expanded into a more comprehensive program by determining how various insecticides and natural enemies for other pests interact with one another.

6. Conclusions

Growers around the world have experienced significant problems from *Liriomyza* leafminers. They continue to invest considerable resources in the management of these pest flies. Despite the long history of problems with leafminers, many of the lessons that have been learned in one area at one time have, unfortunately, had to be relearned elsewhere. Leafminers are classic secondary pests. If the parasitoid complex that attacks leafminers is conserved, economic damage from leafminers can be mitigated. Still, there are clearly circumstances where insecticides are needed to suppress leafminer populations below economically damaging levels. In particular, there may be cases where the lag in the increase in parasitoid populations may allow leafminer populations to exceed economic threshold levels. In such situations, growers should select insecticides that will minimally disrupt the parasitoid complex. First and foremost, though, it is imperative that researchers provide growers with realistic economic action thresholds for different cropping systems so that growers have a clear understanding of when their crop may be at risk. Indeed, insecticide treatments may not always be warranted for seemingly high populations of leafminers. Marketable yield for a crop like tomato may not be lowered until exceedingly high levels of leafmines are reached (Levins et al., 1975).

In a similar vein, when other pests reach economic threshold levels and require therapeutic insecticide treatments, growers are encouraged to consider the effect of those insecticide treatments on leafminer management. Proactive management decisions will reduce the likelihood of inducing severe outbreaks of leafminers. It is possible to produce a crop with few, if any insecticide treatments for leafminers, but this will best be realized if all growers in a community adopt similar IPM programs so that any one grower does not adversely affect neighboring growers. Continuing forward, the basic strategies for leafminer management are clear. However, the practical implementation of such strategies will remain a challenge. There is an ongoing need for development of selective, reduced risk insecticides to incorporate into leafminer management programs and to ensure that appropriate resistance management programs are developed. Further, there is a clear need for improved diagnostic methods and characterization of biological variation among biotypes and cryptic species of pest *Liriomyza.* Because other species share traits with the major pest species, it may be possible that new species of *Liriomyza* may emerge as global threats, as have *L. huidobrensis, L. sativae* and *L. trifolii.*

Because invasions are most likely to continue into the future, it will be critical to accurately identify new invasive species and populations, and to monitor changes in leafminer population dynamics following invasions. As these leafminers will continue to be important pests of high value crops, insecticides will continue to be an important component of leafminer management. Therefore, it is imperative to continue to refine the use of insecticides that target leafminers. Improving application timing and methods will help to conserve insecticide susceptibility and maintain efficacy by mitigating the evolution of resistance. Insecticide resistance management must remain as a critical component of IPM. Furthermore, improving strategies for the conservation and augmentation of leafminer parasitoids will help reduce the need for insecticide applications. Knowledge gaps in regard to the effects of insecticides on various leafminer parasitoids should continue to be addressed. Leafminer management will best be accomplished through research on, and implementation of, comprehensive IPM strategies.

Author details

Stuart R. Reitz[1], Yulin Gao[2] and Zhongren Lei[2]

*Address all correspondence to: stuart.reitz@oregonstate.edu

1 Malheur County Extension, Department of Crop and Soil Sciences, Oregon State University, USA

2 State Key Laboratory for Biology of Plant Disease and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, PR China

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Section 3

Non-Chemical Alternatives to Insecticides

Chapter 9

Plant–Derived Products for Leaf–Cutting Ants Control

Juliana Cristina dos Santos, Ronald Zanetti,
Denilson Ferreira de Oliveira,
Giovanna Cardoso Gajo and Dejane Santos Alves

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/55035

1. Introduction

1.1. Leaf–cutting

Leaf-cutting ants of the genera *Atta* sp. Fabricius (Hymenoptera: Formicidae) and *Acromyrmex* sp. Mayr (Hymenoptera: Formicidae) are among the best known species of the family Formicidae in the New World, mainly due to their behaviour of cutting live plants to grow the symbiotic fungus *Leucoagaricus gongylophorus* (Möller) Singer (Agaricales: Agaricaceae) [1] (Figure 1). This interaction, which emerged more than 50 million years ago [2] has evolved to such a complex level that the ants and fungi cannot survive separately; they live in symbiosis. The fungus supplies the ants with nutrients obtained from metabolising plant materials that can be easily assimilated. In exchange, its environment is highly protected by the ants, which remove contaminants and secrete antibiotics from their metapleural glands [3, 4].

The symbiotic fungus, which exhibits high carbohydrate and protein content but low content, constitutes the main food source for leaf-cutting ant colonies [5] and is the single nutrient source for the queen, larvae, and temporary alate castes. Only 9% of the energy requirements of adult workers, which ingest plant sap when handling plant fragments, are obtained from the fungus [6]. Moreover, the symbiotic fungus produces large amounts of enzymes, which are ingested by the ants and are returned to the fungal garden through faecal liquid to facilitate the digestion of plant tissue [7, 8].

Leaf-cutting ants are considered the main agricultural and forest pest in countries such as Brazil, as they attack plants at any stage of their development, cutting their leaves, flowers, buds, and branches, which are then transported to the interior of their underground nest [9]. A colony of *Atta laevigata* (F. Smith) (Hymenoptera: Formicidae) can cut approximately 5 kg

Figure 1. *Atta sexdens* and its fungal garden: mycelial care. Source: Arnhold, 2012.

of plant material/day [10]. Thus, these ants cause direct losses, such as the death of seedlings and reduction of tree growth. Indirect losses also occur as a result of the decreased resistance of trees to other insects and pathogenic agents [11].

Leaf-cutting ant control has been performed almost exclusively through the application of conventional insecticides, including cyfluthrin (pyrethroid), imidacloprid (neonicotinoid), furathiocarb (carbamate), sulfluramid (fluoroaliphatic sulfonamide), and fipronil (phenyl pyrazole) [12]. Due to the problems these products may cause to the environment and humans, their use has been restricted by governments and forest product certification bodies, which have demanded and encouraged the development of alternative control strategies to these insecticides, such as the use of plant-derived products, entomopathogenic fungi, and pheromones [13].

Plant-derived products can be used to control ant populations through several mechanisms. Some of these substances can act directly against the ant, leading to its death, such as citrus seed oils obtained from *Citrus sinensis* (L.) Osbeck, *Citrus limon* (L.) Burm. f. or *Citrus reticulata* Blanco (Rutaceae) [14] and extracts from the castorbean (*Ricinus communis* L.) (Euphorbiaceae) [15], timbo ((*Ateleia glazioviana* Baill.) (Leguminosae) [16] and eucalyptus ((*Eucalyptus* *urophylla* S.T. Blake) (Myrtaceae) [17]. Certain plant-derived substances can promote aggressive behaviour of ants towards their sisters, as reported for β-eudesmol extracted from eucalyptus leaves. [18, 19, 20] This sesquiterpene is able to modify the chemical composition of the worker's cuticle, impairing nest recognition, which triggers warning and aggressive behaviours among ants [20]. Plant extracts can also be toxic to the symbiotic fungus (*L. gongylophorus*), which represents an interesting target for new products for ant control. Such effects can be observed for extracts of *R. communis*, *Helietta puberula* R.E.Fr. (Rutaceae), *Simarouba versicolor* St. Hill (Simaroubaceae), and *Canavalia ensiformis* (L.) DC. (Fabaceae) [15, 21-22 ,23].

2. Leaf–cutting ants of the genera *Atta* and *Acromyrmex* and their symbiotic fungus, *Leucoagaricus gongylophorus*

Leaf-cutting ants comprise over 12,000 species and are considered social insects because they participate in parental care, reproductive castes, overlapping generations, and a division of labour [24]. These insects live in permanent colonies and are holometabolous. Taxonomically, they belong to the order Hymenoptera, family Formicidae, subfamily Myrmecinae, and tribe Attini [25]. Leaf-cutting ants belong to the genera *Atta* Fabricius and *Acromyrmex* Mayr as well as the basal genera *Apterostigma* Mayr, *Kalathomyrmex* Klingeberg and Brandão, *Mycetagroicus* Brandão and Mayhé-Nunes, *Mycetarotes* Emery, *Mycetaphylax* Fr. Smith, *Paramycetophylax* Kusnezov, *Sericomyrmex* Mayr, *Trachymyrmex* Forel, *Pseudoatta* Gallardo, and *Attaichnus* Lazã. [21] The Attini tribe is found only in the Neotropical region and is distributed between southern South America and the southern United States [26].

The leaf-cutting ants of the genera *Atta* and *Acromyrmex* (known as *saúvas* and *quenquéns* in Brazil, respectively) build nests composed of hundreds of underground chambers that are connected to each other and to the surface through galleries. The exterior of the nest usually exhibits a loose soil mound originating from the chambers formed by the nest [27]. Holes can be found in the loose soil mound or outside of it. According to Mariconi (1970), a large anthill of approximately 200 m^2 may contain an estimated population of six million ants [28].

Leaf-cutting ants cultivate the symbiotic fungus *L. gongylophorus,* which is used as a source of food and of auxiliary enzymes that function in the ants digestive process [29]. After the origination of the ant-fungus symbiosis, a subsequent evolutionary step involved the acquisition of staphylae-rich monophyletic cultivars by the highly derived Attini (Figure 2), particularly the *Atta* and *Acromyrmex* species, which collect only fresh vegetation as the fungal substrate. The conversion of vegetation into substrate requires several different operations and specialisations. The plants are cut by workers who have a head width of 1.6 mm or greater (other tasks, such as defence, involve larger workers), while care of the fungus requires very small workers, and intermediary steps in the garden are performed by medium-sized workers. Among ant species, this great evolutionary change seems to have placed the derived Attini on the path of producing larger and larger colonies due to the increased differences between the sizes of reproductive females and workers. Relatively larger reproductive ants show an increased ovariole count, enabling rapid colony growth [30], which, due to the increased size variation among nestmate workers, are able to execute all activities involved in fungal cultivation.

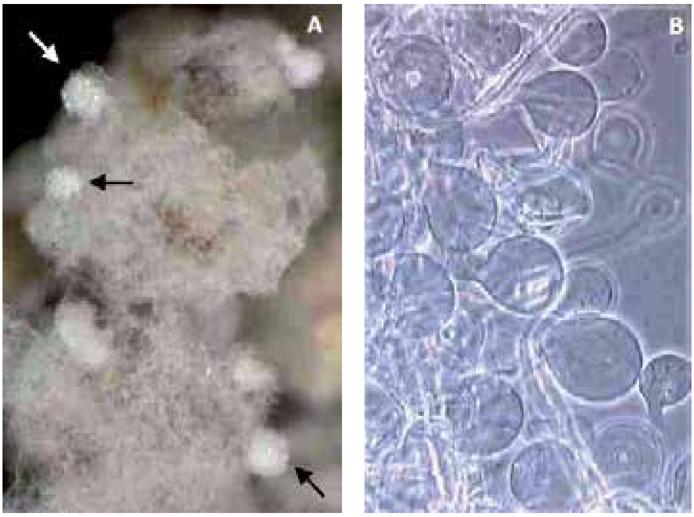

Figure 2. Microscopic images of *Leucoagaricus gongylophorus*, the symbiotic fungus of *Atta sexdens*. (A) Staphylae (arrows) contrasting with the hyphae (50x magnification). (B) Staphylae viewed under an optical microscope: each sphere corresponds to a gongylidia (1,000x magnification). Source: Schneider and Odair, 2003 [78].

Leaf-cutting ants from the genera *Atta* and *Acromyrmex* are the only ants within the group Attini that exhibit polyandry [31]. The development of this behaviour in the Attini coincided with the practice of cutting the leaves and live parts of plants [30]. The use of leaves, which are a widely available resource, would have allowed the leaf-cutting ants to achieve large colonies, leading to the appearance of long-lived queens and more complex forms of social organisation. Polyandry could favour the development of disease resistance as it increases the genetic diversity of the colony [32].

The consecutive matings between *Atta* and *Acromyrmex* species occur at the regional level throughout Brazil. In the southeast region, the nuptial flight of *Atta* species occurs between October and December; during this period, adult nests (approximately 38 months old) produce alate and fertile ants, and during the nuptial flight, referred to as swarming, the males fertilise the females [33]When leaving the nest for the nuptial flight, the virgin queen carries a small fragment of mycelium in her infrabuccal pocket to start a new fungus garden in a new nest [28]After mating, the reproductive females walk on the soil surface, remove their wings and search for a site to begin the excavation of the new nest. [34] In general, 48 hours after the excavation of the initial chamber [28], the future queen regurgitates the fungal fragment from her infrabuccal pocket. When starting the new colony, this small portion of the fungus is sterilised and fertilised with faecal droplets. Five to six days after the beginning of nest excavations, the queen begins oviposition [35]. The queen in a new colony lays both reproductive and trophic eggs, which are used as a food source for both the queens and their first offspring [36]. Early larvae originating from the reproductive eggs emerge 24 to 25 days after soil excavation by the queen. Ant larvae have been described as the digestive caste of the colony [25] and can actively contribute to the integration of the colony not only as a source of new adults but also by providing nutrients and enzymes for the workers, thus ensuring that the larvae will be cared for, which is essential for their survival. At 62 to 66 days after the nuptial flight, the first adults emerge, which feed on sap or obtain liquid food through trophallaxis or regurgitation [35]. On average, 87 days pass between the initial excavation and the appearance of the first opening created by workers. The second opening is only built 14 months after the first one. Additional openings appear quickly; on average, after 82 days, eight openings are present. The tenth opening is created approximately 20 months after the colony is fertilised. When the nest reaches three years of age (±38 months), it reaches adulthood and produces the first nuptial flight [37].

The use of plants as a substrate for the growth of the symbiotic fungus has led these ants to become the main pest on Brazilian forest plantations, despite playing a very important ecological role [38,39,40]. Leaf-cutting ants remove and modify the soil during the nest construction process, promoting changes in the chemical-physical proprieties of the soil and nutrient cycling that are favourable to plant growth [34]. However, their position as a key pest is generally cited more often due to the large quantities of vegetation these ants collect for growing the symbiotic fungus.

Leaf-cutting ants show a preference for certain plant species, which are consistently defoliated, while other species are not attacked, despite being abundant and located close to their nests. Some ant species attack only dicotyledonous plants, others target monocotyledons, and some ants collect both plant types. The preference for particular plant species may be related to the nutritional demands of the symbiotic fungus *L. gongylophorus* grown by these ants [41]. Ants can attack plants at any stage of their development, cutting their leaves, flowers, buds, and thin branches, which are then transported to the interior of their underground nests [42,43].

The complete defoliation of one-month-old *Pinus taeda* L. causes 25% mortality of these plants. For the plants that survive, defoliation affects their diameter more than their height [44]. Previous studies have demonstrated that there is a significant reduction in the development of *P. taeda* during the first 24 months of life when attacked by leaf-cutting ants [45]. Total defoliation reduces the growth of *Eucalyptus grandis* W. Hill ex Maiden in both diameter and height as well as the profit of the producer at the end of the rotation, even if the defoliation occurs only once during the beginning of the planting. The decreases in production and profits are enhanced according to the frequency of defoliation, which can make the maintenance of totally defoliated areas economically unviable. The damage caused by ants is more harmful to the plants in their first three years. A single total defoliation event delays the growth of the plant [46] while two or three consecutive defoliation events may lead to plant death [47,48].

Due to the damage they cause, control of these pests is essential to avoid large losses in agricultural and forest crops [33]

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3. Plants toxic to leaf–cutting ants and their symbiotic fungi

Plants exhibit several mechanisms to prevent herbivory, including producing alcohols, aldehydes, esters, phenols, and hydrocarbons, among other substances, which can be classified as secondary metabolites and can be toxic to leaf-cutting ants and/or their symbiotic fungi. As a result, many studies have been conducted in this field using different plant families, as listed in Table 1; some of these studies are described in more detail below.

Plant species	**Family**	**Identified substance**	**Reference**
Hymenaea courbaril	Fabaceae	Caryophyllene epoxide	Hubbel et al., 1983[80]
Sesamum indicum	Pedaliaceae	Sesamin and/or sesamolin	Pagnocca et al., 1990[49]; Pagnocca et al; 1996[50]; Ribeiro et al., 1998[51]; Morini et al., 2005[52]; Peres Filho and Dorval, 2003[53]
Canavalia ensiformes	Fabaceae	Fatty acids; canavalin, canatoxin	Monteiro et al., 1998[55]; Hebling et al., 2000[81]
Pilocarpus grandiflorus	Rutaceae	Vanillic acid, syringaldehyde	Godoy et al., 2002[82]
Eucalyptus maculata	Myrtaceae	elemol, β-eudesmol	Marsaro Junior et al., 2004[18]; Marinho et al., 2005[19]; Marinho et al., 2008[20]; Marinho et al., 2006[83]
Ricinus communis	Euphorbiaceae	Palmitic acid; ricin	Bigi et al., 2004[15]; Caffarini et al., 2008[84]; Cazal et al., 2009[85]
Dimorphandra mollis	Fabaceae	Astilbin	Cintra et al., 2005[86]
Raulinoa echinata	Rutaceae	Limonoid, limonexic acid	Biavatti et al., 2005[57]
Cedrela fissilis	Meliaceae	3*β*-acetoxicarapine limonoid, oleanolic acid, oleanic acid, cipadesin A, ruageanin A, cipadesin, khayasin T, febrifugin, mexicanolide	Bueno et al., 2005[60]; Leite et al., 2005[61]
Carapa guianensis	Meliaceae	6α-acetoxygedunin	Ambrozin et al., 2006[87]
Azadirachta indica	Meliaceae	Azadirachtin	Santos-Oliveira et al., 2006[62]; Brugger et al., 2008[63]
Helietta puberula	Rutaceae	Kokusagine, anthranilic acid, dictamnine	Almeida et al., 2007[20]

Plant species	Family	Identified substance	Reference
Ageratum conyzoides	Asteraceae	-	Ribeiro et al., 2008[88]
Mentha piperita	Lamiaceae	-	Ribeiro et al., 2008[88]
Simarouba versicolor	Simaroubaceae	4,5-dimethoxy-canthin-6-one, 5-methoxy-canthin-6-one	Peñaflor et al., 2009[22]
Citrus sp.	Rutaceae	Xanthyletin	Cazal et al., 2009[85]
Spiranthera odoratissima	Rutaceae	Furoquinolines, 2-arylquinolin-4-one, limonexic acid, limonin	Terezan et al., 2010[89]
Tithonia diversifolia	Asteraceae	-	Valderrama-Eslava et al., 2009[23]
Tabebuia vellosoi	Bignoniaceae	-	Souza et al., 2010[90]
Magonia pubescens	Sapindaceae	-	Souza et al., 2010[90]
Annona reticulata	Annonaceae	-	Souza et al., 2010[90]
Amburana acreana	Leguminosae	-	Souza et al., 2010[90]
Virola sebifera and Virola sp.	Myristicaceae	Sesamin and Epigalgavrin; (+)-sesamin, (-)-hinoquinin, (-)-kusunokinin	Bicalho et al., 2012[54]; Pagnocca et al., 1996[91]

Table 1. Plant species with toxic effects against leaf-cutting ants and/or their symbiotic fungi and the associated isolated substances.

3.1. *Sesamum indicum*

Crude extracts of the leaves, fruits, and seeds of sesame, *Sesamum indicum* L. (Pedaliaceae), were tested *in vitro* against the symbiotic fungus (*L. gongylophorus*) of *A. sexdens*, isolated from previously established nests. Bioassays were performed according to methodology developed by Pagnocca et al. (1990; 1996) [49,50]. The extracts were added to the culture medium described by Pagnocca et al. (1990) until reaching final concentrations between 7.5 and 60 mg/mL. Ten test tubes were used for each sample, with three replicates for leaf extracts and two replicates for the other extracts. Fungal growth was estimated macroscopically based on the surface area and density of the mycelium after 30-35 days of incubation. The control sample received the same amount of solvent, and the relative growth observed was characterised as follows: 5 + = growth equal to the control; 4 + = growth equivalent to 80% of the control; 3 + = growth equivalent to 60% of the control; 2 + = growth equivalent to 40% of the control; and 1 + = growth equivalent to 20% or less of the control. The crude extracts of sesame leaves, fruits, and seeds inhibited the growth of the symbiotic fungus, which suggests that this species produces compounds with antifungal proprieties (Tables 2 and 3).

Solvent	Final concentration (mg leaf dry weigh/mL)	Relative fungal growth (30-35 days)
Chloroform	7.5	5+
	15.0	5+
	30.0	4+
	60.0	2+
Methanol	7.5	5+
	15.0	5+
	30.0	4+
	60.0	2+
Chloroform+Methanol	30.0+30.0	2+
	60.0+60.0	<1+
Water	60.0	>5+

5+=growth identical to control; 4+=growth equivalent to 80% of control; 3+=growth equivalent to 60% of control; 2+=growth equivalent to 40% to control; 1+=growth equivalent to 20% of control of less. Source: Pagnocca et al., 1990 [49].

Table 2. Antifungal activity of sesame leaf extracts

Material and developmental stage	Final concentration mg dry weight/mL	Relative fungal growth (30-35 days)
L1 (leaves 30 days old)	60.0	2+
L2 (leaves 60 days old)	60.0	2+
L3a (green leaves 90 days old)	60.0	3+
L3b (yellow leaves 90 days old)	60.0	2+
Gfr (green fruit)	30.0	3+
RFr (ripe fruit)	30.0	2+
GS (green seed)	30.0	2+
RS (ripe seed)	30.0	2+

Control=5+

Table 3. Antifungal activity of chloroform extracts of sesame leaves, fruits, and seeds at different stages of development. Source: Pagnocca et al., 1990 [49].

In another study, Pagnocca et al. (1996) determined the number of bacteria and yeast in the organic matter within ant colonies reared in the laboratory with *Eucalyptus alba* Reinw. ex Blume (control) or *S. indicum* (experiment). Transparent plastic pots (2.5 L), connected to each other through transparent tubes (1.5 cm of diameter), were used in these bioassays. In this setup, one chamber was used to supply leaves, a second chamber housed the fungal garden (sponge), and the third chamber contained the residues (waste) from the ants. Fresh leaves (10 to 20 grams) were offered at 48-h intervals after removal of the waste from the previous treatment. In the older sponges in nests treated with *Eucalyptus*, 1.4×10^5 bacterial colony forming units/g (CFU/g) were recorded, while the average in the waste deposits reached 7.3×10^7 CFU/g. The most probable numbers (MPNs) of yeast per gram of the material analysed were 1.3×10^5 and 2.2×10^4 MPN/g for older sponges and waste deposits, respectively, while in ant colonies treated with *S. indicum* leaves, these values were 3.3×10^7 CFU/g and 6.7×10^5 MPN/g. This increase in the numbers of bacteria and yeast led to visible changes in the colouration and humidity of the fungal sponges of nests treated with sesame, which resulted in fungal death.

The application of fractions of the extracts from sesame leaves at a 2.5 mg/mL concentration completely inhibited the development of the symbiotic fungus of the leaf-cutting ants, and 50% inhibition of fungal development was observed for some fractions at a 1.25 mg/mL concentration [51] (Table 4). Chromatographic analysis of the hexanic extracts of leaves revealed the presence of a mixture of tetradecanoic, hexadecanoic, octadecanoic, icosanoic, docosanoic, and 9,12,15-octadecatrienoic acids. Separation of the compounds in the mixture by fractionation resulted in a loss of or decrease in inhibitory activity against the fungus, indicating that the observed inhibition may be a consequence of the joint action of several compounds in the leaves, rather than of a single substance.

Fractions	**Extracts**		
	Hexane	**Dichloromethane**	**Methanol**
Hexane	-	[1.25]/(90)	[1.25]/(60)
	-	[2.50]/(NG)	[2.50]/(NG)
	[5.00]/50	[5.00]/(NG)	[5.00]/(NG)
Dichloromethane	-	[1.25]/(50)	-
	-	[2.50]/(NG)	-
	[5.00]/(90)	[5.00]/(NG)	[5.00]/(10)
Ethyl Acetate	[1.25]/(50)	[1.25]/(50)	[1.25]/(70)[2]
	[2.50]/(NG)	[2.50]/(NG)	[2.50]/(NG)
	[5.00]/(NG)	[5.00]/(NG)	[5.00]/(NG)
Methanol	[1.25]/(90)	[1.25]/(70)	[1.25]/(50)[2]
	[2.50]/(NG)	[2.50]/(NG)	[2.50]/(NG)
	[5.00]/(NG)	[5.00]/(NG)	[5.00]/(NG)
Acetic Acid	[1.25]/(100)	[1.25]/(100)	[1.25]/(100[2])
	[2.50]/(40)	[2.50]/(NG)	[2.50]/(NG)
	[5.00]/(10)	[5.00]/(NG)	[5.00]/(NG)

[1] Thirty days of incubation/25°C; 2dry mix; NG=no growth

Table 4. Fungal growth (%) of *Leucoagaricus gongylophorus* in culture medium containing different concentrations [mg/mL] of the hexane, dichloromethane, and methanol extracts from sesame. Control= (100). Source: Ribeiro et al., 1998 [51].

Extracts from ripe sesame seeds were tested to investigate their toxicity through contact with *A. sexdens* workers. Ripe seeds of *Sesamum indicum* L. (Pedaliaceae) were triturated and pressed, yielding sesame butter. A known mass of this sesame butter was macerated for three days three times at room temperature and then extracted with solvents of increasing polarity (dichloromethane and methanol), resulting in a dichloromethane crude extract (SD) and a methanol crude extract (SM). The SD crude extract was subjected to liquid chromatography in a vacuum syntherised plate funnel with silica gel as the stationary phase and eluents of increasing polarity, which yielded the following fractions: hexane (SD-H), dichloromethane (SD-D), ethyl acetate (SD-E), and methanol (SD-M). The SD-E fraction was produced through successive chromatographic columns, with silica as the stationary phase and hexane/dichloromethane/methanol as the eluent, in gradient mode. A total of 11 sub-fractions were obtained from this process, only four of which (A, B, C, D) contained a sufficient amount of material to be tested. At the tested concentrations, the same proportion as in the original SD-E fraction was maintained in the sub-fractions, and samples at double these concentrations were also tested (Figure 3). The SD-E sub-fractions were combined in amounts necessary to equal that of the original fraction. The seven sub-fractions (E-K) that were isolated in only small amounts were not tested. Tests were also performed in which the concentration of each sub-fraction was reduced by 50% in two combinations: A+B+C+D and A+B+C. To identify the compounds present in SD-E, hydrogen nuclear magnetic resonance (H NMR) and gas chromatography-mass spectrometry (GC-MS) were used. The results demonstrated that *A. sexdens* workers that received the crude dichloromethane extract from sesame seeds (SD) on their *pronoto* exhibited high mortality. This crude extract was then fractionated, and the ethyl acetate fraction (SD-E) was found to be responsible for the toxic effect. However, no toxicity was observed when the SD-E sub-fractions (A, B, C, and D) were tested in the same proportions as found in the original fraction (Table 5). These results could be explained by three hypotheses: 1) each isolated sub-fraction is only toxic at concentrations above the concentration found in the ethyl acetate fraction; 2) the sub-fractions are only toxic when combined through a synergistic effect between their components; and 3) toxic compounds are be present in the untested sub-fractions (E-K), which corresponded to 26.77% of the ethyl acetate fraction. Experiments were conducted to determine why the formicidal activity was lost. First, the authors doubled the concentration of each sub-fraction, and only one fraction, composed of triglycerides, was found to be toxic (Table 5). Then, when sub-fractions A, B, C, and D were combined, the formicidal effect reappeared, even at concentrations reduced to 50% of the original concentration (Table 6). A mixture containing 73.23% (A + B + C + D) of the ethyl acetate fraction contains chemical compounds that reduce the survival of *A. sexdens*. [52]

The results shown in Table 6 indicate that five of the 11 possible combinations of the SD-E sub-fractions were toxic to leaf-cutting ants (A + B + C + D; A + B + C; A + C + D; A + C; B + C), and all of the toxic combinations contained sub-fraction C, which was composed of diglycerides and furfuranic lignans (sesamin and sesamolin). The observed effects are likely due to the presence of lignin furfuranic, which is used as a synergistic factor in insecticides. However, sesamolin exhibited a biological activity that was five times stronger than that of sesamin. Moreover, sub-fraction D, which was composed only of sesamin, either had an in-

Material	Concentration (mg mL^{-1})	% Mortality/Day										S_{50}
		1	2	3	6	8	10	14	17	21	25	
Control D	-	0	0	2	10	35	53	72	90	93	100	10a
SD	200	2	7	18	47	73	90	93	95	97	100	7c
SM	200	3	10	17	45	67	75	87	90	97	100	7c
Control H	-	0	5	7	23	32	57	75	78	85	100	9a
SD H	200	8	53	63	77	85	88	88	92	97	100	2c
Control D	-	5	5	15	37	62	78	85	97	97	100	7b
SD-D	200	0	5	5	27	53	63	78	82	92	100	8a
Control E	-	2	3	5	27	58	68	88	95	97	100	8a
SD-E	200	0	5	7	23	32	57	75	78	85	100	9a
Control M	-	27	47	57	67	70	75	88	93	100	-	3c
SD-M	200	0	5	5	27	53	63	78	82	92	100	8a
Control E	-	0	5	5	20	37	72	88	92	98	100	9a
A	57	2	10	10	37	62	65	77	82	92	100	8a
A	14	0	5	7	23	32	57	75	78	85	100	9a
Control E	-	23	38	53	83	87	87	97	98	98	100	3c
B	53	0	5	8	18	35	53	70	80	85	100	10a
B	106	0	5	5	27	53	63	78	82	92	100	10a
C	25	2	2	2	28	65	83	95	97	100	-	8b
C	50	0	2	8	25	42	60	83	88	90	100	10a
Control E	-	0	5	7	23	32	57	75	78	85	100	9a
D*	12	0	2	3	18	30	63	85	93	98	100	9a
D*	24											

S_{50}= Survival median 50%. Different letters after the S_{50} values indicate a significant difference according to the log-rank test (b=0.01>p<0.05; c=p<0.01). Concentrations: A= 57 mg mL^{-1}; B=53 mg mL^{-1}; C=25 mg mL^{-1}; D*= 12 mg mL^{-1}; ( )*= concentration reduced to 50%

Table 5. Toxicity of SD-E sub-fraction combinations in *Atta sexdens* workers. Source: Morini et al., 2005 [52].

hibitory effect on the action of other sub-fractions (B + C + D; C + D) or was unable to modify their actions (A + D; B + D), showing that the factor responsible for the synergistic toxic effect of sesame seeds is either sesamolin or the combination of sesamin + sesamolin, rather than sesamin alone [52] (Table 6).

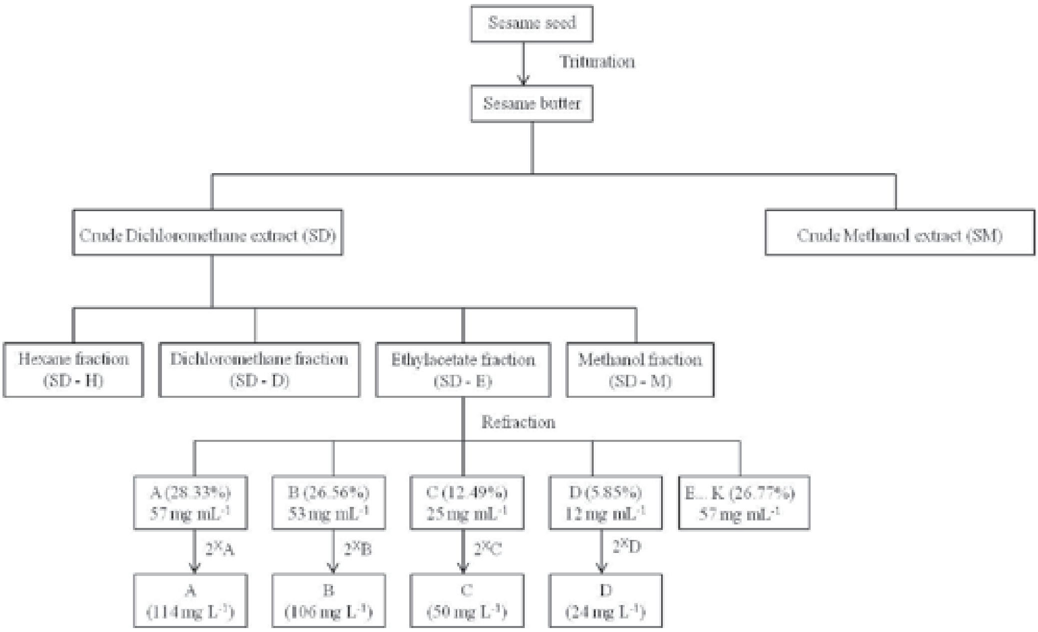


Figure 3. Diagram showing the procedure for obtaining crude extracts, fractions, and sub-fractions from sesame seeds (*S.indicum*) and the sequence of topical application on *A. sexdens rubropilosa* workers. Source: Morini et al., 2005 [52].

The efficiency of different commercial chlorpyrifos- sulfluramid- and fipronil-based formicidal baits as well as others that are manually manufactured using the leaves (15%) and seeds (10%, 20% and 30%) of *S. indicum* against *A. sexdens* Forel. control were assessed in the field. The nest activity was monitored at 30, 60, 90, and 150 days after treatment. The most efficient baits were sulfluramid- and fipronil-based, followed by the formulation derived from sesame leaves (15%). The sulfluramid- and fipronil-based baits caused colony activity to cease at 30 days, while the sesame leaf-based baits (15%) resulted in an 80% inhibition of activity at 90 days, confirming that *S. indicum* has great potential for the development of new products to control leaf-cutting ants [53].

Sub–fraction combination	**% Mortality/Day**										S_{50}
	1	2	3	6	8	10	14	17	21	25	
Control	0	0	2	10	35	53	72	90	93	100	10a
A+B+C+D*	2	7	18	47	73	90	93	95	97	100	7c
(A+B+C+D*)*	3	10	17	45	67	75	87	90	97	100	7c
Control	0	5	7	23	32	57	75	78	85	100	9a
A+B+C	8	53	63	77	85	88	88	92	97	100	2c
(A+B+C)*	5	5	15	37	62	78	85	97	97	100	7b
Control	0	5	5	27	53	63	78	82	92	100	8a
A+B+D*	2	3	5	27	58	68	88	95	97	100	8a
Control	0	5	7	23	32	57	75	78	85	100	9a
A+C+D*	27	47	57	67	70	75	88	93	100	-	3c
Control	0	5	5	27	53	63	78	82	92	100	8a
B+C+D*	0	5	5	20	37	72	88	92	98	100	9a
A+B	2	10	10	37	62	65	77	82	92	100	8a
Control	0	5	7	23	32	57	75	78	85	100	9a
A+C	23	38	53	83	87	87	97	98	98	100	3c
A+D*	0	5	8	18	35	53	70	80	85	100	10a
Control	0	5	5	27	53	63	78	82	92	100	10a
B+C	2	2	2	28	65	83	95	97	100	-	8b
B+D*	0	2	8	25	42	60	83	88	90	100	10a
Control	0	5	7	23	32	57	75	78	85	100	9a
C+D*	0	2	3	18	30	63	85	93	98	100	9a

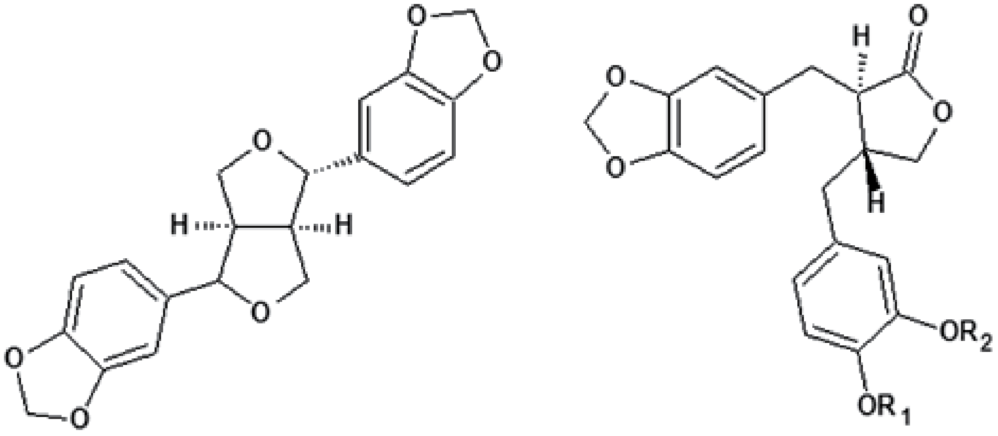
S_{50}= Survival median 50%. Different letters after the S_{50} values indicate a significant difference according to the log-rank test ($b=0.01>p<0.05$; $c=p<0.01$). Concentrations: A= 57 mg mL^{-1}; B=53 mg mL^{-1}; C=25 mg mL^{-1}; D*= 12 mg mL^{-1}; ( )*= concentration reduced to 50%

B=53 mg mL^{-1}; C=25 mg mL^{-1}; D*= 12 mg mL^{-1}; ( )*= concentration reduced to 50%

Table 6. Toxicity of SD-E sub-fraction combinations in *Atta sexdens* workers. Source: Morini et al., 2005 [52].

3.2. *Virola sebifera*

Phytochemical analysis of the leaves of *Virola sebifera* Aubl. (Myristicaceae) resulted in the isolation of three lignans, (+)-sesamin (1), (-)-hinoquinin (2), and (-) – kusunokinin (3) (Figure 4), and three flavonoids, quercetin-3-O-α-L-rhamnoside, quercetin-3-O-β-D-glucoside, and quercetin-3-methoxy-7-O-β-D-glucoside. Techniques such as high-speed counter-current chromatography and high-performance liquid chromatography were employed in this process. The isolated substances were added to the artificial diet and tested against *A. sexdens* leaf-cutting ants at a concentration of 200 or 400 μg mL^{-1}. Diets (0.4-0.5 g per dish) treated with the compounds (experimental treatment) or without (control) were offered daily in a small plastic cap. The percentage of survival was plotted as a function of time in a survival curve that was then used to calculate the median survival time (S_{50}, the time at which 50% of the ants in each experiment remained alive). The lignin (-) - kusunokinin (3) resulted in 90% mortality of *A. sexdens* workers after 25 days of monitoring compared to the controls fed with an untreated diet. Although the other substances did not show biological activity against the ants, the (+)-sesamin (1), (-)-hinoquinin (2) and (-)-kusunokinin (3) lignans inhibited the growth of the symbiotic fungus by 74%, 72%, and 100%, respectively [54] (Figure 4).



(2) R1-R2=CH2
(3)R1=R2=CH3

(1)

Figure 4. Chemical structures of the compounds isolated from *Virola sebifera,* (+)-sesamin (1), (-)-hinoquinin (2), and (-) – kusunokinin (3). Source: Bicalho et al., 2012 [54].

3.3. *Canavalia ensiformis*

In vitro tests showed inhibitory effect on the symbiotic fungus of a hexanic extract of *Canavalia ensiformis* (L.) DC. (Fabaceae) leaves, applied at a 1,000 μg mL^{-1} concentration. This extract was fractionated by column chromatography using silica gel as the stationary phase. A total of 11 fractions were obtained and used in fungal bioassays at a concentration of 500 μg mL^{-1}. Only one fraction (fraction 9) was active; all fractions were esterified with diazomethane and analysed by gas chromatography-mass spectrometry (GC-MS) to identify the active components. The main compounds identified in the active fraction were long-chain saturated fatty acids. In these experiments, it was not possible to identify which of the fatty acids was responsible for the fungicidal action. However, comparison of the different fractions showed that the fatty acids with chains containing 11, 17, 19, 22, and 23 carbon atoms were likely the most active (Table 7), as the fractions in which these fatty acids were not among the major components showed no fungicidal activity [55].

Fraction	Major compounds	Minor compounds
9*	C_{11}, C_{16}, C_{17}, C_{18}, C_{19}, C_{20}, C_{22}, C_{23}, C_{24}, C_{26}	C_8, C_{14}, C_{15}, C_{21}, C_{25}, C_{27}, C_{28}, C_{30}
2	C_{10}, C_{16}, C_{18}, C_{24}, C_{26}	C_{14}, C_{15}, C_{17}, C_{22}, C_{23}, C_{25}, C_{28}, C_{29}
3	C_{16}, C_{18}	C_{10}, C_{14}, C_{15}
4	C_{16}, C_{20}	C_{18}, C_{21}, C_{22}
5	C_{16}	C_{12}

*Active fraction

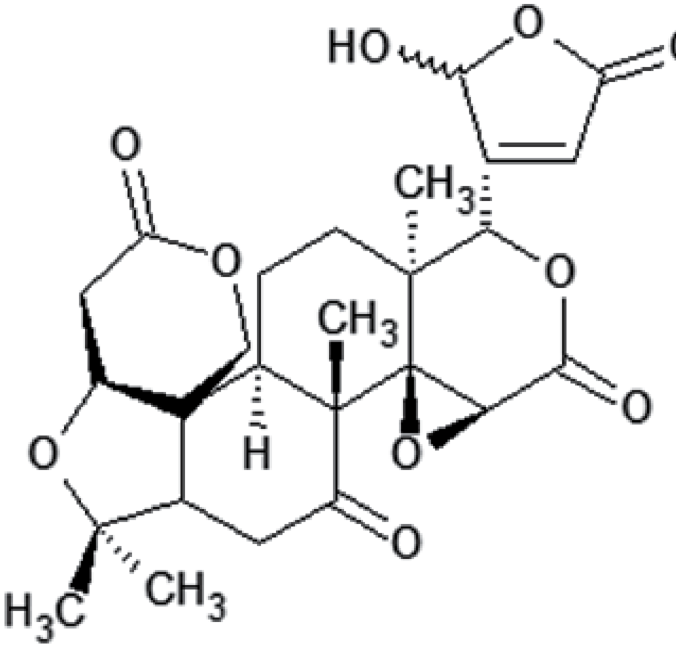
Table 7. Fatty acids found in *Canavalia ensiformis*. Source: Monteiro et al., 1998 [55].

3.4. *Raulinoa echinata*

Phytochemical analyses of the roots of *Raulinoa echinata* R.S.Cowan (Rutaceae) resulted in the isolation and identification of the following limonoids: fraxinellone, fraxinellonone, and epoxy-fraxinellone. Limonexic acid was isolated from the stem of the plant. The toxicity of the compounds against *A. sexdens* was determined in ingestion bioassays according to the protocol described by Bueno et al. (1997) [56]. The ants in the treatment groups received a diet enriched with epoxy-fraxinellone or limonexic acid at a concentration of 200 $\mu g\ mL^{-1}$. Control ants were fed with a component-free diet. Over 25 days, the number of dead ants in each Petri dish was counted, the survival curve of the leaf-cutting ants in each treatment was estimated, and their average longevity was calculated. Limonexic acid (4) (Figure 5) reduced the longevity of *A. sexdens* considerably (11 days) compared to the control (22 days) [57]. *R. echinata* was also able to produce substances that were active against the symbiotic fungus of the leaf-cutting ants; several furoquinoline alkaloids (skimmianine (5), kokusaginine (6), maculine (7) and flindersiamine (8)) and quinolones (2-n-Nonyl-4-quinolone (9), 1-Methyl-2-n-nonyl-4-quinolone (10), 1-Methyl-2-phenyl-4-quinolone (11)) (Figure 6; Table 8) that exhibited fungicidal activity against *L. gongylophorus* were isolated from extracts of its stems and leaves [58].

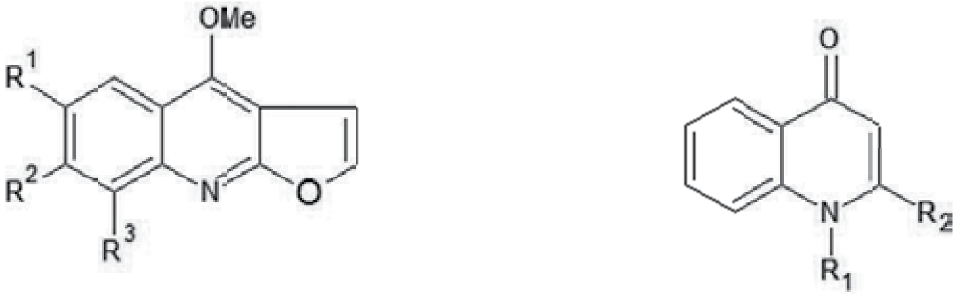
3.5. *Helietta puberula*

Methanolic, hexanic, and dichloromethane extracts obtained from the stems, leaves, and branches of *Helietta puberula* R. E. Fr. (Rutaceae) were tested against *A. sexdens* workers and the symbiotic fungus of this ant species. Experimental diets were prepared by mixing plant



(4)

Figure 5. Limonexic acid isolated from *Raulinoa echinata* stems. Source: Biavatti et al., 2005 [57]



(5) R^1=H, R^2=R^3=OMe

(6) R^1=R^2=OMe, R^3=H

(7) R^1=R^2=OCH_2O, R^3=H

(8) R^1=R^2=OCH_2O, R^3=OMe

(9) R^1=H, R^2=*n*-nonyl

(10) R^1=Me, R^2=*n*-nonyl

(11) R^1=Me, R^2=phenyl

Figure 6. Compounds identified as skimmianine (5), kokusagine (6), masculine (7), flindersiamine (8), 2-n-Nonyl-4-quinolone (9), 1-Methyl-2-n-nonyl-4-quinolone (10), and 1-Methyl-2-phenyl-4-quinolone (11) based on comparison with spectral data presented in the literature. Source: Biavatti et al., 2002 [58]

Extract/fraction/compound	% growth inhibition of *L. gongylophorus*					
	µg mL^{-1}	50	100	250	500	1000
MSE[a]		-	-	-	-	80
Skimmianine (1)		60	80	NT	NT	NT
Kokusagine (2)		20	100	NT	NT	NT
Maculine (3)		10	50	NT	NT	NT
Flindersiamine (4)		-	50	NT	NT	NT
MLE[c]		-	-	-	-	80
MLE (hexane fraction)					80	100
2-n-Nonyl-4-quinolone (5)		20	50	NT	NT	NT
1-Methyl-2-n-nonyl-4-quinolone (6)		-	-	-	-	-
1-methyl-2-phenyl-4-quinolone (7)		NT	NT	NT	NT	NT

[a]MSE: Methanol stem extract, [b]NT: not tested, [c]MLE: methanol leaf extract

Table 8. Evaluation of the growth inhibitory activity of crude extracts, fractions, and compounds of *Raulinoa echinata*

material (crude extract, partially purified extract, or pure compound) and the basic formula described by Bueno et al. (1997) [56]. The final concentrations of crude extracts, fractions, and isolated substances from *H. puberula* in the diet were 2.0, 1.6, and 0.3 mg mL^{-1}, respectively. Blocks of 0.4 g of the experimental diets per plate (control or experimental) were offered daily to the workers. Evaluations were conducted over 25 days, and the number of dead ants was recorded daily. The following substances were isolated from *H. puberula:* anthranilic acid (12), flindersiamine (13), dictamnine (14), kokusaginin (15), maculine (16), and sitosterol. The anthranilic acid, kokusaginine, and dictamnine resulted in 90%, 86%, and 88% mortality, respectively, compared with 68% mortality in the control. The substances anthranilic acid, kokusaginine, masculine, and dictamnine caused fungal inhibition (≥80%) at a concentration of 0.1 mg mL^{-1} [21] (Figure 7).

3.6. *Eucalyptus* sp.

Leaf-cutting ants may exhibit behavioural changes when exposed to plant extracts; Anjos and Santana (1994) [59] observed bites and mutilations among *A. sexdens* and *A. laevigata* nestmates subjected to contact with leaves of four *Eucalyptus* sp. belonging to the family Myrtaceae. With the aim of isolating and identifying the compounds responsible for these changes, *E. maculate* leaves were subjected to extraction with hexane, followed by chromatographic fractionation, resulting in the isolation of six active sesquiterpenes (elemol, β-eudesmol, α-eudesmol, guaiol, hinesol, and γ-eudesmol).

Fragments of filter paper in a rectangle, square, or triangle shape were prepared and (a) impregnated with solvent alone as a control (square), (b) left blank (rectangle), or (c) impregnated with the treatment to be tested using 100 µL of the extract solution or pure compound (triangle).

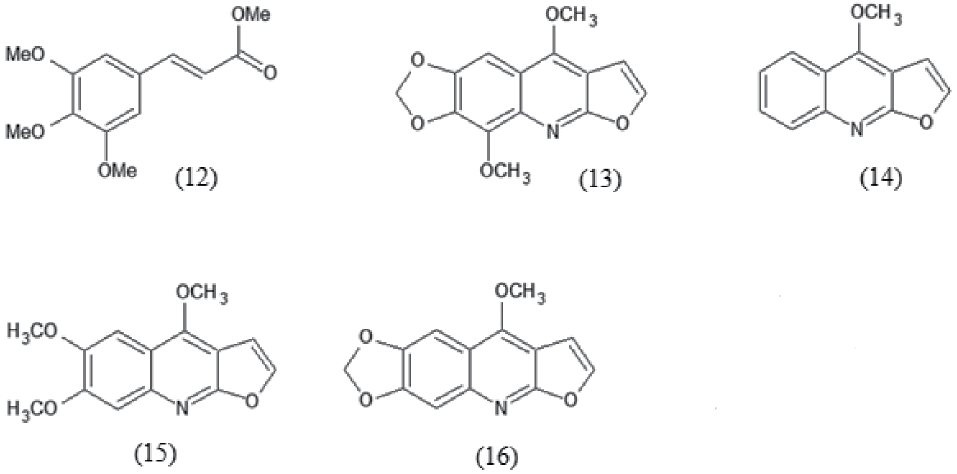


Figure 7. Molecular structures of substances from *Helietta puberula*: anthranilic acid (12), flindersiamine (13), dictamnine (14), kokusaginine (15), and maculine (16). Source: Almeida et al., 2007 [21].

After solvent evaporation, two of the filter paper fragments of each of the three different geometric shapes were placed on three glass slides, which were then transferred to the colonies. Monitoring was performed for 30 minutes after placement of the filter paper, and the number of groups of attackers, the number of ants in each group, and the number of mutilated ants in each group were counted. Elemol (17) and β-eudesmol (18) (Figure 8) were the most active ingredients, and the latter substance was associated with greater numbers of groups of attackers (84.2) and mutilated ants (285.8). After contact with the filter paper impregnated with β-eudesmol, the ants exhibited alarm behaviour and held their mandibles open. When encountering nestmates that had previously contacted the filter paper, they touched their antennas and then attacked each other, frequently on the legs, but also on other parts of the body [18].

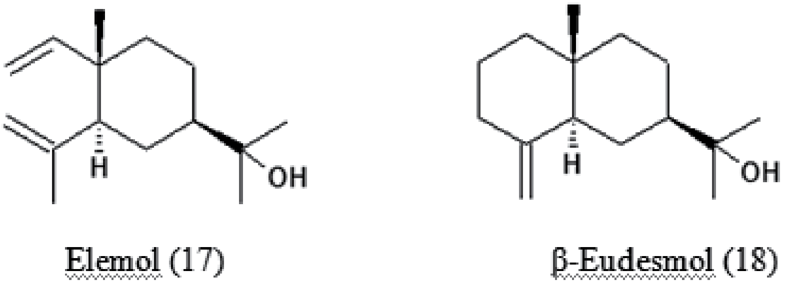


Figure 8. Chemical structures of the sesquiterpenes identified in the most active fraction of the *Eucalyptus maculate* leaf extract. Source: Marsaro Junior et al., 2004 [18].

Upon analysis, the composition of the chemical profile of the cuticles of the workers that had contact with β-eudesmol was different than that found in the other workers. (E)-β-farnesene, busenol, and (E,E)-farnesol were present in the cuticles of ants exposed to β-eudesmol [20]. The changes in the composition of the cuticle interfered in the process of recognition between nestmates. The ants triggered an alarm behaviour when they did not recognise the workers exposed to β-eudesmol.

3.7. *Cedrela fissilis*

The survival of *A. sexdens* workers was significantly reduced when they were fed diets containing hexane or dichloromethane-soluble extracts of the root and leaves of *Cedrela fissilis* Vell. (Meliaceae). These extracts and those derived from fruits and branches, which were hexane- or dichloromethane-soluble, respectively, also inhibited the growth of the *L. gongylophorus* fungus [60,61].

The limonoid 3β-acetoxicarapin and the triterpenes oleanoic and oleanonic acid were isolated from roots of *C. fissilis*. These compounds and six other mexicanolide-type limonoids (cipadesin A, ruageanin A, cipadesin, khayasin T, febrifugin, and mexicanolide) that were previously isolated from *Cipadessa fruticosa* Blume exhibited insecticidal activity against *A. sexdens* leaf-cutting ants. The median survival period (S_{50}) was significantly different from that of the control, confirming activity against *A. sexdens* [61] (Table 9).

Compounds	Days										Survival median (S_{50})/days
	1	2	3	6	8	10	14	17	21	25	
1- cipadesin B	0	0	6	22	40	58	76	88	92	98	9[a]
2- swietemahonolide	0	0	4	40	54	58	82	90	96	98	8[a]
3- 3β-acetoxycarapin	0	2	12	34	50	76	98	100	-	-	8[a]
4- oleanolic acid	0	0	10	52	70	90	100	-	-	-	6[a]
5c- oleanonic acid	0	0	8	38	60	86	100	-	-	-	8[a]
6- cipadesin A	0	2	8	34	46	72	100	-	-	-	9[a]
7- ruageanin A	0	4	18	50	62	74	96	98	98	100	6[a]
8- cipadesin	0	8	12	46	68	76	100	-	-	-	7[a]
9- khayasin T	0	4	10	54	72	86	98	100	-	-	6[a]
10- febrifugin	0	2	6	38	58	70	88	94	98	100	7[a]
11- mexicanolide	0	4	16	50	60	70	100	-	-	-	6[a]
Control (ethyl acetate)	0	0	6	22	36	50	74	90	94	96	10

[a] Significant difference according to the log-rank test ($p<0.05$).

Table 9. Mortality (%) of *Atta sexdens* workers fed on compounds 1-11 at a concentration of 100 $\mu g\ mL^{-1}$. Source: Leite et al., 2005 [61].

3.8. *Azadirachta indica*

Seeds of *Azadirachta indica* were tritured and pressed, yielding a neem paste. After one week, the floating material was isolated, which was referred to as crude extract of neem oil. A known mass of the remaining material, referred to as crude extract of seed neem paste, was macerated for three days three times at room temperature and extracted with solvents of increasing polarity (hexane, dichloromethane and methanol), resulting in three crude extracts. When incorporated in an artificial diet, the crude extract of neem seed oil caused significant toxicity to *A. sexdens* workers at all of the concentrations tested. The survival of the ants was significantly reduced in the diets containing the neem seed paste hexane extract at concentrations of 10 and 20 μg mL^{-1}, the dichloromethane extract at all concentrations tested (2, 10, and 20 μg mL^{-1}), and the methanol extract at concentrations of 10 and 20 μg mL^{-1}.

There was a negative relationship between the neem oil concentration and the frequency of contact of ants with the artificial diet. The lowest frequency of contact was obtained with the highest concentration tested (30 μg mL^{-1}). Moreover, the initial contact with the diet was dependent on the presence of neem. Thus, the period required for the ants to feed on the artificial diet for the first time was 8 seconds in the control, 4 minutes and 36 seconds at a concentration of 5 μg mL^{-1}, 19 minutes at a concentration of 10 μg mL^{-1}, and 55 minutes at a concentration of 30 μg mL^{-1}. Some changes in the behaviours of the ants were observed when the workers contacted the diets containing neem seed oil. Contact between the antenna or legs and the diet caused instantaneous retraction of these body parts. The ants positioned themselves offensively with open mandibles and performed self-grooming. The workers that cleaned themselves by licking showed symptoms of intoxication, such as slow movements, disorientation, and prostration [62].

The hexanic extract of *A. indica* neem was tested against *Acromyrmex rugosus* F. Smith (Formicidae) workers. Two colonies of *A. rugosus* were used, and from each colony, 30 groups of 20 workers each were isolated. A citrus pulp containing neem at concentrations of 0.1, 1.0, and 10% was offered to these groups. In the treatments, pastes composed of hexanic extracts of neem (from leaves, branches and seeds) were prepared with the following composition: pure glucose (10%), citrus pulp powder and soybean oil (10%). The sulfluramid treatment was offered in the form of a paste, in which 0.3% sulfluramid was dissolved in 10% soybean oil and mixed with citrus pulp powder and 10% pure glucose. The positive control was prepared in the same manner, but the active sulfluramid was not added to the paste. Treatments were performed with 5 g of paste per replicate, which was removed from the jars after 48 hours. Monitoring lasted five minutes and was performed immediately, 30 minutes, and 24 hours after treatment. The relative frequencies of each of the workers' behaviours and the number of deaths were recorded. High mortality was observed within the first 24 h in the treatments with neem (>20 workers) compared to the control (5 workers), which is not the slow type of action desired for formicides. The delayed action of the active ingredients in formicide formulations is an essential feature because colonies of leaf-cutting ants are very populous, and the control of their nests depends on contamination of all individuals. If ants detect that the presented substrate is not adequate, they will stop carrying it and can even remove parts of the symbiotic fungus contaminated with this substrate and isolate it in waste chambers [63].

3.9. *Simarouba versicolor*

The dichloromethane-soluble fraction of methanolic extracts of the leaves, stems, and branches of *Simarouba versicolor* St. Hill (Simaroubaceae) was tested *in vitro* on ants through ingestion bioassays and with the symbiotic fungus in culture medium. The median survival period for workers was significantly reduced (S_{50}=4 days) compared to the control (S_{50}=16 days), and 100% inhibition of *L. gongylophorus* growth was observed. From these fractions, two alkaloids were isolated, 4,5-dimethoxy-canthin-6-one (19) and 5-methoxy-canthin-6-one (20) (Figure 9), both of which were toxic to the symbiotic fungus and completely inhibited growth at a concentration of 0.1 mg mL^{-1}. However, only the alkaloid 5-methoxy-canthin-6-one reduced the median survival period of the workers from 14 days (control) to seven days at a 0.3 mg mL^{-1} concentration (Table 10). The triterpenes isolated from the other extracts of the plant (lupenone and lupeol) showed no deleterious effects on the leaf-cutting ants of the symbiotic fungus [22].

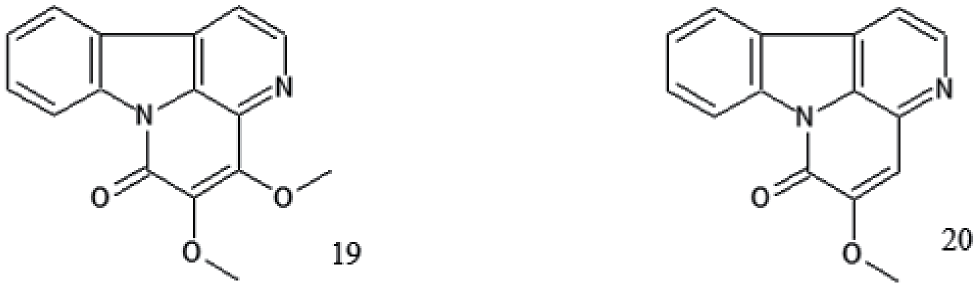


Figure 9. Chemical structures of substances isolated from *Simarouba vesicolor*: 4,5-dimethoxycanthin-6-one (19) and 5 methoxycanthin-6-one (20). Source: Peñaflor et al., 2009 [22].

Treatment[1]	**Day of experiment**										
	1	**2**	**3**	**6**	**8**	**10**	**14**	**17**	**21**	**25**	**S_{50}** [2]
Control	0	2	8	22	30	38	52	62	74	76	14a
Lupenone	4	4	6	12	24	30	46	54	64	76	16a
4,5-dimethoxycanthin-6-one	2	2	8	34	36	48	56	58	68	82	13a
Lupenol	2	2	10	26	26	28	34	36	44	54	19a
5-methoxycanthin-6-one	0	0	10	46	52	56	76	78	80	88	7b

[1] Isolated substances at a concentration of 0.3 mg mL^{-1};

[2] S_{50}=Survival median 50%. Different letters after the S_{50} values indicate a significant difference according to the log-rank test. Different letters after the S_{50} values compared to the respective control indicate a significant difference according to the log-rank test ($P<0.05$). Source: Peñaflor et al., 2009 [22].

Table 10. Toxicity (% mortality and S_{50}) of substances isolated from *Simarouba versicolor* in *Atta sexdens* workers.

3.10. *Ageratum conyzoides*

An assessment of the formicidal activity of a hexanic extract from the leaves of goatweed, *Ageratum conyzoides* L. (Asteraceae), against leaf-cutting ants was performed using the acetone-diluted extract at a concentration of 1.0 mg mL^{-1}. Each worker was topically treated with 1.0 μL of this solution, which was applied on the pronoto of the insect. In the control treatment, the insects were treated with an equal volume of pure acetone. The numbers of living and dead individuals were counted 24 and 48 hours after treatment. The crude extract of goatweed caused increased mortality of *Atta laevigata* F. Smith (Hymenoptera: Formicidae) and *Atta subterraneus subterraneus* Forel (Hymenoptera: Formicidae) workers. The goatweed extract was then fractionated, resulting in the isolation of the compound coumarin. Coumarin was tested against ants at different concentrations (0.5, 4.0, 7.0, 16.0, 50.0, and 100.0 mg mL^{-1} in acetone) to determine its toxicity among the two species of leaf-cutting ants. The median lethal concentration (LC_{50}) decreased (10.9-fold) with increased application time for *A. subterraneus subterraneus*. The LC_{50} was 55.42 mg mL^{-1} at 24 hours and decreased to 5.07 mg mL^{-1} at 48 hours. For *A. laevigata*, the LC_{50} decreased 1.8-fold, from 23.20 mg mL^{-1} at 24 hours to 12.70 mg mL^{-1} at 48 hours. Thus, coumarin is a potential agent for ant control in the form of granulated attractive baits because it has a delayed insecticidal effect [64].

3.11. *Ricinus communis*

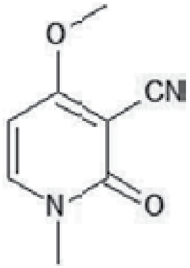
Dry *R. communis* leaves (2 kg) were ground in a Willey mill, and crude extracts were prepared via sequential maceration (3 litres for 7 days for each solvent) with hexane (24.8 g of extract), dichloromethane (32.8 g), ethyl acetate (18.8 g), methanol (54.0 g), and water. With the exception of the water extract, all extracts were subjected to chromatography on silica gel 60 as the stationary phase under vacuum (0.040-0.063 mm, 400 g; column with a sinterised filter in the bottom, internal diameter 10 cm, length 25 cm) with hexane, dichloromethane, ethyl acetate, and methanol (1 litre each) as eluents, yielding four fractions for each extract. The water extract was not fractionated. A portion of the methanol fraction of the hexane extract was refractionated, yielding 12 fractions (MFHE 1-12). These extracts were tested against the symbiotic fungus according to the methodology of Pagnocca et al. (1990) [49]. The sub-fractions MFHE-6, MFHE-9, and MFHE-10 inhibited fungal growth by 80% at a concentration of 0.5 mg mL^{-1}. The same result was observed for the MFHE-11 sub-fraction at a 1.0 mg mL^{-1} concentration. Sub-fraction MFHE-9 contained a mixture of two glycosidic steroids (β-sitosterol-3-*O*-*β*-D-glucoside and stigmasterol-3-*O*-*β*-D-glucoside) and fatty acids (decanoic, myristic, pentadecanoic, palmitic, heptadecanoic, estearic, eicosanoic, docosanoic, tricosanoic, and tetracosanoic acids). Among the above-mentioned compounds, only palmitic acid exhibited antifungal activity and inhibited the growth of the symbiotic fungus by 80% (Table 11).

The methanolic fraction of the dichloromethane-soluble extract of *R. communis* leaves was also re-fractionated, resulting in the isolation of ricin (21) (Figure 10) and monoglyceride (1-palmitic acid glycerol ester). Ricin caused significant death of *A. sexdens* workers when added to their artificial diets. The median survival periods (S_{50}) were 6.93 and 5.27 days at 0.2 and 0.4 mg mL^{-1}, respectively, compared to 10.82 days in the control. However, the effect on mortality was

Acid	%
Decanoic	1.2
Myristic	0.5
Pentadecanoic	6.4
Palmitic	81.0
Heptadecanoic	0.3
Estearic	6.6
Eicosanoic	1.1
Docosanoic	0.2
Tricosanoic	0.7
Tetracosanoic	0.2

Table 11. Activity of fatty acids present in the methanol fraction of hexane extracts from *Ricinus communis* leaves (MFHE) against *Leucoagaricus gongylophorus*. Source: Bigi et al., 2004 [15].

dose dependent. Symptoms of intoxication could be perceived after 24 hours and consisted of a reduction or cessation of movement, followed by disorientation, lack of coordination, and death [15].

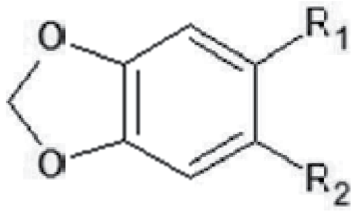


(21)

Figure 10. Ricinin isolated from leaf extracts of *Ricinus communis.* Bigi et al., 2004 [15].

3.12. Synthetic analogues of plant origin

The development of the symbiotic fungus *L. gongylophorus* is inhibited *in vitro* by synthetic compounds containing a piperonyl group: 1-(3,4-methylenedioxybenzyloxy)methane (22); 1-(3,4-methylenedioxybenzyloxy)ethane (23); 1-(3,4-methylenedioxybenzyloxy)butane (24); 1-(3,4-methylenedioxybenzyloxy)hexane (25); 1-(3,4-methylenedioxybenzyloxy)octane (26); 1-(3,4-methylenedioxybenzyloxy)decane (27); and 1-(3,4-methylenedioxybenzyloxy)dodecane (28) (Figure 11). Moreover, *A. sexdens* workers fed daily with an artificial diet containing these compounds showed high mortality compared to controls. The inhibition of fungal growth increased with the number of carbon atoms in the lateral chain, which varied from 1 to 8 (substances 22 to 26). Compounds containing 10 or 12 carbon atoms in the lateral chain did not inhibit fungal growth (substances 27 and 28) (Figure 11). Compound 26, 1-(3,4-methylenedioxybenzyloxy)octane, was the most active and inhibited fungal development by 80% at 15 μg mL^{-1}. In workers, a toxic effect was caused by compound 26 (C8); this effect increased with an increase in the number of carbon atoms in the lateral chains (C10 and C12). Thus, at the same concentration (100 μg mL^{-1}), the mortality rates after eight days of ingestion were 82%, 66%, and 42% under treatment with 1-(3,4-methylenedioxybenzyloxy)decane (compound 28), 1-(3,4-methylenedioxybenzyloxy)dodecane (compound 27), and compound 26, respectively, while for piperonyl butoxide, the observed mortality was 68%. The last compound, which is known as a synergistic insecticide, inhibited the symbiotic fungus with an intensity that was statistically similar to that observed for synthetic compound 26. The results indicate that a formulation can be designed to attack both ants and their symbiotic fungus; such a formulation could represent an advantage over the chemical products used for leaf-cutting ant control, which are directed only towards the ants [65].



22- $R_1=CH_2OCH_3$, $R_2=H$

23- $R_1=CH_2OCH_2CH_3$, $R_2=H$

24- $R_1=CH_2OCH_2(CH_2)_2CH_3$, $R_2=H$

25- $R_1=CH_2OCH_2(CH_2)_4CH_3$, $R_2=H$

26- $R_1=CH_2OCH_2(CH_2)_6CH_3$, $R_2=H$

27- $R_1=CH_2OCH_2(CH_2)_8CH_3$, $R_2=H$

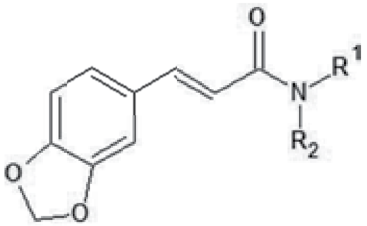
28- $R_1=CH_2OCH_2(CH_2)_{10}CH_3$, $R_2=H$

29- $R_1=CH_2(OCH_2CH_2)_2O(CH_2)_3CH_3$,
$R_2=CH_2CH_2CH_3$

Figure 11. Structures of the synthesised compounds 1-(3,4-methylenedioxybenzyloxy)methane (22); 1-(3,4-methylenedioxybenzyloxy)ethane (23); 1-(3,4-methylenedioxybenzyloxy)butane (24); 1-(3,4-methylenedioxybenzyloxy)hexane (25); 1-(3,4-methylenedioxybenzyloxy)octane (26); 1-(3,4-methylenedioxybenzyloxy)decane (27); 1-(3,4-methylenedioxybenzyloxy)dodecane (28); and the commercial compound piperonyl butoxide (29). Victor et al., 2001 [65].

Several studies have suggested that the amides found in species of the *Piper* genus show potential for insecticidal use due to their effectiveness and knockdown effects. Therefore, the natural amides N-pyrrolidine-3-(4,5-methylenedioxyphenyl)-2-(E)-propenamide and N-piperidine-3-(4,5-methylenedioxyphenyl)-2-(E)-propenamide, found in the roots of *Piper piresii* Yunck (Family: Piperaceae), were used as a model for the synthesis of analogous amides.

The 3-(3,4-methylenedioxyphenyl)-2-(E)-propenamide (30) portion was maintained, and only groups R_1 and R_2 linked to the nitrogen (Figure 12) were altered. Thus, nine amides were synthesised, and the yield varied between 36 and 86% (Table 12; Figure 13). Compounds 3 (S_{50}= 11 days) and 8 (S_{50}=7.5 days) significantly reduced the median survival period (S_{50}) for workers compared to the control (S_{50}= 14 days) at 100 μg mL^{-1} when added to the artificial diet offered daily. Compounds 1, 2, 4, 5, 6, 7, and 9 had no effect on the median survival period at any of the concentrations tested (25, 50, and 100 μg mL^{-1}). At 100 μg mL^{-1}, compounds 1, 2, and 3 completely inhibited fungal growth, and partial inhibition was observed for compounds 4 (80%), 5 (40%), and 6 (20%), while compounds 7, 8, and 9 had no effect on the growth of the symbiotic fungus [66].



(30)

Figure 12. E)-3-(3,4-methylenedioxyphenyl)-2-propenamide group. Source: Pagnocca et al., 2006 [66].

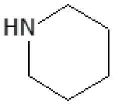
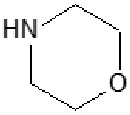
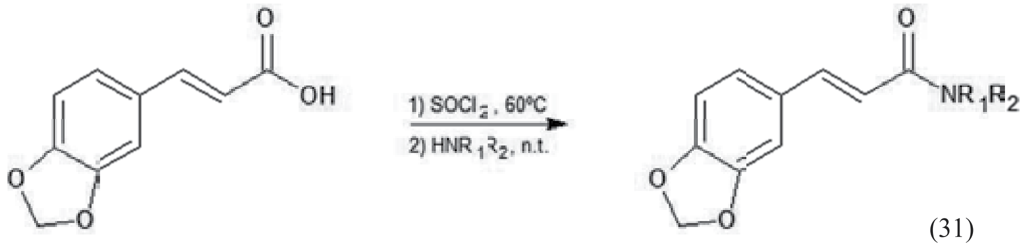
Amide	Amine	NR_1R_2	Yield (%)	Eluent (hexane/ethyl acetate)
1	Piperidine		44	4:6
2	Diethylamine	$N(CH_2CH_3)_2$	42	6:4
3	Pyrrolidine		68	1:1
4	2-Methylbutylamine	$NHCH_2CH$ $(CH_3)CH_2CH_3$	38	7:3
5	Morpholine		40	5.5:4.5
6	Aniline	NHC_6H_5	39	5.5:4.5
7	Disopropylamine	$N[CH(CH_3)_2]_2$	36	2:1
8	Benzylamine	$NHCH_2C_6H_5$	36	4:1
9	Dicyclohexylamine	$N(C_6H_{11})_2$	86	7.5:2.5

Table 12. Amines, the respective yields of the amides, and the eluents used in the chromatographic separation. Source: Pagnocca et al., 2006 [66].



(31)

Figure 13. Synthesis of amides 1-9. Source: Pagnocca et al., 2006 [66].

4. Control of leaf–cutting ants via enzymatic inhibition

When the cut plant fragments reach the colony, a complex process of preparation of the plant substrate for its incorporation into the fungal garden begins. During this processing, the workers may ingest the plant's sap while cutting and pressing the borders of the plant fragment. By scraping the surface, they remove the epicuticular wax layer and facilitate the decomposition of the substrate by the fungus [42,67] indicating the importance of the hydrolytic enzymes in this process. This behaviour is also related to the decontamination of the substrate [68].

Ultra-structural studies of the colonisation of the plant substrate by the fungus have demonstrated that the fungus can only use the portions that have had their border cut. In addition, the cuticular surface of the leaf at the time of colonisation appears to be intact, which suggests an absence of cutinases (enzymes that catalyse the hydrolysis of cutin, a structural component of the cuticle of the plant) in this process. Therefore, it is believed that this symbiotic fungus is a saprophyte that is unable to penetrate into plants that are not damaged [69] (Figure 13).

Therefore, maceration aids in the destruction of the physical barrier of the leaf cuticle, increasing permeability to allow fungal growth, which is assisted by enzymes present in the faecal fluid. [70] The symbiotic fungus is an important mediator involved in providing nutrition to the ants via the hydrolysis of polysaccharides from plant [71] as it produces large amount of enzymes, particularly pectinases, that are ingested by ants, concentrated in the intestine, returned to the fungal garden via faecal fluid, and utilised for the digestion of plant tissues [7]. Therefore, this association is also essential for fungal access to the nutrients in the plant material that is transported by the ants to the nest [71].

The profile of the hydrolytic enzymes involved in this relationship between leaf-cutting ants and fungi has been studied. The extracts from the fungal hyphae of garden fungi exhibit a wide range of activities involving carbohydratases (pectinase, laminarinase, α-glucosidase, β-glucosidase, α-galactosidase), with the proteinase chitinase presenting the highest activity (Erthal et al., 2009).

Extracts from fungal gardens contain a wide variety of digestive enzymes, including carbohydratases (e.g., pectinase, laminarinase, and β-1,3 glucanase) and proteinases (trypsinase and chymotrypsinase) [69]. Interestingly, although the fungus (hyphal extracts) produces chitinase, the plants do not contain chitin. It has been suggested that the chitinases present in the rectal fluid of primitive genera (*Cyphomyrmex*, *Mycocepurus*, and *Myrmicocrysta*) are important in the degradation of substrates such as the carcasses of leaf-cutting ants, which can be used as a substrate for the growth of new hyphae from the symbiotic fungus. It has also been proposed that these enzymes may play an important role in the lysis of competitive funguses, many of which have cellular walls made of chitin [72].

The results from analysis of the labial glands of larvae and adults of *A. subterraneus* indicate that they secrete chitinases. Chromatographic tests to detect chitinolytic activity in the labial glands of the larvae revealed profiles similar to those observed for the glands of the workers, indicating that this enzymatic activity may have a fungal origin. Interestingly, the peak of chitinolytic activity in the middle intestine of the larvae does not coincide with the peak in the labial gland, indicating that the chitinase produced in the labial gland is not active in the middle intestine [73, 74].

In addition to chitinases, pectinases, xylanases, and cellulases derived from the fungus have been detected in the faecal fluid of *Atta colobica tonsipes* [75]. Pectinases present in the faeces of *Acromyrmex echinatior* and *Atta colombica* have been suggested to be of fungal origin [7]. Interestingly, no pectinase, xylanase, or cellulase activity was detected in *A. subterraneus* adults. However, elevated α-glycosidase activity was detected in fractions from the middle intestine and rectum of adult leaf-cutting ants, indicating the importance of this enzyme in the assimilation of glucose and, most likely, of saccharose and maltose present in the plant material [73]. Pectinases and xylanases were detected in the middle intestine of the larvae, where their only source of nutrients is the symbiotic fungus [74].

Febvay and Kermarrec (1984) suggested that digestion of the walls of the fungal cells occurs in the infrabuccal pocket of *Acromyrmex octospinosus*. It is possible that the adult garden ants regurgitate chitinases through their labial glands onto the fungal material to feed the larvae, initiating the partial digestion of fungal parts, without the adult benefiting from ingestion. The larvae can regurgitate their own secretions from their labial glands, providing the energetic needs of the adults, who receive these liquids through trophallaxis [76].

Other authors have reported that the secretions offered by the larvae originate in a liquid produced from the anal region, in a process referred to as proctodeal trophallaxis (Figure 14). The workers contact the anal region of the larvae, which may be of different sizes, and the larvae release a small transparent drop that is immediately ingested by the workers. This observation reveals that the larvae of the leaf-cutting ants play a fundamental role in the nutrient flux in the colony because, although adult individuals are incapable of feeding on the solid portions of the fungus, the larvae are able to feed on these portions, digest their walls, and transfer nutrients to the haemolymph, where they are absorbed by the Malpighian tubules, transferred to the posterior intestine, and offered to the workers, making them available to other individuals in the colony [77].

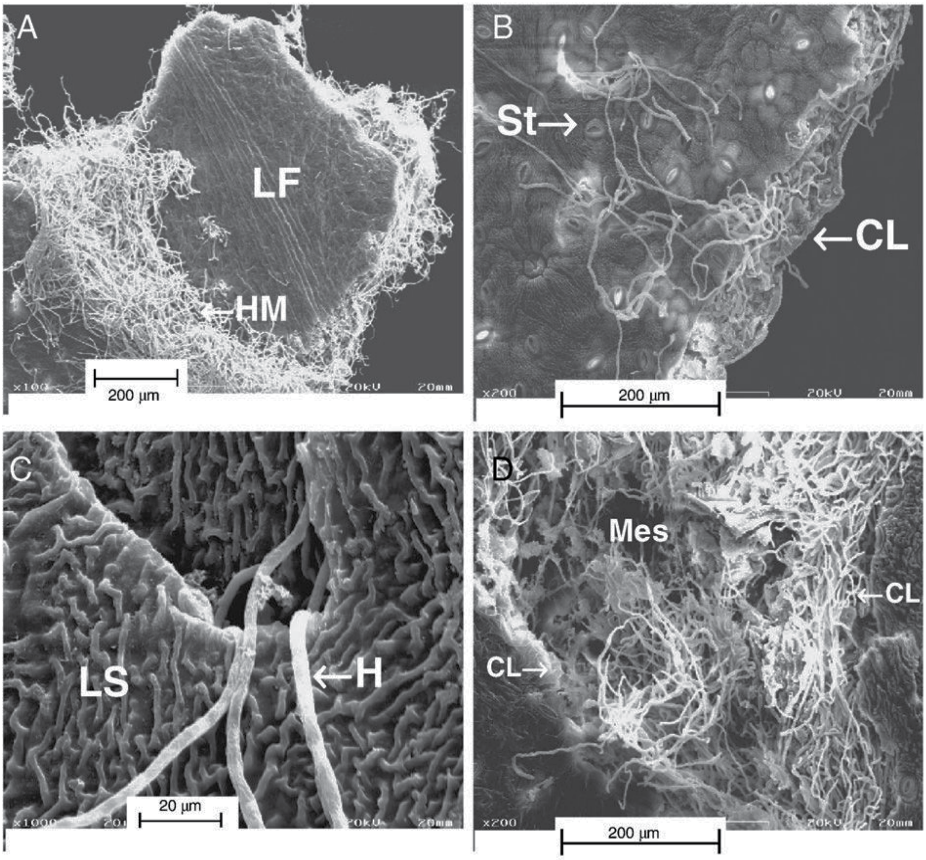


Figure 14. Colonisation of freshly cut leaves by *Leucoagaricus gongylophorus*. Panel A: LF—leaf fragment; HM—hyphal mass. Magnification: ×100. Panel B: St—stomata; CL—cut leaf edge exposing mesophyll tissue. Magnification: ×200. Panel C: H—hyphae; LS—leaf surface. Magnification: ×1000. Panel D: CL—cut leaf edge; MS—exposed mesophyll tissue. Magnification: ×200. Source: Erthal Jr. et al., 2009 [69].

Hydrolytic enzymes are directly involved in this energy transfer within the colony. Thus, studies that seek new insecticides have been conducted with an emphasis on plant extracts or pure substances that exhibit fungicidal, insecticidal, or enzyme inhibitory actions. The integrated application of these three types of functions should lead to the development of a new product with an effective control capacity. In this context, crude extracts of *Cedrela fissilis*, *Tapirira guianensis*, and *Simarouba versicolor* were evaluated and found to inhibit the activity of the pectinase enzyme present in the faecal liquid of *A. sexdens rubropilosa* [61,78]. These enzymes appear to be essential for the nutrition of the ants and the fungus in plant materials [71].

Plant extracts that inhibit enzymatic activity may be useful for the control of leaf-cutting ants and constitute a new approach with respect to methods for controlling these insects. This type of control should be evaluated further to determine the viability and effectiveness of its use in the field and confirm its suggested potential. Phytochemical analyses of active extracts are being conducted with the purpose of isolating the substances associated with enzymatic inhibition.

5. Conclusions

Data from the literature clearly demonstrate that several plants are capable of producing substances with direct action against leaf-cutting ants and/or their symbiotic fungi, such as ricinine (*Ricinus communis*; Euphorbiaceae); β-eudesmol (*Eucalyptus maculata*; Myrtaceae), the limonoid limonéxico acid (*Raulinoa echinata*; Rutaceae), sesamin and sesamoline (Sesamum indicum; Pedaliaceae), anthranilic acid, kokusaginine and dictamine (*Helietta puberula*; Rutaceae), 4.5- dimetoxicantin-6-one and 5-metoxicantin-6-one (*Simarouba versicolor*; Simaroubaceae), (-)-hinokinin and (-) kusunokin (*Virola sebifera;* Myristicaceae), among others. The active substances extracted from these plants may provide the basis for studies aimed at the synthesis of organic molecules and the development of new commercial products that are stable and show low persistence in the environment. In Brazil, these studies intensified after the establishment of restrictive policies by government entities and certifying institutions regarding the use of the active ingredients that are currently available in the market. Several molecules have already been synthesised, although they are not yet available for use by farmers; however, expectations for the use of plant-derived products in the control of leaf-cutting ants are high.

Acknowledgements

To "Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)", "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)", "Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG)" and "Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG)".

Author details

Juliana Cristina dos Santos[1*], Ronald Zanetti[1], Denilson Ferreira de Oliveira[2],
Giovanna Cardoso Gajo[2] and Dejane Santos Alves[1]

*Address all correspondence to: ciencias_biologicas@hotmail.com

1 Department of Entomology, Federal University of Lavras (Universidade Federal de Lavras - UFLA/Lavras), Minas Gerais, Brazil

2 Department of Chemistry, Federal University of Lavras (Universidade Federal de Lavras - UFLA/Lavras), Minas Gerais, Brazil

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Chapter 10

Use of Botanicals and Safer Insecticides Designed in Controlling Insects: The African Case

Patrick Kareru, Zacchaeus Kipkorir Rotich and
Esther Wamaitha Maina

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53924

1. Introduction

Insecticides are toxic substances that are used to kill or control insects. Insects pests affect humans directly by transmitting diseases or indirectly by attacking cultivated plants in farms or in storage, thus affecting food security. It is documented that the use of insecticides by man dates as far back as 1000 B.C, or earlier when burning of stone containing sulfur (brimstone) was used as a fumigant.

Substances used to kill or control insect pests can also be referred to as pesticides, though the latter word has a wider scope of application, since other non-insect pests also exist.. Insecticides in wide use are mostly synthesized organic compounds, though there are some organic compounds of plant origin referred to as "botanicals", in addition to inorganic compounds of natural and synthetic origin. Certain insecticides of synthetic, organic or inorganic origin function as insect repellents, causing little or no harm at all to the target insects. In most situations insecticides are applied by spraying or dusting onto plants and other surfaces traversed or fed upon by insects. However insecticides/pesticides of chemical origin can affect human health directly or indirectly by disrupting ecological systems that exist in rivers, lakes, oceans, streams, wetlands, forests and fields. Release of chemicals into the environment can have global impacts and there is therefore need to use safer analogues designed with safety in mind. A review of some "safe" insecticides used in Africa is presented.

Pesticides in general are toxic chemicals which adversely affect human health when mishandled. Their effects may be direct, for example, during application or when consumed in suicide bids. Also their effects may be indirect when the environment is contaminated either

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due to non-specificity of the target or when higher dosages are used either accidentally or due to ignorance. Such pitfalls may be overcome by use of "smart insecticides". The latter may be designed by incorporating a delivery system so as to release an insecticide over an extended time at a controlled rate. Such insecticides, therefore, target the intended pests without adversely affecting the human health or the environment.

One of the novel techniques in recent use is to encapsulate the insecticides within a macromolecular network. Biopolymers have in recent times been used for this purpose. For example, hydrogels of natural polymers such as sodium alignate, starch, gelatin, carboxymethyl cellulose etc, have been used for encapsulation of insecticides (Anamika et al, 2008).

The future development and use of safer pesticides in Africa will need to address safety concerns using functionalized polymers as delivery systems. Such technology will increase the efficiency of insecticides by targeting the specific pests while indirectly protecting the environment by reducing pollution and safety to end users. This will impact positively on health by controlling disease causing vectors and food security as well.

2. Genetically engineered plant insecticides

The technology of 'genetically engineered insecticides' is based on the development of plants or viruses genetically engineered to produce insect-selective toxins. This involves transferring naturally occurring poison-coding genes from microorganisms into crops. Such insecticides may be referred to as biopesticides or biological pesticides. The latter are based on pathogenic microorganisms specific to a target pest and offer an ecologically sound and effective solution to pest problems. The most commonly used biopesticides are living organisms, which are pathogenic for the pest of interest. Biopesticides fall into three major categories namely: biofungicides (*Trichoderma*), bioherbicides (*Phytophthora*) and bioinsecticides (*Bacillus thuringiensis*). Biopesticides contain a microorganism such as bacterium, fungus, virus, protozoa or alga, as the active ingredient.

The most widely known microbial insecticides are based on the bacterium *Bacillus thuringiensis* (Bt.), which is incorporated into plants to produce genetically modified (GM) crops or genetically modified organisms (GMO). Bt is a soil dwelling Gram-positive bacterium, discovered in 1901 by a Japanese biologist, Shigetane Ishiwatari. Later it was rediscovered in Germany by Ernst Berliner in flour moth caterpillars. The spores and crystalline insecticidal proteins produced by Bt have been used for insect control since 1920s (Lemaux, 2008).). In 1995 potato plants, incorporating Bt, were first introduced in the USA (Romeis et al, 2008) and by 1996 Bt maize, potato and cotton were grown. GMO technology is claimed to alleviate poverty by ensuring high incomes from insect prone cash crops such as cotton, maize or rice. Some Bt-based insecticides are often applied as liquid sprays on crops, where the insecticide is expected to be ingested by pests for it to be effective. A Bt strain, *Bacillus thuringiensis serovar israelensis*, is widely used against mosquito larvae.

Crops are genetically modified with *Bacillus thuringiensis* (Bt) so as to develop insect resistance. *B. thuringiensis* produces a diverse group of insecticidal protein toxins with narrow specificity towards different insects (Santie et al, 2011). Bt bacterium contains insecticidal protein crystal that is eaten by insects. The crystal then dissolves in the midgut of the insect. The toxin mixture is released and the proteins are cleaved into active forms. The toxins bind to the midgut cells, assembling a pore that leads to disintegration of the cells, gut paralysis and death. The Bt strains are known to have toxins specific for insects such as caterpillars, beetles, flies and mosquitoes and have little or no effect on mammals. South Africa has been reported to grow more than 85% of the countries cotton and some maize and is the only African country reported so far to grow 67% of the country's total maize production for food (James, 2007) using the Bt insecticides technology. Outside of South Africa, only Burkina Faso and Egypt allow commercial cultivation of GM crops. Accessibility of these products is, however, relatively restricted, especially in developing countries such as in Africa, due to vocal opposition to GM technology and lack of regulatory mechanisms to deploy such technology (Sentie and David, 2011). South African farmers and consumers have already shown a willingness to embrace biotechnology (cotton, maize, and soybean) resulting in improved yield or reduced cost, however, the Bt potato would be the first publicly-funded bioengineered crop to be released in Africa. Some commercially available Bt varieties and target pests include: *Bacillus thuringiensis*, var. *tenebrionis*- for control of Colorado potato beetle and elm leaf beetle larvae; var. *kurstaki* - for caterpillars; var. *israelensis* – for mosquito, black fly, and fungus gnat larvae; var. *aizawai* for wax moth larvae and various caterpillars, especially the diamondback moth caterpillar.

3. Synthesis of pesticides from plant botanicals

Plant extracts are commonly referred to as plant botanicals and are the secondary plant metabolites synthesized by the plant for protective purposes. Some of these compounds are toxic to insects. These plant compounds are called botanical pesticides, plant pesticides or simply botanicals. Many of the plant botanicals are used as insecticides both in homes, in commercial as well as in subsistence agriculture by small-scale farmers (Table 1). They may be contact, respiratory or stomach poisons. Botanicals are not very selective because they target a broad range of insect pests.

Plant insecticides act in several ways: as repellents by driving the insects away due to smell or taste, as antifeedants which cause insects on the plants to reduce their food intake and hence starve them to death; as oviposition deterrents, by preventing insects from laying egg; or as inhibitors by interfering with the life cycle of the insects.

Plant insecticides have several advantages. Among them are short life spans once applied and are not poisonous to humans and livestock. Secondly, botanicals do not harm the natural enemies of the pests, such as the lady bird beetle. They are cheap, easy to prepare and in most cases readily available and have more than one active ingredient which work synergistically making it difficult for pests to develop resistance. Figures 1-5 shows some structures of some compounds from some of the plants used.

Botanical insecticides role in insect pest management and crop protection in Africa play a minor role due to continued use of effective but 'toxic' commercial pesticides. However, the regulatory environment and public health needs should create opportunities for the use of safer botanicals in since human and animal health is paramount. Botanicals may also find use in organic food production, both in the field and in controlled environments for export to developed countries where strict pesticide levels are strictly monitored in horticultural products before export. In addition the greatest benefits from botanicals might be achieved in developing countries, where human pesticide poisonings are most prevalent. In Africa extracts of locally available plants have been traditionally used as crop protectants, when used alone or in mixtures. In fact indigenous knowledge and traditional practice can make valuable contributions to domestic food production in countries where strict enforcement of pesticide regulations is not applied.

Plant	Pests/Diseases
Neem tree	Armyworms, Stemborers, Bollworms, Leaf miners, Diamond blackmoth, Caterpillars, Storage pests(moth), Aphids, whiteflies, Leaf hoppers, Psyllids, Scales, Maize tassel, Beetle, Thrips, Weevils and Flour beetle
Garlic/Onions	Caterpillars, Cabbage worms, Aphids
Stinging nettle	Caterpillars
Tithonia diversifolia	Caterpillars, aphids
Spider weed	Aphids
Aloe spp. Ash	Storage moths, Storage beetles
Chilies + Hot pepper	Diamond blackmoth, Stemborers, Bollworms, Cutworms, weevils, Aphids, Beetles
Tobacco	Stemborers, Cutworms, Caterpillars, Grain weevils
Pyrethrum + Mexican marigold	Caterpillars, Aphids, bugs, Beetles
Chilies + Mexican marigold	Armyworms, Stemborers, Bollworms, Cutworms, Leaf miner, Diamond blackmoth, caterpillars, Aphids

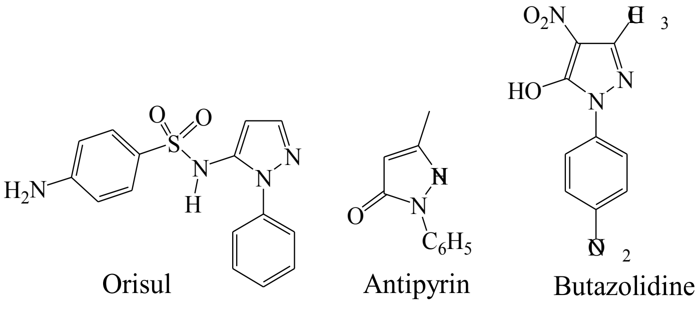
Table 1. Some plants traditionally used to control crop pests and diseases in Kenya (Mureithi J G, 2005).

Studies in some Africa countries suggest that extracts of locally available plants can be effective as crop protectants (Isman, 2008). Among the botanicals used are natural pyrethrins, the neem extract, *Azadirachta indica* (A. Juss), *Khaya senegalensis* against cotton bollworm (in Benin) and extracts from marigold against bruchid beetles from cowpeas in storage in Uganda (Kawuki et al, 2005), among others. M. Mugisha-Kamatenesi et al (2008) have documented a survey of botanical extracts used as insecticides within the Victoria Basin. The study has demonstrated that usage of botanical pesticides pest management by the subsistence farmers is normal around Lake Victoria. Among the plants used are *Capsicum frutescens, Tagetes* spp, *Nicotiana tabacum*, *Cypressus spp.*, *Tephrosia vogelii*, *Azadirachta indica*, *Musa spp* Eucalyptus spp and *Carica papaya*. In Benin, West Africa, the bushmint, *Hyptis suaveolens* extract has been used for the control of pink stalk borer, *Sesamia calamistis* on maize. Also, botanical insecticides have tried for the protection of cowpeas in Ghana (Abatania et al, 2010). Ogunsina et al (2010) has also investigated plant extracts from *Lantana camara* (Verbenaceae), African nutmeg [*Monodora myristica* (Gaerth) Dunal] and Enuopiri [*Euphorbia lateriflora*, Schum and Thonner] against bean weevil *Callosobruchus maculatus* (F.) *and* maize weevil, *Sitophilus zeamais)* Motsch. The overall results showed that bean weevil was much more susceptible to all the extracts than maize weevil.

Some of the reasons for the poor market penetration of botanical insecticides in developing countries are their relatively slow action, variable efficacy, lack of persistence and inconsistent availability (Isman, 2008). But plant botanical extracts may be used as a source of lead compounds in the synthesis of effective and safe insecticides. An example is the synthesis of insecticides from nitrophenols of plant or synthetic origin (Ju and Parales, 2010), Figure 5. One of the approaches is to prospect for insecticides of plant origin. Synthesis of the botanical analogues guarantees higher yields of the insecticide that ordinarily may not be obtained when extracted from the plant parts. The chemical synthesis of botanical insecticide analogues has long been achieved (Benner, 1993). Lu et al (2007), reported synthesis of twelve 1,5-diphenyl-1-pentanone analogues similar to those derived from *Stellera chanaejasme* (Figure 6). These compounds were found to be effective against *A. gossypii* Glov.

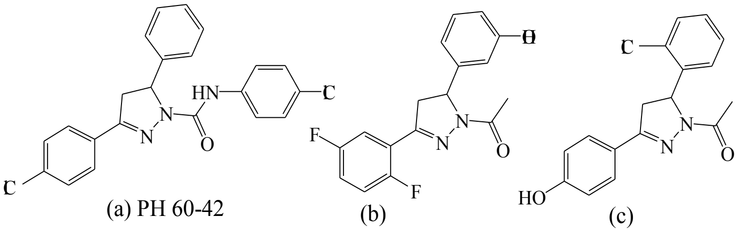
Recent studies have resulted in synthesis of novel esters with insecticidal activity using plant lead compounds (Ji et al, 2011). Gao et al (2012) has demonstrated syntheses twenty three new fraxinellone-based hydrazone derivatives from fraxinellone. Flaxinellone (Figure 5) is a compound from *Dictamnus dasycarpus* Turcz. dried root bark.

Modification of biologically active pyrazoline derivatives of plant origin have produced 1,3,5-trisubstituted-2-pyrazoline derivatives, thought to have insecticidal activity (Kareru and Rotich, 2012). Some of these compounds have the structures below.



Scheme 1. Chemical Structures of Biologically Active Pyrazoline Derivatives

Synthesis and biological activities of various 1,3,5-trisubstituted-2-pyrazoline derivatives have been reported in literature. According to Deng *et al.* (2012), among the existing various pyrazoline type derivatives, 2-pyrazoline has been identified as one of the most promising scaffolds. In the area of medicinal chemistry, 2-pyrazoline derivatives have been found to display anti-cancer and anti-inflammatory activity. 2-Pyrazoline type derivatives such as (code: PH 60-42) shown in Figure 1.(a) have also been known to possess insecticidal activity since 1970s though it was not commercially exploited due to their environmental properties (Deng *et al.,* 2012). Figure 1.(b) and (c) shows some of the examples of biologically active 2-pyrazoline derivatives used in the field of medicine in the treatment of cancer and Alzheimer disease respectively (Gokhan-Kelekci *et al.,* 2007).



(a) PH 60-42 (b) (c)

Figure 1. Examples of biologically active 2-pyrazoline derivatives used in the field of medicine

Synthesis of biologically active compounds from the botanicals lead compounds have advantages of being produced in large amounts unlike the yields obtained by from plant parts using the solvent. Synthesis of insecticides using plant lead compounds is an ongoing research in our laboratories. Toxicity of synthesized compounds will be determined to assess efficacy and safety.

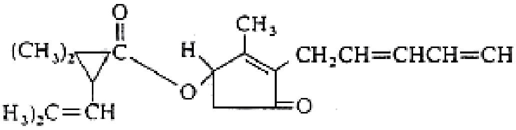


Figure 2. Pyrethrin I structure from Pyrethrum

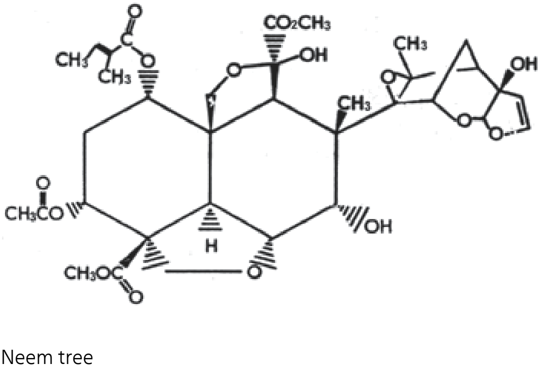


Figure 3. Azadirachtin from Neem tree

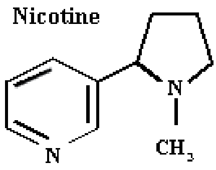


Figure 4. Nicotine structure (from Tobacco)

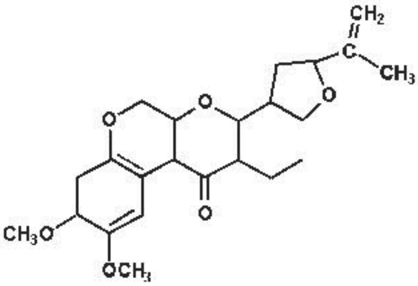


Figure 5. Rotenone molecular structure

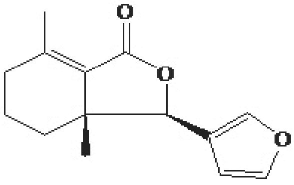


Figure 6. Fraxinellone from *Dictamnus dasycarpus* Turcz. dried root bark

4. Stabilization of pyrethrin insecticides with botanical oils

Pyrethrins are the six esters produced in the Chrysanthemum plant, *Chrysanthemum cinerariaefolium*. The esters are found in high concentration within flower structures known as achenes which are located in the flower head of the Chrysanthemum plant. Pyrethrins have a toxic effect in insects when they penetrate the cuticle and reach the nervous system. Pyrethrins bind to sodium channels that occur along the length of nerve cells and are responsible for nerve signal transmission along the length of the nerve cell. When pyrethrins bind to sodium channels, a loss of function of the nerve cell most often leads to death after pyrethrins exposure. Since insects have evolved detoxification mechanisms to pyrethrins, synergists are added to circumvent the detoxification mechanism of insects. Synergists are chemicals which directly increase the toxicity of insecticides. Usually, the synergist, piperonyl butoxide, is added and a lower concentration of pyrethrins is required to achieve insect control.

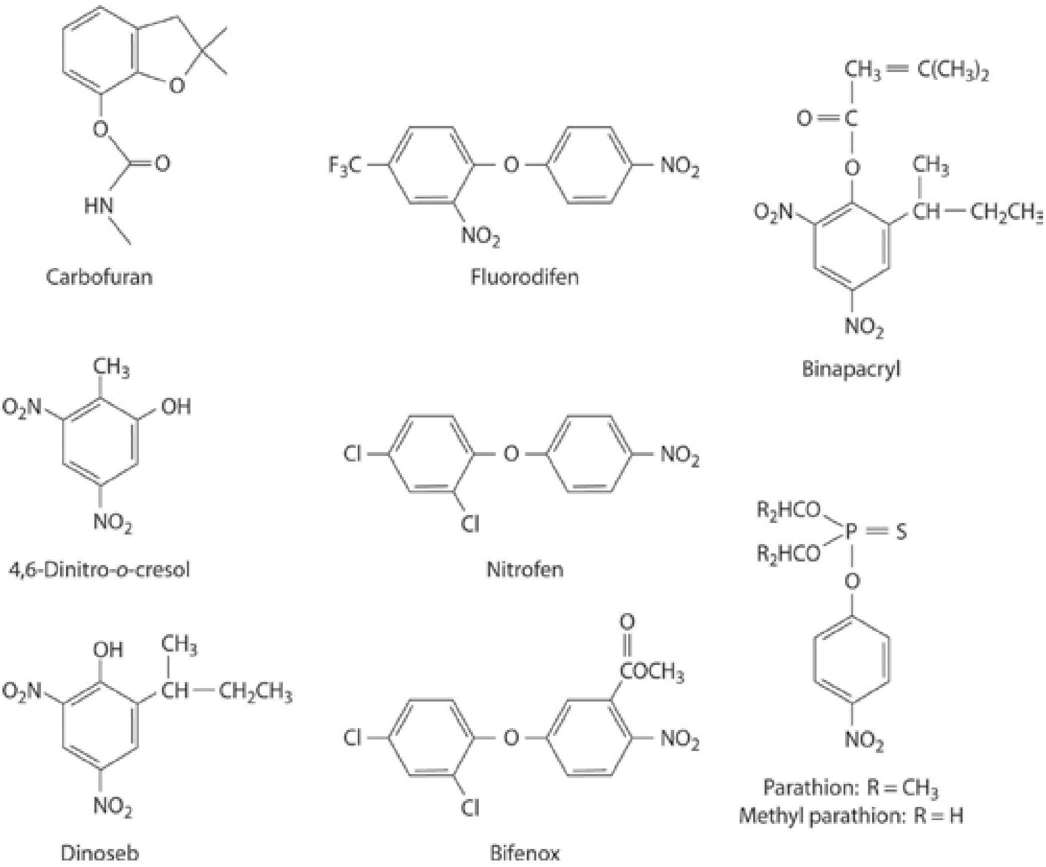
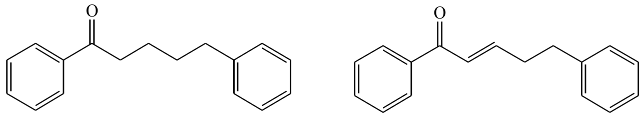


Figure 7. Pesticides synthesized from nitrophenols (Ju et al, 2010)



1,5-diphenyl-1-pentanone 1,5-diphenyl-2-penten-1-one

Figure 8. Lead Compounds from *Stellera chamaejasme* L

Mostly pyrethrins may be used as a contact insecticide for household insects such as flies, mosquitoes or applied as aerosols or space sprays. Some formulations can be applied to agricultural crops and due to their safety, pyrethrum extracts are used extensively in areas such as in households, industrial sanitation and in warehouses to protect stored food. They have little or no hazard to birds and mammals, are relatively nonhazardous to honey bees but toxic to fish. Synthetic analogues are referred to as pyrothroids and have similar insecticidal properties. Both types have minimal residual period in the environment. Pyrethrum pyrethrins and synthetic pyrethroids are stable for a limited time period because they are subject to photochemical degradation by Ultraviolet light. To counteract photochemical degradation chemical additives are added to increase their potency and enhance the mode of action. The addition of synergists causes these insecticides to be more toxic to insects, mammals and humans as well (Berger-Preiss et al, 1997).

Recent studies demonstrated that stabilized natural pyrethrins have shown contact toxicity against adult maize weevils (*Sitophilus zeamais*) in a time dependent manner. Natural pyrethrum extract was stabilized against ultraviolet (UV) light by blending with fixed oils extracted from *Azadirachta indica* A. Juss (neem tree), *Thevetia peruviana* (yellow oleander) and *Gossypium hirsutum* L. (cotton) seeds. In the study, the fixed seed oils enhanced the stabilization of the natural pyrethrum insecticide (Wanyika et al, 2009). The results indicated that natural pyrethrum extract blended with cottonseed oil exhibited the highest mean mortality against the maize weevils. This implied that cottonseed oil had the highest stabilization effect on natural pyrethrum among the botanical oils used. On the other hand the UV stabilization due to neem oil generally increased with the concentration in the insecticide blend. Oleander oil was found to have moderate stabilization effect which decreased with the amount of oil added to the insecticide. It was noted, however, that synergism contributed by vegetable oils in bio-pesticide formulations might have contributed to the enhanced activity of the pyrethrum blends investigated. Pyrethrum extracts stabilized with cotton and neem oils showed a marked increase in bio-efficacy against the maize weevils while the yellow oleander seed oil had a moderate stabilizing effect on the pyrethrum insecticide. Cotton seed oil, however, had the highest stabilizing effect on the pyrethrum extract exposed to UV light at 366 nm compared to the other botanical oils used and the control.

5. Acaricidal effect of bee propolis extracts

Propolis (bee glue) is a strongly adhesive resinous bee-hive product collected by honeybees *(Apis mellifera* Linnaeus) from leaf buds and cracks in the bark of various plants and is used in the hives to exclude draughts, to protect against external invaders and to mummify their carcasses. It typically consists of waxes, resins, water, inorganics, phenolics and essential oils. Chemical analysis of bee propolis from Europe is reported to contain various phytochemicals: phenolic acids and esters, flavanones, flavones and flavonols, cinnamic acids, phenylated p-coumaic acids and furofuran lignans, among others (Bankova V, 2005; Bankova et al, 2002).

A number of researchers have reported insecticidal effect of bee propolis. Solvent extracts of propolis samples from Brazil and Bulgaria exhibited leishmanicidal activity against different species of *Leishmania* (Gerzia et al, 2007). In Nigeria, Osipitan et al (2010) tested propolis ethanolic extracts against the larger grain borer, *Prostephanus truncates* (Horn) in maize grains. A reduction of the borer population in maize was observed. Interestingly, pesticides commonly used in agriculture were detected in honey and propolis samples (Lucia et al, 2011) in Uruguay.

Recently bee propolis extracts have been reported to have acaricidal effect on red spider mites (*Tetranychus spp*.), which attack tomatoes, (Kareru and Wamaitha, 2012, unpublished work).

Figure 9. The four stages of a red spider mite life cycle (egg, larva, nymph and adult). Source: http://www.biobee.com

Tomato is a vegetable crop grown worldwide and its selection and preference as a crop is due to its nutritional value and economic importance. Crop production losses to pests are estimated to exceed 35% annually. Red spider mites (*Tetranychus* species) are polyphagous, parenchyma cell feeding pests and have a serious economic impact on many crops, especially tomatoes. Synthetic pesticides used for control of red spider mites end up in the environment where they may affect non-target species, have adverse effects on wildlife, pollute soil and water and in addition are usually very expensive and beyond the reach of resource of poor African farmers.

Compounds present in propolis can provide potential alternative in the place of currently used insect pest control agents because they constitute a rich source of bioactive chemicals and may act in many way on various types of pest complex. They also have no or little harmful effects on non target organisms such as pollinators, natural enemies and are biodegradable.

Both ethanolic and ethyl acetate extracts of bee propolis acted on red spider mites in a concentration and time dependent manner. The activity of ethanolic extracts at concentrations of 75 and 100 mg/ml was not significantly different with that of the positive control used. Ethanolic and ethyl acetate extracts acted on tomato red spider mites in a concentration and time dependent manner, and had no significant differences in activity.

Bee propolis extracts could thus be used as a safe insecticide in the control of red spider mites. However, further research need to be done on its potential on other life stages of red spider mites and other common tomato pests. The insecticidal activity was thought to be due to bioactive phytochemicals of plant origin ingested by the bees during pollination.

6. Conclusion

While development of safer insecticides is a noble idea in Africa, safety concerns are paramount. Widespread misuse of pesticides, some of which are banned, affects farmers' health with fatal consequences. Due to ignorance on their part, farmers may keep pesticides near food stores where seepage of food is inevitable; over-spray food crops, inhalation and skin contact while in use, have adverse effects to their health. And although an alternative to pesticides use could be achieved through organic farming and integrated pest management practices, the future lies in investing in technology which will afford safe application of insecticides.

Author details

Patrick Kareru[1*], Zacchaeus Kipkorir Rotich[2] and Esther Wamaitha Maina[1]

*Address all correspondence to: pgkareru@yahoo.com

1 Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

2 Presbyterian Church of East Africa University, Kikuyu, Kenya

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Section 4

Insecticides and Human Health

Insecticide Residuality of Mexican Populations Occupationally Exposed

María-Lourdes Aldana-Madrid,
María-Isabel Silveira-Gramont,
Fabiola-Gabriela Zuno-Floriano and
Guillermo Rodríguez-Olibarría

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53980

1. Introduction

In this chapter the information generated by two research projects conducted in Sonora, Mexico (2003-2010) with the objective to assess the presence of insecticide residues (organochlorine and organophosphates) in corporal fluids of two population groups that live and work in agricultural areas (agricultural workers) are described. The presence of residues in body fluids (blood, urine, semen and breast milk) will be related to social, labor, environmental factors, health status (obtained from surveys, and clinical histories), and biochemical and biological indicators, with the purpose of elucidating the degree and persistence of the exposure to these insecticides.

In the countries in development as Mexico, the handling of toxic compounds as pesticides is inadequate; it is possible that people can be exposed to higher concentrations than that allowed by the maximum limits (LMP), as they demonstrate it studies carried out with children in San Luis Potosí state [1], in some endemic areas of malaria in Mexico like Quintana Roo [2], Chiapas and Oaxaca [3-5], and in labor exposed people of Sonora state [6,7]. This evidence suggests that the populations that work in agricultural fields, as well as those that inhabit the surrounding area could have higher exposure risk, as well as chronic contamination that the populations with a basal exposure.

The state of Sonora is amongst the regions of Mexico with more pesticide use; it is calculated that 80% of the total applied in the country is for the production of grains and export vegetables [8]. There are not reliable statistics of pesticide intoxications in rural areas, and there are not epidemiologic studies to detect chronic effects of the pesticides; those should exist at least for the agricultural journeymen and for vulnerable groups, since they lack elementary protection, and don’t have the correct information about pesticide toxicity.

The exposure doses can be small but persistent, causing chronic health problems [8]. DDT (bis[4-chlorophenyl]-1,1,1-trichloroethane, also called dichlorodiphenyl trichloroethane); was first used to protect military areas and personnel against malaria, typhus, and other vector-terminal diseases [9]. In Mesoamerica (Mexico, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua and Panama) DDT was used until the year 2000; Mexico and Nicaragua being the last nations that applied the insecticide in agriculture and for the control of malaria (the amount used for Mexico is approximately 69,545 tons between 1957 and 2000) [10].

Technical-grade DDT contains 65-80% p,p′-DDT, 15-21% o,p′-DDT, and up to 4% p,p'-DDD (bis[4-chlorophenyl]-1,1,-dichloroethane)[11]. When sprayed, DDT can drift, sometimes for long distances. In the soil, the compound can evaporate or attach to wind-blown dust. In the environment, DDT breaks down to p,p′-DDE (bis[4-chlorophenyl]-1,1-dichloroethene) [12], an extremely stable compound that resists further environmental breakdown or metabolism by organism; DDE is the form usually found in human tissue in the highest concentration, especially in areas where there has been no recent use of the parent compound [9]. DDT and DDE can also be transferred from the placenta and breast milk to fetuses and infants. Although some ingested DDT is converted to DDA (bis[4-chlorophenyl]-acetic acid) and excreted, any non-metabolized DDT and any DDE produced is stored in fat, as is all absorbed DDE, which cannot be metabolized. DDT and DDE are highly soluble in lipid; their concentrations are much higher in human adipose tissues (about 65% fat) than in breast milk (2.5-4% fat), and higher in breast milk than in blood or serum (1% fat) [13]. DDT and DDE concentrations increase with age [14].

The use of DDT in Central and South America, Mexico, Africa, and some Asian countries where this has been used for vector control in the past 5-10 years, DDT concentrations in human tissues remain high. For example, in Mexico, the total DDT concentration in breast milk fat was 5.7 μg/g in 1994-95 and 4.7 μg/g in 1997-98 [15]. Others Mexican data where workers used DDT to control mosquitoes, have very high DDT concentrations. Mexican data revealed that the geometric mean of total DDT was 104.48 μg/g in adipose tissue of 40 DDT sprayers in 1996 [16]; however in Finland, USA, and Canada, the value was less than 1 μg/g in adipose tissue in the general population [17]. In another Mexican study, the serum concentration of p,p′-DDE was much higher in DDT sprayers (188 μg/L) than in children (87 μg/L); also in adults (61 μg/L) who lived in sprayed houses, but were not otherwise exposed to DDT [3].

The organophosphate insecticides have the advantage of low environmental persistence over the chlorinated pesticide compounds. However, studies carried out in individuals with exposure to insecticides in Mexico, and other countries, associate the pesticide exposure with adverse health effects, as much in the humans as in experimental animals. These damages can be evident by the presence of certain biochemical indicators in the different biological fluids, and for the detection of morphological, histological, and molecular changes in target organs [2-4,6,7].

The degree of pesticide contamination depends on several factors, such as the formulation of the pesticide, the active ingredient, the time of exposure, the direct or indirect contact, the quantity used, the pesticides mixtures, the climate and season of the year when it's applied, and the person's age, amongst others [18, 19]. There are environmental indicators, health indicators, and other elements that help determine the exposure risk, such as the person residence and occupational history, the clinical history, as well as the presence of the pesticides studied in drinking water, in the ground, in the atmosphere, and in the fresh or processed foods in the region where the studied populations inhabit. The exposure can be increased with the daily time dedicated to the activity, as well as the years of work, the exposure form, the use of protective gear, and/or the physical proximity of the housing to agricultural fields [20-22].

Due to the previously mentioned situations it was considered important to study the degree of exposure to pesticides on workers in those agricultural areas that also reside in their proximity.

2. Materials and methods

2.1. Site description

Sonora State territory has 179,355 km^2 and it is located in Sierra Madre Occidental; geographically it is north 32° 29′, to south 26° 18′ of north latitude, to east 108° 25', to west 115° 03'. The weather in coastal of Sonora is dry. The average annual temperature is around 22°C, being the average maximum 38°C (June and July) and the average minimum temperature is 5°C (January). Only 7% of the land is appropriate for agricultural use and ninety five percent of this area is irrigated. In July and August the rain reaches 450 mm. The weather in Sonora State restricts the agricultural activities. However, in villages Yaqui and Mayo, valleys of Hermosillo, Caborca and Guaymas the major crops with irrigation are wheat, cotton, safflower, watermelon, sesame, garbanzo, sorghum, corn and vine [23]. The agriculture in the south of Sonora is based on 90% of crops such as corn, wheat, oleaginous and cotton [24].

2.2. Population study

Group 1, field workers. The municipal headboards included in this group are localized in the following coordinates: Obregon city 27º 29' north latitude and 109º39' west latitude with a height of 10 m above sea level and Navojoa 27º 05' north latitude and 109º39' west latitude with 40 m above sea level [23].

The town council Cajeme has a population of 175,177 men, from this population, 6,983 live in Yaqui town. In Huatabampo there are 38,563 men, specifically in "Jupare" (1,026 men). Navojoa has a population of 69,341 from this population, 445 men live in 5 de Junio cooperative [25]. In this study participated 37 men from Yaqui town, 19 from "Jupare" and 21 from "5 de Junio" cooperative, this is 0.53%, 1.85% and 4.7% of total population of men, respectively.

2.3. Group 2, nursing mothers

The women that participated in this study were located at Pesqueira community. The community is located between 28 and 30º parallel north latitude in Sonora, Mexico [23]. The weather in this region is dry with rain in summer [23]. The women are dedicated to cultivating and packing table grape. When this study was conducted the population in Pesqueira was 3,648 residents; 47.8% women, from this percentage only 10% were in reproductive age [25]. In the Health center there was a record of 20% women included in the breastfeeding program. However, 26% women in reproductive age (exposed or working with pesticides) participated in the present study, being 1.4% of total population. It is probably that permanent residents of agricultural areas are chronically exposed to chemical residues through wind, drinking water and even clothing from field workers.

2.4. Participation and surveys

Men and women (nursing period) that voluntarily participated in this study, signed a format according to the norms of Mexican Secretary of Health (SS). Participants filled up a survey; they provided demographic data (age, marital status, residence, residence time) and also information related to work history, pesticides exposure, clinical history, issues related to sexuality, pathology and drugs addiction (alcohol, tobacco, cocaine and marihuana, among others). People with drugs addictions or health issues that could have influence with the biochemical determinations of the body fluids were excluded from this research.

2.5. Blood, urine and semen sampling

Blood, urine and semen samples were collected from members of group 1. For hematic biometry analysis, blood chemistry and biochemical indicators, samples were taken with empty stomach. Blood samples were taken by venous puncture and collected in to vacuum tubes (Vacutainer^{MR}) with and without anticoagulant. Once blood was coagulated, it was centrifuged and supernant was transferred in to a new tube for analysis.

Urine samples were collected in a sterile container and kept at 4 °C until analysis. Semen samples were collected in sterile container (including a code, date and time when were collected) by the participant at home. Samples were analyzed no more than 24 h after sampling.

2.5.1. Breast milk sampling

Breast milk was collected either manually or with a breast milk collector in to a 50 mL conical glass tubes (wrapped with aluminum foil). Samples were kept at -20ºC until analysis.

2.5.2. Blood analysis

Blood samples were analyzed in laboratory of General Hospital SS in Cd. Obregon and the laboratory of General Hospital of Navojoa. Blood analysis included hematic biometry using an analyzer Sysmex K-4500, blood chemistry test (glucose, urea, creatinine, uric acid, cholesterol and triglycerides) and total proteins (albumin and globulin) using an analyzer HITACHI 911 and also determination of enzymes in serum such as levels of plasma cholinesterase (Randox^{MR}), alkaline phosphatase (Roche^{MR}), transaminases, and superoxide dismutase (SOD, Randox^{MR}).

2.5.3. Urine analysis

Urine was analyzed by two types of analysis; biochemical and microscopic analysis. Biochemical analysis included glucose, proteins, bilirubin, kenotic bodies, urobilinogen (combo test-10). The macroscopic analysis included number of bacteria, erythrocytes, leucocytes, crystals, epithelial cells, etc.

2.5.4. Semen analysis

Analysis of semen was carried out by using international standardized techniques [26]. Macroscopic and microscopic analyses were included; in the first one liquefaction, aspect, viscosity, pH and volume were determined. In the microscopic analysis motility, viability, presence of leucocytes, erythrocytes, germinal cells, dendrites, agglutination (specific and unspecific), number of spermatozoa and morphology were determined.

2.5.5. Insecticides extraction

Blood, urine and semen. Samples were analyzed following the methodology proposed in [27]. Briefly, 0.5 mL of sample were taken and added 4 mL hexane; the mixture was shaken for 15 seconds and then centrifuged for 2.30 minutes at 2500 rpm. The supernant was transferred to a tube and added 1 mL of 5% K_2CO_3 and 4 mL hexane. Centrifuged for 2.30 minutes at 2500 rpm, supernant was transferred to a tube and evaporated to dryness. Extract was dissolved with 100 μL hexane and analyzed by gas chromatography. *Breast milk* was analyzed using a matrix solid-phase dispersion technique [28].

2.6. Insecticides residues analyses in body fluids

The analytical standards were from Chem Service (West Chester, PA). Quantitation of insecticides was by comparison of five –point calibration curve. The detection ranges used for calibration curves were 50-0.1 μg/L. The average percent recoveries for organochlorine pesticides were p,p'-DDD 95%, p,p'-DDE 98% and p,p'DDT 105%, for organophosphates were diazinon 99%, clorpyrifos 91%, malathion 106% and parathion 92%.

Quantitative analyses were performed by gas chromatography (GC) using a Varian model CP-3800 equipped with an electron capture detector (ECD)(USA). The insecticides were separated using VA-1701 (Varian, 30 m x 0.25 mm) capillary column. The injection volume was

1 μL. Nitrogen (purity 99.999 %) was used as the carrier gas at a flow of 1.5 mL/min. The injector temperature was 180°C and the detector temperature was 300°C. The temperature program was as follows: initial temperature 220°C, increasing temperature at 9°C min^{-1} until the final temperature of 300°C was reached. Data was analyzed using a program Star Chromatography Workstation 5.51.

2.7. Design and analysis of the studies

The design of the study 1 was of the type "Case/Control", where 77 men integrated case group and 17 the control group. Participants of both groups were the same ages (18 to 70 years old). Control group did not have evidence of pesticides exposure. The sample size for the cases group represented approximately 5% of the total male population's in the range of ages selected in the study.

The study 2 was integrated by 39 nursing mothers between 17 and 39 years old, selected randomly among those that accepted to participate. All the other characteristics were similar to study 1.

The nominal data were analyzed by group (in study 1) using contingency tables and Chi-square statistics. Continuous numeric data (age, height, weight, etc.) were reported with descriptive statistics (minimum, maximum, mean, median, and standard deviation). In study 1 there were analysis of variance comparing groups (cases *vs* controls) for the numeric and nominal variables. Several exposure indicators (reported by the literature, erythrocytes, VCM, and RDW) were analyzed by linear regression versus time of exposure, and pesticide amount on a particular body fluid. Also, multiple correlation coefficients were estimated between several biological indicators.

For the study 2 beside of the descriptive statistics, some relationships were evaluated such as the use of protective gear, age, number of years of exposure, children's number, among others. The pesticide residues in breast milk were compared with the maximum residual limits stated by international organizations.

3. Results and discussion

Based on the data obtained in the present study, it was observed that the case group had similar demographic characteristics to the control group but the last one without any pesticide exposure.

3.1. Description of the population based on the surveys and clinical history

Group 1. Field workers. 94 personal surveys were made from which 68% provided data related to medical history. Only 71% of the participants provided blood samples, 69% urine samples and 46% seminal fluid samples. A small number of medical histories and samples were obtained since the participants had the liberty to leave the study at any time.

In the case group, a total of 77 men participated; the majority maintained contact with organophosphate pesticides; the average age of this group was 40 years and 11 years of residency in the site of the study with a maximum of 45 years. The average work years with pesticide exposure was 28, with a maximum of 50 years working in agriculture. Based on data from work history a 62% of the cases had contact with pesticides; from this percentage 43% applied them, 27% works in the places where they were applied and 30% works where they are applied. Only 18% of the field workers uses protection while applying pesticides (like gloves, special clothing for welding fumes, paint fumes o foundry fumes). These results suggest that field workers are chronically exposed to pesticides due to few safety precautions are taken to handle them. There for, it is of importance that the field workers receive training to be aware of the possible health issues related to pesticides exposure.

In the case group besides being in contact with pesticides during work activities they are also in their place of residency; considering this background, the time of exposure is 16 years in average and 65% of them apply insecticides in their homes; 27% are applied with an annual frequency, 23% are applied semiannually, while 10% every 3 months and 8% monthly.

Some factors can exacerbate the toxicological effects caused by pesticide exposure, such as the consumption of alcohol and drugs. The present study found that 69% of the individuals of the case group consume alcohol with a monthly frequency, 16% consume less than 5 cigarettes daily, 8% and 6% less than 20 cigarettes and the rest only 1 cigarette, 4% consumes cocaine, none of the cases consumes marihuana, nor intravenous drugs. Erection and ejaculation problems (4%) were found in case group. Case and control groups had problems having children (6%). Unlike the control group, the case group presented sexual transmission diseases; around 4% had gonorrhea. Both groups have children with congenital health problems (approximately 6%).

Some of the reported symptoms in the case group were cramps (61%), tiredness and weakness (53%), blurred vision (45%), sweating (45%), tearing (43%), nervousness (38%), dizziness (37%) and tingling in the extremities (37%). According to literature it can be considered that pesticide intoxication is nonspecific and produce the subclinical symptoms identified in the present study in addition to anorexia, insomnia, digestive alterations and itching of skin and mucous [11]. Mostly the symptoms caused by pesticides exposure are diagnosed as common cold or flu [29]. This symptomatology is not produced at the same time because every chemical product acts in a different way and will differ in each of the persons with a chronic exposure. In the present study, during the physical auscultation, the average weight and height in the case group and control group was 82 and 81 kg and 1.72 m, respectively. The vital signs were normal for both groups; 70 and 80 pulsations/min (normal value 70-80 pulsations/min), respiratory rate 20 breaths/min (normal 12-20 breaths /min) and blood pressure 120/180 (normal 120/180).

Group 2. Nursing women, description based on surveys. A total of 51 surveys were made to nursing women, 79% of them have been living in this community for more than five years. The average age was 24 years while the median was 23. The highest age was 39 and the lowest 16. The average body weight of women was 82 kg (±15.9), with an average height of 1.72 m (±0.07). The average number of children was three, 98% were married (including the ones that live in free union) and only 2% were single (this includes also the ones that are divorced). The 72% of the women were housewives, but 92% of them mentioned to have worked in agriculture. The 77% of the participants were in contact with pesticides; 69% applied in more than one occasion and only 34% used protective clothing while applying (gloves, special clothing for their work and mask).

The 53% applied pesticides in their home; 38% with an annual application, 43% monthly and 19% weekly. Insecticides applied at home were pyrethroids (20%), and organophosphates (14%).

According to the literature, intoxication by pesticides is nonspecific and produce symptoms like: excitability, tremors, sweating, tiredness, dizziness, headache and convulsions; in women they can also cause a decrease in the duration of breastfeeding [11]. The symptoms present in the participants of this study were fatigue (70%), headache (62%) and perspiration (46%). Around 76% of women (39 of 51) agreed to donate breast milk, 14% (7 of 51) of the women decided to retire and not to collaborate more in the research and the remaining 10% (5 of 51), were not producing the necessary amount for analysis.

3.2. Blood analysis

Significant differences were observed between both groups (case and control) regarding the number of erythrocytes, mean corpuscular hemoglobin concentration (MCHC), and red blood distribution width (RDW); these values were lower for the case group (Table 1). Regarding the obtained results in the chemical blood analysis, both groups presented levels within the normal values for the measured biochemical indicators. However, it is important to mention that statistical differences were observed between the groups with respect to the concentration of total protein, albumin, alkaline phosphatase, glutamic oxaloacetic transaminase (SGOT or AST) and glutamic pyruvic transaminase (SGPT or ALT); the found levels in the case group were superior to control. In a study performed with pesticide factory workers exposed to carbamates, organophosphates and organochlorines superior values were observed in total proteins [30]. However, in studies conducted with experimental animals, total protein values were not altered by the presence of pyrethroid such as cypermethrin, but the albumin levels decreased at the fifth day of intoxication [31]. Some studies performed to determine the influence of pesticide residue on biochemical indicators have reported that glucose levels increased after expose experimental animals to malathion (20 μg/mL) and after few hours levels went back to normal [32]. On the other hand, it has been reported that cholesterol and triglycerides levels are inhibited after applying a daily dose of cypermethrin (a pyrethroid insecticide) to Wistar rats [31]. A study referring to the toxicological effect of polychlorinated biphenyls (PCB's) in fish, reported that PCB's caused lipid peroxidation, increased cholesterol levels in serum and in some species caused hepatic toxicity and hypertension [33]. Recent studies about the indiscriminate use of pesticides in Tasmania, Australia, have reported effects in the health of its habitants (obesity, hypertension and high cholesterol levels) [34]. Researchers have confirmed that acetylcholinesterase is an indicator of damage by organophosphate and carbamate pesticides [35], in this study the case group mentioned having contact with this substances and the levels of cholinesterase were below normal values. In previous research it was observed that chronic exposure to organophosphate insecticides is related to an increase of catalase, superoxide dismutase and glutathione peroxidase [36]. A study performed in the South India, related to the effect of pesticides on SOD, observed an increase in the levels of this enzyme parallel to the severity of the poisoning with organophosphates [37].

Analyses	**Case group**	**Control group**	**Normal levels***
Leucocytes *(cells/µL)*	6,966.20	6,664.71	5,000 – 10,000
Erythrocytes *(millions of cells/µL)*[1]	4.96	5.13	4.6 - 6.2
Hemoglobin *(g/dL)*	14.83	15.24	13.5 - 18
Mean corpuscular hemoglobin concentration (MCHC) *(%)*[2]	33.06	33.52	32 - 36
RDW *(fL)*[3]	44.39	44.62	35 – 55

[1] $p<0.06$, [2] $p<0.04$, [3] $p<0.009$.

* [50].

Table 1. Results of hematic biometry analyses conducted on men exposed to pesticides from Sonora, Mexico

Analyses	**Case group**	**Control group**	**Normal levels**
Serum glucose *(mg/dL)*	105.38	102	55 - 115
Serum urea *(mg/dL)*	27	27	10 - 50
Creatinin *(mg/dL)*	0.86	0.86	0.7 - 1.2
Cholesterol *(mg/dL)*	195.40	180.05	< 200
Triglycerides *(mg/dL)*	137	131.29	< 150
Total proteins *(g/dL)*[1]	7.95	7.5	6.4 - 8.3
Albumin *(g/dL)*[2]	4.87	4.51	3.5 - 5
Globulin *(g/dL)*	3.09	2.98	2.3 - 3.5
Relation Albumin/Globulin	1.69	1.55	2.5
Total bilirrubin *(mg/dL)*[3]	0.67	0.93	< 1.1
Alkaline phosphatase *(U/L)*[4]	106.20	80.31	40 - 129
Cholinesterase *(U/L)*[5]	968-3940	3,382-8,108	4,300-10,500
Dismutase superoxide *(U/mL)*	273.38	275.08	164 - 240

[1] $p<0.03$, [2] $p<0.0002$, [3] $p<0.039$, [4] $p<0.0013$, [5] $p<0.026$.

Table 2. Levels of blood chemistry test conducted on men exposed to pesticides from Sonora, Mexico

3.3. Urine analysis

The results of the testing performed on urine for both groups were very similar and were within the normal values, it can be indicate that no abnormalities were observed in the corporal fluid that can be attributed to pesticide exposure. Besides, no statistical differences were observed amongst the study group (Table 3).

Analyses	Case group	Control group
Leucocytes *(cells/field)*	2	2
Uric acid crystals	53.33% (poor)	25% (moderate)
Calcium oxalate crystals	20% (abundant) 26.67% (moderate)	25% (abundant) 25% (moderate)
Amorphous salts	50% (abundant) 37.5% (poor) 12.5% (moderate)	57.14% (abundant) 28.57% (poor) 14.29% (moderate)
Epithelial cells	95% (poor) 5% (moderate)	87.5% (poor) 12.5% (moderate)
Bacteria	80.77% (poor)	54.55% (poor)
Mucine	59% (poor)	75% (moderate)

Table 3. Results of microscopic analyses of urine conducted in men exposed to pesticides from Sonora, Mexico

3.4. Semen analysis

Mostly all the differences between groups (case and control) were observed in the semen analyses. In table 4, it can be observed that the case group presented a lower volume, pH, sperm viability, fast and slow progressive motility and abnormalities in sperm morphology (spermatozoa macrocephalia, microcephalia, pyriform, band-like, pin-shaped, double head, tail coiled cytoplasmic droplets and amorphous). Additionally, this same group had a higher viscosity and immobility of spermatozoa. In previous studies [6], it was observed that liquefaction was affected by 32% in insecticide applicators, while the present study did not show abnormalities or significant differences between groups. In this study we observed that in the controls there were more live sperm (one third more than the majority of the cases). The percentage of the abnormalities detected in the present study was superior represented by a 35% that the one found in the control group, comparing this result with the study regarding the insecticide applicators in Hermosillo, Sonora, there was a similar behavior [6].

Analyses	Case group	Control group	Normal levels*
Liquefaction	6.9% (normal)	Normal	
Volume *(mL)*[1]	2.48	3.73	"/> 2
pH[2]	8.05	8.35	7.2 – 8.0
Viscosity *(Filament bigger than 2 cm)*	44.83% (normal)	28.57% (normal)	
Aspect	96.55% (normal) 3.45% (anormal, yellow color)	100% (normal)	Opalescent gray color (normal)
Sperm viability	45%	75%	
Sperm fast progressive motility	42.5%	52.80%	
Sperm slow progressive motility	16.88%	21.07%	
Sperm mobility	57.13%	28.93%	
Bacteria	12%	7%	
Germinal cells *(Dentritus)*	30.30%	7.14%	
Specific sperm agglutination espermatozoides	24.24%	-	
Normal sperm morphology	41.08%	75.92%	

[1]p<0.03, [2]p<0.01, [3]p<0.0201, [4]p<0.0305.

* [26].

Table 4. Results of semen analysis conducted on men exposed to pesticides from Sonora, Mexico

Biological fluid	Diazinon	Chlorpyrifos	Malathion	Parathion
Semen[1]	17.6	32.3	53	44
Blood[2]	0	9.1	0	9.1
Urine[3]	0	9.1	5.5	20

[1]n=11, [2]n=33, [3]n=55

Table 5. Organophosphate pesticide residues in biological fluids of field workers from Sonora, Mexico

Biological fluid	p,p′-DDT	p,p′-DDE	p,p′-DDD
Semen[1]	27.3	36.4	36.4
Blood[2]	0	15.2	3
Urine[3]	5.5	9.1	14.5

[1]n=11, [2]n=33, [3]n=55.

Table 6. Organochlorine pesticide residues in biological fluids of field workers from Sonora, Mexico

3.5. Association of exposure indicators

In the regression analysis a relationship was observed between biochemical indicators and pesticide exposure time. The biochemical indicators involved were erythrocytes, mean corpuscular volume, red blood distribution width and urea. For every year of pesticide exposure there was a decrement of 0.082 million of erythrocytes and 0.088 fL of VCM. For every year of pesticide exposure there was an increment of 0.134 fL RDW and 0.163 mg/ dL of urea. In a research performed in Spain with workers chronically exposed to pesticides, the affected biochemical indicators were urea, TGO enzymes and lactate dehydrogenase (LDH) [38]. In the figure 1, there are the associations studied between biochemical indicators and pesticides exposure time. It is important to mention that the determination of cholinesterase in this study was carried out using butyrylcholinesterase or plasma cholinesterase. In previous studies cholinesterase was associated with other biochemical indicators. The results showed that it bound to cholesterol, triglycerides and others as transaminases [38]. This was not possible to observe in the present study, due to the difference on diet and alcohol consumption.

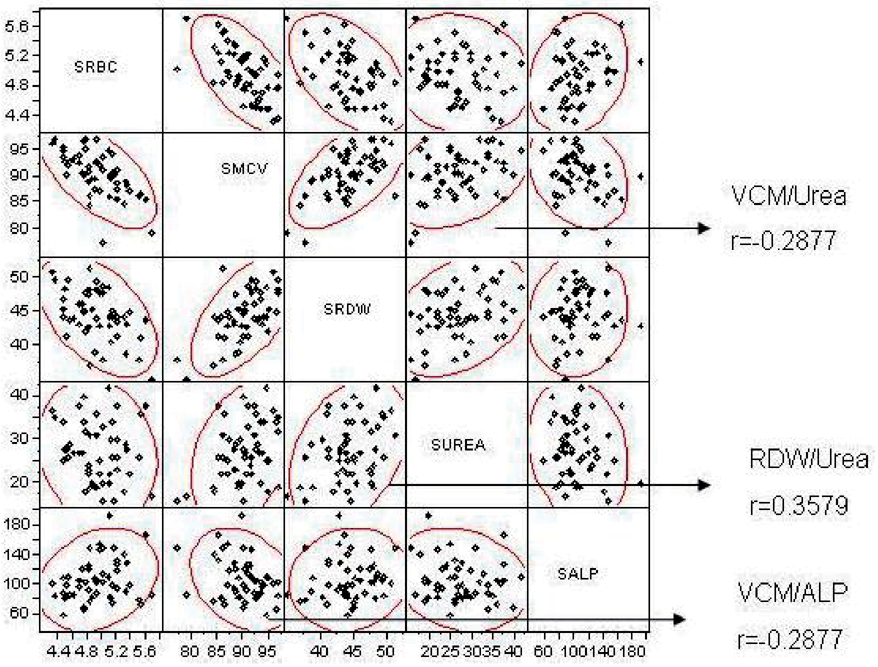

Figure 1. Correlations of blood analyses for case group

3.6. Determination of insecticides and corporal fluids

Blood, urine and semen. A total of 103 samples were analyzed: 73 (71%) were positive to some pesticide. Around 44 (60%) had organochlorine insecticides residues and 29 (40%) had organophosphorus insecticides residues. The organochlorine insecticides detected were 41%

p,p′-DDE, 39% p,p′-DDD and 20% p,p′-DDT. Regarding the organophosphate insecticides analyzed 52% had parathion, 28% chlorpyrifos, 14% malathion and 7% parathion.The highest concentration found was 7.1 μg/L of p,p′-DDE in serum and 6.4 μg/L of p,p′-DDD in urine. The highest concentration found in semen was 2.3 μg/L of p,p′-DDE. Regarding the organophosphate insecticides (chlorpyrifos, malathion and parathion) in the field workers urine the highest concentration were 3.4, 2.2 y 2.0 μg/L, respectively. This levels were considered lower in relation to other studies [39].

Breast milk. There was not detected DDT and DDT metabolites in 85.6% of breast milk samples. Although, 15.4% of samples had p,p′-DDT, p,p′-DDD and p,p′-DDE residues. The most persistent metabolite was p,p′-DDE due to its stability amongst the DDT metabolites [40-42]. The highest level found in breast milk was 9.0 μg/kg (p,p′-DDE) and the lowest level was 0.1 μg/kg (p,p′-DDT). It is important to mention that the infants fed with this contaminated breast milk were in the range of 2-6 month old and their diet was based exclusively on breast milk. Although, according to the American Academy of Pediatrics [43], from the six months onwards, milk is substituted by solid. Other author [44], mentions that breast milk is the primary route where the infants are expose to certain lipophilic toxics that are accumulated by decades in the maternal adipose tissue. If we compare the residues found in this study with the highest levels found in other studies, like the ones reported in [40] for DDE (1.06 mg/kg), and DDT (1.11 mg/kg) in breast milk. The same happened by comparing them with similar works performed in Veracruz with p,p′-DDT and p,p′-DDE (1.27 y 5.02 mg/kg, respectively) [42], and those in the peripheral zone of Mexico City [41], were 108 samples of human milk were analyzed. The content of p,p′-DDT found was 0.117 mg/kg and for p,p′-DDE 2.31 mg/kg. The decrease in the values found was associated to a possible restriction in the use of DDT, although the presence of p,p′-DDE is evident. Specifically, the studies performed in Pueblo Yaqui (Sonora) [45] in breast milk, found levels of p,p′-DDE disturbingly high (6.25 mg/kg), considering that is one of the most important agricultural areas in Mexico. At the present time, in the same zone, the authors reported the presence of p,p′-DDE in 66.66% of the samples of serum from children (between 6 and 12 years of age), with the levels of 0.1 a 443.9 μg/L [46]. These results suggest that DDT is present in the environment and the residues found in biological samples could be due to many factors such as contaminated food consumption. In the present study, the most frequent found metabolite in biological samples was p,p´-DDE due to a its degradation by enzyme system in mammals. According to literature, 50% of p,p′-DDT in the environment could be degrade in 6 years, 67% in 12 years being p,p′-DDE the only product for its degradation [11]. Therefore the contamination present in the studied breast milk can be due to an exposition for more than 12 years. The World Health Organization [47] reports maximum residue limits in foods for DDT and its metabolites of 1.25 mg/kg. While the levels of tolerance established by the FAO/WHO in 1998 [48] for the same compounds in cow milk are 0.05 mg/kg fatty base, in the present study the found levels in breast milk were below the established levels (less than 82%). It is important to mention that DDT levels and other organochlorine compounds in breast milk could be different based on the number of births, age and other factors such as diet, occupation and social status [49, 40]. It is known that levels of these compounds are higher in the breast milk from younger women. In this research, it not was possible to find a correlation between the presence of pesticides residues in breast milk with age, number of children and occupation; this due to a this studied group did not have the necessary characteristics to determine the possible correlations.

4. Conclusions

The results found in this study indicate that the exposed group symptoms are due to pesticide exposure through agricultural activities, and residence on areas near the fields. Some serum biochemical and biometric indicators such as erythrocytes, VCM, RDW, and cholinesterase were affected by pesticide exposure. The most frequent detected pesticide found in semen on the case group was DDT; amongst the indicators affected on the exposed group were sperm mobility, viability and morphology. Although urine analysis did not show significant differences between groups, chlorpyrifos and malathion residues were higher in urine from the exposed group.

The pesticide detected in highest concentration in breast milk was p,p′-DDE (9.0 μg/kg), and the most frequent metabolite was p,p′-DDE.

There were significant differences between groups for some exposure indicators, mainly for erythrocyte count, mean corpuscular hemoglobin and RDW. Also, there was a significant association between exposure time (working or living near agricultural fields) and erythrocyte count. However, associations between pesticide residues in blood or urine with changes on the main pesticide exposure indicators were none significant.

It is important to train field workers on how to protect themselves when handling or being exposed to pesticides, and also inform them about the possible health ill effects caused by inadequate and frequent exposure to pesticides.

On the analytic part, it is recommended to use erythrocyte cholinesterase, because is a better and more specific indicator on chronic pesticide intoxications.

Acknowledgements

This study was carried out with financial support from “Fondos Mixtos-CONACyT- Sonora State Government (No. SON-2004-C01-004). The authors sincerely thank “Secretaria de Salud (SS)” for letting conduct part of the analysis in laboratories of General Hospitals in Obregon city and Navojoa as well “Centro Avanzado de Atención Primaria a la Salud (CAAPS)” in Hermosillo, Sonora. The authors express recognition to the contribution of graduate students from University of Sonora (2010-2004): Enoc Hernández Montiel, Yessica Fabiola Gómez Hernández, Lidia Sarai Güitrón Ochoa, Ana Arely Villegas Castro, Mizar Ethzalé Martínez Romero, Graciela Lizeth Pérez González, Luis Jesús Villarreal Gómez and M. Sc. Edna Delia Molina Romo.

Author details

María-Lourdes Aldana-Madrid[1*], María-Isabel Silveira-Gramont[1],
Fabiola-Gabriela Zuno-Floriano[2] and Guillermo Rodríguez-Olibarría[1]

*Address all correspondence to: laldana@guayacan.uson.mx

1 Departamento de Investigación y Posgrado en Alimentos, Universidad de Sonora. Hermosillo, Sonora, México

2 Department of Environmental Toxicology, University of California, Davis, California, USA

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Chapter 12

DDT as Anti-Malaria Tool: The Bull in the China Shop or the Elephant in the Room?

Mauro Prato, Manuela Polimeni and
Giuliana Giribaldi

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53241

1. Introduction

Malaria is a parasitic disease confined mostly to the tropical areas, caused by *Plasmodium* parasites and transmitted by *Anopheles* mosquitoes. In 2010, nearly 655.000 human deaths, mainly of children ≤5 years of age, were registered among more than 200 million cases worldwide of clinical malaria; the vast majority of cases occurred in the African Region (81%) and South-East Asia (13%), and 91% of them were due to *P. falciparum*, the most virulent among *Plasmodia* strains (WHO, 2011a).

In order to achieve malaria eradication, an ambitious objective which has been prosecuted since 2007 by the Bill and Melinda Gates Foundation, the World Health Organization (WHO) and the Roll Back Malaria association, several strategies are currently adopted, and a major role is played by vector control (Roberts & Enserink, 2007; Greenwood, 2008; Khadjavi et al., 2010; Prato et al., 2012). Dichlorodiphenyltrichloroethane (DDT), one of the insecticides recommended by the WHO for indoor residual spraying or treated bednets approaches against *Anopheles* mosquitoes, is currently used by approximately fourteen countries, and several others are planning to reintroduce it as a main anti-vector tool; however, it strongly polarizes the opinion of scientists, who line up on the field as opponents, centrists or supporters, highlighting DDT health benefits or putative risks depending on their alignment (Bouwman et al., 2011). In this context, the present chapter will review the current knowledge on DDT use, and will suggest some possible future directions to be taken for malaria vector control.

The chapter will open on a short illustration of the *Plasmodium* life cycle, which occurs either in mosquito vector (sexual reproduction) or in human host (asexual replication). Since anti-vector control measures are directed to mosquito killing, *Plasmodium* sexual cycle will be prioritized. Therefore, the insecticides currently allowed for malaria vector control, including organochlorines (OCs), organophosphates (OPs), carbamates (Cs), and pyrethroids (PYs), will be briefly described. After such a brief introduction, a special attention will be paid to DDT. Formulation, cost-effectiveness, mechanisms of action, resistance and environmental issues will be discussed. The big debate among pro-DDT, DDT-centrist, or anti-DDT scientists will be examined. In this context, the state-of-the-art of knowledge on DDT toxicity will be analyzed, and few tips on possible alternatives to DDT will be given.

Taken altogether, these notions should help the reader to arise his own opinion on such a hot topic, in order to feed the ongoing debate. In areas endemic for malaria, is DDT dangerous as the bull in a China shop? Or perhaps is it worth using DDT, since its advantages related to malaria prevention are self-evident as the elephant in the room? Any answers aimed at finding the most practicable way to fight malaria through vector control are urgently required.

2. Materials and methods

All data were obtained from literature searches, by using the search engines Scopus and Pubmed. Because of the complexity of the subject, only the most relevant studies were selected, and reviews were prioritized. Old literature was accessed electronically, or hard copies were obtained from libraries. Information on human exposure and health effects was based on reviews published over the past ten years and supplemented with recent studies on exposure due to indoor spraying and treated bednets.

3. *Plasmodium* life cycle

Malaria parasites have evolved a complicated life cycle alternating between human and *Anopheles* mosquito hosts, as represented in Figure 1. Five *Plasmodium* strains (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*) can affect humans in more than 90 countries, inhabited by 40% of the global population. In some of these areas, over 70% of residents are continuously infected by the most deadly form of the parasite, *P. falciparum*. Surviving children develop various levels of natural immunity; however, it does not protect them from repeated infections and illness throughout life.

3.1. *Plasmodium* life cycle in *Anopheles* mosquitoes

Plasmodium is transmitted to humans by female mosquitoes of *Anopheles* species. There are approximately 484 recognised species, and over 100 can transmit human malaria; however, only 30–40 commonly transmit *Plasmodium* parasites in endemic areas. *Anopheles gambiae* is one of the best known malaria vectors that lives in areas near human habitation (Rogier &

Hommel, 2011). The intensity of malaria parasite transmission varies geographically according to vector species of *Anopheles* mosquitoes. Risk is measured in terms of exposure to infective mosquitoes, with the heaviest annual transmission intensity ranging from 200 to >1000 infective bites per person. Interruption of transmission is technically difficult in many parts of the world because of limitations in approaches and tools for malaria control. In addition to ecological and behavioral parameters affecting vectorial capacity, *Anopheles* species also vary in their innate ability to support malaria parasite development. Environmental conditions such as temperature in mosquito microhabitats serve to regulate both the probability and timing of sporogonic development (Rogier & Hommel, 2011).

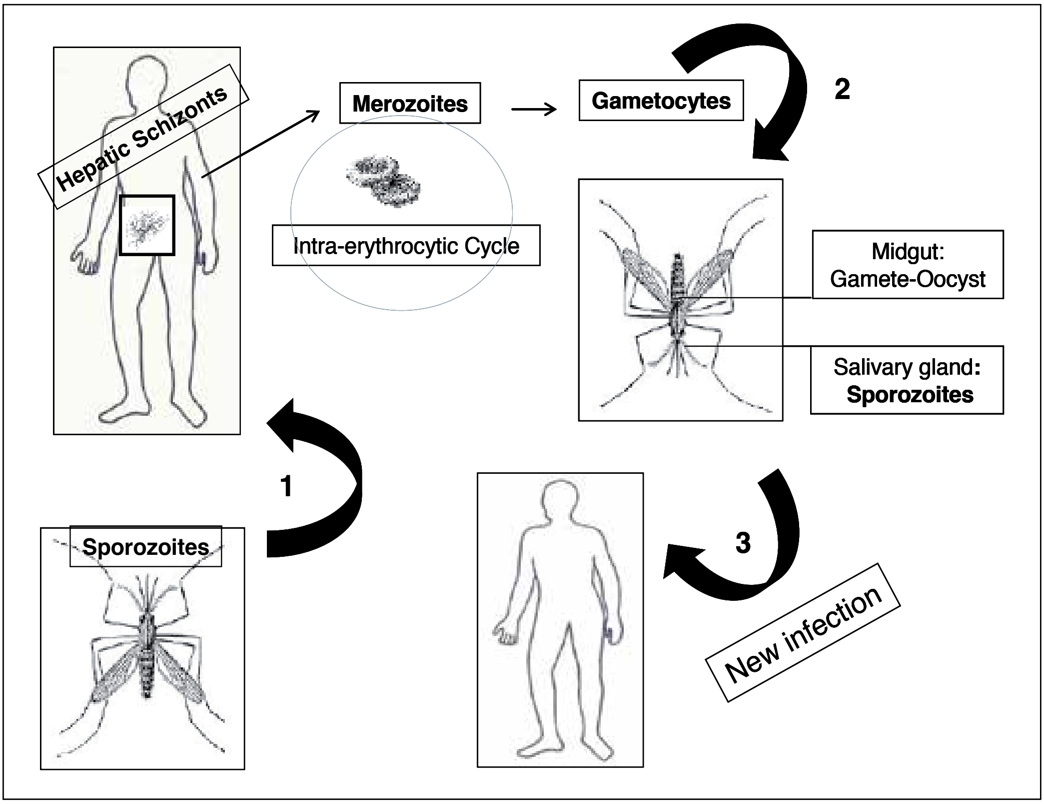

Figure 1. *Plasmodium* parasite life cycle.

In the mosquito, three phases of life of the parasite involve developmental transitions between gametocyte and ookinete stages, between ookinetes and mature oocysts, and between oocysts and sporozoites. When a female Anopheles sucks the blood of a malaria patient, the gametocytes also enter along with blood. They reach the stomach, and gamete formation takes place (Aly et al., 2009). Two types of gametes are formed: the microgametocytes (male) originate active microgametes, and the megagametocyte (female) undergoes some reorganization forming megagametes. Fertilization of the female gamete by the male gamete occurs rapidly after gametogenesis. The fertilization event produces a zygote that remains inactive for some time and then elongates into a worm-like ookinete. The ookinete is one of the most important stages of *Plasmodium* development in the mosquito. It is morphologically and biochemically distinct from the earlier sexual stages (gametocytes and zygote), and from the later stages (oocyst and sporozoites). Development to ookinete allows the parasite to escape from the tightly packed blood bolus, to cross the sturdy peritrophic matrix, to be protected from the digestive environment of the midgut lumen, and to invade the gut epithelium. The success of each of these activities may depend on the degree of the biochemical and physical barriers in the mosquito (such as density of blood bolus, thickness of peritrophic matrix, proteolytic activities in the gut lumen etc.) and the ability of the ookinete to overcome these barriers. Ookinete motility, resistance to the digestive enzymes, and recognition/invasion of the midgut epithelium may play crucial roles in the transformation to oocyst. At the end of the process oocysts produce sporozoites, which can navigate successfully to the salivary glands, where they will be ready for further infection of human beings, and continuation of their life cycle (Beier, 1998).

3.2. *Plasmodium* life cycle in humans

The transmission of the parasite to humans starts when the mosquito injects a small amount of saliva containing 5-200 sporozoites (resident in the salivary gland of the vector) into the skin of the human vector (Menard, 2005). Once in the bloodstream, sporozoites reach the liver and infect the hepatocytes (Trieu et al., 2006). In the liver district, sporozoites grow and change into a new structure of parasite called schizont, a large round cell. The schizont divides through an asexual reproduction (schizogony) resulting in the formation of a thousand small cells called merozoites. After a developmental period in liver, during which patients do not show any clinical symptoms of disease, merozoites are released from liver schizonts into the blood, entering host erythrocytes and starting the intraerythrocytic stage of parasite development (Banting et al., 1995).

This occurs inside a parasitophorous vacuole, the membrane of which separates the cytosol of the erythrocyte from the plasma membrane of the parasite. In the erythrocyte young 'ring' forms of the parasite grow to become trophozoites. lntraerythrocytic development is completed by the formation of new plasma membranes after multiple nuclear divisions (schizogony). Infectious merozoites are then released from the erythrocyte and a new cycle restart (Cowman & Crabb, 2006). One erythrocytic cycle is completed in 48 hours. The toxins are liberated into the blood along with merozoites. The toxins are then deposits in the liver, spleen and under the skin. The accumulated toxins cause malaria fever that lasts for 6 to 10 hours and then it comes again after every 48 hours with the liberation of new generated merozoites. During the erythrocytic stage, some merozoites increase in size to form two types of gametocytes, the macrogametocytes (female) and microgametocytes (male). This process is called gametocytogenesis. The specific causes underlying this sexual differentiation are largely unknown. These gametocytes take roughly 8–10 days to reach full maturity. The gametocytes develop only in the appropriate species of mosquito. If this does not happen, they degenerate and die (Rogier & Hommel, 2011).

4. Vector control as a key strategical approach for malaria eradication

The historical successful elimination of malaria in various parts of the world has been achieved mainly by vector control (Harrison, 1978). Since early nineteenth century (Breman, 2001), vector control has remained the most generally effective measure to prevent malaria transmission and therefore is one of the four basic technical elements of the Global Malaria Control Strategy. The principal objective of vector control is the reduction of malaria morbidity and mortality by reducing the levels of transmission. Vector control methods vary considerably in their applicability, cost and sustainability of their results.

4.1. Classification of insecticides used for vector control

The most prominent classes of insecticides act by poisoning the nervous system of insects, which is very similar to that of mammals. They are often subclassified by chemical type as organochlorines (OCs), organophosphates (OPs), carbamates (Cs) and pyrethroids (PYs) (Prato et al., 2012).

OCs belong to a larger class of compounds called chlorinated hydrocarbons, containing chlorine and including DDT. They have various chemical structures, and are cheap and effective against target species. OCs can alter and disrupt the movement of ions (calcium, chloride, sodium and potassium) into and out of nerve cells, but they may also affect the nervous system in other ways depending on their structure. OCs are very stable, slow to degrade in the environment and soluble in fats: unfortunately, due to persistence and fat solubility, OCs can bioaccumulate in the fat of large animals and humans by passing up the food chain.

OPs were developed in the 1940s as highly toxic biological warfare agents (nerve gases). On the other hand, Cs feature the carbamate ester functional group. OPs and Cs are very different at a chemical level; however, they have a similar mechanism of action. OPs and Cs block a specific enzyme, the acetylcholinesterase, which is able to remove an important neurotransmitter, the acetylcholine, from the area around the nerve cells stopping their communication. Hence, these insecticides are called acetylcholinesterase inhibitors. Structural differences between the various OPs and Cs affect the efficiency and degree of acetylcholinesterase blockage, highly efficient and permanent for nerve gases, temporary for commonly used pesticides. Many different OPs have been developed in order to replace DDT and find compounds that would be less toxic to mammals. Unfortunately, OP Parathion acute toxicity is greater than DDT, and this characteristic causes a significant number of human deaths.

Finally, synthetic PYs, developed in the 1980s, represent one of the newer classes of insecticides. Although their chemical structure is quite different from that of other insecticides, the target of action is also the nervous system. PYs affect the movement of sodium ions (Na^+) into and out of nerve cells that become hypersensitive to neurotransmitters.

4.2. Indoor Residual Spraying (IRS) and Insecticide-Treated Nets (ITNs)

Indoor residual spraying (IRS) with insecticides continues to be the mainstay for malaria control and represents the process of spraying stable formulations of insecticides on the inside walls of certain types of dwellings, those with walls made from porous materials such as mud or wood but not plaster as in city dwellings. Mosquitoes are killed or repelled by the spray, preventing the transmission of the disease. The main purpose of IRS is to reduce malaria transmission by reducing the survival of malaria vectors, life span of female mosquitoes, thereby reducing density of mosquitoes (WHO, 2006b). Several pesticides have historically been used for IRS, the first and most well-known being DDT.

Space spraying, or fogging, relies on the production of a large number of small insecticidal droplets, that resemble smoke or fog by rapidly heating the liquid chemical, intended to be distributed through a volume of air over a given period of time. When these droplets impact on a target insect, they deliver a lethal dose of insecticide. It is primarily reserved for application during emergency situations to rapidly reduce the population of flying insects in a specific area resulting in decrease of transmission (CDC, 2009). It is effective as a contact poison with no residual effect, thus it must be repeated at intervals of 5-7 days in order to be fully effective. The application must coincide with the peak activity of adult mosquitoes, because resting mosquitoes are often found in areas that are out of reach to the applied insecticides. The best moment to kill adult mosquitoes by fogging is at dusk, when they are most active in forming swarms. The most commonly used products are natural pyrethrum extract, synthetic PYs, and Malathion.

Mosquito nets treated with insecticides—known as insecticide treated nets (ITNs) or bednets—were developed in the 1980s for malaria prevention. Properly used, a mosquito net effectively offers protection against mosquitoes and other insects, and thus against the diseases they may carry. Two categories of ITNs are available: conventionally treated nets and long-lasting ITNs (LLINs). ITNs are estimated to be twice as effective as untreated nets, and offer greater than 70% protection compared with no net. These nets are impregnated with PYs, which will double the protection over a non-treated net by killing and repelling mosquitoes, and are proved to be a cost-effective prevention method against malaria (D'Alessandro et al., 1995). Washing and the associated regular retreatment of the nets determine a rapid loss of efficacy of ITNs, thus limiting the operational effectiveness of an ITN program (Lines, 1996).

Biological activity of LLINs, a relatively new technology, generally retains the efficacy for at least 3 years (WHO, 2005), and can reduce human–mosquito contact, which results in lower sporozoite and parasite rates. Different types of long-lasting insecticide impregnated materials are under field trials in different countries. Treatments of screens, curtains, canvas tents, plastic sheet, tarpaulin, etc., with insecticides may provide a cheap and practical solution for malaria vector control. Particularly, the residual insecticides in insecticide-treated wall lining (ITWL) are durable and can maintain control of insects significantly longer than IRS by providing an effective alternative or additional vector control tool (Munga et al., 2009).

5. Dichlorodiphenyltrichloroethane (DDT)

DDT is an OC insecticide; it is white, crystalline solid, tasteless, and almost odorless (PAN, 2012). It is a highly hydrophobic molecule, nearly insoluble in water but with good solubility in most organic solvents, such as fats and oils. DDT is not present naturally, but is produced by the reaction of chloral (CCl_3CHO) with chlorobenzene (C_6H_5Cl) in the presence of sulfuric acid, which acts as a catalyst. DDT was originally synthesised in 1874, but its action as an insecticide was not discovered until 1939. It was the first widely used synthetic pesticide, employed extensively by allied forces during the Second World War for the protection of military personnel from malaria and typhus, released commercially only in 1945. The Swiss chemist Paul Hermann Müller was awarded the Nobel Prize in Physiology or Medicine in 1948 for his discovery of the high efficiency of DDT as a contact poison against several arthropods.

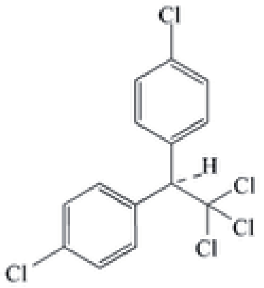

Figure 2. DDT

5.1. Production and use

While the post-war period also saw the introduction of most of the other major families of insecticides still in use today, DDT remained the most extensively used insecticide throughout the world until the mid 1960s. By this time, it had been credited with a number of significant public health successes, including the eradication of malaria from the United States and Europe (Attaran & Maharaj, 2000). DDT is currently being produced in three countries: India, China, and the Democratic People's Republic of Korea (North Korea). By far the largest amounts are produced in India for the purpose of disease vector control. In China, the average annual production during the period 2000–2004 was 4,500 metric tons of DDT, but 80–90% was used in the production of Dicofol, an acaricide, and around 4% was used as additive in antifouling paints. The remainder was meant for malaria control and was exported (PAN, 2012).

5.2. Cost-effectiveness

Both the effectiveness and costs of DDT are dependent on local settings and deserve careful consideration in relation to alternative products or methods (Walker, 2000). DDT has been known as the only insecticide that can be used as a single application in areas where the malaria transmission season is > 6 months. However, information is lacking on the potential variability in residual action of DDT (e.g., due to sprayable surface, climatic conditions, social factors). Direct costs of IRS are the procurement and transport of insecticide, training of staff, operations, awareness-raising of communities, safety measures, monitoring of efficacy and insecticide resistance, monitoring of adverse effects on health and the environment, and storage and disposal. Apart from the direct costs, it is essential that the unintended costs of DDT to human health and the environment are included in the cost assessment. In addition, contamination of food crops with DDT could negatively affect food export. A comprehensive cost assessment of DDT versus its alternatives should include the potential costs of atmospheric transport and chronic health effects.

5.3. Mechanism of action

The basic mechanism of action for most pesticides is an alteration in the transfer of a signal along a nerve fiber and across the synapse from one nerve to another or from a nerve to a muscle fiber. The transfer of a signal along a nerve occurs by changes in the electrical potential across the nerve cell membrane which is created by the movement of ions in and out of the cell. At the terminal end of a nerve, the signal is transferred across the synapse to the next nerve cell by the release of neurotransmitters. Different classes of pesticides inhibit this process in different ways, but the end result is an alteration in normal nerve signal propagation. OCs pesticides act primarily by altering the movement of ions across the nerve cell membranes, thus changing the ability of the nerve to fire.

The WHO has designated DDT as a Class II pesticide, based on its LD_{50} of 250 mg/kg (WHO, 1996). The mechanism by which DDT causes neurotoxicity is well studied. In insects DTT opens sodium ion channels in neurons, causing them to burn spontaneously. By causing repetitive firing of nerve cells, the cells eventually are unable to fire in response to a signal. DDT produces tremors and incoordination at low doses, convulsions at higher doses caused by the repetitive discharge (over-firing) of the nerves. Effects of chronic exposures to DDT are difficult to identify because they are general nervous systems alterations that can occur through many causes (apathy, headache, emotional lability, depression, confusion and irritability).

5.4. Resistance issues

As the number and size of programs that use DDT for indoor spraying increase, insecticide resistance is a matter of growing concern. Insects with certain mutations in their sodium channel gene are resistant to DDT and other similar insecticides. DDT resistance is also conferred by up-regulation of genes expressing cytochrome P450 in some insect species (Denholm et al., 2002).

Many insect species have developed resistance to DDT. The first cases of resistant flies were known to scientists as early as 1947, although this was not widely reported at the time (Metcalf, 1989). Since the introduction of DDT for mosquito control, DDT resistance at various levels has been reported from > 50 species of anopheline mosquitoes, including many vectors of malaria (Hemingway & Ranson, 2000). Unless due attention is paid to the role of insecticide resistance in the breakdown of the malaria eradication campaign of the 1960s, resistance may once again undermine malaria control.

In the past, the use of DDT in agriculture was considered a major cause of DDT resistance in malaria vectors, as many vectors breed in agricultural environments. By 1984 a world survey showed that 233 species, mostly insects, were resistant to DDT (Metcalf, 1989). Today, with cross resistance to several insecticides, it is difficult to obtain accurate figures on the situation regarding the number of pest species resistant to DDT. At present, DDT resistance is thought to be triggered further by the use of synthetic PYs (Diabate et al., 2002). This is due to a mechanism of cross-resistance between PYs and DDT, the so-called sodium channel mutation affecting neuronal signal transmission, which is governed by the *kdr* (knock-down resistance) gene (Martinez-Torres et al., 1998). The *kdr* gene is being reported from an increasing number of countries; thus, even in countries without a history of DDT use, resistance to DDT is emerging in populations of malaria vectors (WHO, 2006a). Contemporary data from sentinel sites in Africa indicate that the occurrence of resistance to DDT is widespread, especially in West and Central Africa. In Asia, the resistance to DDT is particularly widespread in India.

5.5. Environmental issues

Part of the success of DDT can be attributed to its persistence in the environment, thus reducing the need for frequent application. DDT is one of nine persistent organic pollutants (POPs) which bioaccumulate and are transported by air and water currents from warmer climates to temperate zones, where they have never been used. DDT has low to very low rates of metabolism and disposition, depending on ambient temperatures: the process of degradation is dramatically slowed down in cooler climates. It is degraded slowly into its main metabolic products, 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE) and dichlorodiphenyldichloroethane (DDD), which have similar physicochemical properties but differ in biological activity.

DDT is emitted through volatilization and runoff. It is more volatile in warmer than in colder parts of the world, which through long-range atmospheric transport results in a net deposition and thus gradual accumulation at high latitudes and altitudes (Harrad, 2001). Loss through runoff is low because DDT is extremely hydrophobic and has a strong affinity for organic matter in soils and aquatic sediment but is virtually insoluble in water. However, when applied to aquatic ecosystems, DTT is quickly absorbed by organisms and by soil or it evaporates, leaving little amount of DDT dissolved in the water itself (Agency for Toxic Substances and Disease Registry, 2002). Half-lives of DDT have been reported in the range of 3–7 months in tropical soils (Varca & Magallona 1994; Wandiga, 2001) and up to 15 years in temperate soils (Ritter et al., 1995). The half-life of each of its metabolic products is similar or longer.

The global risk of adverse effects to human health and the environment has led the international community to mandate the UN Environment Programme (UNEP) to convene an intergovernmental negotiating committee (INC) for a POPs Convention to phase out production and use (UNEP, 1997a; UNEP, 1997b). As a result of these environmental concerns, the use of DDT was increasingly restricted or banned in most developed countries after 1970.

DDT and its metabolic products present in the global environment have originated mostly from its previous large-scale use in agriculture and domestic hygiene. Because DDT is currently allowed only for indoor spraying for disease vector control, its use is much smaller than in the past. Nevertheless, DDT sprayed indoors may end up in the environment (e.g., when mud blocks of abandoned houses are dissolved in the rain). Even today, DDT remains so widespread in the environment that it is likely that exposure to it is unavoidable. While exposure in the industrialised world has fallen dramatically, exposure remains high in some developing countries where DDT continues to be used in vector control.

DDT is very fat-soluble and could therefore be found in fatty foods such as meat and diary products. Even in countries across North America and Northern Europe, where its use has been banned for over a decade DDT residues are still often found in food. This is because of environmental persistence, illegal use, or importation of contaminated food from regions where DDT is still used.

6. The big debate on DDT as anti-malaria tool

In 1955, the WHO commenced a program to eradicate malaria worldwide, relying largely on DDT. The program was initially very successful, eliminating the disease in Taiwan, much of the Caribbean, the Balkans, parts of northern Africa, the northern region of Australia, and a large swath of the South Pacific and dramatically reducing mortality in Sri Lanka and India (Harrison, 1978). However, widespread agricultural use led to resistant insect populations. In many areas, early victories partially or completely reversed, and in some cases rates of transmission even increased (Chapin & Wasserstrom,1981). The program was successful in eliminating malaria only in areas with "high socio-economic status, well-organized healthcare systems, and relatively less intensive or seasonal malaria transmission" (Sadasivaiah et al., 2007). In tropical regions, DDT was less effective due to the continuous life cycle of mosquitoes and poor infrastructure. It was not applied at all in sub-Saharan Africa due to these perceived difficulties.

Moreover, the adverse health effects of DDT versus the health gains in terms of malaria prevention require more attention. For example, a gain in infant survival resulting from malaria control could be partly offset by an increase in preterm birth and decreased lactation, both of which are high risk factors for infant mortality in developing countries. The WHO is conducting a re-evaluation of health risks of DDT, but progress has been slow (PAN, 2012). Nevertheless, in 2006 it approved the use of DDT, particularly indoor residual spraying of walls, in areas endemic for malaria for health-related reasons (WHO, 2006a; WHO, 2006b), although it also carefully drew up major guidelines (WHO 2000). Currently, DDT represents one the main stays to achieve goals of Global Eradication Program launched in 2007 by the Bill and Melinda Gates Foundation, the World Health Organization (WHO) and the Roll Back Malaria association (Roberts & Enserink, 2007; Greenwood, 2008; Khadjavi et al., 2010; Prato et al., 2012). However, in the recent years the possible effects of DDT on human health have been a hot topic of discussion inside malaria research community, as certified by the large number of available publications and intense correspondence among scientists (e.g., Blair et al., 2009; Burton, 2009; van den Berg, 2009; Tren & Roberts, 2010; Bouwman et al., 2011; Tren & Roberts, 2011). The debate is heavily polarized, and three main viewpoints can be identified, as suggested by Bouwman et al. (Bouwman et al., 2011): anti-DDT, centrist-DDT, and pro-DDT.

6.1. Anti-DDT point of view

DDT opponents usually claim for DDT elimination because of environmental and health concerns. However, Tren & Roberts (Tren & Roberts, 2011) pointed that the "activist groups currently promote an anti-DDT agenda routinely hyping supposed human health and environmental harm from DDT and ignoring studies that find no association between DDT and such harm". As an example, Tren & Roberts mentioned the Biovision's "Stop DDT" project engaged to achieve a world-wide ban on DDT (Biovision, 2011), which apparently was connected to the Secretariat of the Stockholm Convention's promotion of an arbitrary deadline for cessation of DDT production by 2020 (United Nations Environment Programme, 2007). Another representative example of a recent anti-DDT action is given by a court case occurred in Uganda (Lewis, 2008): a petition filed in Kampala's High Court accused the Ugandan government of not following DDT spraying guidelines, whether those of the WHO or those of Uganda's National Environment Management Authority. In that case, it appears evident that the big matter was not DDT itself as a molecule, but its incorrect use. In this context, a major point questioned by anti-DDT scientists is that also IRS workers are highly exposed to DDT, since prescribed personal protection procedures and safe practices are not always followed, because of uncomfortable working conditions. Not wearing masks or gloves and frequent wiping of sweaty faces with the same cloth increases dermal and inhalation uptake leading to very high exposure (Bowman et al., 2011). Indeed, DDT serum levels in IRS workers in South Africa were high compared with the general population living in DDT-sprayed houses (Bouwman et al., 1991). On the other hand, Bimenya et al. (Bimeneya et al., 2010) did not found any DDT increase in serum of Ugandan DDT applicators over an entire spray season, stating that effective exposure reduction is possible when protective clothing is used and strict adherence to WHO guidelines (WHO, 2000) is observed. Nevertheless, the WHO's review of human health aspects of DDT use in IRS concluded that "for households where IRS is undertaken, there was a wide range of DDT and DDE serum levels between studies. Generally, these levels are below potential levels of concern for populations" (WHO, 2011b), and none of the thousands of studies conducted to find possible human health effects of

DDT satisfied even the most basic epidemiological criteria to prove a cause-and-effect relationship (Tren & Roberts, 2011).

6.2. Centrist-DDT point of view

According to Bouwman et al. (Bouwman et al., 2011), "the centrist-DDT point of view adopts an approach that pragmatically accepts the current need for DDT to combat malaria transmission using indoor residual spraying (IRS) but at the same time recognizes the risks inherent in using a toxic chemical in the immediate residential environment of millions of people". Thus, scientists sharing a centrist-DDT point of view such as Bouwman and colleagues suggest caution in using DDT because of insufficient investigation whether DDT is safe or not; however, they do recognize its undoubted benefits in areas endemic for malaria and its major role as a life-saving tool. In this context, DDT-centrists call for alternative chemicals, products, and strategies, eventually in order to terminate in the future any use of DDT in IRS for malaria control. As it will be discussed in paragraph 6, some vector control methods are already available as alternatives to DDT. Two of these, the use of alternative insecticides in IRS and the use of insecticide-treated bed nets (ITNs), are mainstreamed because of their proven impact on the malaria burden; other alternatives are receiving limited attention to date, but may play an important role in the future (van den Berg, 2009).

6.3. Pro-DDT point of view

DDT supporters consider DDT safe to use in IRS when applied correctly, and promote DDT to be used for IRS in malaria control where it is still effective. In their perspective, in a risk-benefit comparison, the eventual toxic effects of DDT would be far less than those caused by malaria (Africa Fighting Malaria, 2010; Roberts et al., 1997). Apparently, this is the point of view of WHO itself, since it approved in 2006 the use of DDT, particularly indoor residual spraying of walls, in areas endemic for malaria for health-related reasons (WHO, 2006a; WHO, 2006b), although it also carefully drew up major guidelines (WHO 2000). Moreover, several national malaria control programs and ministers of health repeatedly proclaimed the importance of DDT for disease control programs in countries with high incidence of malaria. These include Namibia and the Southern African Development Community (SADC), which recently reasserted that DDT is a major tool for malaria vector control and announced their intention to produce DDT locally (SADC, 2011). Similarly, the 35 heads of state of the countries members of the African Leaders Malaria Alliance (ALMA) recently endorsed use of DDT in indoor residual spraying (IRS) (ALMA 2010). As a matter of fact, as a consequence of the global eradication program recently launched by charity foundations, which invested relevant amounts of money in DDT-based vector control (Roberts & Enserink, 2007; Greenwood, 2008; Khadjavi et al., 2010; Prato et al., 2012), in 2010 World Health Organization (WHO) officially registered - for the first time in the last decade - a decline in estimated malaria cases and deaths, with 655.000 deaths counted among more than 200 million clinical cases worldwide (WHO 2011a).

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7. Studies on DDT toxicity

Despite the concerns of DDT opponents (see par. 6.1), to date there is no consistent evidence that DDT or its metabolite DDE can be toxic for humans. Indeed, despite the large number of studies performed in this context, results are highly contradicting, probably due to different analytical conditions and approaches used by different researchers. On the other hand, DDT toxic effects on animals have been demonstrated quite convincingly. This should be taken in account in the context of general environmental issues (par. 5.5) which led to DDT ban in malaria-free countries. In the following sub-sections, current knowledge on DDT effects on animal and human health will be reviewed.

7.1. Animals

Due to its lipophilicity, DDT readily binds with fatty tissue in any living organism, and because of its chemical stability, bioconcentrates and biomagnifies with accumulation of DDT through the food chain, in particular in predatory animals at the top of the ecological pyramid (Jensen et al., 1969). By the mid 1950s, experimental studies on animals have demonstrated chronic effects on the nervous system, liver, kidneys, and immune systems in experimental animals attributable to DDT and DDE (Turusov et al., 2002), and it quickly became apparent that this could extend to the broader environment (Ramade, 1987). However, dose levels at which effects were observed are at very much higher levels than those which may be typically encountered in humans.

DDT is highly toxic to fish. The 96-hour LC50 (the concentration at which 50% of a test population die) ranges from 1.5 mg/litre for the largemouth bass to 56 mg/litre for guppy. Smaller fish are more susceptible than larger ones of the same species. An increase in temperature decreases the toxicity of DDT to fish (PAN, 2012).

DDT and its metabolites can lower the reproductive rate of birds by causing eggshell thinning which leads to egg breakage, causing embryo deaths. Sensitivity to DDT varies considerably according to species. Predatory birds and fish-eating birds at the top of the food chain are the most sensitive. The thickness of eggshells in peregrine falcons was found to have decreased dramatically following the pesticide's introduction (Ratcliffe, 1970), likely due to hormonal effects and changes in calcium metabolism (Peakall, 1969). Colonies of brown pelicans in southern California plummeted from 3000 breeding pairs in 1960 to only 300 pairs and 5 viable chicks in 1969. In the US, the bald eagle nearly became extinct because of environmental exposure to DDT. According to research by the World Wildlife Fund and the US EPA, birds in remote locations can be affected by DDT contamination. Albatross in the Midway islands of the mid-Pacific Ocean show classic signs of exposure to OCs chemicals, including deformed embryos, eggshell thinning and a 3% reduction in nest productivity. Researchers found levels of DDT in adults, chicks and eggs nearly as high as levels found in bald eagles from the North American Great Lakes (PAN, 1996).

7.1.1. Reproductive and teratogenic effects (birth defects)

DDT causes adverse reproductive and teratogenic effects in test animals. In one rat study, oral doses of 7.5 mg/kg/day for 36 weeks resulted in sterility. In rabbits, doses of 1 mg/kg/day administered on gestation days 4-7 resulted in decreased foetal weights. In mice, doses of 1.67 mg/kg/day resulted in decreased embryo implantation and irregularities in the oestrus cycle over 28 weeks (Agency for Toxic Substances and Disease Registry, 1994). Many of these observations may be the result of disruptions to the endocrine (hormonal) system.

In mice, maternal doses of 26 mg/kg/day DDT from gestation through to lactation resulted in impaired learning in maze tests.

7.1.2. Cancer

The evidence relating to DDT and carcinogenicity provides uncertain conclusions. It has increased tumour production, mainly in the liver and lungs, in test animals such as rats, mice and hamsters in some studies, but not in others. In rats, liver tumours were induced in three studies at doses of 12.5 mg/kg/day over periods of 78 weeks to life, and thyroid tumours were induced at doses of 85 mg/kg/day over 78 weeks. Tests have shown laboratory mice were more sensitive to DDT. Life time doses of 0.4 mg/kg/day resulted in lung tumours in the second generation and leukaemia in the third generation, and liver tumours were induced at oral doses of 0.26 mg/kg/day in two separate studies over several generations (PAN, 2012).

7.2. Humans

The US Department of Health and Human Services (DHHS) has determined that "DDT may reasonably be anticipated to be a human carcinogen". DHHS has not classified DDE and DDD, but the US Environmental Protection Agency (EPA) has stated that they are probable human carcinogens (PAN, 2012), suspecting DDT, DDD and DDE of being environmental endocrine disrupters (Colburn et al., 1996) which may affect human health. Based on the results of animal studies, DDT was suspected to cause cancer, diabetes, neurodevelopmental deficits, pregnancy and fertility loss (Beard, 2006). However, available epidemiological studies reject DDT contribution in the development of these diseases and results are still unclear (Beard, 2006).

7.2.1. Reproductive disorders

In vitro studies have shown DDT and its metabolites to have human estrogenic activity (Chen et al., 1997) and DDE to act as an androgen antagonist (Kelce et al., 1995). Some researchers have also hypothesized a trend for decreasing semen quality in the general human community following the introduction of DDT (Carlsen et al., 1992; Sharpe & Skakkebaek, 1993) suggesting that environmental exposure to OCs may be causing human endocrine disruption. However, the observed patterns may simply reflect geographic variations and lifestyle factors (Hauser et al., 2002).

http://dx.doi.org/10.5772/53241

Much of the epidemiologic research about the possible influence of pesticide exposure in general on pregnancy outcome suffers from significant methodological problems. The largest and most rigorous study of DDT and adverse reproductive outcomes was conducted in a US perinatal cohort of over 44,000 children born between 1959 and 1966 (Longnecker et al., 2001). DDE concentration was estimated in stored serum taken during pregnancy from mothers of 2380 children. Increasing concentrations of serum DDE were statistically and significantly related to preterm births, intra-uterine growth retardation (Siddiqui et al., 2003) and maternal diastolic blood pressure (Siddiqui et al., 2002). On the other hand, other studies have failed to find any relationship between maternal DDT exposure and birth weight (Gladen et al., 2003).

Both animal models and early human studies have suggested a link with exposure to the DDT and the most common adverse pregnancy outcome (spontaneous abortion) (Saxena et al., 1980). However, the results of recent research are inconsistent. One small case-control study nested in a longitudinal study of Chinese textile workers found significantly higher levels of DDE in women with spontaneous abortion than full term controls. (Korrick et al., 2001) On the other hand, other studies have been unable to find an association (Gerhard et al., 1998). Unclear findings have been identified about the impact of DDT on fertility (Cohn et al., 2003): the probability of daughters' pregnancy fell with increasing levels of DDT in maternal serum, but it increased with increasing levels of DDE. Finally, OCs appear to transfer freely across the placenta from mother to foetus and could be also excreted in human milk (PAN, 2012).

In the late 1960s, concentrations of DDE in animals and first-trimester human fetal tissues correlated with reproductive abnormalities in male offspring such as hypospadias and undescended testes (Gray et al., 2001). A case-control study nested in a US birth cohort (1959–1966) (Longnecker et al., 2002) showed small increases in crypt-orchidism, hypospadias, and polythelia among boys with the highest DDE maternal serum levels when compared with those with the lowest maternal levels, although none of these were statistically significant. On the other hand, other studies failed to find a significant association between influence of DDT exposure on hormone levels in adult men, or DDT levels and sperm concentration/mobility in male partners of sub-fertile couples (Hauser et al., 2003).

7.2.2. Other endocrine conditions

Bone mineral density, which is regulated by the antagonistic effect of androgens and oestrogens, may be another possible target of endocrine disruption. DDT has been shown to modulate trophoblast calcium handling functions *in vitro* (Derfoul et al., 2003) and two small cross-sectional studies have suggested there may be a weak association between serum DDE levels and reduced bone mineral density (Beard et al., 2000; Glynn et al., 2000). However, a third study failed to demonstrate any correlation (Bohannon et al., 2000).

In vitro studies suggest that DDT and its metabolites do not influence thyroid metabolism (Langer et al., 2003; Rathore et al., 2002). Other research has failed to find a significant association with endometriosis, a hormone dependant pelvic inflammatory disease (Lebel et al., 1998).

7.2.3. Cancer

Breast cancer has been studied most rigorously; even though the majority of results showed no causative association with DDT exposure (Beard et al., 2006), the latest evidence indicates an increased risk in women who were exposed at a young age. It was hypothesised that DDT co-genres and metabolites might act as tumour promoters in hormonally sensitive cancers due to their oestrogenic and anti-androgenic properties (Iscan et al., 2002). More recently, larger and better designed studies have generally not supported this hypothesis (Calle et al., 2002; Snedeker, 2001). Other hormonally sensitive cancers include cancer of the endometrium and prostate. Two case-control studies have explored the possibility that DDT may be related to endometrial cancer with neither finding a significant association (Sturgeon et al., 1998; Weiderpass et al., 2000). On the other hand, an Italian hospital-based multisite case-control study of prostate cancer found an increased risk among farmers exposed to DDT (Settimi et al., 2003), although exposure assessment in this study relied on self-report, leaving these findings susceptible to recall bias. Rates of prostate cancer were also found to be increased among male applicators using chlorinated pesticides in the Agricultural Health Study cohort (Alavanja et al., 2003) and in a Swedish cohort of pesticide applicators (Dich & Wiklund, 1998).

Pesticides have been associated with pancreatic cancer (Beard, 2006). A large Norwegian prospective study of lifestyle factors and pancreatic cancer identified a higher risk among men occupied in farming, agriculture or forestry (Nilsen & Vatten, 2000). Recent research lends a physiological plausibility to a possible association between DDT and pancreatic cancer by suggesting that DDT may modulate oncogene expression or provide a growth advantage to mutated cells, for example, through its actions as an endocrine disrupter (Porta et al., 1999).

Case control studies using self-reported exposure have found significant associations between DDT exposure and lung cancer, leukaemia and non-Hodgkins lymphoma (NHL) (Beard, 2006). However a nested case-control study using stored serum identified a dose response relationship for NHL with PCB exposure but not DDT. A small case-control study using serum levels drawn at diagnosis has suggested an association between DDT exposure and colorectal cancer.

7.2.4. Nervous system

Animal studies have suggested DDT may cause central nervous system (CNS) toxicity (Eriksson & Talts, 2000). Exposure to DDT may be associated with a permanent decline in neurobehavioral functioning and an increase in psychiatric symptoms, but the few studies and limited exposure information made it impossible to be confidant about this potential relationship (Colosio et al., 2003). These findings are also complicated by potential confounding from exposure to other pesticides, such as organophosphates, that are known to have neurological effects. One recent case study suggested that DDT may be related to neurological impairment (Hardell et al., 2002). Another recent study of retired malaria-control workers found various neurobehavioral functions and performance deteriorated significantly with increasing years of DDT application (van Wendel de Joode et al., 2001). Subjects exposed to pesticides including DDT also scored worse than non-exposed subjects on a self-reported neuropsychological questionnaire of surviving members of a historical cohort of pesticide applicators (Beard, 2006).

7.2.5. Immune system

At least one cross-sectional study has associated DDT and other pesticide exposures with suppression or induction of several immune parameters (Daniel et al., 2002).

7.2.6. Diabetes

Diabetes has been associated with OC exposure in at least one study. An Australian cohort study of mortality in staff working as part of an insecticide application program also found increased mortality from pancreatic cancer in DDT-exposed subjects and from diabetes in subjects working with any pesticide (Beard, 2006).

7.3. Epidemiological studies

It is only in the last 25 years that more rigorous epidemiological research has focused on the possible adverse effects of exposure to DDT in humans. Unfortunately, they are not easily answered since epidemiologic research in this field is plagued by methodological challenges (Blondell, 1990). Fewer early human studies have been undertaken specifically on DDT, moreover they were small and limited in scope. A major methodological challenge is the difficulty in getting accurate information on subject exposure since many of the possible adverse effects of DDT (for example, cancer) may not become evident until many years after a causative exposure. Moreover, since it is rare for past exposure to have been accurately recorded at the time, exposure estimation has often been based on the response by subjects to questioning. However, subjects may have been unaware of significant past exposures to DDT through the food chain and even occupationally exposed subjects are unlikely to accurately remember and quantify exposures faced 20–30 years in the past. In the absence of a recorded exposure history, biological sampling of subjects may give some measure of their past exposure. Unlike other pesticides, DDT and DDE are only very slowly eliminated, making biological monitoring a relatively accurate, easy and cheap means of assessing past exposure. Serum levels of DDT and DDE are closely correlated with levels in adipose tissue and thus provide a relatively non-invasive measure (Mussalo-Rauhamaa, 1991). Unfortunately, biological monitoring of DDT presents its own potential for epidemiological bias since levels can also be influenced by factors that relate directly to the outcome of interest, in particular weight change.

Since DDT and its metabolites are so persistent in the environment and human tissues, humans are not excluded from this ecological trends raising questions about the possible impact of widespread pesticide exposure on human communities. Biological sampling near the time of peak use during the 1960s showed increasing DDT levels in most human communities, mainly due to exposure to residues in food. High levels of human exposure to DDT among those living in sprayed houses, most of whom are living under conditions of poverty and often with high levels of immune impairment, have been found in studies in South Africa and Mexico (Aneck-Hahn et al., 2007; Bouwman et al., 1991; De Jager et al., 2006; Yanez et al., 2002), but contemporary peer-reviewed data from India, the largest consumer of DDT, are lacking. The simultaneous presence of, and possible interaction between, DDT, DDE and PYs in human tissue is another area of concern (Bouwman et al., 2006; Longnecker, 2005). In North America, rather high levels of exposure have been recorded in biological samples collected in the 1960s (Eskenazi et al., 2009). DDT accumulates in fatty tissue and is slowly released. The half-life of DDT in humans is > 4 years; the half-life for DDE is probably longer (Longnecker, 2005).

8. Possible alternatives to DDT

Several vector control methods are currently available as alternatives to DDT, while others are under development. As previously stated, the use of alternative insecticides in IRS and the use of insecticide-treated bed nets (ITNs), are mainstreamed because of their proven impact on the malaria burden. Moreover, several non-chemical approaches could play a pivotal role in the future. Table 1 summarizes some possible alternative methods to DDT.

Alternatives to DDT	Chemical (yes/no)	Vector stage	Availability	Delivery/Resources	Risk
attractants	yes	adult	under development	local, private sector	resistance, toxicity
botanicals	no	larva adult	available	local	toxicity
chemical larviciding	yes	larva	available	spray teams	resistance, effect on ecosystems
design of irrigation structures	no	larva	available	irrigation sector	negligible
elimination of breeding sites	no	larva	available	local	negligible
fungi	no	adult	under development	not applicable	negligible
genetic methods	no	adult	under development	not applicable	to be studied
habitat manipulation	no	larva	available	local, agricolture sector	negligible
house improvement	no	adult	available	local, development programs	resistance

Alternatives to DDT	Chemical (yes/no)	Vector stage	Availability	Delivery/Resources	Risk
indoor residual spraying	yes	adult	available	spray teams	resistance, toxicity
insecticide-treated bednets	yes	adult	available	free distribution, social marketing, private sector	resistance, toxicity
irrigation management	no	larva	available	local, irrigation sector	negligible
microbial larvicides	no	larva	available	programs, private sectors	resistance
polystyrene beads	no	larva	available	local	negligible
predation	no	larva	available	local, programs, agriculture sector	negligible
repellents	yes	adult	under development	local, private sector	resistance, toxicity

Table 1. Alternative methods for malaria vector control. Adapted from (van den Berg, 2009)

8.1. Chemical methods

The strength of IRS with insecticides lies in its effect on shortening the life span of adult mosquitoes near their human targets (MacDonald, 1957). Two new approaches are currently being developed with regard to IRS, including some existing insecticides not currently available for public health (chlorfenapyr and indoxacarb), potentially effective in areas with pyrethroid resistance (N'Guessan et al., 2007a; N'Guessan et al., 2007b), and new formulations of existing insecticides with prolonged residual activity (Hemingway et al., 2006).

The main alternative to IRS are ITNs, which have been shown convincingly to substantially reduce all-cause child mortality, under both experimental (Lengeler, 2004) and operational conditions (Schellenberg et al., 2001; Fegan et al., 2007). Various new developments in ITN technology have spread recently. At least one nonpyrethroid insecticide with novel chemistry has been developed for ITNs (Hemingway et al., 2006) to cope with the problem of resistance; however, safety issues are still a concern. Other new ITN products are not expected to come to market in the short term.

Chemical insecticides as larvicides can play an important role to control mosquito breeding in urban settings, but they are a concern to the integrity of aquatic ecosystems.

Moreover, in order to push away mosquitoes, which usually are attracted by the moisture, warmth, carbon dioxide or estrogens from human skin, a large spectrum of repellents have been developed and are currently used; these substances, manufactured in several forms, including aerosols, creams, lotions, suntan oils, grease sticks and cloth-impregnating laundry emulsions, are usually applied on the skin or clothes, and produce a vapor layer characterized by bad smell or taste to insects (Brown & Hebert, 1997). The ideal repellent should satisfy several criteria: a) have long-lasting effectiveness; b) do not irritate human skin; c) have a bad odor only to mosquitoes but not to people; d) have no effects on clothes; e) be inert to plastics commonly used, such as glasses or bracelets; f)be chemically stable; and g) be economical (Brown & Hebert, 1997). The list of main insect repellents, some of which are also used as insecticides, includes N,N-diethyl-3-methylbenzamide (DEET), permethrin, picaridin, indalone, and botanicals (Prato et al., 2012). Additionally, innovative work is in progress on the attractiveness of human odors to malaria vectors, with potential applications as mosquito attractants and repellents for use in trapping and personal protection (Zwiebel & Takken, 2004).

8.2. Nonchemical methods

The development of non-chemical strategies alternative to insecticides and repellents is already available or currently on study. Before the advent of synthetic insecticides, vector control depended primarily on environmental management, and a meta-analysis of data mostly from that period indicated that it substantially reduced malaria risk (Keiser et al., 2005).

Elimination of vector-breeding habitats and managements of water bodies plays a key role in vector suppression, (Walker & Lynch, 2007). In irrigated agriculture, vector breeding can be controlled, through land leveling and intermittent irrigation (Keiser et al., 2002).

The role of aquatic predators as control agents of malaria vectors is potentially enhanced through conservation or through the introduction of agents from outside. Larvivorous fish have frequently been reared and released for controlling vector breeding in small water tanks and wells, but successes have generally been limited to more or less permanent water bodies (Walker & Lynch, 2007).

Microbial larvicides such as *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* produce mosquito-specific toxins associated with a low risk of resistance development (Lacey, 2007). Recent field trials and pilot projects have shown good potential of both bacteria to manage mosquito breeding and to reduce biting rates in certain settings (Fillinger et al., 2008).

Also, insect pathogenic fungi have shown promising results for controlling adult *Anopheles* mosquitoes when sprayed on indoor surfaces and have potential to substantially reduce malaria transmission (Scholte et al., 2005).

Novel methods under development are genetically engineered mosquitoes and the sterile insect technique (Catteruccia, 2007). Genetic control appears a promising tool, comprising all methods by which a mechanism for pest or vector control is introduced into a wild population through mating. These include the sterile insect release method or the sterile insect technique (SIT), through which males are sterilized by irradiation or other means and released to mate with wild females, leading them to lay sterile eggs. Additionally, the introduction of genetic factors into wild populations aimed to make pests harmless to humans might be relevant (Pates & Curtis, 2005).

Finally novel approaches against vector borne diseases include transgenesis and paratransgenesis to reduce vector competence (Coutinho-Abreu et al., 2010). For vector transgenesis, the goal is to transform vectors with a gene (or genes) whose protein(s) impair pathogen development. Several mosquito species vectors of different parasites and viruses have been transformed. Some of the transformed mosquitoes were shown capable of blocking pathogen development via tissue-specific expression of molecules impairing the pathogen attachment to the midgut (Ito et al., 2002), or activating some biochemical pathways detrimental to pathogen survival (Franz et al., 2006). Paratransgenesis aims to reduce vector competence by genetically manipulating symbionts. Transformed symbionts are spread maternally or via coprophagy across an insect population (Durvasula et al., 1997).

Unfortunately, although these approaches are potentially promising, they remain a complex approach with a limited use (Coutinho-Abreu et al., 2010). Also, data on the cost-effectiveness of nonchemical methods are scarce. In a retrospective analysis of data from Zambia, environmental management was as cost-effective as ITNs (Utzinger et al., 2001). Moreover, environmental management can benefit from local resources, reducing the need for external funds.

9. Conclusion

To date, DDT represents a major tool for vector control in areas endemic for malaria, and in 2010 it was the main stay contributing to reduce malaria burden. Despite the big ongoing debate whether improve or ban its use, no convincing evidence on long-term toxic effects of DDT on humans is currently available. In the future, further constructive research aimed at ascertaining DDT effects on human health will be certainly welcome; also, the concurrent use of safe DDT alternatives (as long as they are effective as DDT, of course), should not be neglected. Nevertheless, DDT benefits appear self-evident up to now, thereby justifying its current use as an effective anti-malaria tool.

Author details

Mauro Prato[1,2], Manuela Polimeni[1] and Giuliana Giribaldi[1]

1 Dipartimento di Genetica, Biologia e Biochimica, Facolta’ di Medicina e Chirurgia, Universita’ di Torino, Italy

2 Dipartimento di Neuroscienze, Facolta’ di Medicina e Chirurgia, Universita’ di Torino, Italy

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Section 5

Insecticides and Environment

Chapter 13

Impact of Systemic Insecticides on Organisms and Ecosystems

Francisco Sánchez-Bayo, Henk A. Tennekes and
Koichi Goka

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/52831

1. Introduction

Systemic insecticides were first developed in the 1950s, with the introduction of soluble organophosphorus (OP) compounds such as dimethoate, demeton-S-methyl, mevinphos and phorate. They were valuable in controlling sucking pests and burrowing larvae in many crops, their main advantage being their translocation to all tissues of the treated plant. Systemic carbamates followed in the 1960s with aldicarb and carbofuran. Since then, both insecticidal classes comprise a large number of broad-spectrum insecticides used in agriculture all over the world. Nowadays, OPs are the most common pesticides used in tropical, developing countries such as the Philippines and Vietnam, where 22 and 17% of the respective agrochemicals are ‘extremely hazardous’ [126], i.e. classified as WHO class I. Systemic insect growth regulators were developed during the 1980-90s, and comprise only a handful of compounds, which are more selective than their predecessors. Since 1990 onwards, cartap, fipronil and neonicotinoids are replacing the old hazardous chemicals in most developed and developing countries alike [137].

Through seed coatings and granular applications, systemic insecticides pose minimal risk of pesticide drift or worker exposure in agricultural, nurseries and urban settings. Neonicotinoids and fipronil are also preferred because they appear to be less toxic to fish and terrestrial vertebrates. Initially proposed as environmentally friendly agrochemicals [129], their use in Integrated Pest Management (IPM) programs has been questioned by recent research that shows their negative impact on predatory and parasitic agents [221, 258, 299]. New formulations have been developed to optimize the bioavailability of neonicotinoids, as well as combined formulations with pyrethroids and other insecticides with the aim of broadening the insecticidal spectrum and avoid resistance by pests [83]. Indeed, as with any other chemical used in pest control, resistance to imidacloprid by whitefly (*Bemisia tabaci*), cotton aphids (*Aphis gossypii*) and other pests is rendering ineffective this and other neonicotinoids such as acetamiprid, thiacloprid and nitenpyram [247, 269].

This chapter examines the negative impacts that systemic insecticides have on organisms, populations and ecosystems. The efficacy of these products in controlling the target pests is assumed and not dealt with here – only the effects on non-target organisms and communities are considered.

2. Exposure to systemic insecticides

Unlike typical contact insecticides, that are usually taken up through the arthropod's cuticle or skin of animals, systemic insecticides get into the organisms mainly through feeding on the treated plants or contaminated soil. Thus, monocrotophos and imidacloprid are more lethal to honey bees (*Apis mellifera)* through feeding than contact exposure [143]. Residual or contact exposure affects also some pests and non-target species alike.

Systemic insecticides are applied directly to the crop soil and seedlings in glasshouses using flowable solutions or granules, and often as seed-dressings, with foliar applications and drenching being less common. Being quite water soluble (Table 1), these insecticides are readily taken up by the plant roots or incorporated into the tissues of the growing plants as they develop, so the pests that come to eat them ingest a lethal dose and die. Sucking insects in particular are fatally exposed to systemic insecticides, as sap carries the most concentrated fraction of the poisonous chemical for a few weeks [124], whereas leaf-eating species such as citrus thrips and red mites may not be affected [30]. Systemic insecticides contaminate all plant tissues, from the roots to leaves and flowers, where active residues can be found up to 45-90 days [175, 187], lasting as long as in soil. Thus, pollen and nectar of the flowers get contaminated [33], and residues of imidacloprid and aldicarb have been found at levels above 1 mg/kg in the United States [200]. Guttation drops, in particular, can be contaminated with residues as high as 100-345 mg/L of neonicotinoids during 10-15 days following application [272]. Because these insecticides are incorporated in the flesh of fruits, the highly poisonous aldicarb is prohibited in edible crops such as watermelons, as it has caused human poisoning [106].

As with all poisonous chemicals spread in the environment, not only the target insect pests get affected: any other organism that feeds on the treated plants receives a dose as well, and may die or suffer sublethal effects. For example, uptake of aldicarb by plants and worms results in contamination of the vertebrate fauna up to 90 days after application [41], and honey bees may collect pollen contaminated with neonicotinoids to feed their larvae, which are thus poisoned and die [125]. Newly emerged worker bees are most susceptible to insecticides, followed by foraging workers, while nursery workers are the least susceptible within 72 h of treatment [80]. Insects and mites can negatively be affected by systemic insecticides whenever they feed on:

1. pollen, nectar, plant tissue, sap or guttation drops contaminated with the active ingredient (primary poisoning);
2. prey or hosts that have consumed leaves contaminated with the active ingredient (secondary poisoning).

Parasitoids may be indirectly affected because foliar, drench or granular applications may decrease host population to levels that are not enough to sustain them. Furthermore, host quality may be unacceptable for egg laying by parasitoid females [54]. Small insectivorous animals (e.g. amphibians, reptiles, birds, shrews and bats) can also suffer from primary poisoning if the residual insecticide or its metabolites in the prey are still active. It should be noticed that some metabolites of imidacloprid, thiamethoxam, fipronil and 50% of carbamates are as toxic as the parent compounds [29]. Thus, two species of predatory miridbugs were negatively affected by residues and metabolites of fipronil applied to rice crops [159]. However, since systemic insecticides do not bioaccumulate in organisms, there is little risk of secondary poisoning through the food chain.

Apart from feeding, direct contact exposure may also occur when the systemic insecticides are sprayed on foliage. In these cases, using a silicone adjuvant (Sylgard 309) reduces the contact exposure of honey bees to carbofuran, methomyl and imidacloprid, but increases it for fipronil [184]. In general the susceptibility of bees to a range of insecticides is: wild bees > honey bee > bumble bee [185]. In reality a combination of both contact and feeding exposure occurs, which is more deadly than either route of exposure alone [152, 218].

In soil, residues of acephate and methomyl account for most of the cholinesterase inhibition activity found in mixtures of insecticides [233]. Fortunately, repeated applications of these insecticides induces microbial adaptation, which degrade the active compounds faster over time [250]. Degradation of carbamates and OPs in tropical soils or vegetation is also faster than on temperate regions, due mainly to microbial activity [46]. Some neonicotinoids are degraded by soil microbes [172], and the yeast *Rhodotorula mucilaginosa* can degrade acetamiprid but none of the other neonicotinoids [63], which are quite persistent in this media (Table 2).

Chemical	Group	Vapour Pressure (mPa, 25°C)	Solubilityin water (mg/L)	Log Kow#	GUS index*	Leaching potential
aldicarb	C	3.87	4930	1.15	2.52	moderate
bendiocarb	C	4.6	280	1.72	0.77	low
butocarboxim	C	10.6	35000	1.1	1.32	low
butoxycarboxim	C	0.266	209000	-0.81	**4.87**	high
carbofuran	C	0.08	322	1.8	**3.02**	high
ethiofencarb	C	0.5	1900	2.04	**3.58**	high
methomyl	C	0.72	55000	0.09	2.20	marginal
oxamyl	C	0.051	148100	-0.44	2.36	moderate
pirimicarb	C	0.43	3100	1.7	2.73	moderate

Chemical	Group	Vapour Pressure (mPa, 25ºC)	Solubilityin water (mg/L)	Log Kow#	GUS index*	Leaching potential
thiodicarb	C	5.7	22.2	1.62	-0.24	low
thiofanox	C	22.6	5200	2.16	1.67	low
triazamate	C	0.13	433	2.59	-0.9	low
cartap	D	1.0×10^{-10}	200000	-0.95	-	high
halofenozide	IGR	<0.013	12.3	3.34	**3.75**	high
hexaflumuron	IGR	0.059	0.027	**5.68**	-0.03	unlikely to leach
novaluron	IGR	0.016	0.003	**4.3**	0.03	low
teflubenzuron	IGR	0.000013	0.01	**4.3**	-0.82	low
acetamiprid	N	0.000173	2950	0.8	0.94	low
clothianidin	N	2.8×10^{-8}	340	0.905	**4.91**	high
dinotefuran	N	0.0017	39830	-0.549	**4.95**	high
imidacloprid	N	0.0000004	610	0.57	**3.76**	high
nitenpyram	N	0.0011	590000	-0.66	2.01	moderate
thiacloprid	N	0.0000003	184	1.26	1.44	unlikely to leach
thiamethoxam	N	0.0000066	4100	-0.13	**3.82**	high
acephate	OP	0.226	790000	-0.85	1.14	low
demeton-S-methyl	OP	40	22000	1.32	0.88	low
dicrotophos	OP	9.3	1000000	-0.5	**3.08**	high
dimethoate	OP	0.25	39800	0.704	1.06	low
disulfoton	OP	7.2	25	3.95	1.29	low
fenamiphos	OP	0.12	345	3.3	-0.11	low
fosthiazate	OP	0.56	9000	1.68	2.48	moderate
heptenophos	OP	65	2200	2.32	0.26	low
methamidophos	OP	2.3	200000	-0.79	2.18	moderate
mevinphos	OP	17	600000	0.127	0.19	low
monocrotophos	OP	0.29	818000	-0.22	2.3	moderate
omethoate	OP	3.3	10000	-0.74	2.73	moderate
oxydemeton-methyl	OP	2.0	1200000	-0.74	0.0	low
phorate	OP	112	50	3.86	1.4	low
phosphamidon	OP	2.93	1000000	0.79	2.39	moderate
thiometon	OP	39.9	200	3.15	0.37	low
vamidothion	OP	1.0×10^{-10}	4000000	-4.21	0.55	low
fipronil	PP	0.002	3.78	3.75	2.45	moderate

Table 1. Physicochemical properties of systemic insecticides. C = carbamates; D = dithiol; IGR = Insect growth regulator; N = neonicotinoid; OP = organophosphate; PP = phenylpyrazole

Partition coefficients between n-octanol and water (Kow) indicate bioaccumulation potential when Log Kow > 4.

*The Groundwater Ubiquity Score (GUS) is calculated using soil half-life (DT50) and organic-carbon sorption constant (Koc) as follows: GUS = log(DT50) x (4-log Koc). A compound is likely to leach if GUS > 2.8 and unlikely to leach when GUS < 1.8; other values in between indicate that leaching potential is marginal.

Chemical	**Group**	**Water**		**Field**	
		Photolysis (pH 7)	Hydrolysis (pH 5-7)	Water-sediment	Soil (range)
aldicarb	C	8	**189**	6	10 (1-60)
bendiocarb	C	13	25	2	4 (3-20
butocarboxim	C	Sta ble	stable	-	4 (1-8)
butoxycarboxim	C	Stable	18 (510-16)	-	42
carbofuran	C	71	37 (46-0.1)	9.7	14 (1-60)
ethiofencarb	C	-	16	52	37 (34-131)
methomyl	C	Stable	stable	4	7 (5-30)
oxamyl	C	7	8	<1	11
pirimicarb	C	6	stable	**195**	9 (5-13)
thiodicarb	C	9	30 (69-0.3)	<1	18 (1-45)
thiofanox	C	1	30	-	4 (2-6)
triazamate	C	301	2	<1	<1
cartap	D	-	-	-	3
halofenozide	IGR	10	stable	-	**219** (60-219)
hexaflumuron	IGR	6	stable	-	**170**
novaluron	IGR	Stable	stable	18	97 (33-160)
teflubenzuron	IGR	10	stable	16	14 (9-16)
acetamiprid	N	34	**420**[a]	-	3 (2-20)
clothianidin	N	0.1	14[a]	56	**545** (13-1386)
dinotefuran	N	0.2	stable	-	82 (50-100)
imidacloprid	N	0.2	~ **365**[a]	**129**	**191** (104-228)
nitenpyram	N	NA	2.9[a]	-	8
thiacloprid	N	stable	stable	28	16 (9-27)
thiamethoxam	N	2.7	11.5[a]	40	50 (7-72)
acephate	OP	2	50	-	3
demeton-S-methyl	OP	-	56 (63-8)	-	2.7
dicrotophos	OP	-	-	-	28
dimethoate	OP	175	68 (156-4)	15	7 (5-10)
disulfoton	OP	4	**300**	15	30
fenamiphos	OP	<1	**304**	60	2 (1-50)
fosthiazate	OP	Stable	**104** (178-3)	51	13 (9-17)
heptenophos	OP	-	13	7	1
methamidophos	OP	90	5	24	4 (2-6)
mevinphos	OP	27	17	21	1 (1-12)
monocrotophos	OP	26	**134**	-	30 (1-35)
omethoate	OP	Stable	17	5	14
oxydemeton-methyl	OP	222	73 (96-41)	3	5
phorate	OP	1	3	-	63 (14-90)

Chemical	Group	Water		Field	
phosphamidon	OP	-	36 (60-12)	13	12 (9-17)
thiometon	OP	-	22	-	2 (2-7)
vamidothion	OP	-	**119**	7	1 (<1-2)
fipronil	PP	0.33	stable	68	65 (6-135)

Table 2. Degradation of systemic insecticides expressed as half-lives in days. Compounds with half-lives longer than 100 days are considered persistent (Sources: Footprint database & [284]. [a] for pH 9C = carbamates; D = dithiol; IGR = Insect growth regulator; N = neonicotinoid; OP = organophosphate; PP = phenylpyrazole

Aquatic organisms take up easily whatever residues reach the waterbodies, through runoff from treated fields or contaminated groundwater. Some 20% systemic insecticides are prone to leaching, and 45% are mobile in wet soils (Table 1). For example, acephate leaches more easily than methamidophos [305], and so acephate should be restricted or avoided in tropical areas and rice crops [46]. Residues of aldicarb and methomyl in groundwater can have sublethal effects in mammals [215]. Even if residue levels of systemic insecticides in rivers and lakes are usually at ppb levels (μg/L), persistent compounds such as fipronil, neonicotinoids and growth regulators can have chronic effects due to their constant presence throughout several months in the agricultural season [123]. For example, about 1-2% of imidacloprid in treated soil moves into runoff after rainfall events, with the highest concentrations recorded at 0.49 mg/L [12]. Systemic carbamates and OPs do not last long in water because they breakdown through photolysis or hydrolysis in a few days, or are taken up and degraded by aquatic plants [100]. In any case, their presence and frequency of detection in water depends on local usage patterns [39, 171]. The acute toxicity of most systemic compounds is enhanced in aquatic insects and shrimp under saline stress [22, 253].

A characteristic feature of most systemic insecticides –except carbamates– is their increased toxicity with exposure time, which results from a constant or chronic uptake through either feeding or aquatic exposure (Figure 1). Effects are more pronounced some time after the initial application [16], and could last up to eight months [286]. Also, as a result of chronic intoxication, there may not be limiting toxic concentrations (e.g. NOEC or NOEL) in compounds that have irreversible mechanism of toxicity, since any concentration will produce an effect as long as there is sufficient exposure during the life of the organism [274]. This is precisely their main advantage for pest control: any concentration of imidacloprid in the range 0.2-1.6 ml/L can reduce the population of mango hoppers (*Idioscopus* spp.) to zero within three weeks [291]. However, it is also the greatest danger for all non-target species affected, e.g. predators, pollinators and parasitoids. By contrast, contact insecticides act usually in single exposures (e.g. spray droplets, pulse contamination after spraying, etc.) and have the highest effects immediately after application.

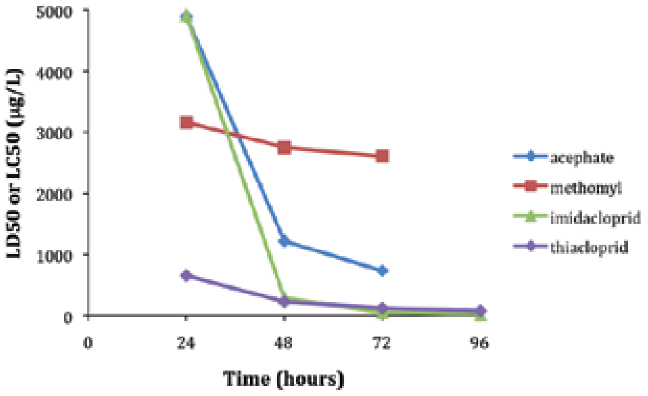

Figure 1. Increasing toxicity of several systemic insecticides with time of exposure. LD50 for acephate to *Episyrphus bateatus* and for methomyl to *Bombus terrestris* [75]; LC50 for imidacloprid to *Cypridopsis vidua* [234] and thiacloprid to *Sympetrum striolatum* [28].

3. Modes of action of systemic insecticides

Before describing their impacts on organisms and ecosystems, a description of the mechanisms of toxicity of systemic insecticides is briefly outlined.

3.1. Acetylcholinesterase inhibitors

Carbamates and organophosphorus compounds are inhibitors of the acetylcholinesterase enzyme (AChE), thus blocking the transmission of the nervous impulse through the neuronal synapses. The binding of carbamates to the enzyme is slowly reversible and temporary, i.e. < 24 h [197], whereas that of alkyl OPs is irreversible. The binding of methyl-OPs does not last as long as that of alkyl-OPs, and this feature is compound specific [182]. Given their mode of action, all these compounds are broad-spectrum insecticides, extremely toxic to most animal taxa, from worms to mammalian vertebrates. Avian species are often more susceptible to these compounds due to relatively low levels of detoxifying enzymes in birds [207, 297]. Thus, recovery of ducklings exposed to a range of carbamate and OP insecticides occurred within eight days after being depressed 25-58% following dosing [91].

3.2. Insecticides acting on nicotinic acetylcholine receptors (nAChR)

Neonicotinoids are derived from nicotine, which is found in the nightshade family of plants (Solanaceae), and particularly in tobacco (*Nicotiana tabacum*). They all are agonists of the nicotinic acetylcholinesterase receptor (nAChR), which mediate fast cholinergic synaptic transmission and play roles in many sensory and cognitive processes in invertebrates. Binding of neonicotinoids to these receptors is irreversible in arthropods [40, 307]. Given that nAChRs are embedded in the membrane at the neuronal synapses, their regeneration seems unlikely because neurons do not grow. The lower affinity of neonicotinoids for mammalian nAChRs has been attributed to the different ionic structure of the vertebrate subtypes [283]. The high toxicity of neonicotinoids to insects and worms is comparable to that of pyrethroids, but aquatic crustaceans, particularly waterfleas, are more tolerant [119, 136].

Cartap is a dithiol pro-insecticide that converts to nereistoxin, a natural toxin found in marine *Nereis* molluscs. Both cartap and nereistoxin are antagonists of the nAChR in insects and other arthropods [164], blocking irreversibly the neuronal functions of these receptors. Unlike neonicotinoids, cartap appears to be very toxic to fish and amphibians [235].

3.3. GABA-R antagonists (fipronil)

Fipronil is a phenylpyrazole antagonist of the γ-aminobutyric acid (GABA)-gated chloride channel, binding irreversibly to this receptor and impeding the nervous transmission [56]. Its mode of action, therefore, appears to be identical to that of cyclodiene organochlorins (e.g. endosulfan), but fipronil is mostly systemic whereas all cyclodienes are insecticides with contact activity. Interestingly, while aquatic organisms (e.g. cladocerans, fish) are quite tolerant of fipronil, vertebrates are more susceptible to this compound than to the old organochlorins [235].

3.4. Insect growth regulators (IGR)

Hexaflumuron, novaluron and teflubenzuron are the only systemic benzoylureas in the market. They are chitin inhibitors, blocking the biosynthesis of this essential component of the arthropod's exoskeleton. As a consequence, insects and other arthropods cannot moult and die during their development. Since their mode of action is restricted to arthropods, benzoylureas are not very toxic to any other animal taxa, e.g. molluscs, vertebrates, etc. [235].

Halofenozide is the only systemic compound among the hydrazines, a group of chemicals that mimic the steroidal hormone ecdysone, which promotes moulting in arthropods [71]. The premature moulting in larvae of some insect taxa, particularly in Lepidoptera, prevents them from reaching the adult stage. Toxicity of halofenozide is selective to insects only.

4. Effects on organisms and ecosystems

4.1. Direct effects on organisms

Mortality of non-target organisms exposed to insecticides is mostly due to acute toxicity, particularly in the case of carbamates. However, with systemic compounds there are many observations of long-term suppression of populations that suggest a chronic lethal impact over time. The latter impacts are likely due to persistence of residual activity in the soil, foliage or water in the case of reversible toxicants (i.e. carbamates), or to irreversible and persistent binding in other cases. (note: all application rates and concentrations here refer to the active ingredient).

4.1.1. Acetylcholinesterase inhibitors

These compounds can have serious impacts on soil organisms of various taxa. Aldicarb and phorate applied to a cotton crop soil at 0.5 and 1 kg/ha, respectively, eliminated or reduced significantly non-target mesofauna, including mites and springtails. Populations of the latter taxa were reduced for more than 60 days (phorate) and 114 days (aldicarb) [17, 225], with the highest effects peaking after 18 days [16]. Granular applications of phorate (250 mg/kg dry soil) killed almost all earthworms, Collembola, Acarina, free-living saprophytic and parasitic nematodes and Protozoa, with populations of Collembola recovering only when residues went below 2 mg/kg [300]. After a single aldicarb application to soil at 2.5 g/m^2, Gamasina predatory mites went to extinction within a year [148]. Bendiocarb impacts on predaceous arthropods and oribatid mites were less severe and temporary compared to the impacts of non-systemic OPs, but increased trap catches of ants two weeks after application [55], possibly as a result of a longer-term effect. Many soil arthropods, in particular mites and springtails, were the most affected by dimethoate –and its metabolite omethoate– residues in soil after sprays of 1-2 ml/L in the farms of the Zendan valley, Yemen [4]. Similar observations were made when dimethoate was sprayed on vegetation of arable fields [85] or in soil microcosms [180]; the springtail populations recovered but attained lower densities a year later, while their dominance structure had changed. However, dimethoate or phosphamidon applied in mustard fields produced only a temporary decline, compared to the long-lasting effect of monocrotophos [141]. Collembola populations do not seem to be affected by pirimicarb applications on cereal crops [95].

Earthworm populations were affected initially after application of phorate and carbofuran to turfgrass, but not thiofanox, and their numbers recovered subsequently [53]. Reduction of earthworm populations by bendiocarb was the highest (99% in one week) among 17 insecticides applied at label rates on turfgrass, with significant effects lasting up to 20 weeks [216]. Juveniles and species living in the surface layers or coming to the soil surface to feed (e.g., *Lumbricus terrestris*) are most affected, since a high degree of exposure is usually found in the first 2.5 cm of soil [288]. However, systemic carbamates can be selective to plant-parasitic nematodes without affecting fungal or microbial communities [296]. Thus, cholinesterase inhibitors do not have significant impacts on bacteria, fungi and protozoa in soil [133], and consequently do not alter the soil biochemical processes [79]. Nevertheless, a combined dimethoate-carbofuran application reduced active hyphal lengths and the number of active bacteria in a treated forest soil [58].

Populations of beneficial predators can be decimated initially as much as the target pests, but they usually recover quickly. For example, thiodicarb or its degradation product, methomyl, applied at 0.5 kg/ha on soybean crops, significantly reduced populations of the predatory bugs *Tropiconabis capsiformis* and *Nabis roseipennis* within two days after treatment only [25]. Demeton-S-methyl reduced populations of predatory insects on strawberry patches, whereas pirimicarb and heptenophos had no significant effect on spiders, staphylinids and anthocorids, or on hymenopteran parasitoids [76]. While populations of web spiders and carabid beetles are severely reduced by dimethoate applied to cabbage fields and cereal crops [144], pirimicarb does not seem to have much impact on these taxa [97, 195], affecting mainly aphids [131]. Pirimicarb on wheat crops does not impact on ladybirds, but larvae of *Episyrphus balteatus* are affected [135]. By contrast, longer impacts have been observed with acephate applied at 0.5 kg/ha on rice paddies, which reduced populations of predatory bugs (*Cyrtorrhinus lividipennis* and *Paederus fuscipes*) for at least 10 days [155]. Similar rates of acephate on rice and soybean crops reduced spiders populations for three weeks, but they recovered afterwards [181]. In addition, acephate is deadly to three species of whitefly parasitoid species [267].

Direct mortality of bumble bees (*Bombus terrestris*) in short exposures to dimethoate is much higher than for heptenophos or ethiofencarb [132]. However, what matters most is the chronic toxicity to the entire bee colony not just the workers. For example, methamidophos contaminated syrup (2 mg/L) produced significant losses of eggs and larvae of honey bees without any appreciable loss of workers after one week of exposure; the colonies would recover completely within 13 weeks if the insecticide was applied only once [301], indicating a long-term impact on the colony. Similarly, the mortality of non-target adult chrysomelid beetles (*Gastrophysa polygoni*) after foliar treatment with dimethoate on the host plants was low (1.9-7.6%), but because this insecticide was most toxic to the egg stage, the overall beetle population decreased over time due to hatching failure [146].

Primary poisoning of birds and mammals by ingestion of OP and carbamate granules or coated seeds is still a problem despite the many attempts to reduce these impacts [189, 190]. For example, mortality of birds that ingested granules of carbofuran in a corn field was extensive, affecting waterfowl, small songbirds and mice within 24 hours. Residues up to 17 mg/kg body weight (b.w.) were found in the dead animals [19]. The granular formulation of this carbamate was banned in the mid-1990s by the US EPA after numerous cases of direct poisoning by animals; however, the liquid formulation applied to alfalfa and corn is just as deadly to bees, because this systemic insecticide is present in the pollen of those plants [208]. Phosphamidon sprayed at 1 kg/ha to larch forests in Switzerland caused many bird deaths [243]; large bird mortality was also observed in Canadian spruce forests sprayed with phosphamidon (0.55 kg/ha), particularly among insectivorous warblers. There was good evidence that birds picked up the insecticide from sprayed foliage within a few hours of application [94]. Carbofuran and phosphamidon were the most common pesticides implicated in deaths of wild birds in Korea between 1998-2002 [157], and ducklings died in large numbers when phorate was applied to South Dakota wetlands [73]. Usually birds die when their brain AChE depression is over 75% [92, 114]. Thus, 11 out of 15 blue jays (*Cyanocitta cristata*) which had depression levels ranging 32-72% after disulfoton was sprayed to pecan groves would die [302], but their carcasses would probably not be found. In orchards sprayed with methomyl, oxamyl or dimethoate, the daily survival rates for nests of Pennsylvania mourning dove (*Zenaida macroura*) and American robin (*Turdus migratorius*) were significantly lower than in non-treated orchards, and the species diversity was also lower. Repeated applications of these and other insecticides reduced the reproductive success of doves and robins and may have lowered avian species diversity [93].

Secondary poisoning with bendiocarb was attributed to 22 birds that had depressed AChE activity after eating contaminated mole crickets and other soil organisms on the applied turfgrass [224]. Several species of raptors were killed or debilitated after consuming waterfowl contaminated with phorate – the fowl had ingested granules of the insecticide that were applied to potato fields a few months earlier [84]. Equally, ladybugs (*Hippodamia undecimnotata*) fed upon *Aphis fabae,* which were reared on bean plants treated with carbofuran, experienced a 67% population reduction due to secondary poisoning [206]. Pirimicarb caused 30-40% mortality of Tasmanian brown lacewing (*Micromus tasmaniae*) larvae when feeding on contaminated 1st instar lettuce aphid (*Nasonovia ribisnigri*) for three days [298].

Impacts on aquatic organisms usually do not last more than a month. For example, thiodicarb applied at 0.25-1.0 kg/ha had severe impacts on copepods, mayflies and chironomids in experimental ponds for three weeks, but not so much on aquatic beetle's larvae; eventually there was recovery of all populations [7]. Pirimicarb can be lethal to common frog (*Rana temporaria*) tadpoles, but does not appear to have chronic effects [139]. However, vamidothion and acephate are most lethal to non-target organisms in rice crops, and are not recommended in IPM programs [153]. Carbofuran and phorate are very toxic to aquatic invertebrates [140], particularly amphipods and chironomids but not so much to snails, leeches or ostracods [72, 249]. Small negative effects in zooplankton communities (cladocerans copepods and rotifers) were observed in rice paddies treated with carbofuran at recommended application rates, but fish were not affected [107]. Carbofuran should not be used in rice paddies, whether in foliar or granular formulations: not only induces resurgence of the brown planthoppers (*Nilaparvata lugens*) [122], but it is also more toxic to the freshwater flagellate *Euglena gracilis* than the non-systemic malation [15]. It reduces populations of coccinellid beetles, carabid beetles, dragonfly and damselfly nymphs, but does not impact much on spiders [255]. However, it appears that carbofuran at 0.2% per ha can double the densities of *Stenocypris major* ostracods in rice paddies, whereas other insecticides had negative effects on this species [168]. Repeated applications of carbofuran can also have a significant stimulation of the rhizosphere associated nitrogenase activity, with populations of nitrogen-fixing *Azospirillum* sp., *Azotobacter* sp. and anaerobic nitrogen-fixing bacteria increasing progressively up to the third application of this insecticide [142].

4.1.2. Insecticides acting on nAChR

Direct toxicity of cartap to fish species is not as high as that of other neurotoxic insecticides, with 3-h LC50s between 0.02 and 6.8 mg/L [161, 308]. However, cartap affects negatively several species of Hymenoptera and aphid parasitoids used to control a number of crop pests [14, 77, 147, 270]. This insecticide also inhibits hatching of eggs of the nematode *Agamermis unka,* a parasite of the rice pest *Nilaparvata lugens* [50], and reduces significantly the populations of ladybugs and other predatory insects in cotton crops when applied at the recommended rates, i.e. 20 g/ha [109, 169]. In rice paddies, cartap hydrochloride reduced populations of coccinellid beetles, carabid beetles, dragonflies and damselflies by 20-50% [255]. Pollinators such as honey bees and bumble bees can also be seriously reduced in numbers when feeding on crops treated with cartap hydrochloride, which is included among the most toxic insecticides to bees after neonicotinoids and pyrethroids [179, 278]. For all its negative impacts on parasitoids and predatory insects it is hard to understand why cartap was the third most common insecticide (19% of all applications) used in IPM programs in Vietnam a decade ago [31], and is still among the most widely used in rice farms in China [308].

Cumulative toxicity of neonicotinoids over time of exposure results in long-term pest control compared to the impact of cholinesterase inhibitor insecticides. For example, soil treated with clothianidin at 0.05-0.15% caused increasing mortality in several species of wireworms (Coleoptera: Elateridae), reaching 30-65% after 70 days, whereas chlorpyrifos at 0.15% produced 35% mortality within 30 days but no more afterwards [292]. Soil application of imidacloprid did not eliminate rapidly Asian citrus psyllid (*Diaphorina citri*) and leafminer (*Phyllocnistis citrella*) populations, but resulted in chronic residues in leaf tissue and long-term suppression of both pests [245]. Also, soil applications of neonicotinoids are very effective in controlling soil grubs and berry moths (*Paralobesia viteana*) in vineyards provided there is no irrigation or rain that washes off the insecticide [289]. For the same reason, however, the impact of neonicotinoids on non-target organisms is long-lasting. For example, repeated corn-seed treatement with imidacloprid caused a significant reduction in species richness of rove beetles in three years, even though the abundance of the main species was not affected [88]. In addition to long-term toxicity, acute toxicity of acetamiprid, imidacloprid and thiomethoxam to planthopper and aphid species is similar to that of synthetic pyrethroids, and higher than that of endosulfan or acetylcholinesterease inhibitors [219, 246]. Thus, combinations of pyrethroid-neonicotinoid have been hailed as the panacea for most pest problems as it suppresses all insect resistance [70]. Mixtures of imidacloprid and thiacloprid had additive effects on the toxicity to the nematode *Caenorhabditis elegans* but not on the earthworm *Eisenia fetida* [108].

Acute toxicity of imidacloprid, thiamethoxam, clothianidin, dinotefuran and nitenpyram to honey bees is higher than that of pyrethroids, while toxicity of acetamiprid and thiacloprid is increased by synergism with ergosterol-inhibiting fungicides [134, 242] and antibiotics [116]. Thus, neonicotinoids can pose a high risk to honey bees, bumble bees [176, 263] and wasps [90]. Bees can be killed immediately by direct contact with neonicotinoid droplets ejected from seed drilling machines. Thus, numerous worker bees were killed when seed was coated with clothianidin during drilling of corn in the Upper Rhine Valley (Germany) in spring 2008 [102]. The same problem happened in Italy with thiamethoxam, imidacloprid and clothianidin [105, 285], leading to the banning of this application method on sunflower, canola and corn during 2008-09 [20]. However, most of the time bee colonies are intoxicated by feeding on contaminated pollen and nectar [9, 228]. It has been observed that bee foraging was notably reduced when Indian mustard was treated with 178 mg/ha imidacloprid [10]. Imidacloprid residues in sunflowers are below the no-adverse-effect concentration to honey bees of 20 μg/kg at 48-h [241], with surveys in France showing residue levels in pollen from treated crops in the range 0.1-10 μg/kg and average in nectar of 1.9 μg/kg [33]. However, bees feeding on such contaminated pollen or nectar will reach first sublethal and later lethal levels, with 50% mortality occurring within 1-2 weeks [228, 266]. Such data was disputed [89, 240] as it was in conflict with some long-term field observations of honey bees feeding on sunflowers grown from imidacloprid-treated seeds at 0.24 mg/seed [256]. However, recent evidence suggest that chronic lethality by imidacloprid is implicated in the colony collapse disorder (CCD) that affects honey bees [174]. Based on the fast degradation of imidacloprid in bees (4-5 hours), it is assumed that honey bees which consume higher amounts of imidacloprid die already outside of the hive, before the colony's demise and before samples are taken, though residues of imidacloprid in bees at 5-8 μg/kg have been found in some cases [111]. Clothianidin residues of 6 μg/kg in pollen from canola fields reduced the number of bumble bee (*Bombus impatients*) workers slightly (~20%) [96], but exposure to clothianidin-treated canola for three weeks appeared not to have affected honey bee colonies in Canada [61]. Thiamethoxam applied to tomatoes (~150 g/ha) through irrigation water does not have impacts on bumble bees (*Bombus terrestris*) [244], whereas pollen contaminated with this insecticide causes high mortality and homing failure [125].

Negative impacts of neonicotinoids on non-target soil arthropods are well documented. A single imidacloprid application to soil reduced the abundance of soil mesofauna as well as predation on eggs of Japanese beetle (*Popillia japonica*) by 28-76%, with impacts lasting four weeks. The same level of impact was observed with single applications of clothianidin, dinotefuran and thiamethoxam, so the intended pest control at the time of beetle oviposition runs into conflict with unintended effects – disruption of egg predation by non-target predators [210]. Among several insecticides applied to home lawns, only imidacloprid suppressed the abundance of Collembola, Thysanoptera and Coleoptera adults, non-oribatid mites, Hymenoptera, Hemiptera, Coleoptera larvae or Diptera taxonomic groups by 54-62% [209]. Imidacloprid applied to the root of eggplants (10 mg/plant) greatly reduced most arthropod communities and the species diversity during the first month. Small amounts of soil residues that moved into the surrounding pasture affected also some species; however, non-target ground arthropods both inside and outside the crop showed significant impacts only in the two weeks after planting [238], probably due to compensatory immigration from nearby grounds.

Foliar applications of thiamethoxam and imidacloprid on soybean crops are preferred to seed treatments, as neonicotinoids appear to have lesser impacts on non-target communities than pyrethroids [204]. However, a foliar application of thiacloprid (0.2 kg/ha) to apple trees reduced the population of earwigs (*Forficula auricularia*), an important predator of psyllids and woolly apple aphid, by 60% in two weeks, while remaining below 50% after six weeks [294]. Branchlets of hemlock (*Tsuga canadensis*) treated with systemic imidacloprid (1-100 mg/kg) reduced the populations of two non-target predators of the hemlock woolly adelgid (*Adelges tsugae*) and had both lethal and sublethal effects on them [78]. Clothianidin, thiamethoxam and acetamiprid were as damaging to cotton crop predators as other broad-spectrum insecticides and cartap [169]. All neonicotinoids are lethal to the predatory mirid *Pilophorus typicus*, a biological control agent against the whitefly *Bemisia tabaci*, since their residual activity can last for 35 days on the treated plants [201]. The ladybug *Serangium japonicum*, also a predator of the whitefly, is killed in large numbers when exposed to residues of imidacloprid on cotton leaves applied at the recommended rate (40 ppm) or lower; apparently, the predator was not affected when imidacloprid was applied as systemic insecticide [120]. Clothianidin is 35 times more toxic to the predatory green miridbug (*Cyrtorhinus lividipennis* 48-h LC50 = 6 μg/L) than to the main pest of rice (*Nilaparvata lugens* 48-h LC50 = 211 μg/L), thus questioning seriously its application in such crops [221]. Not surprisingly, populations of predatory miridbugs and spiders suffered an initial set back when rice paddies were treated with a mixture of ethiprole+imidacloprid (125 g/ha), and their recovery was slow and never attained the densities of the control plots [154]. Mixtures of ethiprole+imidacloprid and thiamethoxam+l λ-cyhalothrin on rice paddies are also highly toxic to mirid and veliid natural enemies of rice pests, with 100% mortalities recorded in 24 h [159].

Secondary poisoning with neonicotinoids reduces or eliminates eventually all predatory ladybirds in the treated areas, compromising biological control in IPM programs. Indeed, exposure of larval stages of *Adalia bipunctata* to imidacloprid, thiamethoxam, and acetamiprid, and adult stages to imidacloprid and thiamethoxam, significantly reduced all the demographic parameters in comparison with a control –except for the mean generation time–, thus resulting in a reduced coccinellid population; adult exposures produced a significant population delay [162]. Eighty percent of 3rd and 4th instar larvae of the ladybug *Harmonia axyridis* died after feeding for 6 hours on corn seedlings grown from seeds treated with clothianidin, compared to 53% mortality caused by a similar treatment with thiamethoxam; recovery occurred only in 7% of cases [196]. Survival of the ladybird *Coleomegilla maculata* among flower plants treated with imidacloprid at the label rate was reduced by 62% [251], and *Hippodamia undecimnotata* fed upon aphids reared on bean plants treated with imidacloprid, experienced a 52% population reduction [206]. Equally, 96% of Tasmanian brown lacewing (*Micromus tasmaniae*) larvae died after feeding on 1st instar lettuce aphid (*Nasonovia ribisnigri*) for three days. Low doses did not increase mortality but from days 3 to 8, lacewing larvae showed significant evidence of delayed developmental rate into pupae [298]. Grafton-Cardwell and Wu [110] demonstrated that IGRs, neonicotinoid insecticides, and pyrethroid insecticides have a significant, negative impact on vedalia beetles (*Rodolia cardinalis*), which are essential to control scale pests in citrus; neonicotinoids were toxic to vedalia larvae feeding on cottony cushion scale that had ingested these insecticides, and survival of adult beetles was also affected but to a lesser extent than other insecticides.

Recent evidence of the negative impacts of neonicotinoids on parasitoids reinforces that these insecticides are not suitable for IPM [271]. All neonicotinoids are deadly to three whitefly parasitoid species (*Eretmocerus* spp. and *Encarsia formosa*), with mortality of adults usually greater than the pupae [267]. Thiamethoxam appears to be less toxic to whitefly parasitoids compared to imidacloprid [202]. Imidacloprid, thiamethoxam and nitenpyram appeared to be the most toxic to the egg parasitoids *Trichogramm*a spp. [231, 299]. For example, the acute toxicity of thiomethoxam and imidacloprid to *Trichogramma chilonis*, an egg parasitoid of leaf folders widely used in cotton IPM, is about 2000 times higher than that of other insecticides used in rice crops in India, such as acephate or endosulfan [220]. Acute toxicity of imidacloprid is more pronounced on Braconidae parasitoids than on *T. chilonis*, whereas thiacloprid only reduced the parasitization on *Microplitis mediator* [192]. Thiacloprid is as toxic to the cabbage aphid *Brevicoryne brassicae* as to its parasitoid (*Diaeretiella rapae*), whereas pirimicarb and cypermethrin are more toxic to the aphid and are, therefore, preferred in IPM [3].

Neonicotinoids pose also risks to aquatic taxa. The synergistic toxicity of imidacloprid+thiacloprid on *Daphnia magna* [173] implies the combined effect of neonicotinoids on aquatic arthropods would be higher than expected, even if *Daphnia* is very tolerant of neonicotinoids [119]. Other contaminants, such as the nonylphenol polyethoxylate (R-11) act also synergistically with imidacloprid [49]. Thiacloprid causes delayed lethal and sublethal effects in aquatic arthropods, which can be observed after 4 to 12 d following exposure to single 24-h pulses [28]. Thus, its 5% hazardous concentration (0.72 μg/L) is one order of magnitude lower than predicted environmental concentrations in water [35]. Also, thiacloprid LC50 for survival of midges (*Chironomus riparius*) is only 1.6 μg/L, and EC50 for emergence 0.54 μg/L [160], so both acute and chronic toxicity reduce the survival and growth of *C. tentans* and *Hyalella azteca* [265]. Acute toxicity of neonicotinoids to red swamp crayfish (*Procambarus clarkii*) is 2-3 orders of magnitude lower than that of pyrethroids [23]; comparative data such as this gives the neonicotinoids an apparent better environmental profile. However, experimental rice mesocosms treated with imidacloprid at label rates (15 kg/ha) eliminated all zooplankton communities for two months, and their recovery did not reach the control population levels four months later. Equally, mayflies, coleoptera larvae and dragonfly nymphs were significantly reduced while residues of imidacloprid in water were above 1 μg/L [117, 237]. Similarly, streams contaminated with a pulse of thiacloprid (0.1-100 μg/L) resulted in long-term (7 months) alteration of the overall invertebrate community structure [27]. However, while aquatic arthropods with low sensitivity to thiacloprid showed only transient effects at 100 μg/L, the most sensitive univoltine species were affected at 0.1 μg/L and did not recover during one year [167].

4.1.3. Fipronil

Fipronil is very efficient in controlling locust outbreaks, but causes more hazards than chlorpyrifos and deltamethrin to non-target insects in the sprayed areas, although it is more selective to specific taxa [214, 252]. Thus, abundance, diversity and activity of termites and ants were all reduced in northern Australia after spraying several areas with fipronil for locust control [262], and 45% of the termite colonies died within 10 months of a spraying operation with fipronil for controlling locusts in Madagascar [214]. Reducing the recommended application rates by seven times (0.6-2 g/ha) still achieves 91% elimination of locusts while having lesser impacts on non-target organisms, comparable to those inflicted by carbamate and OP insecticides [18].

Despite its selectivity, fipronil in maize crops reduced the abundance of arthropod populations of the soil mesofauna more significantly than other systemic insecticides, i.e. carbofuran [59], although springtails are little affected as they avoid feeding on litter contaminated with fipronil and are more tolerant of this insecticide [232]. When applied to citrus orchards, fipronil was among the most detrimental insecticides affecting two *Euseius* spp. of predatory mites [112]. In rice crops, the effectiveness of fipronil in controlling pests was overshadowed by its negative impact on the predatory miridbugs *Cyrtorhinus lividipennis* and *Tytthus parviceps* [159].

Of greater concern is the impact of this systemic chemical on honey bees and wild bee pollinators. With an acute contact LD50 of 3.5 ng/bee [166] and acute oral LD50 of 3.7-6.0 ng/bee [2], fipronil is among the most toxic insecticides to bees ever developed. Even more worrying is the finding that the adjuvant Sylgard, used to reduce the toxicity of most insecticidal products on bees, increases the toxic effects of fipronil [184]. The systemic nature of this chemical implies that chronic feeding of the bees on nectar contaminated with fipronil caused 100% honey bee mortality after 7 days, even if the residue concentration was about 50 times lower than the acute lethal dose [8]. Residues of fipronil in pollen have been measured as 0.3-0.4 ng/g, which are 30-40 times higher than the concentration inducing significant mortality of bees by chronic intoxication [33]. Unlike neonicotinoids, no residues of fipronil have been found in guttation drops [272].

The acute toxicity of fipronil to cladocerans is similar to the toxicity to estuarine copepods, with 48-h LC50 in the range 3.5-15.6 μg/L [47, 259], but the chronic toxicity with time of exposure is what determines the fate of the populations exposed. For example, populations of *Daphnia pulex* went to extinction after exposure to 80 μg/L for 10 days, equivalent to LC75 [259], and 40% of a population of grass shrimps (*Palaemonetes pugio*) died in 28 days after being exposed to fipronil concentrations of 0.35 μg/L in marsh mesocosms, and none of the shrimps survived when exposed to 5 mg/L during the same period [303]. Such impacts on zooplankton are likely to occur in estuaries, where waters have been found to contain 0.2-16 μg/L of fipronil residues [45, 163], even if no apparent effect on amphipods, mussels nor fish has been observed [37, 303]. Fipronil sprays on water surfaces to control mosquito larvae have negative impacts not only on cladocerans but also on chironomid larvae exposed to chronic feeding on contaminated residues [183, 264]. Studies on rice mesocosms have shown that significant population reductions due to fipronil application at the recommended rates (50 g per seedling box) are not restricted to zooplankton and benthic species, but affect most species of aquatic insects. Moreover, fipronil impacts on aquatic arthropods were more pronounced after a second application in the following year [118], indicating persistence of this insecticide in rice paddies. Chronic toxicity over time explains the long-term toxicity of this systemic compound, so it is not surprising that concentrations of 1.3 μg/L in paddy water were sufficient to kill 100% of dragonfly (*Sympetrum infuscatum*) nymphs in nine days [138].

4.1.4. Insect growth regulators

There is little information about the effect of systemic chitin inhibitors on non-target organisms. Obviously these compounds are harmless to fish at levels above 1 mg/L for a week-long exposures [290], and to all vertebrates in general. IGRs affect mainly the larval stages of Lepidoptera, Coleoptera and Hymenoptera, and their activity last longer than that of other pest control products [178]. The effectiveness of these compounds in controlling target pests is demonstrated by comparing the dietary LC50 of hexaflumuron (0.31 mg/L) to the target cotton worm (*Helicoverpa* sp.), which is 35 times lower than that of the systemic carbamate thiodicarb and less damaging to non-target predators [64]. Aquatic communities of non-target arthropods in rice fields (e.g. Cladocera, Copepoda, Odonata, Notonectidae, Coleoptera and Chironomidae taxa) were not affected by teflubenzuron applied at rates to control mosquitoes (5.6 mg/ha), even though this IGR remained active for several weeks during autumn and winter periods [239].

After application of IGRs to a crop, affected insect pests are prey to many species of spiders, some of which are also susceptible to the toxicity of these products, in particular the ground hunter spiders [211]. Larvae and eggs of pests contaminated with systemic IGR are consumed by a number of predators, including earwigs, which undergo secondary poisoning and stop growing beyond the nymph stage [226]. Chitin inhibitors only show effects on the larvae of predatory insects that had consumed treated-prey, not on the adult insects. As a consequence, predatory populations collapse, as it happened with the ladybeetle *Chilocorus nigritus* that fed on citrus red scales (*Aonidiella aurantii*) in African orchards that had been treated with teflubenzuron [177]. Teflubenzuron sprayed at 16.4 g/ha for locust control in Mali did not affect the non-target arthropods in the herb layer, whereas ground-living Collembola, Thysanura, Coleoptera and Lepidoptera larvae were reduced by about 50% [151]. Moreover, teflubenzuron has multigenerational impacts: experiments with springtails exposed to artificial soil contaminated with this IGR showed that the F2 generation suffered significantly from its effects even when only the F0 generation had been exposed for 10 days [42]. Secondary poisoning with chitin inhibitors can be detrimental also to parasitoids such as *Diadegma semiclausum*, which may fail to produce enough cocoons in the treated hosts, but do not seem to affect the parasitism of other Hymenoptera [98]. For instance, novaluron did not affect the parasitisation of *Trichogramma pretiosum* on mill moth's caterpillars, a pest of tomato crops [44]. On the other hand, teflubenzuron appears to be harmless to predatory mites [32]. IPM programs must always consider the implications of using systemic chitin inhibitors to control specific pests without destroying their natural predators in the first place.

Halofenozide does not appear to cause any acute, adverse effects through topical, residual, or dietary exposure of the ground beetle *Harpalus pennsylvanicus*. In contrast to the negative effects of other systemic insecticides (i.e. imidacloprid), the viability of eggs laid by females fed halofenozide-treated food once, or continuously for 30 days, was not reduced [156].

4.2. Sublethal effects

Very often, sublethal effects of systemic insecticides are a first step towards mortality, as they are caused by the same neurotoxic mechanisms. Apart from these, there may be other effects on reproduction, growth, longevity, etc. when organisms are exposed to low, sublethal doses or concentrations. These effects are only observable in individuals that survive the initial exposure, or in species that are tolerant to insecticides. For a review see [69].

4.2.1. Acetylcholinesterase inhibitors

Longevity of the parasitoid *Microplitis croceipes* that fed on nectar from cotton treated with aldicarb was affected for at least 10 days after application, and its foraging ability of the parasitoid's host was severely impaired for 18 days [257]. Carbofuran caused a significant reduction of adult weight and longevity of the predator ladybug *Hippodamia undecimnotata*, as well as a 55% reduction in fecundity when fed on aphids contaminated with this insecticide [206]. Longevity and survival of *Aphidius ervi*, an important parasitoid of the pea aphid (*Acyrthosiphon pisum*), were significantly reduced after treating with LC25 concentrations of dimethoate or pirimicarb [11]. A significant reduction in body size of females of the predator carabid *Pterostichus melas italicus* and altered sexual dimorphism were observed after long-term exposure in olives groves treated with dimethoate at a rate that caused 10% mortality after three days [104]. Unlike other insecticides, no behavioural effects of dimethoate or triazamate on honey bees were recorded [67].

Earthworms (*Lumbricus terrestris*) experienced significant reduction in growth rate and total protein content after soil applications of aldicarb at LC10 or LC25, but only small amounts of residues were detected in the worms [198]. Aldicarb and phorate can also increase infections by *Rhizoctonia* stem canker in potato fields [280].

A typical pattern of sublethal intoxication was revealed when red-winged blackbirds (*Agelaius phoeniceus*) were exposed to increasing doses of dimethoate: 2 mg/kg b.w. doses produced ataraxia, defecation and diarrhoea; neuromuscular dysfunctions and breathing complications appeared at 3 mg/kg, and by 5 mg/kg muscle paralysis and death occurred. The estimated LC50 was 9.9 mg/kg, and all birds died at doses above 28 mg/kg [38]. Although sublethal AChE depression by acephate (25% brain) did not affect the attack behaviour in American kestrels (*Falco sparverius*) [229], nor did alter breeding behaviour in American robins (*Turdus migratorius*) [65], exposure to 256 mg/kg b.w. acephate impaired the migratory orientation of the white-throated sparrow (*Zonotrichia albicollis*) [295]. Similarly, low doses of demeton–S-methyl did not affect starlings (*Sturnus vulgaris*) behaviour [279], but doses of 2.5 mg/kg b.w. of dicrotophos administered to female starlings significantly reduced their parental care and feeding of nestlings [113]. Carbofuran orally administered to pigeons (*Columba livia*) had profound effects on flight time, with pigeons falling off the pace of the flock when doses were between 0.5 and 1.0 mg/kg b.w. [36].

AChE activities in adductor muscle were depressed in freshwater mussels (*Elliptio complanata*) exposed for 96 h at concentrations as low as 0.1 mg/L and 1.3 mg/L of aldicarb and acephate respectively, while increasing the water temperature from 21 to 30 ^{o}C resulted in mortality [199]. High AChE inhibition (70%) by acephate was not associated with immobility of *Daphnia magna*, but increasing the concentration of acephate further had a strong detrimental effect on mobility, suggesting that binding sites other than AChE may be involved in acephate toxicity [222].

Exposure of bluegill fish (*Lepomis macrochirus*) to 30 μg/L carbofuran decreased significantly adenylate parameters in gill, liver, muscle and stomach tissues after 10 days, and then returned to normal [128]. Also, concentrations of carbofuran at half the LC50 dose for fathead minnow (*Pimephales promelas*) larvae caused reductions in swimming capacity, increased sensitivity to electric shocks, and a reduction in upper lethal temperature [121]. Enzymes of protein and carbohydrate metabolism were altered (some increased, others decreased) in liver and muscle tissues of the freshwater fish, *Clarias batrachus* when exposed to 7.7 mg/L of carbofuran for six days, recovering later to normal levels [26]. Exposure of guppies (*Brachydanio rerio*) to half the recommended dose for dimethoate (0.025 μl/L) caused morphological changes in hepatocytes within three days, as well as necrosis and other abnormalities [227]. When exposed to a range of monocrotophos concentrations (0.01-1.0 mg/L), male goldfish (*Carassius auratus*) showed higher levels of 17-β-estradiol and vitollogenin and lower levels of testosterone than normal, interfering with gonadotropin synthesis at the pituitary gland [281]. Eggs of the toad *Bufo melanostictus* exposed to acephate hatched normally, but the tadpoles exhibited deformities such as tail distortions and crooked trunk; decreased pigmentation, peeling of the skin, inactivity, delay in emergence of limbs and completion of metamorphosis were also apparent [103].

Insecticide mixtures can enhance not only the acute but also the sublethal effects. For example, disulfoton together with endosulfan caused cytological and biochemical changes in liver of rainbow trout (*Oncorrhynchus mykiss*), independently of their respective modes of action [13]. Mixtures of aldicarb and other insecticides enhanced significantly the establishment of parasitic lungworm nematodes (*Rhandias ranae*) in leopard frogs (*Rana pipiens*) some 21 days after infection [101], as the frog's immune response was suppressed or altered [51]. Similarly, laboratory rats exposed to sublethal mixtures of aldicarb, methomyl and a herbicide (metribuzin) showed learning impairment, immune response and endocrine changes [215].

4.2.2. Insecticides acting on nAChR

Laboratory experiments have shown a number of abnormalities such as less melanin pigmentation, wavy notochord, crooked trunk, fuzzy somites, neurogenesis defects and vasculature defects in zebrafish (*Danio rerio*) embryos exposed to a range of cartap concentrations. The most sensitive organ was the notochord, which displayed defects at concentrations as low as 25 µg/L [308]. It is obvious that essential enzymatic processes are disturbed during embryo development, among which the inhibition of lysyl oxidase is responsible for the notochord undulations observed.

Imidacloprid does not cause high mortality among eggs or adults of the preparasite nematode *Agamermis unka,* but impairs the ability of the nematode to infect nymphs of the host brown planthopper (*Nilaparvata lugens*) [50]. Contrary to this, a synergistic effect of imidacloprid on reproduction of entomopathogenic nematodes against scarab grubs may increase the likelihood of infection by subsequent generations of nematodes, thereby improving their field persistence and biological potential to control grubs. Acetamiprid and thiamethoxam, however, do not show synergist interactions with nematodes [149]. Imidacloprid at 0.1-0.5 mg/kg dry soil disturbs the burrowing ability of *Allolobophora* spp. earthworms [43], and the highest concentration can also induce sperm deformities in the earthworm *Eisenia fetida* [306]. Reduction in body mass (7-39%) and cast production (42-97%) in *Allolobophora* spp. and *Lumbricus terrestris* have also been observed after 7 days exposure to relevant environmental concentrations of imidacloprid [74]. Residues of imidacloprid in maple leaves from treated forests (3–11 mg/kg) did not affect survival of aquatic leaf-shredding insects or litter-dwelling earthworms. However, feeding rates by aquatic insects and earthworms were reduced, leaf decomposition (mass loss) was decreased, measurable weight losses occurred among earthworms, and aquatic and terrestrial microbial decomposition activity was significantly inhibited, thus reducing the natural decomposition processes in aquatic and terrestrial environments [150].

The dispersal ability of the seven-spotted ladybirds (*Coccinella septempunctata*) sprayed with imidacloprid was compromised, and this may have critical consequences for biological control in IPM schemes [21]. A significant reduction of adult weight and longevity of the ladybug *Hippodamia undecimnotata*, as well as 33% reduction in fecundity were observed when this predatory bug fed on aphids contaminated with imidacloprid [206]. Imidacloprid and fipronil had adverse effects on the immune response of the wolf-spider *Pardosa pseudoannulata*, reducing significantly its phenoloxidase activity, the total number of hemocytes and encapsulation rate [282]; the implications of such effects on this natural enemy of rice pests are unknown. When applied in the egg-larval or pupal stages, acetamiprid or imidacloprid reduced the parasitisation capacity of F1 and F2 generation females of *Trichogramma pretiosum* on mill moth's caterpillars (*Anagasta kuehniella*), a pest of tomato crops [44]. Longevity of females of the parasitoid *Microplitis croceipes* that fed on nectar from imidacloprid-treated cotton was affected for at least 10 days after application, while the parasitoid's host foraging ability was severely affected from day 2 onwards [257]. Exposure of western subterranean termites (*Reticulitermes hesperus*) to acetamiprid (1 mg/kg sand) or imidacloprid also impaired locomotion of termites within 1 hour [230].

Bumble bees (*Bombus terrestris*) interrupt their activity for several hours when exposed to imidacloprid sprayed on plants [132], and soil treatment at the highest recommended doses extended the handling times of *B. impatiens* on the complex flowers [194]. Such an impairment affects the bees foraging behaviour and can result in a decreased pollination, lower reproduction and finally in colony mortality due to a lack of food [193]. Although Franklin et al. [96] found that clothianidin residues of 6 μg/kg in canola pollen reduced the production of queens and increased the number of males in *B. impatients*, their study did not find significant differences with controls due to a high variability in the results. Larval development in wild bees (*Osmia lignaria* and *Megachile rotundata*) was delayed significantly when fed pollen contaminated with either imidacloprid or clothianidin at 30 or 300 μg/kg [1]. Honey bees are more sensitive to neonicotinoids than bumble bees: at 6 μg/kg, imidacloprid clearly induced a decrease in the proportion of active bees [57], and 50-500 μg/L affect significantly their activity, with bees spending more time near the food source [273]. Other authors found that lower activity of honey bees during the hours following oral exposure to 100-500 μg/L imidacloprid in syrup is transitory [186]. In any case, that may explain the delayed homing behaviour of honey bees exposed to 100 μg/L imidacloprid in syrup and their disappearance at higher doses [34, 304]. Honey bees fed on syrup contaminated with acetamiprid increased their sensitivity to antennal stimulation by sucrose solutions at doses of 1 μg/bee and had impaired long-term retention of olfactory learning at 0.1 μg/bee. Contact exposure at 0.1 and 0.5 μg/bee increased locomotor activity and water-induced proboscis extension reflex but had no effect on behaviour [82]. Similar response was obtained with honey bees exposed to thiomethoxam by contact, having impaired long-term retention of olfactory learning at 1 ng/bee [8]. Winter bees surviving chronic treatment with imidacloprid and its metabolite (5-OH-imidacloprid) had reduced learning performances than in summer: the lowest-effect concentration of imidacloprid was lower in summer bees (12 μg/kg) than in winter bees (48 μg/kg), indicating a greater sensitivity of honey bees behaviour in summer bees compared to winter bees [68].

Honey bees infected with the microsporidian *Nosema ceranae* experienced 7 or 5 times higher mortality than normal when fed syrup contaminated with sublethal doses of thiacloprid (5 mg/L) or fipronil (1 μg/L), respectively [293]. *N. ceranae* is a key factor in the CCD in honey bees [127], and the synergistic effect of these systemic insecticides on *Nosema* is probably its underlying cause [213]. Suppression of the immune system is not restricted to bees, as a massive infection of medaka fish by a protozoan ectoparasite (*Trichodina* spp.) when exposed to imidacloprid in rice mesocosms has been documented [236].

Imidacloprid residues in water as low as 0.1 μg/L are sufficient to reduce head and torax length in mayfly nymphs of *Baetis* and *Epeorus*, whether applied as pulses or in continuous exposures for 20 days [6]. At 1 μg/L the insecticides caused feeding inhibition. However, 12-h pulses induced emergence because of stress, whereas constant exposure reduced survivorship progressively. Also, the aquatic worm *Lumbriculus variegatus* experienced immobility during 4 days when exposed to 0.1-10 μg/L imidacloprid [5].

4.2.3. Fipronil

Apart from the extreme acute toxicity of this insecticide to bees, honey bees fed on sucrose syrup contaminated with fipronil (2 μg/kg) reduced significantly their attendance to the feeder [57]. It has also been demonstrated that sublethal concentrations of this insecticide as low as 0.5 ng/bee, whether orally or topically applied, reduce the learning performance of honey bees and impair their olfactory memory but not their locomotor activity [67, 82]. Furthermore, chronic feeding exposure at 1 μg/kg or 0.01 ng/bee reduced learning and orientation, whilst oral treatment of 0.3 ng/bee reduced the number of foraging trips among the exposed workers [66]. In addition to their activity, honey bees fed with sucrose syrup containing 1 μg/L fipronil increased significantly the mortality of bees infected with the endoparasite *Nosema ceranae*, suggesting a synergistic effect between the insecticide and the pathogen [293]. All these sublethal effects reduce the performance of the hive and help explain the decline in honey bee and wild bee pollinators in many countries [205], although fipronil is not alone in causing this demise – neonicotinoids are equally implicated.

Female zebra finches (*Taeniopygia guttata*) fed with single sublethal doses of fipronil (1, 5, and 10 mg/kg b.w.) failed to hatch 6 out of 7 eggs laid. The only chick born was underdeveloped and had fiprole residues in the brain, liver and adipose tissues. By contrast, 12-day-old chicken eggs injected with fipronil (5.5 to 37.5 mg/kg egg weight) hatched normally although the chicks from the highest dose group showed behavioural and developmental abnormalities [145].

Low residues of fipronil in estuary waters (0.63 μg/L) inhibited reproduction of the copepod *Amphiascus tenuiremis* by 73-89%, and this effect seems to be more prevalent on males than on females [45]. Even lower residue levels (0.22 μg/L) halted egg extrusion by 71%, whereas exposure to 0.42 μg/L nearly eliminated reproduction (94% failure) on this species. Based on these results from chronic and sublethal toxicity, a three-generation Leslie matrix model predicted a 62% decline in population size of *A. tenuiremis* at only 0.16 μg/L [47]. Unlike other insecticides, the stress on *Ceriodaphnia dubia* caused by predatory cues of bluegill fish (*Lepomis macrochirus*) was significantly exacerbated when the cladocerans were exposed to 80-160 μg/L of fipronil [223]; however, these concentrations are much higher than the residue levels usually found in waters [99, 163].

While fipronil applied at the recommended rates in rice fields induces biochemical alterations in carp (*Cyprinus carpio*), such metabolic disturbances do not appear to have any effect on growth nor mortality of this fish after 90 days exposure at <0.65 μg/L [52]. However, similar residue levels (<1 μg/L) reduced significantly the growth of adult medaka fish (*Oryzias latipes*) after two weeks of exposure, as well as growth of their offspring in the first 35 days, even if residues of fipronil by that time were below the analytical detection limit (0.01 μg/L) [117].

4.2.4. Insect growth regulators

Longevity of predatory bug *Podisus maculiventris* was reduced after preying on Colorado potato beetles that fed on foliage treated with novaluron at 85 g/ha. Females produced fewer eggs and their hatching was significantly suppressed, while 5th instars that also preyed on the beetles failed to moult into adults [62]. Novaluron and hexaflumuron significantly decrease (<30%) the total protists population in the guts of termites (*Reticultermes flavipes*), thus upsetting their digestive homeostasis [165].

4.3. Indirect effects on populations and communities

Indirect effects result from the dynamics of ecosystems. Thus, applications of granular phorate to soil eliminate most soil invertebrates (see 4.1) except for Enchytraeidae worms, which increase in large numbers and take over the leaf-litter decomposition function carried out by the eliminated springtails [300].

Resurgence or induction of pests by altering the prey-predator relationships in favour of the herbivore species is most common. When carbofuran was applied to corn plantations in Nicaragua, the population levels of the noctuid pest *Spodoptera frugiperda* increased because of lesser foraging activity by predatory ants [212]. Methomyl eliminated the phytoseiid predatory mite *Metaseiulus occidentalis* for 10 days, thus causing an increase in Pacific spider mites (*Tetranychus pacificus*) and leafhopper (*Eotetranychus willamettei*) populations in the treated vineyards [130]. Unexpected outbreaks of a formerly innocuous herbivore mite (*Tetranychus schoenei*) were observed after imidacloprid applications to elms in Central Park, New York. A three-year investigation on the outbreaks showed that elimination of its predators and the enhanced fecundity of *T. schoenei* by this insecticide were responsible for that outcome [268].

The widespread use of insecticides usually tips the ecological balance in favour of herbivore species. For example, dimethoate sprayed on clover fields indirectly reduced the populations of house mice (*Mus musculus*) in the treated areas as the insect food source was depleted. However, herbivore species such as prairie voles (*Microtus ochrogaster*) and prairie deer mouse (*Peromyscus maniculatus*) increased in density levels [24], since they had more clover available due to either higher clover yields or through less competition with the house mice or both.

A reduction in arthropod populations often implies starvation of insectivorous animals. For example, densities of two species of lizards and hedgehogs in Madagascar were reduced 45-53% after spraying with fipronil to control a locust outbreak, because their favourite termite prey was almost eliminated (80-91%) by this chemical [214]. However, this type of indirect impact is difficult to observe and measure in birds, since they can move to other areas or change their resource diet. For example, hemlock forests treated with imidacloprid to control hemlock woolly adelgid (*Adelges tsugae*) reduced significantly Hemiptera and larval Lepidoptera, but not other insect taxa. Although larval Lepidoptera are the primary prey for insectivorous foliage-gleaning birds, many birds were able to find other food resources in the mixed hemlock-deciduous stands that were not treated [87]. Similarly, post-treatment with fipronil for grasshopper control in Wyoming did not affect bird densities, perhaps due to the large initial insect populations; fipronil plots generally had higher avian population densities (nongregarious, insectivores and total birds) than other areas treated with carbaryl [203]. Although some early studies found that fipronil did not have much impact on aquatic communities of Sahelian ponds [158], nor in predatory invertebrates in the Camargue marshes, herons in the latter region avoid rice fields treated with fipronil because of the scarcity of invertebrate food in there [188].

Food aversion to pesticide-treated seeds or plants is a mechanism that may indirectly ameliorate the toxic effects of systemic insecticides such as carbofuran in mice and other small rodents [170]. Some Collembola species (i.e. *Folsomia fimetaria*) avoid dimethoate sprayed areas [86], and female parasitoids (*Cotesia vestalis*) are discouraged from getting to their host –the diamond-back month (*Plutella xylostella*) – in turnip plants treated with methomyl, whereas clothianidin does not produce aversion [248]. Equally, dimethoate and oxydemeton-methyl sprayed on peach trees discourage honey bees from visiting in the first two days after application, while treatments with imidacloprid, acetamiprid and thiamethoxam allow honey bees visits [246]. This helps explain the high long-term impact of neonicotinoids on bees compared to the effect of OP insecticides, even if imidacloprid at high experimental concentrations in syrup (>0.5 mg/L) may also have repellent effect on honey bees [34].

5. Risk assessment of systemic insecticides

All systemic compounds have effects with time of exposure. However, only the persistent chemicals (fipronil, neonicotinoids, cartap and some OPs) have cumulative effects over time, since the non-persistent compounds are quickly degraded in soil and water.

For risk assessment of these compounds it is important to understand their chronic impacts. Unlike traditional protocols based on acute toxicity, the persistent activity of the parent and toxic metabolites requires that exposure time must be taken into consideration [115]. Concerns about the impacts of dietary feeding on honey bees and other non-target organisms are thus justified [9, 60, 228], because the accumulation of small residue levels ingested repeatedly over time will eventually produce a delayed toxic effect [276]. For example, bees that feed on contaminated nectar and pollen from the treated crops are exposed to residues of imidacloprid and fipronil in the range 0.7-10 μg/kg and 0.3-0.4 μg/kg respectively [33], which appear in 11% and 48% of the pollen surveyed in France [48]. Based on those findings an estimate of the predicted environmental concentrations that bees are ingesting in that country can be made for each insecticide. Since there is a log-to-log linear relationship between concentration and time of exposure [234], the critical levels of residue and time of exposure can be determined.

The declining populations of predatory and parasitic arthropods after exposure to recommended applications of most systemic insecticides are worrying. In view of the above, it not so much the small concentrations they are exposed to but the time of exposure that makes the population decline progressively over weeks, months and even years of treatment, as described in this chapter. Lethal and sublethal effects on reproduction are equally implicated. This is the reason why systemic insecticides should be evaluated very carefully before using them in IPM schemes. Obviously, recovery rates are essential for the populations affected to come back, and this usually occurs by recolonisation and immigration of individuals from non-affected areas. For example, modelling based on recovery data after dimethoate application to wheat fields [277] demonstrates that a non-target organism that is reduced by only 20% but is unable to recover is likely to be far more at risk from exposure to a pesticide than an organism that is reduced 99% for a short period but has a higher recovery potential.

The above is also relevant to the impact of small residues of those systemic insecticides that have cumulative effects (e.g. neonicotinoids, fipronil and cartap) on aquatic ecosystems. Because of the short life-cycle of many zooplankton species, the negative population parameters that result from sublethal and chronic effects on such organisms can lead their local populations to extinction [260]. Immediate reductions in populations and species may not always be apparent due to the small residue concentrations and the delayed effects they cause. For example, in recent surveys of pesticide residues in freshwaters of six metropolitan areas of USA, fipronil appears regularly in certain states [254]. Fipronil and its desulfinyl, sulfide, and sulfone degradates were detected at low levels (≤ 0.18–16 µg/L) in estuary waters of Southern California [163], and make some 35% of the residues found in urban waters, with a median level of 0.2-0.44 µg/L, most frequently during the spring-summer season [99]. Imidacloprid was detected in 89% of water samples in agricultural areas of California, with 19% exceeding the US Environmental Protection Agency’s chronic invertebrate Aquatic Life Benchmark of 1.05 µg/L [261]. In the Netherlands, imidacloprid appeared in measurable quantities in 30% of the 4,852 water samples collected between 1998 and 2007 [287]. These figures indicate there is already a widespread contamination of waterways and estuaries with persistent systemic insecticides.

The first consequence of such contamination is the progressive reduction, and possible elimination, of entire populations of aquatic arthropods from the affected areas. As time is a critical variable in this type of assessment, it is envisaged that should this contamination continue at the current pace over the years to come the biodiversity and functionality of many aquatic ecosystems will be seriously compromised [191]. Secondly, as these organisms are a primary food source of a large number of vertebrates (e.g. fish, frogs and birds), the depletion of their main food resource will inevitably have indirect impacts on the animal populations that depend on them for their own survival. The case of the partridge in England is an example of how a combination of herbicides and insecticides can bring the demise of a non-target species by indirectly suppressing its food requirements [217]. Therefore, warnings about the possible role of environmental contamination with neonicotinoids in steeply declining populations of birds, frogs, hedgehogs, bats and other insectivorous animals are not far fetched and should be taken seriously [275].

6. Conclusions

This review has brought some light on the direct, sublethal and indirect effects that systemic insecticides have on species populations and ecosystems. Some long-term impacts have been known for some time (e.g. carbofuran, phorate), but it is the rapid increase in the usage of neonicotinoids and other systemic products that poses a new challenge to the ecological risk assessment of agrochemicals. Indeed, current risk protocols, based on acute, short-term toxic effects are inadequate to cope with the chronic exposure and cumulative, delayed impacts of the new compounds. Awareness of the increasing contamination of the environment with active residues of these chemicals should help regulators and managers to implement new approaches for risk assessment of these substances.

Author details

Francisco Sánchez-Bayo[1*], Henk A. Tennekes[2] and Koichi Goka[3]

*Address all correspondence to: sanchezbayo@mac.com

1 University of Technology Sydney, Australia

2 Experimental Toxicology Services (ETS) Nederland BV, The Netherlands

3 National Institute for Environmental Sciences, Japan

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Chapter 14

Thiamethoxam: An Inseticide that Improve Seed Rice Germination at Low Temperature

Andréia da Silva Almeida, Francisco Amaral Villela,
João Carlos Nunes, Geri Eduardo Meneghello and
Adilson Jauer

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53207

1. Introduction

The discovery of thiamethoxam has opened new perspectives for the Brazilian agriculture, mainly in seed treatment. The molecule was the center of studies by a group of researchers from official agencies and universities, in order to evaluate its mechanism of action.

Researches were made to establish the activity of the active ingredient on the physiology of the plant, when applied the soybean seed treatment. It was observed that seed germination index and seedling vigor were higher than those of plants in plots without seed treatment. It was also found that, under water stress conditions soybean plants from seed treated with thiamethoxam showed better growth, such as increased length and root volume, faster initial development, higher leaf area, height, number of pods and green colored more intense.

The various pesticides used today can be classified into different classes (CASTRO, 2006), such as:

a. Regulatory Plant or Bio-regulators - organic compounds, non-nutrient, which applied to the plants, at low concentrations promotes, inhibits or modifies some morphological or physiological plant process. The term regulator is restricted to natural or synthetic compounds, applied externally in plants (named exogenous).

b. Plant Hormones - substances produced by plants, which at low concentrations regulate morphological and physiological processes of the plant. Hormones can move within the plant from the generated to the action site, or be produced at the action site. The term hormone is restricted to products that occur naturally in plants (named endogenous).

Belonging to both the previous classes, there are: auxins, gibberellins, cytokinins, abscisic acid and ethylene. It is considered, for the bio-regulator acts, that it must primarily bind to a receptor on the plasmatic membrane of the cell.

c. Plants Stimulants or bio-stimulants - mixtures of plant regulators, occasionally along together nutrients, vitamins, amino acids or miscellaneous debris. They exhibit a different stimulatory effect than if application isolated, creating a synergistic effect between regulators. Some examples of bio-stimulants the Stimulate are Promalin and e mixture GA_3 + 2,4-D.

d. Bioactivators - complex organic substances, that modify the morphology and physiology of plants and are capable of acting in the synthesis and action of endogenous hormones, leading to increase in productivity. In this class some insecticides fit, such as aldicarb and thiamethoxam, besides of the hydrogen cyanamide.

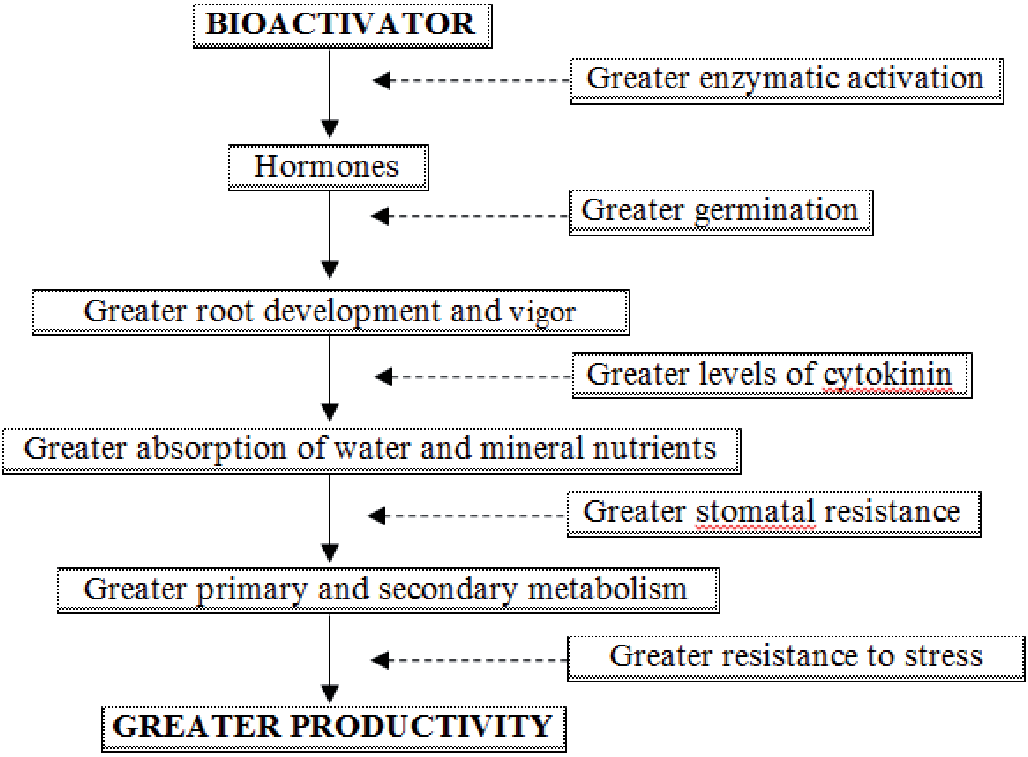

Figure 1. Sequence of events promoted by thiamethoxam (CASTRO, 2006)

Castro (2008), found that the molecule of thiamethoxam is capable of inducing physiological changes in plants. In function of the results obtained, it is concluded that the bioactivator can act in two ways: the first one, is to enable transport proteins from the cell membranes allowing a greater ionic transport, increasing the mineral nutrition of the plant. This increase in the availability of mineral salts promote positive responses in the development and plant productivity (Figure 1). The second one is related to the higher enzymatic activity caused by thiamethoxam, as the seed level or as the plant one. The highest enzymatic activity would increase both the primary and the secondary metabolism. It would increase the synthesis of amino acids, precursors of new proteins. The plant response to these proteins and hormone biosynthesis could be related to important increases in production (Figure 2).

The bioactivators are organic substances, potentially modifying the morphology and physiology of plants, by acting on the synthesis and action of endogenous hormones and may lead to increases in productivity.

In general, insecticides and fungicides are used to control insects and fungi, respectively. However, it has been found that certain chemicals may also exert actions modifying the morphology and metabolism of plants.

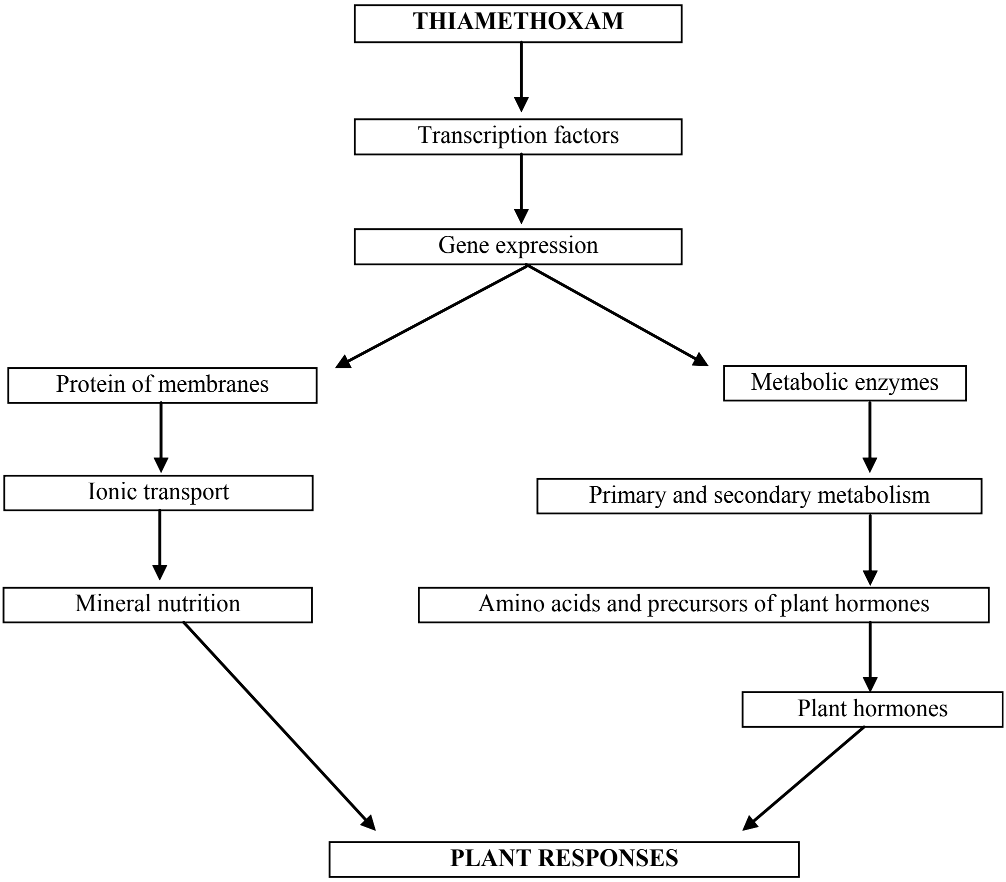

Figure 2. Action mode of thiamethoxam in plants (CASTRO, 2006)

Certain insecticides like aldicarb, carbofuran and thiamethoxam, may cause a physiological effect promoting changes in certain processes in plant physiology, such as growth, morphology or plant biochemistry.

The thiamethoxam can be applied in seed treatment, by spraying on leaves of plants or by soil application, being absorbed by the roots. Applied as a seed treatment, the thiamethoxam can promote the expression of the effect by stimulating root growth and increasing germination rate, consequently, reducing the time for field crop establishment.

2. Physiological changes in rice seeds exposed to low temperature at germination

Rice is grown in diverse environmental conditions, but when compared to other cereals such as oats or wheat, is much more sensitive to low temperatures (Mertz et al., 2009). The occurrence of cold weather is one of the major problems when irrigated rice in Rio Grande do Sul - Brazil, is cultivated since the most of the cultivars in use are from tropical origin. The occurrence of low temperatures, together with the susceptibility of the materials used can cause serious damage to the establishment of the crop, reducing the initial stand and consequently favoring the establishment of weeds. The productivity of irrigated rice in Rio Grande do Sul has suffered strong oscillations over the years, caused in part by climatic conditions, where the occurrence of low temperatures has been one of the major determinants factors of this variability at the productivity levels (Mertz et al., 2009).

On the other hand, hormone controllers have received increasingly more attention in agriculture as the crop techniques develop, especially in high value crops. The bioactivators are complex organic substances that can alter the growth, capable to act on the transcription of DNA in plant, gene expression, membrane proteins, metabolic enzymes and mineral nutrition (Castro and Pereira, 2008). The thiamethoxam insecticide has shown positive effects such vigor expression increase, biomass accumulation, high photosynthetic rate and deeper roots (Cataneo, 2008).

The aim of this work was to evaluate the influence of thiamethoxam in the rice crop and the potential benefits that treatment can provide, when rice seeds are subjected to low temperature during germination and emergence.

3. Material and methods

Three rice cultivars where used: two conventional (BR IRGA 417, BR IRGA 424) and one hybrid (Avax R.). The cultivars had the same physiological quality and were evaluated for tolerance to low temperature through the germination test. The seeds were treated with a commercial product containing 35 grams of thiamethoxam active ingredient per liter of product. The treatments were: Treatment 1 - untreated seeds; Treatment 2 - 100ml of product/100kg of seed; Treatment 3 - 200 ml of product/100kg of seed; Treatment 4 - 300 ml of product/100kg of seed and Treatment 5 - 400 ml of product/100kg of seed, prior to sowing.

The germination test was performed in three replications, eight sub-samples of 50 seeds (400 seeds per replicate) for each cultivar. The seeds were placed to germinate in paper rolls moistened with water equivalent to 2,5 times the weight of the substrate, following the criteria established by the Rules for Seed Testing (Brazil, 2009). Five germination temperatures were used: 25, 20, 18, 15 and 13°C. The germination test at temperatures of 25 and 20ºC were performed in the germinator, and at temperatures 18, 15 and 13°C held in BOD. The counting of normal seedlings was performed seven days after sowing for temperatures of 25, 20 and 18°C and at 21 days for temperatures of 15 and 13ºC.

4. Results and discussion

According to the results, the rice seeds cultivars BR IRGA 417, BR IRGA 424 and Avax R. treated with thiamethoxam, were superior in all tested temperatures, when compared to the values obtained in the zero dose (without application of thiamethoxam), varying only the intensity of this difference due to the dose used and the temperature.

By observing the data shown in Figure 3, it is found that the treated seeds showed significant increases in germination at different temperatures.

The temperatures of 15ºC and 13°C were the most adverse ones, but when the seeds are treated independent from the dose, they showed germination over the zero dose. At the dose of 200 mL/100 kg of seeds at a temperature of 15°C, there was an increase of 21 percentage points, whereas at 13°C this increase was 37 percentage points. At temperatures of 25, 20 and 18ºC this increase was on average 7 percentage points when compared with the zero dose.

Figure 4 shows that seeds treated with thiamethoxam at different temperatures had positive additions in relation to the zero dose. The results of this study confirm those obtained by Castro et al. (2007), working with soybeans, and those by Clavijo (2008) working with rice, when claiming that seeds treated with thiamethoxam had their germination accelerated by stimulating the enzymes activity, besides of showing more uniform emergency and stand more uniform and better initial impulse. Also in soybean seeds, Cataneo (2008) observed that thiamethoxam accelerates germination, and induces further development of the embryonic axis. According to the results, rice seeds, cultivars BR IRGA 417, BR IRGA 424 and Avax R. treated with thiamethoxam, were superior in all the tested temperatures, when compared to the values obtained at the zero dose (without application of thiamethoxam), varying only the intensity of this difference due to the dose used and temperature.

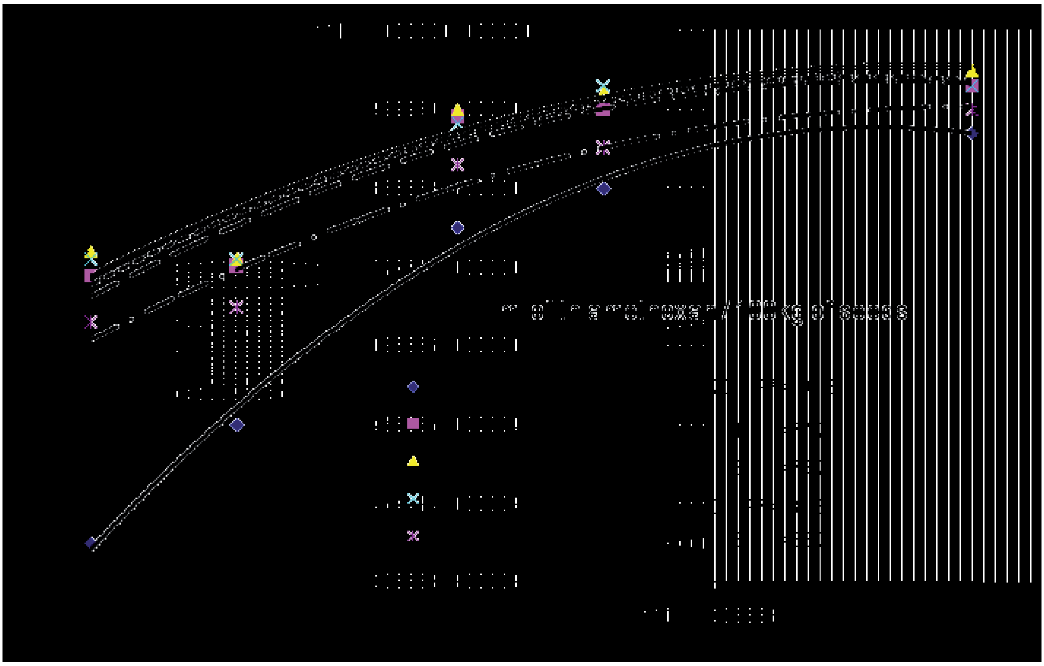

Figure 3. Germination (%) rice seeds, cultivar BR IRGA 417, treated with thiamethoxam at different temperatures.

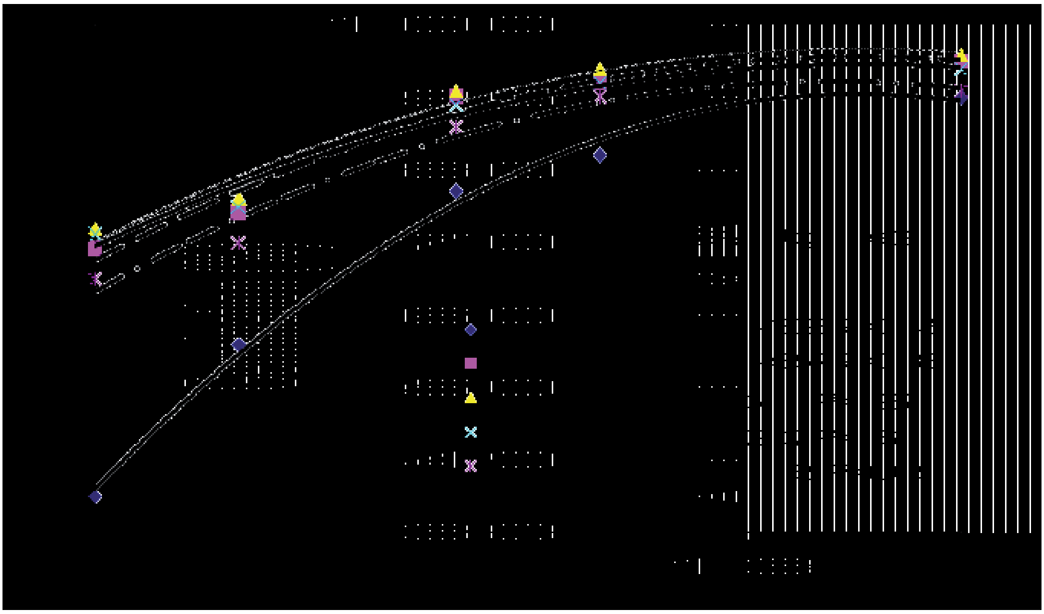

Figure 4. Germination (%) rice seeds, cultivar BR IRGA 424, treated with thiamethoxam at different temperatures.

According to Figure 5, the results of cultivar AVAXI R, hybrid rice seeds when treated with thiamethoxam showed increases in relation to the dose zero. The dose 100mL/100 kg of seeds showed higher increases when compared with other doses at all studied temperatures, being of 28 percentage points at a temperature of 13°C which is the most drastic one, comparing the doses 100mL/100kg of seeds with the zero dose.

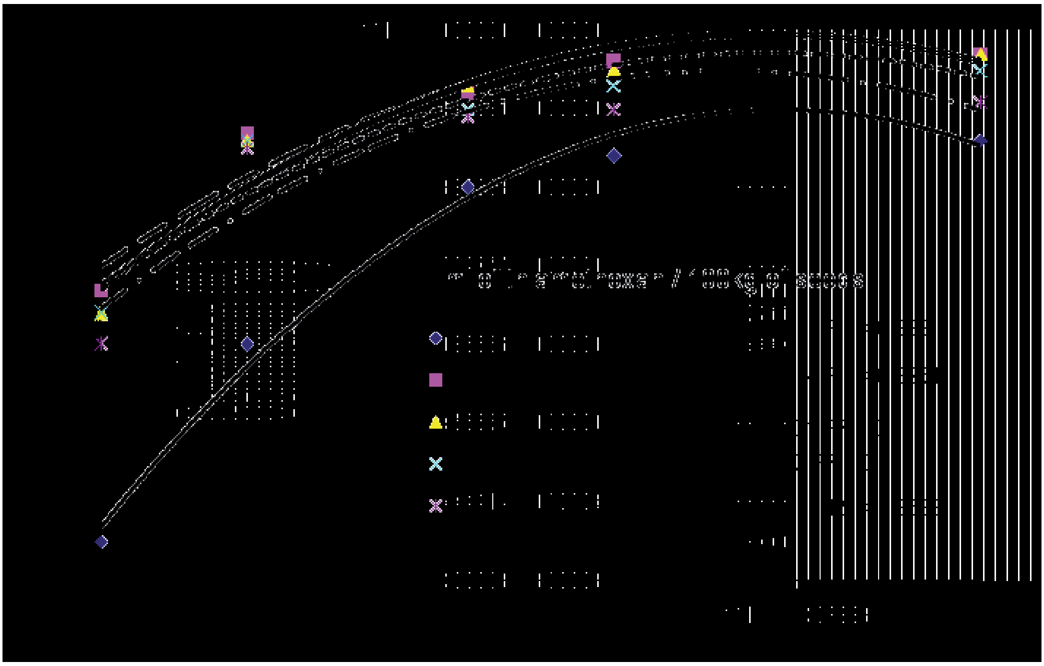

Figure 5. Germination (%) rice seeds, cultivar Avax R., treated with thiamethoxam at different temperatures.

It was observed that at all temperatures studied with product addition there was an increase in germination of the seeds (Figure 6). In average there were increases reaching up to 8 percent germination at 25 º C, 12 percentage points at 20 º C, 17 percentage points at 18 º C and 34 percentage points in the germination test with temperatures of 15 and 13 ° C when compared with seedlings from untreated seeds. Besides increasing the percentage of germination, there is also the activating effect of the product, with the increase in size of roots and shoots (Figure 7). This increase may provide a more rapid and uniform establishment of the crop. According to Clavijo (2008), the thiamethoxam is transported inside the plant through its cells and activates several physiological reactions like protein expression. These proteins interact with various mechanisms of defense related to the plant stresses, allowing to a better deal with adverse conditions such as drought, low pH, high soil salinity, free radicals, stress by high or low temperature, toxic effects of high levels of aluminum injury caused by pests, winds, hail, attack of viruses and nutrient deficiency

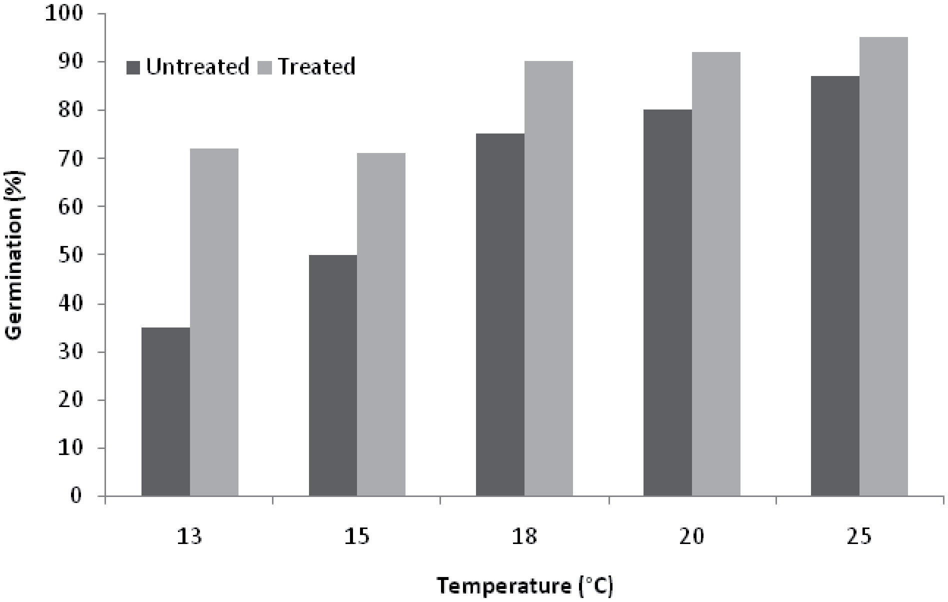

Figure 6. Average germination, cultivar BR IRGA 417, under different doses of thiamethoxam.

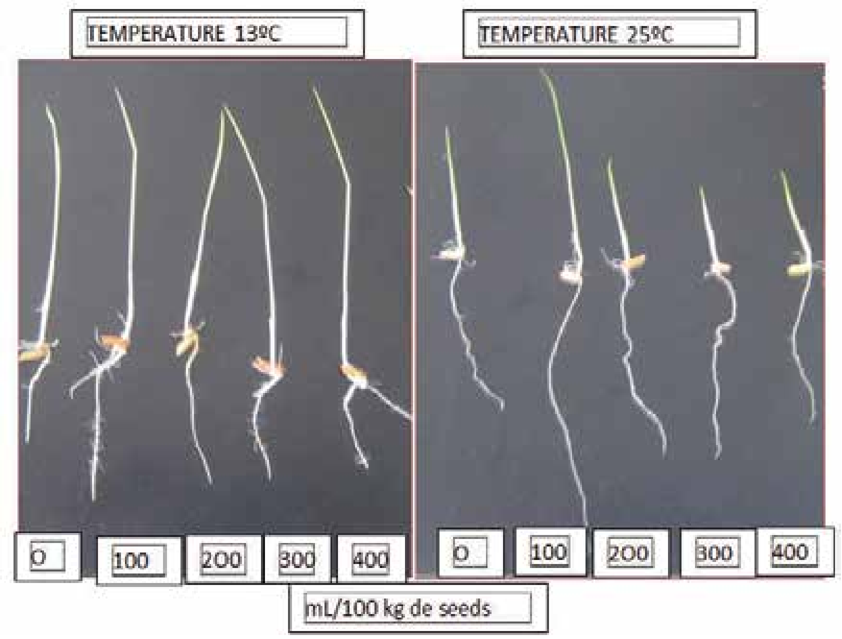

Figure 7. Growth of rice seedlings exposed to different doses of thiamethoxam temperatures of 13 and 25°C

5. Conclusion

The rice seed treatment with thiamethoxam positively favors the physiological quality of seeds.

The doses of 100 and 200 mL of product per 100 kg of rice seed are more effective to improve the physiological performance of rice seeds, in temperatures between 13 and 25 °C.

Author details

Andréia da Silva Almeida[1], Francisco Amaral Villela[2], João Carlos Nunes[3],
Geri Eduardo Meneghello[4] and Adilson Jauer[5]

1 Seed Care Institute- Syngenta, Brazil

2 PPG Ciência e Tecnologia de Sementes, Universidade Federal de Pelotas, Brazil

3 Syngenta Crop Protection- Seed Care Institute, Brazil

4 Universidade Federal de Pelotas, Brazil

5 Syngenta Crop Protection, Brazil

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Spatial and Monthly Behaviour of Selective Organochlorine Pesticides in Subtropical Estuarine Ecosystems

T.S. Imo, T. Oomori, M.A. Sheikh, T. Miyagi and
F. Tamaki

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/54842

1. Introduction

Organochlorine pesticides (OCPs) are one of the most important persistent organic pollutants (POPs) which pose threats to ecosystems and human health. The twelve so-called POPs; nine of which are organochlorine pesticides. The two organochlorine pesticides (HCH and dieldrin) were used in or arise from industry mainly for agriculture purposes. Sediments serve as both a source and a removal mechanisms for contaminants to and from rivers and streams and as a means of contaminant transport downstream. Sediment also provides habitat for benthic biota and can be in the food web around rivers and stream, and some organisms such as fish are consumed by people and birds (Brasher & Anthony, 1998; Laabs et al., 2002). Although the residue levels of the chlorinated compounds in the environments have considerable declined in the past 20 years, recent work has depicted that chlorinated pesticides could be detected in the range of 0.03-25.17 ngg^{-1} (dry weight) (Chang & Doong, 2006; Zhou et al., 1994).

Some OCPs such as DDT and endosulfan are still used in some countries around the tropical and subtropical regions for agricultural and medicinal purposes. These compounds can be deposited into the sediments through long-range atmospheric transport, resulting in a high exposure to OCPs in the area near the pollution source (Tanabe et al., 1994; Doong et al., 2002; Fabricius, 2005). River bed sediments and fish tissues contain higher concentrations of organochlorine compounds than the surrounding water, so analysis of sediment increases the likelihood of detecting compounds that are present in the river.

1.1. A brief history of organochlorine pesticides (OCPs)

A beginning of the twentieth century, early research and studies with chemical pesticides led to the widespread use of inorganic compounds within agriculture containing elements such as sulphur, arsenic, mercury, lead and other metals (Turnbull, 1998). For some natural products such as pyrethrum were also known to be effective pesticides at the time, but were considered to expensive for widespread use (Awofolu and Fatoki, 2003). Between the world wars, the development of the chlor-alkali industry provided the raw material for the mass production of synthetic chlorinated organic molecules. The first and early chlorinated phenoxy acid herbicide (2,4-D) was first discovered in 1932 (Burton and Bennett, 1987). Although this chemical rapidly breaks down in the environment, the seed fungicide hexachlorobenzene (HCB) were introduced in 1933 was found to be far more persistent (Carlsen et al., 1995). The structurally similar insecticides hexachlorocyclohexane or HCH or BHC (also known as benzene hexachloride-BHC) also emerged at this time. The outbreak of war in 1939 and the need to administer malaria and typhus amongst soldiers and civilians has led to the uncovering, unravelling and application of DDT across the world within four and half years from 19430. Related research about the nerve gas agents in Germany led to the discovery of the associated organophosphorus pesticides (Carlsen et al., 1995). Towards the end of the world war, a clear new future for the agrochemical control using these organochlorine chemicals was contemplated. After the world was, the British government considered a practical need to improve agricultural activity and increase food production by the admittance of more complex machinery creation of larger fields, use of chemical fertilizers and the new synthetic pesticides. By 1953, two insecticidal seed dressings, dieldrin and aldrin were being introduced into the UK (Burton and Bennett, 1987). In America, toxaphene was first produced in 1945 as an effective insecticide for cotton plants. This mixture of over 170 chlorinated derivatives known as camphachlor in Europe was recommended as an alternative to DDT before it was banned in the 1980s due to its environmental toxicity (Carlsen et al., 1995). Coupled with other persistent organochlorines such as chlorofluorocarbons, chlorinated biphenyls, dibenzodioxins and dibenzofurans (Doong et al., 2002) the chlorinated pesticides have the potential to cause significant damage to the natural ecosystem by interfering with reproductive processes, this influencing the biodiversity of non-target organisms (Forget et al., 1995). Some aspects of this impairment are now well researched and documented. Whether from past application in developing countries or from continuing current use, these compounds can now be detected in the most remote regions of the planet.

1.2. Definition and importance of organochlorinepesticides (OCPs)

Organochlorines are carbon-based chemicals that contain bound chlorine. These compounds are hydrophobic and lipophilic to varying degrees, meaning their solubility in water is very low, whereas their solubility in fats and oils is relatively high (Cheevaporn et al., 2005). They are noted for their persistence and bioaccumulation characteristics. Some substances may be very persistent in the environment (i.e. with half-lives ($t_{½}$) greater than 6 months). The nature of this persistence needs to be clarified- it is the length of time the compound will remain in the environment before being broken down or degraded into other and less hazardous substances (Forget et al., 1995). The widespread use of these compounds over the past half century has led to their detection in many hydrologic systems world-wide from agricultural and non-agricultural purposes (Monirith et al., 2003). Organochlorine pesticides (OCPs) are considered to be dangerous not only for the environment but for animals and human beings as well. They are very stable substances and it has been cited that the degradation of DDT in soil in 75-100% in 4-30 years (Doong et al., 2002). Other chlorinated pesticides such as Aldrin, Dieldrin, Endrin and Isodrin remain stable in water for many years after their use (Cheevaporn et al., 2005).

1.3. Chemistry

Organochlorine pesticides (OCPs) are organic compounds that highly resistant to degradation by biological, photolytic or chemical means. OCPs are mostly chlorinated. The carbon-chlorine bond is very stable towards hydrolysis and the greater number of chlorine substituted and functional groups, the greater the resistance to biological and photolytic degradation (Doong et al., 2002). Chlorine attached to an aromatic (benzene) ring is more stable to hydrolysis than chlorine in aliphatic structures (Forget et al., 1995). As a result, OCPs are typically ring structures with a chain or branched chain framework. By virtue of their solubility leading their propensity to pass readily through the phospholipids structure of biological membranes and accumulate in fat deposits.

1.4. Human health

As noted for environmental effects, it is also most difficult to establish cause and effects relationships for human exposure of OCPs and incident diseases. As with wildlife species, human encounter a broad range of environmental exposures and frequently to a mixture of chemicals at any time. Much work remains to be done on the study of the human health impact of exposure to OCPs, particularly in view of the broad range of concomitant exposing experienced by humans (Vagi et al., 2005). Previous and present scientific evidences suggest that some OCPs have the potential to cause significant adverse effects to human health at the local level and at the regional and global levels through long-range transport (Doong et al., 2002). For some OCPs, occupational and accidental high-level exposure is of concern for both acute and chronic worker exposure. The risk is greatest in developing countries where the OCPs in tropical agriculture have resulted in a large number of deaths and injuries (Fu et al., 2003). In addition, to other exposure courses, workers exposure to OCPs during waste management is a significant source of high concentration of certain OCP which resulted in illness and death (Doong et al., 2002). For example, a study in the Philippines showed that in 1990, endosulfan became the number one cause of pesticide-related acute poisoning among subsistence rive-farmers and mango sprayers (Forget et al., 1995). Earliest reports of exposures to OCPs related to human health impact include an episode of HCB poisoning of food in south-east Turkey, resulting in the death of 90% of those affected and in other exposure related incidences of hepatic cirrhois, porphyria and urinary arthritic and neurological disorders (Barakat, 2004). Occupational, bystanders and near field exposure to toxic chemicals is often difficult to minimize in developing countries (WHO, 2004). Laboratory and field observations on animals as well as clinical demonstrate that over exposure to certain OCPs may be associated with a wide range of biological effects. These adverse effects may include immune dysfunction, neurological deficits, reproductive anomalies, behavioural adnormalities and carcinogenesis (Forget et al., 1995). The scientific evidence demonstrating a link between chronic exposure to sub lethal concentrations of OCPs (such as that which could occur as a result of long range-transport) and human health impacts is more difficult to establish but gives cause for serious concern (Doong et al., 2002).

1.5. Organochlorine pesticides production and use

The nine OCPs out of twelve POPs compounds were used in or arise from industry, agriculture crops and disease vector control of public health (Chang and Doong, 2006). By the late 1970 all eight OCPs has been either banned or subjected to severe use restriction in the developed world but the major release of these compounds were mostly used by developing countries especially Asia (Hung and Thiemann, 2003), South/Central America (Falco et al., 2003) and Africa (Mwevura et al., 2002). Although the statistics on the use in many areas remained unclear (FAO, 1989). Previous studies revealed that some HCH remains a common compound used in large quantities in India, China, Africa and South America (Turnbull, 1995). It was also recorded that India consumed 25,000 tons of HCH annually over recent years (Davis et al., 1992) and one factory in China was thought to have an annual product of 20,000 tons (Zhang et al., 2002). In Japan, the using of these OCPs has been prohibited in the field in the 1970-1980 (Nakai et al., 2004). It was estimated that the pesticide used in the United States was 550,000 tons during 1995 (Golfinopoulos et al., 2003). In addition, Greece consumed approximately 3500 tons per year of OCPs in the form of insecticides and pesticide (Miliadis, 1993). In Vietnam, approximately 15,000 tons was used from 1957-1972 (Quyen et al., 1995) and 50 tons from the year 1999 (Hung and Thiemann, 2003). In Germany, 36,000 tons was consumed in the year 1991 (Statistisches Bundesamt, 1993, Hung and Thiemann, 2003).

1.6. Characteristics of hexachlorocyclohexane (HCH)

Hexachlorocyclohexane (HCH) is an insecticide that exists in eight different forms. One of its form is known as gamma-HCH (γ-HCH) or commonly called Lindane is produced and used as an insecticide on fruit, vegetables and forest crops. It is a white solid that turns into a vapour when released into the air with a melting point varied with isomeric composition. Its vapour pressure at 4.2 mm Hg at 20°C. (US EPA, 2012).

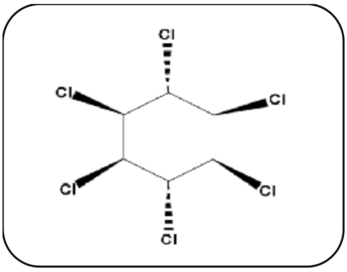

Figure 1. Chemical structure of hexachlorocyclohexane

1.7. Characteristics of dieldrin

Dieldrin is an insecticide which is closely related to aldrin, which reacts further to form dieldrin. It used principally to control textile pests and insects living in agricultural soils. It is a white crystals with a melting point of 175-176°C. Its solubility in water is 140 μgL^{-1} at 25°C with a vapour pressure of 1.78×10^{-7} mm Hg at 20°C (US EPA, 2012).

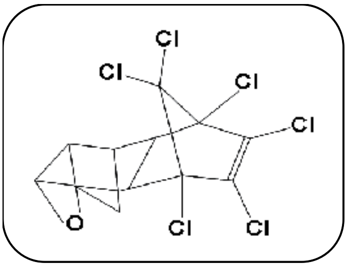

Figure 2. Chemical structure of dieldrin

2. Environmental impact to estuaries

Organochlorine pesticides are carbon-based chemicals that contain bound chlorine. These compounds are hydrophobic and lipophilic to varying degrees, meaning their solubility in water is very low, whereas their solubility in fats and oils is relatively high (Cheevaporn et al., 2005).They are noted for their persistence and bioaccumulation characteristics. The widespread use of these compounds over the past half century has led to their detection in many hydrologic systems world-wide from agricultural and non-agricultural purposes (Monirith et al., 2003). The presence of HCH and dieldrin pesticides in the environment may be related to both past and present land use in a watershed. It enters the aquatic environment from a variety of sources, including the atmosphere, industrial and municipal effluents and agricultural and urban non-point source run-off. HCH and dieldrin are mostly associated with bottom sediments, which can be ingested by benthic organisms. These organisms are then eaten by fish and birds, which can result in higher concentrations through aquatic and terrestrial food chains. Due to the long residence time of these substances in the environment, it is important to examine the pollution they cause not only the environment but also for the lower invertebrates such as corals. Since the ocean is the receiving basin for terrigenous freshwater run-off and its entrained materials, some fractions of these compounds that are used in upland eventually reach the marine ecosystems.

The Manko and Okukubi estuaries are protected wetlands located in a subtropical climate on Okinawa Island. These estuaries are very famous host for migrating birds from South East Asia and mainland Japan. It also plays a great role of species conservation and it was added to the RAMSAR Convention register of wetlands. However, estrogenic activities were detected in sediment samples from these estuaries (Tashiro et al., 2007). Previous studies showed that the coral reef ecosystems and their adjacent environments in and around the Okinawa Island are contaminated with OCPs, OTCs and PCBs (Tashiro et al., 2003; Imo et al., 2007; Sheikh et al., 2002). However, very little is known about the behaviour of HCH and dieldrin in estuarine sediments of subtropical areas. The main objective of this chapter is provide crucial information on the distribution and behaviour of HCH and dieldrin compounds in protected subtropical estuaries in the Okinawa Island.

3. Experimental

3.1. Sample processing

Surface sediment samples were collected with a stainless steel grab. The upper 1-3 cm of the sample were carefully removed and stored in acid rinsed polyethylene 250 mL glass bottles. Samples were transferred to the laboratory and were stored at -20ºC until sample extractions. Details of sampling areas is shown in Table 1 and description of samples as shown in Table 2. Sampling location is shown in Figure 3.

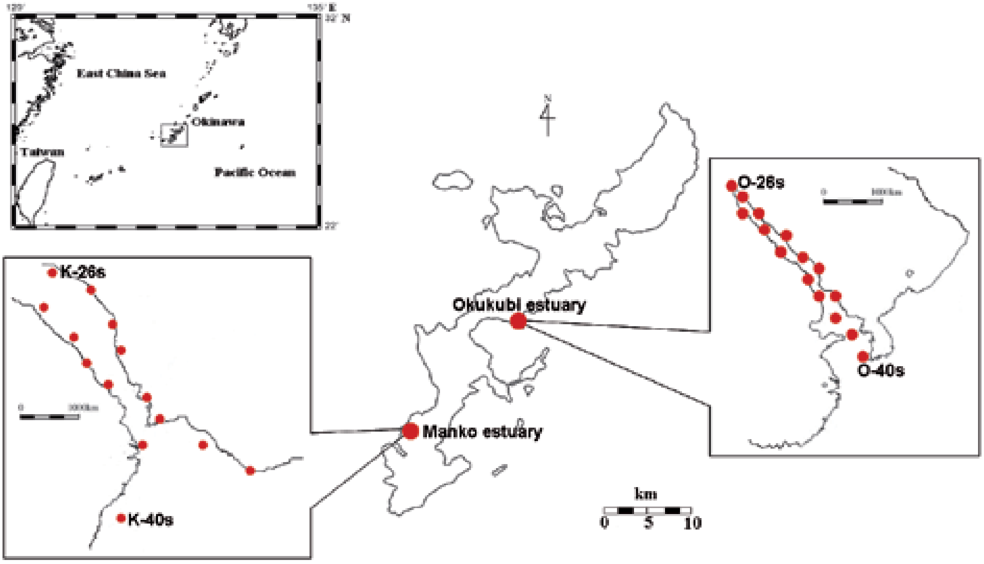

Figure 3. Sampling locations

Estuary	Transect	Sample	Location	Activities
Manko	TM1	K-26s-05		
		K-27s-05	Upstream	Residential area
		K-28s-05		
	TM2	K-29s-05		
		K-30s-05	Mid –stream	Residential area
		K-31s-05		
	TM3	K-32s-05		
		K-33s-05	Mid-stream	Residential area
		K-34s-05		
	TM4	K-35s-05		
		K-36s-05	River mouth	Residential and fishing port
		K-37s-05		
	TM5	K-38s-05		
		K-39s-05	Naha port	Commercial port
		K-40s-05		
Okukubi	TO1	O-26s-05		
		O-27s-05	Upstream	Agriculture
		O-28s-05		
	TO2	O-29s-05		
		O-30s-05	Mid-stream	Agriculture
		O-31s-05		
	TO3	O-32s-05		
		O-33s-05	Mid-stream	Agriculture
		O-34s-05		
	TO4	O-35s-05		
		O-36s-05	Mid-stream	Fishing area
		O-37s-05		
	TO5	O-38s-05		
		O-39s-05	River mouth	Residential, public area
		O-40s-05		

Table 1. Details of sampling areas.

Sample	Surface river sediments
	Sample sketch
K-26s-05	mud
K-27s-05	mud
K-28s-05	mud
K-29s-05	mud
K-30s-05	mud
K-31s-05	mud
K-32s-05	mud
K-33s-05	mud
K-34s-05	mud-sandy
K-35s-05	sandy
K-36s-05	sandy
K-37s-05	sandy
K-38s-05	sandy
K-39s-05	sandy
K-40s-05	sandy
O-26s-05	sandy
O-27s-05	sandy
O-28s-05	sandy
O-29s-05	sandy
O-30s-05	sandy
O-31s-05	sandy
O-32s-05	sandy
O-33s-05	sandy
O-34s-05	sandy
O-35s-05	sandy
O-36s-05	sandy
O-37s-05	sandy
O-38s-05	sandy
O-39s-05	sandy
O-40s-05	sandy

Table 2. Description of surface sediment samples

3.2. Sample extraction

Prior to extraction, surface sediments were freeze-dried, homogenized with a stainless spatula and passed through a 63 μm sieve followed by mixing with anhydrous Na_2SO_4. The sediment samples were extracted by ultrasonication technique as described by (Vagi et al., 2005). The surrogate standard of 5 μgL^{-1} was added to 40 g of sediments. Portion of this amount was used for QC analysis (i.e. each batch contained 1 sample, 1 blank, 4 spiked). For the spiked samples, various concentration of Chlorinated Mix (5, 10, 50 μgL^{-1}) were added to each spiked sample. The samples were extracted by ultra wave sonication for 15 minutes with 10 mL of dichloromethane. The extracts were then filtered using WHATMAN filters (0.45 μm) followed by centrifugation at 3000 rpm for 15 minutes. The clear organic supernants were removed then the combine extracts were evaporated on a rotary evaporator at 30-35°C near to dryness. A 1 mL of hexane was added to the dried residues. For further cleanup, the samples were then added to the florisil ENVI Carb and the samples were eluted with hexane. The residues were dissolved in 1 mL hexane. A 1mL of Internal Standard (Pentachloronitrobenzene, 50 μgL^{-1}) in the amount extracted before GC-MS analysis.

3.3. Environmental parameters

3.3.1. Total Organic Carbon (TOC) in sediments

Approximately 3g sediment sample was weighed (± 0.002 g) and HCl (2*M*) was added to the sample and left over night to remove all carbonates. Milli Q water was added to rinse the acid from the sediments. To ensure that all the acid was removed from the samples, a 6*M* of HCl was added. The acid from the sample was removed by adding 2 mL of distilled water followed by centrifugation. The sediments were then dried at 60°C over night and ready for analysis (US EPA, 2012). The Total Organic Carbon (TOC) was determined using the CHNS analyser (JM 10 Model from J-Science Lab, Co. Ltd, Japan0. Calibration was performed using Antipyrine as Standard with the following compositions: $C_8H_9NO = 135.17$ (C = 70.19%, H = 6.43%, O = 8.50% and N = 14.88 %).

3.3.2. pH

The pH was measured using a portable pH meter at room temperature in the laboratory using PHM 95/ion meter, Radiometer model (± 0.001 pH).

4. Results and discussion

The highest concentration of HCH was found in the sampling month of October (213 ng/g) (Manko estuary) and Dieldrin (98 ng/g) (Okukubi estuary) followed by the month of November (HCH-199 ng/g) (Manko estuary) and (Dieldrin-90 ng/g) (Okukubi estuary). The status of HCH and dieldrin in sediments in this study was compared with those in other rivers. The levels of OCPs in this study are lower than that of Er-jen river, Taiwan (80-8200 ng/g (dw))

(Zhang et al., 2002). River Mataniko, Solomon Island (140 ng/g (dw)) (Iwata et al., 1995) but higher in some rivers in Japan (2.5-12 ng/g (dw)) (Sakar et al., 1997).The basic physico-chemical parameters of sediments such as TOC were also measured. The TOC contents ranged from nd-3.96% (Table 3 and Table 4). Figure 4-Figure 5 shows a positive correlation with the concentration of HCH especially in the Manko estuary. No correlation was shown between HCH and TOC from the Okukubi estuary. It is clear that sediments from the Okukubi estuary were composed of fine particles. This observation is consistent with other studies which demonstrated that fine particles can retain large amounts of organic compound and pose a high pollution potency (Hong et al., 1995). Since HCH and dieldrin exhibit carcinogenic activities, the contamination levels detected may pose a high ecotoxicity for aquatic and marine organisms.

Sample	Temperature (ºC)	pH	Total Organic Carbon (%)
K-26s	26.5	8.01	3.13
K-27s	27.4	7.86	2.74
K-28s	28.4	7.81	1.29
K-29s	27.9	7.43	0.24
K-30s	27.6	7.56	nd
K-31s	27.6	7.66	nd
K-32s	28.9	7.74	nd
K-33s	28.9	7.65	nd
K-34s	28.2	7.55	nd
K-35s	28.2	7.64	nd
K-36s	28.6	7.62	nd
K-37s	28.6	7.58	nd
K-38s	28.9	7.56	nd
K-39s	28.9	7.58	nd
K-40s	28.9	7.56	nd

nd: not detected

Table 3. Summary of Environmental Parameters – Manko Estuary

Sample	Temperature (°C)	pH	Total Organic Carbon (%)
O-26s	25.4	8.06	3.96
O-27s	25.9	8.00	2.82
O-28s	26.0	7.99	2.11
O-29s	26.2	7.99	1.76
O-30s	26.5	7.78	0.54
O-31s	26.9	7.56	nd
O-32s	27.0	7.69	nd
O-33s	27.2	7.69	nd
O-34s	27.0	7.72	nd
O-35s	27.1	7.71	nd
O-36s	27.1	7.60	nd
O-37s	26.8	7.64	nd
O-38s	26.9	7.64	nd
O-39s	26.9	7.58	nd
O-40s	26.9	7.54	nd

nd: not detected

Table 4. Summary of Environmental Parameters – Okukubi Estuary

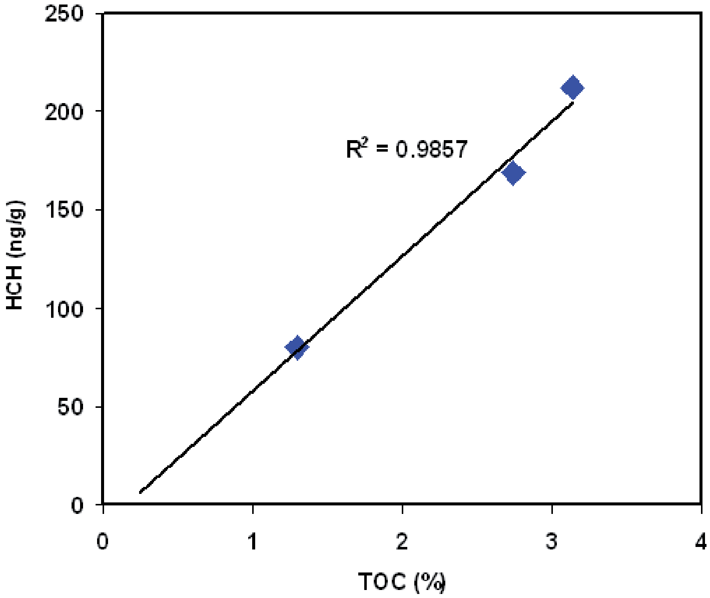

Figure 4. Correlation of HCH with TOC [Manko estuary]

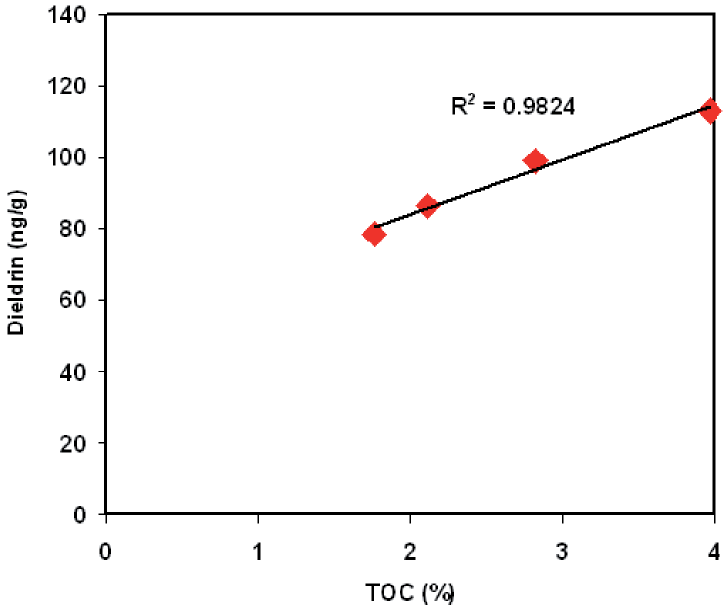

Figure 5. Correlation of Dieldrin with TOC [Okukubi estuary]

4.1. Spatial distribution of HCH and dieldrin in sediments

The highest concentrations of HCH was found in sample K-26s – 213 ng/g (dw) (Manko estuary) and the highest dieldrin concentration was found in sample O-26s – 98 ng/g (dw) (Okukubi estuary). Most samples in the Okukubi estuary had relatively low levels of HCH compared to Manko estuary, where the sediments mainly composed of sand. It may be due to the similar historical input and deposit indicating important sources of these organochlorine pesticides in these areas. The second highest concentration of HCH in the Manko estuary, 99 ng/g (dw) followed by 90 ng/g (dw). The second highest concentration of dieldrin in the Okukubi estuary was 199 ng/g (dw) followed by 95.5 ng/g (dw). The levels of HCH and dieldrin in this study are higher in those found in the sediments of the Mingjiang River Estuary, China (2.99–16.21 ng/g, with a mean value of 8.62 ng/g dw (Kennicutt et al., 1994). the Wushi Estuary, Taiwan (0.99–14.5 ng/g, with a mean value of 3.78 ng/g dw (Iwata et al., 1995) Xiamen Harbor, China (0.14–1.12 ng/g, with a mean value of 0.45 ng/g dw, Hong et al., 1995) and Casco Bay, USA (<0.25– 0.48 ng/g) dw, but lower than the Matanico River and Solomon Islands (140 ng/g) (Walker et al., 1999).

4.2. Monthly variations of HCH and dieldrin in sediments

It clearly revealed that the HCH and dieldrin pesticides residues in October were higher compared to other sampling months. This means that some organochlorine pesticides could be released from the run-off effluents to waters with much rainfall during the rainy season and typhoon season in Okinawa during the summer. In all sampling months, the highest concentration of organochlorine pesticides in the Manko estuary was 213 ng/g (dw) in October and for the Okukubi estuary, the highest concentration of organochlorine pesticides was also detected in the month of October, 213 ng/g (dw). The second highest concentration of HCH was detected in the month of November (199 ng/g (dw)) followed by the month of December (99 ng/g (dw)) for the Manko estuaries. The second highest concentration of dieldrin was detected in the month of October (90 ng/g (dw)), followed by 89.5 ng/g (dw) in the month of November for the Okukubi estuaries.

4.3. Composition analyses in sediments

Composition difference of HCH in the environment could indicate contamination sources (Wu et al., 1999). Technical HCH has been used as broad spectrum pesticides for agricultural purposes, which has been banned in the 1970’s in Japan. Technical-grade HCH consists principally of four isomers, α-HCH, β-HCH, γ-HCH and δ-HCH. The physiochemical properties of these HCH isomers are different. The β-HCH has the lowest water solubility and vapour pressure which is the most stable and relatively resistant to microbial degradation (Strandberg et al., 1998). Also it should be noted that α-HCH can be converted to β-HCH in the environment (Lee et al., 2001). The results showed that a high percentage of HCH isomer was recorded in the sampling months December, January and February. It is possible that HCH may be re-absorbed to surface sediments. There was no strong evidence to prove the recent usage of HCH in Okinawa; however Manko estuary was contaminated with HCH.

5. Conclusion

Generally the distribution of organochlorine pesticides were associated with land use practices including agriculture and urbanization and the sediments from estuary have higher contents of organic matter such as TOC and organochlorine pesticides residues. The concentration and compositions of organochlorine pesticides varied significantly with different sampling sites. The HCH in the surface sediments were well correlated with TOC content. The organochlorine pesticides residues (HCH and dieldrin) were detected due to re-absorption in sediments due to previous deposition. The possible sources of these organochlorine pesticides are still unknown but they may come from residential areas, commercial and naval ports and agriculture activities.

Author details

T.S. Imo[1,5], T. Oomori[1], M.A. Sheikh[1,4], T. Miyagi[2,3] and F. Tamaki[3]

1 Department of Chemistry, Graduate School of Engineering and Science, University of the Ryukyus, Nishihara, Okinawa, Japan

2 Okinawa Prefectural Institute of Health and Environment, Ozato Ozato Nanjo, Okinawa, Japan

3 Water Quality Control Office, Okinawa Prefectural Bureau, Miyagi, Chatan, Okinawa, Japan

4 Research Unit, The State University of Zanzibar, Tanzania

5 Faculty of Science, National University of Samoa, Samoa

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Section 6

Insecticides Against Pests of Urban Area, Forests and Farm Animals

Chapter 16

Bait Evaluation Methods for Urban Pest Management

Bennett W. Jordan, Barbara E. Bayer,
Philip G. Koehler and Roberto M. Pereira

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53421

1. Introduction

Baits are a preferred type of formulation used in urban pest management, especially for the control of cockroaches, ants, and increasingly termites. With precise placement in areas away from contact with human population, especially children, and a reduced rate of active ingredient (AI) application in a given structure area, baits are more economical and pose less risk for consumers and the environment than other formulations. However, baits are very difficult to evaluate for efficacy. For baits, pest acceptance and horizontal transfer of bait are essential in order to control pest populations.

Baits are composed of one or more insecticide active ingredients incorporated into an attractive food matrix, which varies according to the type of target pest, and even according to species within a certain pest type. Although commercial development of species-specific baits may represent a serious commercial problem due to limited market, this has been done in the past, for instance with imported fire ant baits in the USA. However, typically, baits are developed to target a group of similar insects, e.g., cockroaches, which may vary in their response to the bait formulation, resulting in varying degrees of control depending on the pest population composition.

In order to perform successfully, baits must attract the target insect and be ingested in sufficient amount that will cause the desirable level of control in the pest population. For non-social insects, such as cockroaches, transfer of the active ingredient among different segments of the pest population (e.g., adults and immature forms, reproductives, etc) is desirable but not necessarily an essential characteristic of the baits. However, in social insects (ants and termites) the transfer of the active ingredients between the foragers and the remaining of the population, and specially the reproductive caste, is essential in providing adequate control of the pest population within reasonable time.

2. Cockroach bait evaluations

Cockroach consumption of a bait and subsequent control can be complex. More than one of these cockroach pests may occur at a location with each having its own food requirements [1], susceptibility to insecticides [2], and aversion to certain bait formulations [3, 4]. Additionally, within each species, feeding patterns [5] and insecticide susceptibility [6] can vary among stadia.

When cockroach baits are placed close to harborage, they are usually in direct competition with other food and water resources. Therefore, baits, which are often gels with 40-60% moisture [7, 8], need to out-compete other sources of dry food as well as other moisture sources so cockroaches will consume them. Although cockroach control by baits is primarily due to bait consumption, not all insects within a population are actively seeking food, so not all individuals may consume a bait. Besides consumption, cockroaches are exposed to insecticidal active ingredients by contacting baits with antennae or palps. Contact exposure results in some toxicant transfer and mortality [9]. Also, cockroaches can be affected by contact with small amounts of translocated active ingredient (trampling), or when they consume contaminated feces (coprophagy), dead or dying cockroaches (cannibalism), or vomit (emetophagy) [10, 11, 12, 13]. These effects can result in secondary and sometimes even tertiary mortality [14].

The combination of consumption, contact, and secondary exposure results in mortality of various cockroach species and stadia (Figure 1).

Therefore, it is important to consider the consumption of insecticidal baits and the consequent ingestion of active ingredients, the mortality of different life stages within the pest cockroach population, and any possible effect of indirect to the active ingredient in the bait without actual bait consumption.

Consumption. In testing consumption in the laboratory, the use of mixed-age cockroach populations (adults of both sex and nymphs all in the same arena) is important to simulate bait consumption in natural infestations (Figure 2).

Weight-change controls, which are protected from consumption by the insects but otherwise under the same conditions as the bait being tested, must be used so adjustments can be made due to moisture change in bait and any other used in the experiments. To better understand potential differences between different products when consumed by different insect populations, it is important to estimate the consumption of bait in relation to the size of the insect consuming it. Bait consumption (B_{con}) per g of insect can calculated as using the following equation:

$$\text{Consumption (mg)/g cockroach} = \left(\left(F_B - \{F_B * [(WC_B - WC_A) / WC_B]\} - F_A\right) / W_t\right)$$

where F_B is the weight of bait (mg) available to cockroaches at the start of the experiment, WC_B is the weight of weight-change control bait (mg) before the experiment, WC_A is the weight of weight-change control bait (mg) at the end of the experiment, F_A is the weight of bait (mg) remaining after the experiment and consumption by the cockroaches, and W_t is the total weight of cockroaches placed in the arena.

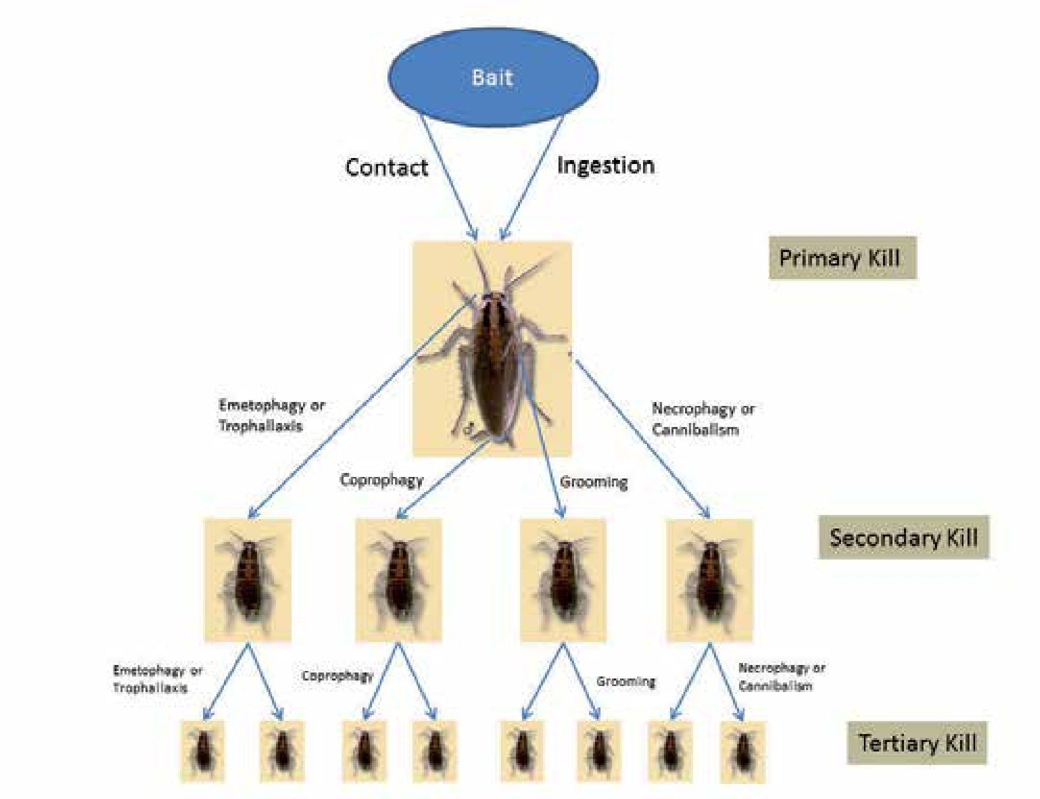

Figure 1. Cockroach baits can control populations in several ways, including primary kill, secondary kill, and tertiary kill.

Precise measurement of gel bait consumption by cockroaches is complicated by several factors:

a. Cockroaches are likely to remove and spread bait that is never eaten, especially in small arenas with large insect populations. Although the contact with the bait without consumption can be an important element in producing the total cockroach mortality, it can cause the measurement of bait consumption to be very unreliable and variable among the different experimental replicates. The use of larger arenas with smaller cockroach populations and plenty of harborage areas away from the bait can help in minimize this effect.

b. Rapid water loss cause gel bait especially, but also other bait forms, to change weight even in the absence of any consumptions. This factor is most severe on baits with very high water content, and formulations that do not limit water loss. Unless changes in water content can be estimated precisely, consumption can be overestimated. Any other calculations that result from the bait consumption (active ingredient consumption, bait preference in relation to alternative food, etc) can be greatly affected by over or underestimation of the bait. Also, an estimate of the amount of bait necessary for control of a population of cockroaches will be greatly affected by any miscalculations due to imperfect water loss estimates. Rapid water loss can also affect the palatability and nutritional content of the baits, which will greatly affect the bait's effectiveness as well as any measurements associated with its consumption.

c. Differential consumption of food by different cockroach life stadia can vary over short periods of time. For experimentation, strict selection of insects within specific age groups can minimize any problems associated with the inclusion of insects that will not consume any bait, or any other food, for some time into the experimental period.

One solution to minimize some of the effects on measurements of consumption is to limit the measurement to a specific time period (e.g., 24 h following the initial exposure). Although water loss and trampling may be more severe during the initial hours after application, some of these problems can be resolved by using weight-loss controls. Limitations on how the cockroaches can reach the bait may be used to limit trampling on the baits but researchers must be careful in not limiting access to the bait, especially if bait stations are crowded.

Figure 2. Cockroach arena set up for bait evaluations. Harborage and water vials are on left, bait and untreated food is in center, and protected water controls are on right.

Active Ingredient Consumption. Because mortality is dependent on the actual amount of active ingredient consumed by the insects, it is important to determine that consumption (Figure 3).

Figure 3. Cockroaches leave harborages in choice tests and can choose to ingest either bait or untreated food, like laboratory chow.

Baits with lower active ingredient content may be more palatable to the insect and be consumed in relatively high amounts, while a bait with high active ingredient content may partially deter consumption by the pest population. The active ingredient (AI) consumption/g cockroach can be calculated as follows:

$$AI / g \text{ cockroach } (\mu g) = B_{con}{}^{*} C_{ai}{}^{*} 1000$$

where B_{con} is bait consumption (mg)/g cockroach, and C_{ai} is the percentage of active ingredient in the formulated bait.

From the point of view of pest control, the delivery of the active ingredients is the most critical factor when using baits. Different active ingredient concentrations combined with varying bait palatability determine the total amount of active ingredient ingested by the pests and their consequent mortality (Figure 4).

In general, when consuming baits, individual cockroaches will consume more active ingredient than necessary to cause death. Depending on the bait product and the cockroach species, the quantity of active ingredient ingested can vary from just above what is needed kill the insect to more than 1000 times the LD_{50}. This excess consumption of AI may be important in avoiding or delaying development of insecticide resistance by killing virtually all cockroaches exposed to these baits [15].

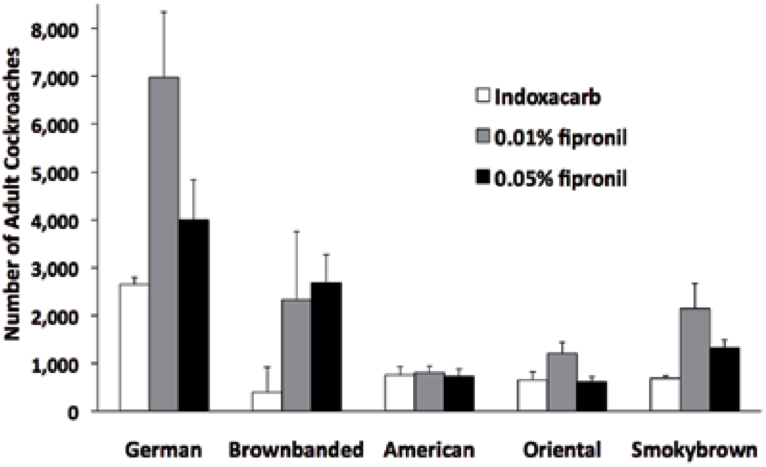

Figure 4. Number of adult cockroaches that could potentially consume and be killed by a 30-g tube of gel bait based on the consumption/g cockroach and the weights of average adult cockroaches from 5 different species (German: 56.7 ± 0.83 mg; brownbanded: 82.8 ± 0.49 mg, American: 959.4 ± 8.44 mg, smokeybrown 679.6 ± 19.02 mg, oriental 499.6 ± 9.51 mg).

Speed of kill is another aspect associated with the amount of active ingredient ingested by the cockroach. Although a quick kill can be advantageous in eliminating the pest problem within short time after the control action, other aspects, such as the possibility of transferring the active ingredient to other insects, can be maximized by limiting the amount of active ingredient that any individual cockroach will consume. Thus, understanding the different factors affecting the amount of active ingredient likely to be consumed by the average cockroach will help in the development of baits with greatest chances for success. Differences in susceptibility to insecticides [2, 6] and in feeding patterns [5] for different life stages of the pest, may also affect how fast bait materials will act. Adult males and non-gravid females are more likely to encounter and consume bait due to more consistent feeding patterns than nymphs.

Percent Bait Consumption. In the development of bait product, it is important that the bait competes well against other preferred food that the insect may find in their habitat. Thus, some measurement of bait preference over other foods is important in the development of baits. An important indication of how well a bait product will perform is the percentage of the total food consumption that is actually represented by the bait. The greater the percentage of bait in the total food consumption, the greater and faster mortality can be expected in the pest population. Percentage bait selection over an alternative preferred food can be determined by calculating the percentage of bait in the total food consumption, as follows:

$\% \text{ Bait Selection} = [B_{con} / (B_{con} + AF_{con})] * 100$

where, B_{con} is the bait consumption (mg) and + AF_{con} is alternative food consumption (mg).

Cockroach baits have been optimized for some of the most common pest species, and minor pest species may be much less attracted to the commercial baits. Preference for the baits may be due to a balanced mixture of moisture and nutrients specially given the high water need by most cockroach species. However, different species of cockroaches have different water needs and this will be reflected in the percent of bait consumed when alternative foods vary widely in water content in relation to the bait. With high water losses [16], high cuticular permeability [17], high metabolic rate [17], cockroaches need to balance moisture and nutritional needs [18] in order to survive and reproduce at optimal levels.

Secondary Effects. Secondary effects to the consumption of bait may be very important, especially in relation to the portion of the cockroach population that may not consume any of the bait. Gravid females and nymphs during the ecdysis may not consume the bait before dries up or is consume by more aggressive bait consumers. The only way to affect the cockroaches that do not consume the bait directly is through secondary effects that result from consumption or other contact with bait contaminated debris, feces, and other materials.

To test these secondary effects, the arenas used in primary consumption studies should be set aside and remain unchanged except for the removal of live and dead cockroaches and any unused bait. Any remaining alternative food as well as harborages, water vials, containers, frass and any debris can be left in the arenas. These arenas can then be supplied with fresh food and water and a new population of cockroaches. Secondary effects (mortality) due to the contact with an environment contaminated by cockroaches consuming insecticidal baits can be evaluated against separate populations of cockroaches which are added to the contaminated arenas immediately after the primary consumption experiment and at different time intervals. A mixed population of cockroaches should also be used for these secondary effect experiments so that mortality in natural infestations can be simulated. Data similar to that collected in primary consumption experiments can be obtained in these experiments.

To reach the portion of the cockroach population that will not consume the bait, perhaps the best solution is the design of baits that offers greater opportunity for secondary mortality through contact with either dying insects or debris moved by insect that visit and consume the baits. The development of baits with these characteristics requires testing under conditions that maximize transfer of the material between segments of the cockroach populations.

Contact Effects With No Consumption. The effect of direct contact with bait without consumption can also provide better understand on how different baits can affect cockroach populations. These experiments are difficult because they require the sealing of mouthparts in cockroaches so they cannot consume the bait. These experiments produce better results when the insect life stage used is sufficiently resistant to lack of water, or is placed in an ambient environment were water loss does not cause serious mortality in the test population. Adult male German cockroaches have been used in such experiment due to superior survivability without feeding.

Once their mouthparts are sealed using a droplet of melted paraffin wax or other non-lethal method, the cockroaches are placed into arenas containing pre-weighed portion of a bait and other materials used for the direct consumption experiments. Mortality and other parameters can be observed during a short period of time (2-5 days) while the insects survive despite the lack of food and water consumption. These experiments have short durations due to the need to evaluate treatment mortalities within the time period when mortality in the control insects is still within reasonable levels. Beyond 3-5 days, mortality in the control insects will increase rapidly, mostly due to the lack of water in insects with their mouthparts sealed, and any results will be heavily influenced by that the control mortality.

Contact mortality with baits, besides being difficult to document, may be of lesser importance in population control, especially because cockroaches cannot survive long without ingesting food or water. Contact mortality relies on cockroaches investigating the bait without consuming it as would occur with bait-averse populations [19, 20]. Certain active ingredients, such as fipronil, are more likely to cause higher contact mortality than others; however, differences in bait matrices may also cause varying levels of contact mortality [19], and some formulations may have the potential for killing high proportions of cockroaches by contact alone.

Although different pest cockroach species have varying food preferences and, within the same species, there is great variability in the amount of bait individual cockroaches may consume, baits remain the most efficient method for control of cockroaches. Although maximization of bait consumption must take priority in bait product development, other factors that enhance secondary mortality and contact toxicity must be considered. Evaluation of these bait products in relation to direct mortality, by bait consumption, as well indirect mortality, by secondary and even tertiary contact with the active ingredient in the bait, are also important in development and evaluation of cockroach baits.

3. Ant bait evaluations

The control of social insects using baits relies greatly on the fact that in these social colonies, mortality of the individual workers has little effect on the survival of the colony. It is only by removing the reproductives, or at least a sufficient number of workers and juveniles to directly affect the reproductive potential of the colony, that an pest ant colony can be eliminated. Some of the active ingredients used in baits formulated for ant control completely bypass any effect on the worker, and concentrate their power with specific chemicals that interfere with the reproductive potential of the queen or queens. Because the reproductive individuals in ant colonies do not normally gather food or consume material that has not been somehow prepared by other colony individuals, reaching the reproductives is the greatest obstacle for any active ingredient formulated in an ant bait.

Because ant workers do not ingest large solid particles, ant bait formulations that target most urban ant pests must contain a liquid component. An ant head dissected shows the structures that prevent solids larger than 0.5 microns from being ingested (Figure 5). Food enters through the mouth and passes into the infrabuccal pocket. The infrabuccal pocket is a location for food particles too large to swallow. Food in the infrabuccal pocket passes through the buccal tube that is lined with setae that serve as filters. Particles too large to pass through this filtering mechanism remain in the infrabuccal pocket. These food particles can later be transferred to larvae for that ingest and digest these particles. Liquids that are ingested pass through the buccal tube into the pharynx and down the esophagus to the crop and midgut for storage and digestion. For baits to be ingested by urban pest ants they are usually liquids or granules that are soaked with liquid baits.

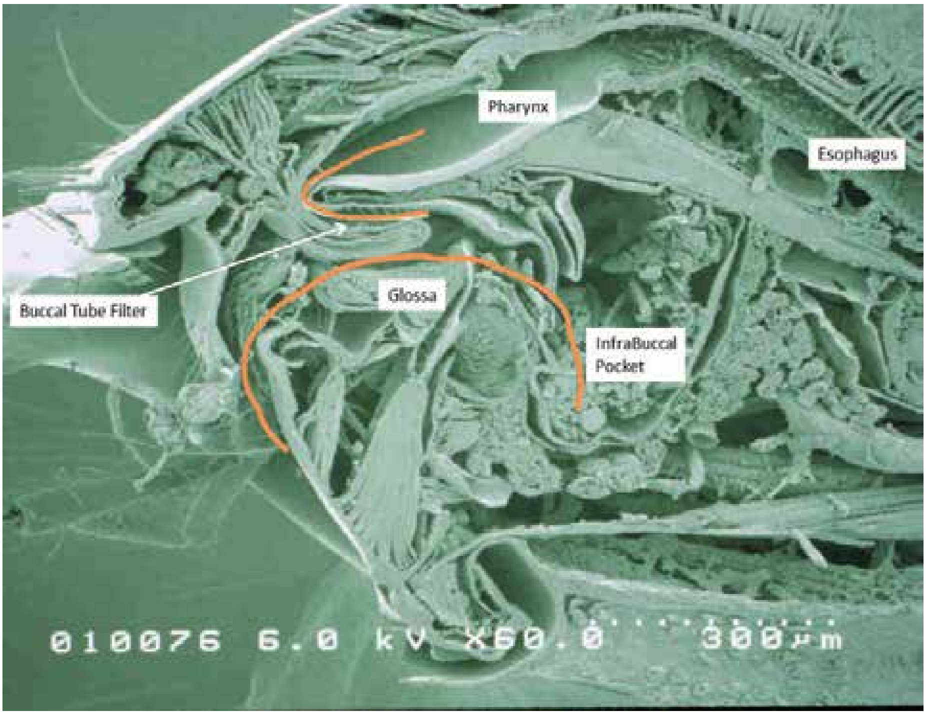

Figure 5. Cross section of an carpenter ant head showing the structures associated with ingestion of food.

Baits that target fungus-gardening ants target ants in a very different way, and are therefore develop following a completely different model. Most urban ant baits come in liquid, gel or granular formulations, but the granular formulation consists of a matrix containing a liquid that can the removed by the ants. Gel baits are only appropriate for indoor use or other special situations where protection from the climatic conditions is possible. Liquid baits normally require application into a holding device which the ants will have access to the bait. Granular baits are more practical for application outdoors, although they are also convenient for indoor applications.

Baits work by taking advantage of ant biology and behavior such as social grooming and trophallaxis. Once the bait is discovered, the foraging ants pick of the bait and transport it back to the colony. The brood, especially late instars, may be important in the digestion solid bait particles into a liquified form that can the transferred to workers and reproductives in the colony. The amount of brood in the colonies, in the laboratory and field, could be responsible for the foraging preference. Fourth instar larvae do most of the protein digestion in the ant colony [21, 22] and their presence in a colony can change ant foraging preference to proteinaceous materials.

It is through food sharing that the toxicant in the bait can be transferred to the rest of the colony. Because the bait is picked up directly by the ant workers and is later shared within the colony, relatively low amounts of the toxicant can be used in targeting a pest ant population [23]. Ant foragers are usually the older workers that first pick up or consume the bait. They share the toxicant with other workers and queen tenders, and eventually after 3-4 days the toxicant reaches the queen, which affects reproduction in the colony. Even if the queen dies, eggs may hatch, larvae may pupate and develop into workers. Eventual control of a large colony may take 1-5 months.

The current baits out on the market for ant control include gel baits, liquid baits, and solid granular baits. A liquid or gel bait is usually one that requires a bait station and constant reapplication due to the elements and are usually used with ants that display mass recruitment to food sources.

In many cases there is little distinction between liquid and solid baits in terms of what the ants actually harvest in the field, as in the instance of popular fire ant baits. Fire ant baits consist of oil placed on a carrier. Foraging worker ants only feed from liquids so workers only remove oil off the bait granule. Thus the granule serves as a vehicle for the toxicant and attractant but it may not be carried into the nest at all. The active ingredient will enter the colony as a liquid.

Because granular baits can be broadcast over larger areas, this is the preferred formulation to reach most of the ant species. Granular baits take advantage of foraging patterns of different ant species. Granular baits consist of attractants, a carrier and active ingredients [24]. Four characteristics are important in a granular bait: 1) delayed toxicity, 2) easy transfer among individuals in the colony, 3) non-repellent active ingredient, and 4) attractive formulation for the target ant species [25, 23].

1. Delayed toxicity: In most ants, only a small percentage of the worker population actively forages outside of the nest. The use of active ingredient with delayed toxicity can guarantee maximum distribution of the bait within the colony before the ants start showing signs of toxicity. If there is enough delay, the active ingredient will be fed to larvae and reproductives before foraging and food sharing activities are shut down in the colony. This guarantees mortality of different castes within the nest, and the elimination of immatures.

2. Easy transfer: This should be applied both to the bait itself, so that it can be handled by a maximum number of individuals within the colony, but especially to the active ingredient. With fast and easy active ingredient transfer, most of the colony can receive a le-

thal dose of the active ingredient before initial gatherers are affected and start showing toxicity effects.

3. Non-repellency: Because baits rely on the pick up and transfer by workers in the ant colony, non-repellent materials will be more easily masked within the bait formulation. Repellent active ingredients could also be used if they could be sufficiently masked by formulations components so not to prevent rejection of the bait by foragers.

4. Attractive formulation: The first step in the bait use process is attracting foragers so they are enticed to seek the bait and carry it back to the nest. Attractants added to the bait can overcome other deterrent characteristics of the bait or avoid any defense behavior that would normally prevent ants from returning toxic components to the nest. A great deal of work is dedicated to examining ant preferences to bait components so formulations can be picked up preferentially by the ants in an environment that will likely have many other food sources.

Several aspects of the granular bait formulation should be considered during the development of new products, including granule composition and size, attractive additives, and active ingredient.

The ideal granular bait should contain granules of similar size that can be easily applied to areas when needed. The carrier of the active ingredient is the most important part of the granular formulation because both the particle size and the materials and components used determine the spreading characteristics, the effectiveness of recruitment and removal of the bait, and the residual life of the active ingredient [26].

Size of the granular carrier may determine the size of ants that can be targeted with a particular formulation (Figure 6). In general, smaller ant species prefer smaller particle sizes to larger ones when given a choice [27]. If the particle size can be matched to a particular target pest ant species, this can increase the efficacy of the granular bait. Ants can normally carry granules with size that roughly match the size of head of foraging workers, but in some species, ant workers may collaborate in carrying larger pieces. Also, ants may subdivide larger bait particles before carrying the bait back to the nest. However, for baits developed for different ant species, this size matching may not be a preferred option.

A large granule size is more convenient because more active ingredient can be added to a larger particle size, allowing more active ingredient to be introduced into the colony with fewer particles collected by the foragers. Ant foraging normally fits what has been described as the optimal foraging theory [28, 29], which states that ants should take the biggest pieces of food particles that they can carry, in order to increase their net energy intake per unit of effort (Figure 7).

However, the difficulty in transport by the ants navigating the larger granular bait into the nest must be considered, especially for ant species that do not cooperate during foraging.

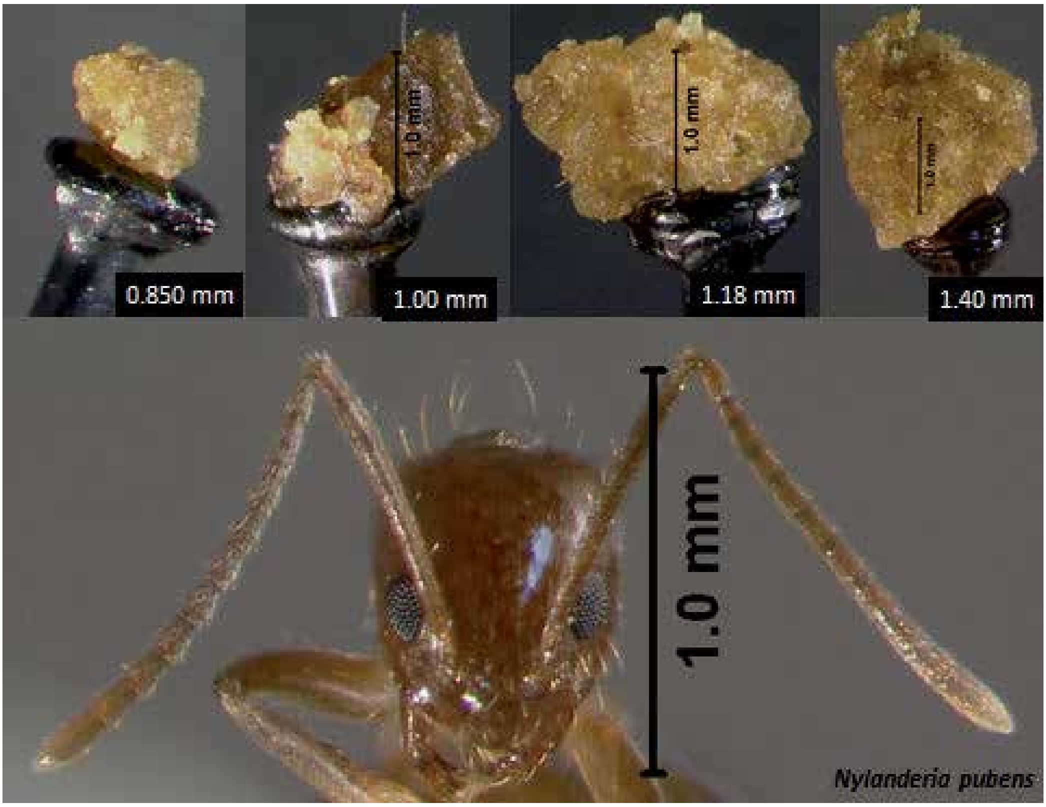

Figure 6. Workers head, *Nylanderia fulva*, in comparison with dog food granules used in size preference experiments.

Because different ants have different requirements for protein, lipids and sugar, and these requirements are likely to vary throughout the year or the life of the ant colony, the composition of the granule can be critical in bait development. Two approaches have been used in the formulation of granular baits for ants: a) use of non-nutritious granule to which food attractants are added, b) use of food particles as the granule matrix to which the active ingredient is added. With either approach, the quality of the food attractant determines which ants are attracted to the bait. Although several bait compositions use sugars as the only attractant, some products have been formulated with protein and lipid attractants, and even insect tissue.

A non-nutritious granule normally used in formulating ant baits is de-fatted corncob, a byproduct from corn processing [30, 24]. It is capable to absorbing relatively large amounts of the liquid additives such as the oil used in many fire ant baits. Fine granules from dog food or other animal diets serve as a nutritious granule for ant baits because it fulfills ant nutrient requirements, is easy to prepare in a uniform granular size and it readily absorbs additives.

Figure 7. *Nylanderia fulva* foraging in a laboratory setting on 1.00-mm (Top) and 1.40-mm (Bottom) dog food granules used in size preference experiments.

The addition of insect tissue [31], which attempts to mimic the natural diet of many ant species, adds complexity to the bait but also to the production process, with inevitable cost consequences. The use of a readily available byproduct, such as silkworm pupae can facilitate production and cut costs. Other insects that can be mass reared at low costs, such as crickets or waxmoth larvae, are also interesting alternatives. Laboratory reared crickets ground into a slurry with addition of small quantity of water have been used in our laboratory to be added to dog food granules tested as a bait to tawny crazy ant (*Nylanderia fulva*).

Traditional baits for *S. invicta*, consist of oil (attractant) on corn cob matrix (carrier) [30, 24]. On the other hand, baits that contain proteins and carbohydrates are very attractive to species such as *L. humile* and *Paratrechina* spp. that are not attracted to the lipid-based fire ant baits [24].

In order to enhance a carrier, it is important to know the ant species food preferences, based on field observations, laboratory experiments, and reports in the literature on similar ant species. Observations of feeding habits such as ants feeding on honeydew from aphids, plant nectaries and insect tissue [32], tending to aphids and mealy bugs [33] can serve as clues in the development of ant baits. Preference studies indicating the balance between components in the ant diets and other aspects of ant nutrition [21, 34, 35, 36, 37, 38, 39] also offer valuable clues that can help the development of new ant baits.

Ant Bait development. Bait development should be initiated with preference tests in the laboratory (Figure 8), but should quickly move into field tests due to the great variability food preference and gathering between laboratory and field populations of the same ant. Laboratory colonies are usually fed a constant diet that is rich in all nutrients need by the ants, while field ant populations are more likely to go through periods when their diet is relatively low in certain nutrients or components such as live insects or sugars. Differences between controlled environment in the laboratory and more variable environment in the field lead to differences in foraging behaviors preference to bait components [40, 41, 42, 43]. Differences in the presence and proportion of different developmental life stages in the colony [44] can also be important factors in determining differences between laboratory and field results.

Field tests should also be conducted at different times of the year in order to characterize the bait preference and effect given different levels of foraging on a specific formulation throughout the season. Because foragers need to move very little between the nest and the foraging arena in laboratory colonies, food choices may be different from those for field ants which usually will travel much further from the nest both in scouting for new food sources and in foraging trails. Distance between the nest and food source can affect choice and quantify of food gathered by an ant colony.

In the laboratory, arena preference tests with multiple bait choices can be used in the elimination of candidate formulations. Later tests with limited choices can be used later to further define preference for specific formulations. Careful design of the foraging arena will avoid preference biases for baits that are found more readily.

If using colony fragments in foraging experiments, careful attention to the size and composition of these experimental colonies is important to preserve the foraging behavior and other characteristics that match those of the full colonies (Figure 9). For instance, foraging of different baits can be drastically affected by the presence or absence of brood, and the brood age structure in an ant colony.

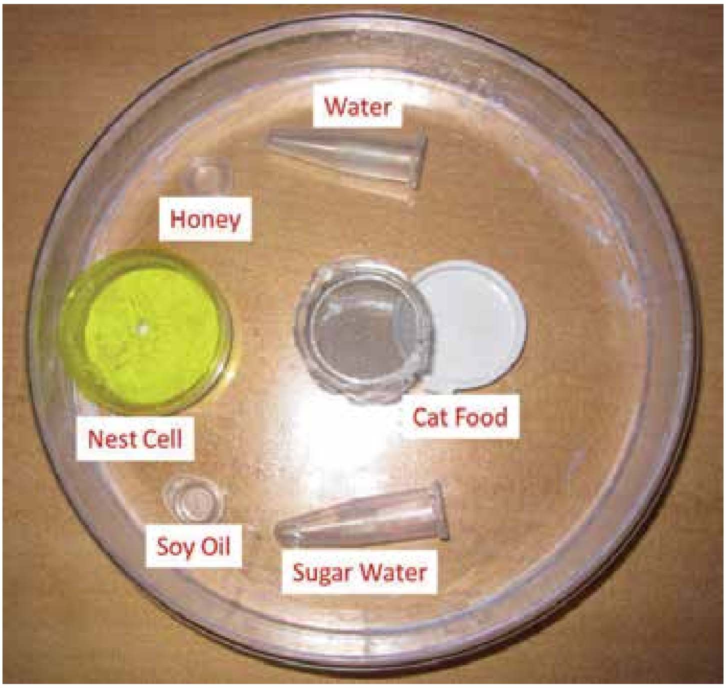

Figure 8. Testing arena used for experiments on *Nylanderia fulva* using granular bait matrix applied with active ingredient.

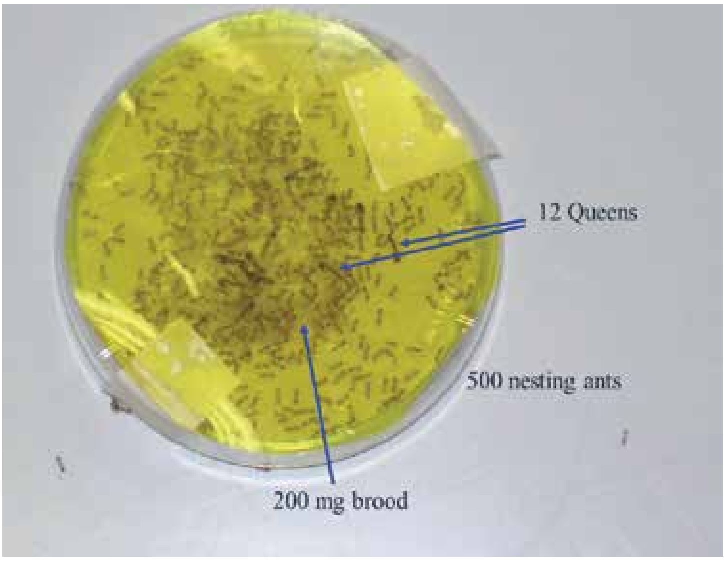

Figure 9. Colony fragments of Pharaoh ants are set up in cells containing brood, workers and queens.

Eventually, bait development must include field experiments to determine the fate and efficacy of the material when applied in situations for which the bait is designed. Careful observations on ant behaviors associated with finding, gathering, and moving the bait material to the ant colony are useful in understanding potential shortcomings of the developed products. Use of fields in different locations that represent the variety of situations where the ant baits can be used is essential in defining clearly the effects of different parameters and factors on the performance on the ant bait.

The data collected from the different experiments will vary, and should be adjusted for each ant species and situation. However, at minimum, the data should allow estimation of the quantity of material necessary to eliminate pest ant colonies, and the total mortality after different periods of time. Of particular importance, is the effect that bait may have on reproductive individuals. In polygyne colonies, it is especially important that the bait achieves maximum distribution within the colony, so that reproductive individuals throughout the colony can be effectively controlled.

4. Termite bait evaluations

Methods of subterranean termite exclusion and prevention of structural infestations have broadened from soil termiticides and barrier treatments to include monitoring and baiting systems. Baiting systems have increased in registration and use since the introduction of the first bait 18 years ago (tradename Recruit, Dow AgroSciences LLC, Indianopolis). The specificity and mode of action of these active ingredients requires much less product to be applied to the environment. Hexaflumeron was the first active ingredient (AI) registered in the United States to be used in a termite bait formulation and there are currently several other AI in use, all of which fall into two classes: insect growth regulators (IGR) and energy production inhibitors. Both classes are considered to be slow-acting and rely on foraging termites to transfer small amounts of consumed bait material thoughout the colony though contact, trophallaxis, grooming, fecal consumption and cannibalism. Baiting systems using IGRs are intended to be used as stand alone treatments. Bait formulations with AI affecting energy production are used in conjunction with soil treatments. There are baits designed to be used in-ground to prevent structural infestation and others for use above-ground in areas with known termite activity. A successful baiting system should be proven to affect termite populations (Figure 10).

Active ingredient evaluation. A non-repellent, lethal and slow-acting active ingredient is required for a termite bait to be effective. When evaluating a potential bait toxicant one must first determine the toxicity of it towards the termite species it will be used againt. For example, at what concentration will it kill 90% of exposed termites (LC_{90})? How long does it take for that 90% to die (LCt_{90})? Termites are highly social and do not fare well when kept in small numbers so great numbers of exposed termites should be placed together

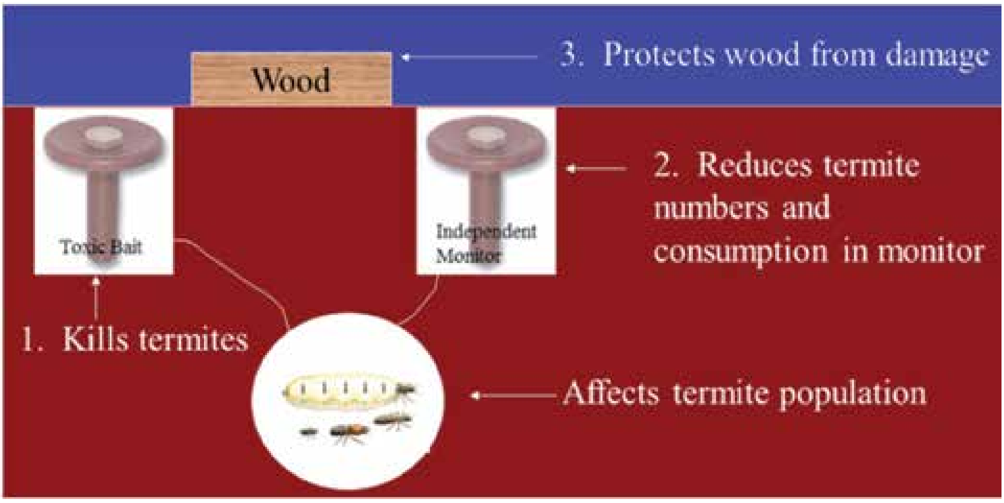

Figure 10. Termite bait efficacy data can document termite populations are affected in 3 ways.

Once the ideal bait toxicant concentration has been determined it must be tested to determine whether it will be readily consumed or show a feeding deterrent effect when adhered to wood or other cellulose containing matrix.

Bait formulation evaluation. The formulation of a palatable bait matrix is essential to the success of a baiting program. In the natural environment of subterranean temites there are a lot of potential food sources providing competition for baits. If there is no preference shown towards a bait it will not be very successful when installed below ground. Above ground baits must be more palatable because the termites present already have a source of wood that they are consuming. Impregnating wood or another cellulose containing material with active ingredient is the most common formulation method for commercially available baits.

Choice tests can be used to determine termite feeding preferences and any foraging biases. In a laboratory setting one can prepare a trial arena filled with moistened soil (10% wt:wt). Each dish will contain one piece of untreated wood and one piece of bait matrix, both of which have the same moisture content, dimensions and orientation within the arena. One thousand subterranean termites are introduced in a Petri dish with a small opening covered by a piece of filter paper at the end. Once the filter paper has been consumed the termites will be free to tunnel and forage throughout the arena. Locations of food choices should be randomized to eliminate any directional biases. Repeating this experiment multiple times with multiple colonies will show which food is preferred by termites. Which source did the termites consume the most of by weight? Was the first food source contacted the only one consumed or was there cross over between both food sources? Statistical analysis will indicate if termites prefer the bait matrix to untreated wood. This simple assay can be repeated and altered to include multiple wood and bait choices, different soil types and moisture levels.

Baiting system evaluation. Using a baiting program to prevent structural infestation may take considerably longer to be effective than soil treatments and thus the methods used to evaluate them are different. In order to gain registration, evaluations of termite baits must ultimately fulfill requirements set forth by state government guidelines (*Florida: 5E-2.0311 Performance Standards and Acceptable Test Conditions for Preventive Termite Treatments for New Construction*). Stand-alone baiting systems must be tested and meet specific requirements in field plot and building tests.

Independent Monitors	Building Monitoring	Reinfestation of Buildings
• >90% reduction in termite activity	• Cessation of live termite activity	• Visual inspection showing no reinfestation within 2 years
• >90% of test biuldings protected	• >90% of test biuldings protected	• Research and visual inspection showing no reinfestation within 1 year
• Protection within 12 months of initiation of feeding on bait active ingredient	• Protection within 12 months of initiation of feeding on the formulated bait	

Table 1. Performance standards for stand-alone termite baits in structures with existing infestations.

Evaluation of below-ground baits: field plot tests. Once it has been determined that feeding on bait has started, infested field plot tests require a reduction in each termite population by at least 50% or a reduction of wood consumption at independent monitors by a minimum of 50% in at least 75% of baited population colonies within one year.

The minimum required thresholds must be maintained for at least 6 months. In order to meet this requirement one can place monitoring stations, which have the same shape, appearance and moisture levels of a baiting station but contain untreated wood instead of bait, throughout a field plot or around a structure known to contain termites. Monitoring stations containing untreated wood are installed in the ground in augered holes at consistent intervals (every 10-20 feet).

Monthly inspections of monitoring stations will continue until live termites are found. Monitoring stations without termites are not switched to bait stations until live termites are found. When live termites are found monitoring stations are deemed ‘active’, wood will be replaced by a bait tube containing active ingredient and termites contained in the wood will be placed in the bait station. Plastic bucket traps (with uniform holes allowing for termite entry) containing wooden blocks may be placed around these stations and checked monthly. The purpose of these bucket traps is to be able to count and assess the nature of termite activity and chart differences over time. Commercially available bait stations require different monitoring intervals but for evaluation it is recommended to be conducted monthly to better determine when control has been achieved.

During monthly inspections, the number of termites present and the amount of bait consumed will be recorded. Bait matrix consumption is typically a visual estimate of the percent consumed as bait weights can be misleading. If baits are completely consumed, compromised or damaged they will be replaced with new bait. Once termite presence and bait consumption ceases monitoring resumes and monthly inspections will continue for at least 6 months. If monitoring stations are found to be active a new bait tube till be installed. The amount of active ingredient consumed can be measured at the end of the study by drying the bait and comparing initial and post-treatment weights using the percentage of active ingredient by weight in the matrix formulation.

The question of whether a colony has been eliminated of merely suppressed can be difficult to answer and may require months to years of monitoring before and after a baiting system is put in place. A suppressed colony will exhibit a period of inactivity in which no termites will be found in monitoring stations yet eventually recover and continue foraging [45]. To better determine the level of control achieved it is recommended to use cuticular dyes and genetic markers as detailed below.

Detecting presence of multiple colonies and foraging areas. Once a monitoring station is attacked by termites they will be collected and keyed to species level. If one is interested in determining the number of colonies present and their respective foraging areas in a field site there are two options: mark-release-recapture or cuticular dyes. Mark-release-recapture involves collecting live termites at monitoring stations, bringing them into the laboratory and feeding them filter paper impregnated with fat-soluble cuticular dyes such as Nile Blue A, Sudan Red 7B, or Neutral Red (Fisher,Pittsburgh, PA) [46]. Once termites are dyed they are placed back into the stations from which they are collected. Mark-release-recapture will also help in estimating population sizes.

A less obtrusive method involves placing a cellulose matrix impregnated with cuticular dyes, Nile Blue A or Neutral Red, in bait tubes which will allow for long-term tracking of termites from station to station. This eliminates the need for termites to be handled. Once the termites are dyed and back in test site the procedure is the same. During monthly monitoring of stations the locations and numbers of dyed termites can be recorded and a map of foraging activities produced [47]. Both methods can be enhanced though the use of genetic markers to help differentiate between colonies [48, 49, 50].

Evaluation of above-ground baits: building tests. Evaluation of above-ground baits can only be conducted in buildings with active subterranean termite infestations. Mud tubes are broken and both monitoring and bait stations are installed in line with the disturbed tube. Weekly monitoring is recommended because bait toxicants are introduced to the colony very quickly. Bait consumption will be visually estimated as a percentage and should be replaced if too much has been consumed or the bait has been compromised. If baits are too dry water may be added but too much water will become a deterrent to consumption. Termites will be counted in monitoring stations but not removed. Once feeding on bait and monitoring stations has ceased, baits will be replaced with monitoring stations.

Above-ground baiting programs must show ≥ 90% reduction in termite activity in ≥ 90% of test buildings within one year from the initiation of bait consumption. A successful above-ground baiting program must show that there has been no re-infestation within one year after activity has ceased. This must be verified by combining a visual inspection with termite detecting tools including infrared devices, moisture meters, radar, chemical detection, bath trap inspection ports, canine detection or fiber optics. The alternative is to wait until two years after the last evidence of termite presence and conduct a visual inspection of the site.

5. Summary

Baits have many advantages for use in urban environments. The advantages extend from use in IPM programs, to non-impact of humans who are living and living or working amid infestations, and to advantages associated with controlling the pests (Table 2).

IPM	Human	Pest Control
• Preserve beneficial organisms involved in biological control	• No odor	• Slow acting
• Reduced risk	• Lower exposure to pesticides	• Non-repellent
• Can be used in sensitive areas	• No mixing needed	• Attractives and phagostimulants to enhance consumption
• Long lasting	• Less preparation prior to pesticide application	• Secondary mortality by transfer
• Application as point sources		• Long lasting
• Less active ingredient		• Transfer of AI within pest population
• Narrow spectrum of insects controlled		• Translocation • Overcome insecticide resistance

Table 2. Advantages of baits in pest management in urban environments in relations to IPM principles, humans and pest control.

Insecticides used in urban environments are almost always in proximity to people, pets, and food. As a result, the safety of products used and efficacy of the formulation in urban pest control are of extreme importance. People can be affected by the use of a wrong formulation, or buildings can be destroyed when ineffective products are used. As a result of screening active ingredients and formulation, a variety of insecticides have been developed for urban pest management (Table 3). Most of the active ingredients are listed by the USEPA as reduced risk products. As reduced risk, there is an expedited registration process for baits containing these actives.

Type/Active Ingredient	Mode of Action	Pest Groups
• Oxadiazine Indoxacarb	• Sodium channel blockage	• Cockroaches, ants
• Neonicotinoid Imidacloprid Dinotefuran	• Acetylcholine receptor stimulation	• Cockroaches, ants, flies
• Spinosins Spinosad	• Acetylcholine receptor stimulation	• Flies
• Phenylpyrazoles Fipronil	• GABA receptor blockage	• Cockroaches, ants
• Avermectins Abamectin Emamectin	• Glutamate receptor stimulation	• Cockroaches, ants
• Chitin synthesis inhibitors Hexaflumuron Noviflumuron Diflubenzuron	• Block chitin formation	• Termites
• Amidinohydrazone Hydramethylnon	• Inhibit energy production	• Cockroaches, ants
• Pyrroles Chlorfenapyr	• Inhibit energy production	• Termites
• Borates Boric acid Sodium borate Disodium octaborate tetrahydrate	• Non-specific metabolic disruption	• Cockroaches, ants, flies

Table 3. Active ingredients, modes of action, and pests controlled with baits used for pest management in urban environments.

Baits have become one of the most popular formulations used by pest management professionals for use against cockroaches, ants, and termites. One of the advantages of bait formulations is that they are usually ready to use, in low concentrations, and can be placed only where and when needed. Hazards of using baits is minimized by using child-resistant bait stations or careful placement directly into harborages. The use of baits requires more time than spraying and costs may be higher because of the use of food-grade ingredients in the formulation.

Overall, baits are a very effective and successful insecticide formulation for urban pest control. As a result, the industry has been expanding testing and screening programs for label expansions so insects other than ants, cockroaches, and termites can be controlled.

Author details

Bennett W. Jordan, Barbara E. Bayer, Philip G. Koehler and Roberto M. Pereira

Department of Entomology and Nematology, Steinmetz Hall, Natural Area Drive, University of Florida, Gainesville, FL, USA

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Advances in Insecticide Tools and Tactics for Protecting Conifers from Bark Beetle Attack in the Western United States

Christopher J. Fettig, Donald M. Grosman and
A. Steven Munson

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/54178

1. Introduction

Bark beetles (Coleoptera: Curculionidae, Scolytinae), a large and diverse group of insects consisting of ~550 species in North America and >6,000 species worldwide, are primary disturbance agents in coniferous forests of the western U.S. Population levels of a number of species (<1%) oscillate periodically, often reaching densities that result in extensive tree mortality when favorable climatic (e.g., droughts) and forest conditions (e.g., dense stands of susceptible hosts) coincide (Table 1). The genera *Dendroctonus*, *Ips* and *Scolytus* are well recognized in this regard. In recent decades, billions of conifers across millions of hectares have been killed by native bark beetles in forests ranging from Alaska to New Mexico, and several recent outbreaks are considered the largest and most severe in recorded history.

Host selection and colonization behavior by bark beetles are complex processes. Following initial attacks and subsequent mating, adults lay eggs in the phloem and larvae excavate feeding tunnels in this tissue and/or the outer bark. Depending on the bark beetle species and the location and severity of feeding, among other factors, this process may result in mortality of the host tree. Top-kill and/or branch mortality are not uncommon. Following pupation, adult beetles of the next generation tunnel outward through the bark and initiate flight in search of new hosts. The lifecycle may be repeated once every several years or several times a year depending on the bark beetle species, geographic location and associated climatic conditions. Extensive levels of tree mortality may result in host replacement by other tree species and plant associations, and may impact timber and fiber production, water quality and quantity, fish and wildlife populations, aesthetics, recreation, grazing capacity, real estate values, biodiversity, carbon storage, endangered species and cultural resources.

Common name	Scientific name	Primary host(s)
Arizona fivespined ips	*Ips lecontei*	*Pinus ponderosa*
California fivespined ips	*I. paraconfusus*	*P. contorta*, *P. jeffreyi*, *P. lambertiana*, *P. ponderosa*
Douglas-fir beetle	*Dendroctonus pseudotsugae*	*Pseudotsuga menziesii*
eastern larch beetle	*D. simplex*	*Larix laricina*
fir engraver	*Scolytus ventralis*	*Abies concolor*, *A. grandis*, *A. magnifica*
Jeffrey pine beetle	*D. jeffreyi*	*P. jeffreyi*
mountain pine beetle*	*D. ponderosae*	*P. albicaulis*, *P. contorta*, *P. flexilis*, *P. lambertiana*, *P. monticola*, *P. ponderosa*
northern spruce engraver	*I. perturbatus*	*Picea glauca*, *Pi. x lutzii*
pine engraver	*I. pini*	*P. contorta*, *P. jeffreyi*, *P. lambertiana*
pinyon ips	*I. confusus*	*P. edulis*, *P. monophylla*
roundheaded pine beetle	*D. adjunctus*	*P. arizonica*, *P. engelmannii*, *P. flexilis*, *P. leiophylla*, *P. ponderosa*, *P. strobiformis*
southern pine beetle	*D. frontalis*	*P. engelmannii*, *P. leiophylla*, *P. ponderosa*
spruce beetle*	*D. rufipennis*	*Pi. engelmannii*, *Pi. glauca*, *Pi. pungens*, *Pi. sitchensis*
western balsam bark beetle	*Dryocoetes confusus*	*A. lasiocarpa*
western pine beetle*	*D. brevicomis*	*P. coulteri*, *P. ponderosa*

*Species for which preventative insecticide treatments have been well studied.

Table 1. Bark beetle species that cause significant amounts of tree mortality in coniferous forests of the western U.S.

While native bark beetles are a natural part of the ecology of forests, the economic and social impacts of outbreaks can be substantial. Several tactics are available to manage bark beetle infestations and to reduce associated levels of tree mortality. While these vary by bark beetle species, current tactics include tree removals that reduce stand density (thinning) and presumably host susceptibility [1]; sanitation harvests [1]; applications of semiochemicals (i.e., chemicals produced by one organism that elicit a response, usually behavioral, in another organism) to protect individual trees or small-scale stands (e.g., <10 ha) [2]; and preventative applications of insecticides to individual trees. The purpose of this chapter is to synthesize information on the efficacy, residual activity, and environmental safety of insecticides commonly used to protect trees from bark beetle attack so that informed, judicious decisions can be made concerning their use.

2. Types and use of preventative applications of insecticides

Preventative applications of insecticides involve topical sprays to the tree bole (bole sprays) or systemic insecticides injected directly into the tree (tree injections) [3]. Systemic insecticides applied to the soil are generally ineffective. In an operational context, only high-value, individual trees growing in unique environments or under unique circumstances are treated. These may include trees in residential (Fig. 1), recreational (e.g., campgrounds) (Fig. 2) or administrative sites. Tree losses in these environments result in undesirable impacts such as reduced shade, screening, aesthetics, and increased fire risk. Dead trees also pose potential hazards to public safety requiring routine inspection, maintenance and eventual removal [4], and property values may be negatively impacted [5]. In addition, trees growing in progeny tests, seed orchards, or those genetically resistant to forest diseases may be considered for preventative treatments, especially if epidemic populations of bark beetles exist in the area. During large-scale outbreaks, hundreds of thousands of trees may be treated annually in the western U.S., however once an outbreak subsides (i.e., generally after one to several years) preventative treatments are often no longer necessary.

Figure 1. Tree mortality attributed to western pine beetle in San Bernardino County, California, U.S. In the wildland urban interface, tree losses pose potential hazards to public safety and costs associated with hazard tree removals can be substantial. Furthermore, property values may be significantly reduced. The value of these trees, cost of removal and loss of aesthetic value often justify the use of insecticides to protect trees from bark beetle attack during an outbreak. Photos: C.J. Fettig, Pacific Southwest Research Station, USDA Forest Service.

Figure 2. Conditions before (left) and after (right) a spruce beetle outbreak impacted the Navajo Lake Campground on the Dixie National Forest, Utah, U.S. Daily use decreased substantially due to reductions in shade, screening and aesthetics associated with mortality and removal of large diameter overstory trees. Photos: A.S. Munson, Forest Health Protection, USDA Forest Service.

Although once common, insecticides are rarely used today for direct or remedial control (i.e., subsequent treatment of previously infested trees or logs to kill developing and/or emerging brood). While remedial applications have been demonstrated to increase mortality of brood in treated hosts, there is limited evidence of any impact to adjacent levels of tree mortality. Furthermore, there are concerns about the effects of remedial treatments on non-target invertebrates, specifically natural enemy communities. Many of these species respond kairomonally to bark beetle pheromones and host volatiles, and their richness increases over time [6], suggesting that the later remedial treatments are applied the more likely non-target organisms will be negatively impacted.

3. Insecticide registrations

Insecticide sales and use in the U.S. are regulated by federal (U.S. Environmental Protection Agency, EPA) and state (e.g., California Department of Pesticide Regulation in California) agencies. Therefore, product availability and use vary by state. EPA regulates all pesticides under broad authority granted in two statutes, (1) the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) that requires all pesticides sold or distributed in the U.S. to be registered; and (2) the Federal Food, Drug and Cosmetic Act that requires EPA to set pesticide tolerances for those used in or on food. EPA may authorize limited use of unregistered pesticides or pesticides registered for other uses under certain circumstances. Under Section 5 of FIFRA, EPA may issue experimental use permits that allow for field testing of new pesticides or uses. Section 18 of FIFRA permits the unregistered use of a pesticide in a specific geographic area for a limited time if an emergency pest condition exists. Under Section 24(c) of FIFRA, states may register a new pesticide for any use, or a federally-registered product for an additional use, as long as a "special local need" is demonstrated.

A complete list of active ingredients and products used for protecting trees from bark beetle attack is beyond our scope as availability changes due to cancellations, voluntary withdraws, non-payment of annual registration maintenance fees, and registration of new products at federal and state levels. Several studies have been published on the efficacy of various classes, active ingredients, and formulations that are no longer registered [e.g., benzene hexachloride (Lindane®)]. Therefore, we limit much of our discussion to the most commonly used and/or extensively-studied products (Fig. 3). A list of products registered for protecting trees from bark beetle attack can be obtained online from state regulatory agencies and/or cooperative extension offices, and should be consulted prior to implementing any treatment. Furthermore, all insecticides registered and sold in the U.S. must carry a label. It is a violation of federal law to use any product inconsistent with its labeling. The label contains abundant information concerning the safe and appropriate use of insecticides (e.g., signal words, first aid and precautionary statements, proper mixing, etc.). For tree protection, it is important to note whether the product is registered for ornamental and/or forest settings, and to limit applications to appropriate sites using suitable application rates.

Figure 3. The carbamate carbaryl and pyrethroids bifenthrin and permethrin are commonly used to protect trees from bark beetle attack in the western U.S. Several formulations are available and effective if properly applied. Residual activity varies with active ingredient, bark beetle species, tree species, geographic location, and associated climatic conditions. Photos: C.J. Fettig, Pacific Southwest Research Station, USDA Forest Service.

4. Experimental designs for evaluating preventative treatments

When evaluating preventative treatments one of three experimental designs is generally used. Each has its own advantages and disadvantages. Laboratory assays require trapping and/or rearing of live bark beetles for inclusion in experiments. Captured individuals are immediately transported to the laboratory, identified and sorted. Damaged (e.g., loss of any appendages), weakened, or beetles not assayed within 48 h after collection should be discarded. Generally, serial dilutions of each insecticide are prepared, and toxicity is determined in filter paper or topic assays [7]. The life-table method is used to estimate the survival probability of test subjects to different doses of each insecticide [7]. Filter paper assays more closely approximate conditions under which toxicants are encountered by bark beetles during host colonization, especially for products other than contact insecticides [7], but both methods ignore important environmental factors (e.g., temperature, humidity and sunlight) and host tree factors (e.g., architecture) that influence efficacy. However, results are rapidly obtained with limited risk and loss of scientific infrastructure compared to field studies.

A second design involves field assays in which insecticides are applied to an experimental population of ~25−35 uninfested trees [8]. Trees are often baited with a bark beetle species-specific attractant to increase beetle "pressure" and challenge the treatment following application. Efficacy is based on tree mortality and established statistical parameters [8]. This design is accepted as the standard for evaluating preventative treatments for tree protection in the western U.S., and provides a very conservative test of efficacy [9]. However, it is laborious, time-consuming (i.e., generally efficacy is observed for at least two field seasons) and expensive. Experimental trees may be lost to woodcutting or wildfire, and ≥60% of the untreated control trees must die from bark beetle attack to demonstrate that significant bark beetle pressure exists in the area or the experiment fails and results are inconclusive [8]. Some have argued that the design is perhaps too conservative as under natural conditions aggregation pheromone components would not be released for such extended periods of time as often occurs with baiting. Finally, bark beetles may initiate undesirable infestations near experimental trees as a result of baiting, which may be unacceptable under some circumstances.

The "hanging bolt" assay [10], "small-bolt" assay [11] and similar variants have received limited attention in the western U.S. Typically, insecticides are applied to individual, uninfested trees that are later harvested and cut into bolts for inclusion in laboratory and/or field experiments. Alternatively, freshly-cut bolts may be treated directly in the laboratory. Efficacy is often based on measures of attack density or gallery construction by adult beetles. Compared to [8], these methods allow for rapid acquisition of data; reduced risk of loss to scientific infrasture; and increased probability that a rigorous test will be achieved as bolts are transported to active infestations or brought into the laboratory and exposed to beetles. While these methods account for some host factors (e.g., bark architecture), others such as host defenses and environmental factors are ignored. Furthermore, the hanging bolt and small bolt assays do not provide an estimate of tree mortality, while the effectiveness of any preventative treatment is defined by reductions in tree mortality.

5. Topical applications to the tree bole

Topical applications to protect trees from bark beetle species such as western pine beetle, *Dendroctonus brevicomis* LeConte, mountain pine beetle, *D. ponderosae* Hopkins, and spruce beetle, *D. rufipennis* (Kirby), are applied with ground-based sprayers at high pressure [e.g., ≥2,241 kPa] to the tree bole. Insecticides are applied on all bole surfaces up to a height of ~10.6 to 15.2 m until runoff generally from the root collar to mid-crown (Fig. 4). For engraver beetles, *Ips* spp., that typically colonize smaller diameter hosts branches >5 cm diameter should also be treated. The amount of material (product + water) applied varies with bark and tree architecture, tree size, equipment and applicator, among other factors, but ranges from ~15 to 30 L per tree under most circumstances [12-14]. Application efficiency, the percentage of material applied that is retained on trees, ranges from ~80 to 90% [14].

Figure 4. A common method of protecting trees from bark beetle attack is to saturate all surfaces of the tree bole using a ground-based sprayer at high pressure. Photos: C.J. Fettig, Pacific Southwest Research Station, USDA Forest Service.

Bole sprays are typically applied in late spring prior to initiation of the adult flight period for the target bark beetle species. However, bole sprays require transporting sprayers and other large equipment, which can be problematic in high-elevation forests where snow drifts and poor road conditions often limit access. Additionally, many recreation sites (e.g., campgrounds) where bole sprays are frequently applied occur near intermittent or ephemeral streams that are associated with spring runoff, limiting applications in late spring due to restrictions concerning the use of no-spray buffers to protect non-target aquatic organisms. For these and other reasons, researchers are evaluating alternative timings of bole sprays and less laborious delivery methods.

5.1. Carbaryl

Carbaryl is an acetylcholinesterase inhibitor that prevents the cholinesterase enzyme from breaking down acetylcholine, increasing both the level and duration of action of the neurotransmitter acetylcholine, which leads to rapid twitching, paralysis and ultimately death. Carbaryl is considered essentially nontoxic to birds, moderately toxic to mammals, fish and amphibians, and highly toxic to honey bees, *Apis mellifera* L., and several aquatic insects [15]. However, carbaryl is reported to pose little or no threat to warm-blooded animals. Several experts report that carbaryl is still the most effective, economically-viable, and ecologically-compatible insecticide available for protecting individual trees from mortality due to bark beetle attack in the western U.S. [9,16]. Today, carbaryl (e.g., Sevin® SL and Sevin® XLR Plus, among others) is commonly used to protect trees from bark beetle attack, and is the most-extensively studied active ingredient registered for use. Failures in efficacy are rare and typically associated with inadequate coverage, improper mixing (e.g., using an alkaline water source with pH >8) [17] or inaccurate mixing resulting in solutions of reduced concentration, improper storage, and/or improper timing (e.g., applying treatments to trees already successfully attacked by bark beetles).

Mountain and western pine beetles. Several rates and formulations of carbaryl have been evaluated, and most research indicates two field seasons of protection can be expected with a single application. The effectiveness of 1.0% and 2.0% Sevimol® was demonstrated in the early 1980s [18-22]. This and other research [23-24] led to the registration of 2.0% Sevimol® as a preventative spray, which was voluntarily canceled in 2006. [22] evaluated the efficacy of 0.5%, 1.0% and 2.0% Sevimol® and Sevin® XLR and found all concentrations and formulations were effective for protecting lodgepole pine, *P. contorta* Dougl. ex Loud., from mortality due to mountain pine beetle attack for one year. The 1.0% and 2.0% rates were efficacious for two years. [9] reported 2.0% Sevin® SL protected ponderosa pine, *Pinus ponderosa* Dougl. ex Laws., from western pine beetle attack in California; ponderosa pine from mountain pine beetle attack in South Dakota; and lodgepole pine from mountain pine beetle attack in Montana (two separate studies) for two field seasons. Similar results have been obtained elsewhere [12]. Ongoing research is evaluating the efficacy of fall versus spring applications of 2.0% Sevin® SL for protecting lodgepole pine from mountain pine beetle attack in Wyoming. Both treatments provided 100% tree protection during the first field season while 93% mortality was observed in the untreated control (C.J.F. and A.S.M., unpublished data). A similar study is being conducted for mountain pine beetle in ponderosa pine in Idaho.

Southern pine beetle. Southern pine beetle, *D. frontalis* Zimmerman, occurs in a generally continuous distribution across the southern U.S., roughly coinciding with the distribution of loblolly pine, *P. taeda* L. However, southern pine beetle also occurs in portions of Arizona and New Mexico where it colonizes several pine species, and is therefore considered here. While preventative treatments have not been evaluated in western forests, carbaryl is ineffective for protecting loblolly pine from mortality due to southern pine beetle attack in the southern U.S. [25-26]. This was later linked to insecticide tolerance in southern pine beetle associated with an efficient conversion of carbaryl into metabolites, and a rapid rate of excretion [27-29]. Therefore, despite important environmental differences between the southern and western U.S., carbaryl is regarded as ineffective for preventing southern pine beetle attacks and subsequent tree mortality in the western U.S. [30].

Spruce beetle. Most research suggests that three field seasons of protection can be expected with a single application of carbaryl. In south-central Alaska, [31] reported that 1.0% and 2.0% Sevin® SL protected white spruce, *Picea glauca* (Moench) Voss, and Lutz spruce, *P. glauca* X *lutzii* Little, from attack by spruce beetle for three field seasons, despite early work indicating carbaryl was ineffective in topical assays [32]. One and 2.0% Sevimol® were effective for protecting Engelmann spruce, *P. engelmannii* Parry ex. Engelm., from spruce beetle attack for two field seasons in Utah [33], which agrees with results from [9] for 2.0% Sevin® SL. However, the two latter studies were concluded after two field seasons. In the case of [9], all Sevin® SL-treated trees were alive at the end of the study.

Red turpentine beetle. Red turpentine beetle, *D. valens* LeConte, usually colonizes the basal portions of stressed, weakened, or dead and dying trees. Therefore, the species is not considered an important source of tree mortality in the western U.S., and limited work has occurred regarding the development of tree protection tools. [34] reported that 2.0% Sevin® XLR and 4.0% Sevimol® 4 were effective for protecting ponderosa pine in California. Several formulations of carbaryl are effective for protecting Monterey pine, *P. radiata* D. Don, [35], but residual activity is generally short-lived (<1 yr).

Engraver beetles. A single application of 2.0% Sevin® SL was effective for protecting single-leaf pinyon, *P. monophylla* Torr. & Frem., from mortality due to pinyon ips, *I. confusus* (LeConte), for two field seasons in Nevada [9]. A similar study in pinyon pine, *P. edulis* Engelm., on the Southern Ute Reservation in Colorado found 2.0% Sevin® SL was efficacious for one field season, but bark beetle pressure was insufficient the second year of the study to make definitive conclusions regarding efficacy [9]. [9] also evaluated the efficacy of 2.0% Sevin® SL for protecting ponderosa pine from pine engraver, *I. pini* (Say), but very few trees were attacked during the experiment. Approximately one year later, trees in this study were harvested and cut into bolts that were then laid on the ground in areas containing slash piles infested with pine engraver, sixspined ips, *I. calligraphus* (Germar), and Arizona five-spined ips, *I. lecontei* Swain [13]. From this and related research, the authors concluded 1.0% and 2.0% Sevin® SL were effective for protecting ponderosa pine from engraver beetle attacks for one entire flight season in Arizona. [36] reached similar conclusions for 2.0% Sevin® 80 WSP for a complex of engraver beetles, including sixspined ips, that colonize loblolly pine in the southeastern U.S.

5.2. Pyrethroids

Pyrethroids are synthesized from petroleum-based chemicals and related to the potent insecticidal properties of flowering plants in the genus *Chrysanthemum*. They are axonic poisons and cause paralysis by keeping the sodium channels open in the neuronal membranes [37]. First generation pyrethroids were developed in the 1960s, but are unstable in sunlight. By the mid-1970s, a second generation was developed (e.g., permethrin, cypermethrin and deltamethrin) that were more resistant to photodegradation, but have substantially higher mammalian toxicities. Third generation pyrethroids (e.g., bifenthrin, cyfluthrin and lambda-cyhalothrin) have even greater photostability and insecticidal activity compared to previous generations. Pyrethroids are one of the least acutely toxic insecticides to mammals, essentially nontoxic to birds, but are highly toxic to fish, amphibians and honey bees [38]. Today, permethrin (e.g., Astro® and Dragnet®, among others) and bifenthrin (e.g., Onyx™) are commonly used to protect trees from bark beetle attack, and following carbaryl are the most-extensively studied active ingredients registered for use.

Mountain and western pine beetles. Several active ingredients and formulations of pyrethroids have been evaluated as preventative treatments, and most research indicates at least one field season of protection can be expected with a single application. [8] evaluated 0.1%, 0.2% and 0.4% permethrin (Pounce®) for protecting ponderosa pine from mortality due to western pine beetle attack, and reported that 0.2% and 0.4% provided control for four months. Permethrin plus-C (Masterline®), a unique formulation containing methyl cellulose (i.e., "plus-C") thought to increase efficacy and stability by reducing photo-, chemical- and biological-degradation of the permethrin molecule, exhibits efficacy similar to that of other formulations of permethrin [12]. [39] examined several rates of esfenvalerate (Asana® XL) and cyfluthrin (Tempo® 20 WP) as preventative treatments. In California, 0.025% and 0.05%

Asana® XL protected ponderosa pine for western pine beetle attack for one field season, but not a second. In Montana, 0.006% and 0.012% Asana® XL were ineffective for protecting lodgepole pine from mountain pine beetle, but 0.025% was effective for one field season. Tempo® 20 WP applied at 0.025% provided protection of ponderosa pine from western pine beetle for one field season in Idaho, but not California [39]. Surprisingly, 0.025%, 0.05% and 0.1% Tempo® 20 WP were effective for protecting lodgepole pine from mountain pine beetle attack for two field seasons [39]. [9] evaluated 0.03%, 0.06% and 0.12% bifenthrin (Onyx™) reporting at minimum one field season of protection for mountain pine beetle in lodgepole pine and two field seasons of protection for western pine beetle in ponderosa pine. This study and related research led to the registration of 0.06% Onyx™ as a preventative spray in the mid-2000s. [40] reported 0.06% Onyx™ failed to provide three field seasons of protection for western pine beetle in ponderosa pine, confirming Onyx™ is only effective for two field seasons in that system.

Southern pine beetle. While limited research has occurred, permethrin (Astro®) appears to have longer residual activity than bifenthrin (Onyx™) at least in small-bolt assays [11].

Spruce beetle. Most research suggests that at least one field season of protection can be expected. [9] reported 0.03%, 0.06% and 0.12% bifenthrin (Onyx™) would likely provide protection for two field seasons in Utah. However, 0.025% cyfluthrin (Tempo® 2) and 0.025% and 0.05% esfenvalerate (Asana® XL) only provided one field season of protection in Utah [33]. Protection of Lutz spruce in Alaska is possible for two field seasons with a single application of 0.25% permethrin (formulation unreported) [41].

Red turpentine beetle. [35] reported 0.5% permethrin (Dragnet®) was effective for protecting Monterey pine, and that it had longer residual activity than carbaryl. [34] reported 0.1%, 0.2% and 0.4% permethrin (formulation unreported) were ineffective for protecting ponderosa pine from red turpentine beetle.

Engraver beetles. Most research suggests that at least one field season of protection can be expected with a single application; however, [9] reported 0.03%, 0.06% and 0.12% bifenthrin (Onyx™) protected single-leaf pinyon from pinyon ips for two field seasons in Nevada. A similar study on the Southern Ute Reservation in Colorado found 0.12% Onyx™ protected pinyon pine for one field season, but bark beetle pressure was insufficient the second year of the study to make conclusions regarding efficacy at that rate. Both 0.03% and 0.06% Onyx™ were ineffective [9]. [13] reported that 0.19% permethrin plus-C (Masterline®) and 0.06% bifenthrin (Onyx™) were effective for protecting ponderosa pine bolts from engraver beetle attack in Arizona. [36] reported 0.06% bifenthrin (Onyx™) significantly reduced colonization of trees by bark and woodboring beetles, including sixspined ips, in the southeastern U.S.

6. Systemic injections to the tree bole

Researchers attempting to find safer, more portable and longer-lasting alternatives to bole sprays have evaluated the effectiveness of injecting small quantities of systemic insecticides directly into the lower bole. Early work indicated that several methods, active ingredients and formulations were ineffective [e.g., 13,42-44]. In recent years, the efficacy of phloem-mobile active ingredients injected with pressurized systems (e.g., Sidewinder® Tree Injector, Tree I.V. micro infusion® and Wedgle® Direct-Inject™) capable of maintaining >275 kPA have been evaluated for engraver beetles, mountain pine beetle, southern pine beetle, spruce beetle, and western pine beetle (Fig. 5). These systems push adequate volumes of product (i.e., generally less than several hundred ml for even large trees) into the small vesicles of the sapwood [45]. Applications take <15 minutes per tree under most circumstances. Following injection, the product is transported throughout the tree to the target tissue (i.e., the phloem where bark beetle feeding occurs). Injections can be applied at any time of year when the tree is actively translocating, but time is needed to allow for full distribution of the active ingredient within the tree prior to the tree being attacked by bark beetles. Under optimal conditions (e.g., adequate soil moisture, moderate temperatures and good overall tree health) this takes ~4 weeks [46], but may take much longer, particularly in high-elevation forests. Tree injections represent essentially closed systems that eliminate drift, and reduce non-target effects and applicator exposure, but efficacy is often less than that observed for bole sprays in high-elevation forests [40]. Significant advancements in the development of this technology have been made in recent years, but tree injections are still rarely used in comparison to bole sprays in the western U.S. With the advent of designer formulations of insecticides specific for tree injection, we suspect that tree injections will become a more common tool for protecting trees from bark beetle attack in the near future, particularly in areas where bole sprays are not practical (e.g., along property lines or within no-spray buffers).

Figure 5. Experimental injections of emamectin benzoate for protecting trees from western pine beetle attack in Calaveras County, California, U.S. (left), and mountain pine beetle attack in the Uinta-Wasatch-Cache National Forest, Utah, U.S. (right). Photos: C.J. Fettig, Pacific Southwest Research Station, USDA Forest Service (left) and D.M. Grosman, Texas A&M Forest Service (right).

6.1. Emamectin benzoate

Emamectin benzoate is a macrycyclic lactone derived from avermectin B1 (= abamectin) by fermentation of the soil actinomycete *Streptomyces avermitilis* that disrupts neurotransmitters causing irreversible paralysis. Emamectin benzoate is highly toxic to fish and honey bees, and very highly toxic to aquatic invertebrates. It is highly toxic to mammals and birds as well on an acute oral basis, but is dermally benign to mammals. In recent years, emamectin benzoate has received the most attention among systemic injections for protecting trees from bark beetle attack in the western U.S. [40].

Mountain and western pine beetles. [40] evaluated an experimental formulation of 4.0% emamectin benzoate mixed 1:1 with methanol for protecting ponderosa pine from mortality due to western pine beetle attack in California. Results of this study indicate three field seasons of protection can be expected with a single application. To our knowledge, this was the first demonstration of a successful application of a systemic insecticide for protecting individual trees from mortality due to bark beetle attack in the western U.S. This and other research led to the registration of emamectin benzoate (TREE-age™) in 2010 for protecting individual trees from bark beetle attack.

The experimental formulation of emamectin benzoate was ineffective for protecting lodgepole pine from mountain pine beetle attack in Idaho [40], which agrees with field studies conducted in British Columbia and Colorado (D.M.G., unpublished data). Site conditions such as ambient temperatures, soil temperatures and soil moistures may help explain the lack of efficacy observed in these studies as these factors may slow product uptake and translocation within trees in high-elevation forests [40]. As such, failures for protecting lodgepole pine from mountain pine beetle attack were initially attributed to inadequate distribution of the active ingredient following injections made ~5 weeks prior to trees coming under attack by mountain pine beetle [40]. The authors commented that injecting trees in the fall and/or increasing the number of injection points per tree could perhaps increase efficacy. Currently, spring and fall applications of TREE-age™ are being evaluated for protecting lodgepole pine from mortality due to mountain pine beetle attack in Utah. Results for fall treatments are very promising (Table 2).

Southern pine beetle. Several studies have evaluated the efficacy of emamectin benzoate for protecting loblolly pine from mortality due to southern pine beetle attack in the southern U.S. [47, D.G.M., unpublished data]. Most have demonstrated a reduction in tree mortality, but few trees were attacked in the untreated controls, presumable due to low population levels.

Spruce beetle. An experimental formulation of 4.0% emamectin benzoate injected in late August was ineffective for protecting Engelmann spruce from mortality due to spruce beetle attack in Utah [40]. However, the commercial formulation TREE-age™ has yet to be evaluated. Studies are planned to evaluate alternative timings of injection of TREE-age™ (i.e., early summer versus late summer) and the number and position of the injection ports in trees, both of which are thought to influence efficacy [40].

Engraver beetles. Several studies have reported that emamectin benzoate is effective for preventing engraver beetle attacks, including sixspined ips, for at least two years in Texas [46, D.M.G., unpublished data].

Treatment[a]	Rate[b] (/2.54 cm dbh)	Percent mortality[c]
Spring injection	10 ml	33%
Fall injection	10 ml	0%
Untreated control (yr 1)	-	80%
Untreated control (yr 2)	-	60%

[a] Injections occurred in spring (i.e., June, ~1 month prior to peak mountain pine beetle that year) and fall (i.e., September, ~10 months prior to peak mountain pine beetle flight the following year).

[b]dbh = diameter at breast height (1.37 m in height).

[c]Based on presence or absence of crown fade in September 2011. Data obtained from Fettig et al. (unpublished data).

Table 2. The effectiveness of injections of emamectin benzoate (TREE-age™) into the lower bole of lodgepole pine for reducing levels of tree mortality due to mountain pine beetle attack, Uinta-Wasatch-Cache National Forest, Utah, U.S., 2009-2011.

6.2. Abamectin

Abamectin (= avermectin B1) is a natural fermentation product of the soil actinomycete *Streptomyces a. vermitilis.* Like emamectin benzoate, abamectin acts on insects by interfering with neural and neuromuscular transmission. Abamectin is relatively non-toxic to birds, but highly toxic to fish, aquatic invertebrates and honeybees. Most formulated products are of low toxicity to mammals. Ongoing studies indicate Abacide™ 2 is effective for protecting lodgepole pine from mortality due to mountain pine beetle attack in Utah for at least one field season (C.J.F. et al., unpublished data). Similarly, efficacy has been demonstrated for a complex of engraver beetles, including sixspined ips, for three field seasons in Texas (D.G.M., unpublished data). A request to add mountain pine beetle and engraver beetles to the label for Abacide™ 2 may be forthcoming.

6.3. Fipronil

Fipronil is a phenyl pyrazole that disrupts the insect central nervous system by blocking the passage of chloride ions through the gamma-aminobutyric acid (GABA) receptor and glutamate-gated chloride channels. This results in hyperexcitation of contaminated nerves and muscles and ultimately death. Fipronil is of low to moderate toxicity to mammals, highly toxic to fish, aquatic invertebrates, honeybees and upland game birds, but is practically non-toxic to waterfowl and other bird species. Fipronil reduced levels of tree mortality due to engraver beetles, including sixspined ips, on stressed trees in Texas [46]. However, fipronil is ineffective for protecting loblolly pine from southern pine beetle [47] and Engelmann spruce from spruce beetle [40,48]. While results are inconclusive [40, 48], fipronil does not appear effective from reducing levels of lodgepole pine mortality due to mountain pine beetle attack in Utah or ponderosa pine mortality due to western pine beetle attack in California. Thus, registration is not being pursued at this time.

7. Environmental concerns

Most data on the deposition, toxicity, and environmental fate of insecticides in western forests come from aerial applications to control tree defoliators, and therefore are of limited applicability to bole sprays or tree injections used to protect trees from bark beetle attack. [49] studied the effects of lindane, chlorpyrifos and carbaryl on a California pine forest soil arthropod community by spraying normal levels of insecticide, and levels five times greater than would be operationally used to protect trees from bark beetle attack. The authors concluded carbaryl was least disruptive to the soil arthropod community [49]. Persistence and movement of 2.0% carbaryl within soils of wet and dry sites has been evaluated [50]. The highest concentrations of carbaryl were detected within the uppermost soil layers (upper 2.54 cm), with levels exceeding 20 ppm 90 d after application on most sites [50].

Carbaryl is relatively nontoxic to *Enoclerus lecontei* (Wolcott) [51] and *E. sphegeus* (F.) [52], and less toxic than either lindane or chlorpyrifos to *Temnochila chlorodia* (Mannerheim) [51], common predators of bark beetles in the western U.S. [32] measured the remedial efficacy of 0.25%, 0.5%, 1.0%, and 2.0% chlorpyrifos (Dursban®), fenitrothion (Sumithion®) and permethrin (Pounce®) on emerged and nonemerged predators and parasites of spruce beetle in Alaska. Two percent Pounce® had the least impact on emerged natural enemies while Dursban® and Sumithion® had the greatest impacts. In many cases, the lowest concentrations resulted in the highest mortality of emerged parasites and predators (74-94% mortality), but lowest mortality of nonemerged individuals. The authors attributed this to higher concentrations resulting in prolong emergence [32]. Mortality of nonmerged parasites and predators was <45% for all active ingredients and concentrations, except 2.0% chlorpyrifos [32].

Werner and Hilgert [53] monitored permethrin levels in a freshwater stream adjacent to Lutz spruce that were treated with 0.5% permethrin (Pounce®) to prevent spruce beetle attack. Treatments occurred within 5 m of the stream. Maximum residue levels ranged from 0.05 ± 0.01 ppb 5 h after treatment to 0.14 ± 0.03 ppb 8-11 h after treatment, declining to 0.02 ± 0.01 ppb after 14 h. Levels of permethrin in standing pools near the stream were 0.01 ± 0.01 ppb. Numbers of drifting aquatic invertebrates increased two-fold during treatment and four-fold 3 h after treatment and declined to background levels within 9 h. Trout fry, periphyton and benthic invertebrates were unaffected [53].

Two studies have been published on the amount of drift resulting from carbaryl applications to protect trees from bark beetle attack. In the early 1980s, [54] used spectrophotofluorometry to analyze ground deposition from the base of the ponderosa pine to 12 m from the bole in California. In a more recent study, [14] used high performance liquid chromatography (HPLC) to evaluate ground deposition occurring at four distances from the tree bole (7.6, 15.2, 22.9 and 38.1 m) during conventional spray applications for protecting individual lodgepole pine from mountain pine beetle attack, and Engelmann spruce from spruce beetle attack. Despite substantial differences in these methods (i.e., spectrophotofluorometry limits detection of finer particle sizes that are accounted for with HPLC), they yielded some similar results. For example, [14] reported application efficiencies of 80.9% to 87.2%, while [54] reported values of >80%. Furthermore, [14] found no significant difference in the amount of drift occurring between lodgepole pine and Engelmann spruce at any distance from the tree bole despite differences in application rate and pressure, while [54] reported drift was similar between two methods applied at 276 kPa and 2930 kPa. However, [14] reported higher levels of ground deposition further away from the tree bole, which is expected given use of HPLC, a more sensitive method of detection.

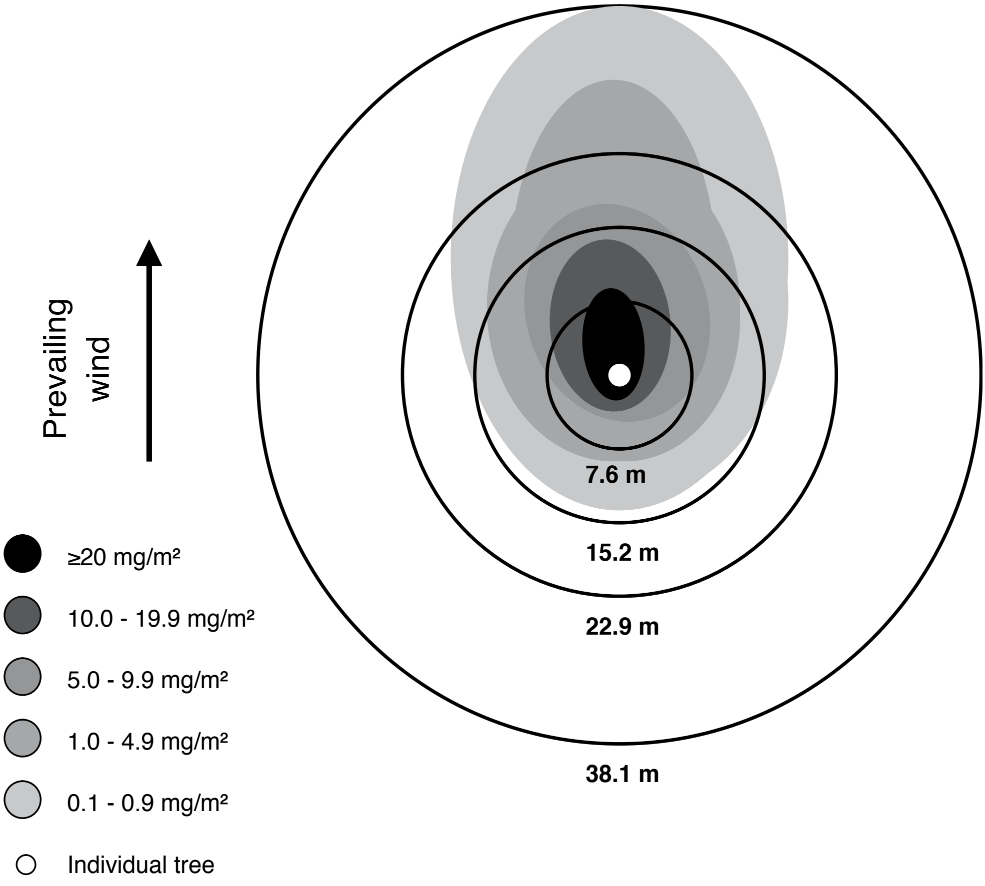

Figure 6. Average drift following experimental applications of carbaryl to protect trees from bark beetle attack, Uinta-Wasatch-Cache National Forest, Utah, U.S. Data obtained from Fettig et al. (2008). Wind speed was correlated with drift up to 22.9 m from the tree bole, and direction largely influenced the direction of prevailing drift. For example, while deposition is detected at 38.1 m on the leeward side of treated trees (maximum wind speeds averaged 3.5 km/h), drift is undetectable less than half that distance on the windward side. Less drift is expected in dense forest stands due to reduced wind speeds and interception by foliage. Studies show no-spray buffers will ensure that adjacent aquatic and terrestrial environments are protected from negative impacts.

Fettig et al. [14] reported mean deposition values from 0.04 ± 0.02 mg carbaryl/m^2 at 38.1 m to 13.30 ± 2.54 mg carbaryl/m^2 at 7.6 m. Overall, distance from the tree bole significantly affected the amount of deposition. Deposition was greatest 7.6 m from the tree bole and declined quickly thereafter. Approximately 97% of total spray deposition occurred within 15.2 m of the tree bole (Fig. 6). To evaluate the potential risk to aquatic environments, the authors converted mean deposition to mean concentration assuming a water depth of 0.3 m selected to represent the average size of lotic systems, primarily small mountain streams, adjacent to many recreational sites where bole sprays are often applied [14]. No adjustments were made for the degradation of carbaryl by hydrolysis, which is rapid in streams or for dilution by natural flow. Comparisons were made with published toxicology data available for select aquatic organisms. No-spray buffers of 7.6 m are sufficient to protect freshwater fish, amphibians, crustaceans, bivalves and most aquatic insects. In laboratory studies, carbaryl was found to be highly toxic to stoneflies (Plecoptera) and mayflies (Ephemeroptera), which are widely distributed and important food sources for freshwater fishes, but negative impacts in field populations are often short-lived and undetectable several hours after contamination [55]. No-spray buffers >22.9 m appear sufficient to protect the most sensitive aquatic insects such as stoneflies.

An advantage of tree injections is that they can be used on environmentally-sensitive sites as these treatments represent an essentially closed system and therefore little or no contamination occurs outside of the tree. However, following injection residues move within the tree and are frequently detected in the foliage [e.g., 44,56-57], which could pose a risk to decomposers and other soil fauna when needles senesce. This has been shown for imidacloprid in maple [57], but injections of emamectin benzoate in pines appear of little risk. For example, [56] reported emamectin benzoate was not detected in the roots or the surrounding soil, but was present at 0.011–0.025 μg/g in freshly fallen pine needles. However, levels gradually declined to below detectable thresholds after 2 months [56].

8. Conclusions

The results of the many studies presented in this chapter indicate that preventative applications of insecticides are a viable option for protecting individual trees from mortality due to bark beetle attack. Bole sprays of bifenthrin, carbaryl and permethrin are most commonly used. Several formulations are available and effective if properly applied. Residual activity varies with active ingredient, bark beetle species, tree species and associated climatic conditions, but generally one to three years of protection can be expected with a single application. Recent advances in methods and formulations for individual tree injection are promising, and further research and development is ongoing. We expect the use of tree injections to increase in the future. In general, preventative applications of insecticides pose little threat to adjacent environments, and few negative impacts have been observed. We hope that forest health professionals and other resource managers use this publication and other reports to make informed, judicious decisions concerning the appropriate use of preventative treatments to protect trees from mortality due to bark beetle attack. Additional technical assistance in the U.S. can be obtained from Forest Health Protection (USDA Forest Service) entomologists (www.fs.fed.us/foresthealth/), state forest entomologists, and county extension agents (www.csrees.usda.gov/Extension/). We encourage use of these resources before applying any insecticides to protect trees from bark beetle attack.

Acknowledgements

We thank Stanislav Trdan (University of Ljubljana) and Romana Vukelic and Danijela Duric (InTech) for their invitation to contribute and guidance during the publishing process. Numerous colleagues from Arborjet Inc., BASF, Bayer ES, Bureau of Land Management (U.S.), FMC Corp., Fruit Grower's Supply Co., Mauget Inc., Nevada Division of Forestry, Sierra Pacific Industries, Southern Ute Reservation, Syngenta Crop Protection, Texas A&M Forest Service, Univar USA Inc., University of Arizona, University of California, University of Georgia, and USDA Forest Service have contributed to the success of much of the research discussed in this chapter. We are thankful for their support and helpful insights.

This publication concerns pesticides. It does not contain recommendations for their use, nor does it imply that the uses discussed here have been registered. All uses of pesticides in the United States must be registered by appropriate State and/or Federal agencies. CAUTION: Pesticides can be injurious to humans, domestic animals, desirable plants, and fish or other wildlife—if they are not handled or applied properly. Follow recommended practices for the disposal of surplus pesticides and their containers.

Author details

Christopher J. Fettig[1], Donald M. Grosman[2] and A. Steven Munson[3]

*Address all correspondence to: cfettig@fs.fed.us

1 Pacific Southwest Research Station, USDA Forest Service, Davis, CA, USA

2 Forest Health, Texas A&M Forest Service, Lufkin, USA

3 Forest Health Protection, USDA Forest Service, Ogden, UT, USA

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The Use of Deltamethrin on Farm Animals

Papadopoulos Elias

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/54839

1. Introduction

Ectoparasites reduce significantly animal production and welfare. They cause nuisance, anaemia, irritation and transfer of pathogens of important diseases, often leading to animal death. Examples of diseases with high mortality transmitted by arthropods include viral diseases, such as the Bluetongue disease, or parasitic diseases, such as piroplasmosis and filariosis. Biting midges of the *Obsoletus* species complex of the ceratopogonid genus *Culicoides* were assumed to be the major vectors of bluetongue virus in northern and central Europe during the 2006 outbreak of bluetongue disease. Most recently, field specimens of the same group of species have also been shown to be infected with the newly emerged Schmallenberg virus in Europe, as other bloodsucking arthropods. Furthermore, ectoparasites may attack humans and threaten public health, such as diseases transmitted by mosquitoes or ticks.

The control of ectoparasites found on animals, i.e. midges, fleas, ticks, lice, flies, is largely based on the use of chemicals (insecticides). The main groups, which have been used as the basis of the common ectoparasiticides, include the synthetic pyrethroids, organochlorines, organophosphates, carbamates, formamidines and others. The macrocyclic lactones (avermectins and milbemycines) have also been shown to have a high activity against a range of ectoparasites. Furthermore, there are also compounds which affect the growth and development of insects, such as the chitin inhibitors, chitin synthesis inhibitors and juvenile hormone analogues. Insect growth regulators (i.e. lufenuron) are used mostly against fleas and certain flies.

2. Deltamethrin

Pyrethroids, synthetic analogues of pyrethrins, were developed to improve stability of the natural pyrethrins since they degraded rapidly by light. The pyrethrin insecticides were originally derived from extracts of the flower heads of *Chrysanthemum cinerariaefolium*. There are six compounds that comprise the natural pyrethrins, namely, pyrethrins I and II, cinerins I and II, and jasmolins I and II.

Pyrethroid insecticides are attractive compounds because of their high potency and ability to reduce disease transmission, selective toxicity, relative stability in the environment and ease of degradation in vertebrates. Compared with organophosphates and carbamate insecticides, pyrethroids are less likely to cause acute and chronic health effects to vertebrates. The common synthetic pyrethroids in use include deltamethrin, permethrin, cypermethrin, flumethrin and others. The main value of these compounds is their repellent effect and since they persist well on the coat or skin, but not in tissue, they are of particular value against parasites that feed on the skin surface such as ticks, lice, some mites and nuisance flies. They can act as contact insecticides due to their property to be lipophilic. Some have the ability to repel and to affect flight and balance without causing complete paralysis (knockdown effect). They pose a strong affinity for sebum. They are widely used in veterinary medicine for agricultural and domestic purposes.

Pyrethroids are primarily targeted on the nervous system. They act as neurotoxins upon sensory and motor nerves of the neuroendocrine and CNS of arthropods. Several mechanisms of action have been proposed, including alterations in sodium channel dynamics in nerve tissues, which polarise membranes and result in abnormal discharge in targeted neurons.

Synthetic pyrethroids are relatively safe. However, if toxicity occurs, it is expressed in the peripheral nervous system of animals as hypersensitivity and muscle tremors. They are extremely toxic to fish and aquatic invertebrates (except for molluscs and amphibians). However, it would appear that, in practice, the risks of deltamethrin are limited. Light, the pH of the water, organic or colloidal molecules in suspension, and the presence of sediment and bacteria, all contribute to a rapid breakdown of the molecule into rapidly decomposed non-toxic products. Deltamethrin does not present any toxicity problems for birds, including game birds. Regarding public health, some adverse effects on humans may occur, with neurotoxicity and developmental toxicity being potential side effects following acute high-dose exposures to pyrethroids.

Among synthetic pyrethroids, Deltamethrin (Butox, MSD) is of particular importance. Contrary to other pyrethroids, it is a single *cis*-isomer (Figure 1), which is considered to be more effective than isomer combinations. Deltamethrin repels ectoparasites by the ''hot foot effect'', which is typical for pyrethroids. An insect after it had a ''touchdown'' on such an animal, redraws its feet suddenly from treated hair. Even after a very short contact, for only a few seconds, to treated hair, a ''knock-down effect'' occurs since insects and ticks die soon after the open nerve ends at their feet got into contact with the insecticide. This efficacy leads to a constant reduction of biting or attacks and same time dead female population stops breeding. On the other hand, studies carried out in 3 generations of rats, using daily doses of 0.15, 1 and 3.75 mg/kg in the feed, did not reveal any differences between treated and control animals with respect to fertility, duration of gestation, fecundity and viability of the litters. Finally, Deltamethrin did not show any mutagenic effects in any of the tests (both *in vivo* and *in vitro*) employed.

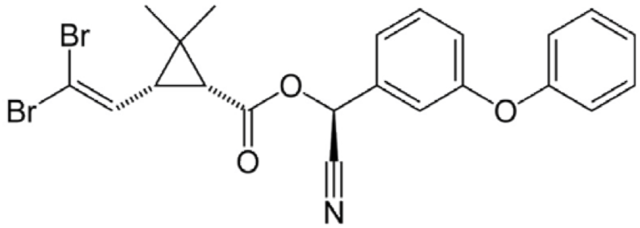

Figure 1. The structure of deltamethrin

3. Efficacy

Several published papers exist in scientific journals demonstrating the strong properties of Deltamethrin (Butox, MSD) to repel or kill arthropods infesting livestock, such as biting midges, nuisance flies, ticks, lice, certain mites etc. Results from these field trials proved the high efficacy of this compound to protect ruminants from midges, i.e. *Culicoides* spp., for periods over 4-5 weeks, even if the animals became wet several times. It has been found to be effective against ticks, including all developmental stages, mosquitoes and many others.

Herein follow, in more details, the results of some studies evaluating the potential use of Deltamethrin (Butox, MSD) on farm animals.

3.1. *Culicoides* midges

One set of such experiments include the investigation of the control of Bluetongue disease of ruminants using this drug, carried out by Schmahl and colleagues in 2008. Bluetongue disease is a viral disease, which harms considerably farm ruminants with high mortality rates in cattle and especially in sheep, while wild ruminants become infected, serve as virus reservoirs, but show only rarely severe symptoms of disease. From several transmission experiments and epidemiological studies in South Africa and in Southern Europe, it was known that the main vector belonged to the midges (Family Ceratopogonidae, genus *Culicoides*). Therefore, protection methods were needed to avoid transmission of the virus from one animal to the other. Thus, the aim of this study was to compare the efficacy of Deltamethrin when *Culicoides* specimens come into contact with hair of cattle and sheep that had been treated for 7, 14, 21, 28, or 35 days before. This study was needed, since it had to be clarified, whether the product in this formulation of Deltamethrin can reach the hair of feet in sufficient amounts when they are applied onto the hair along the back line. The product must arrive in sufficient amounts at the feet and along the belly since there are the predominant biting sites of the very tiny (only 0.8–3 mm long) specimens of *C. obsoletus, C. pulicaris* and *C. dewulfi*, the proven vectors of Bluetongue in Europe. Towards this end, one group of three young cattle of about 400 Kg bodyweight and one group of three young sheep of 60 Kg each were treated by application (pour on) of 30 ml and 10 ml, respectively, of the product Butox® 7.5 onto the skin along the backside of the animals. Butox® 7.5 contains 7.5 g deltamethrin per liter of the ready-to-use solution and is a registered trademark of MSD pharmaceutical company. Seven, 14, 21, 28, and 35 days after treatment, hair was clipped off from the feet of the cattle and sheep (just above the claws), collected in separate, suitable plastic bags, and transported to the institute, where it was mixed with freshly caught midges, which had been caught in the previous night with the aid of an ultraviolet light lamp. Each vial contained at least ten *Culicoides* specimens, besides other insects. The trapped insects were incubated with treated hair or with hair of an untreated animal (control). The exposure periods of the insects to hair lasted for 15, 30, 60, or 120 seconds—a period which was thought to be realistic compared to the field conditions. The insects were thereafter separated from the hair and placed on filter paper inside closed plastic petri dishes, where they were observed at regular intervals (5–10 min) using a stereo microscope to record reactions and the time of death after the first contact with treated hair.

The midges (*Culicoides* species) were apparently highly sensitive to Deltamethrin (Butox® 7.5) since they died even after rather short contacts to hair treated even 35 days ago, which is a very satisfying effect. There were no significant differences between the species of treated animals (sheep or cattle), although the distance from the place of application (back) until the feet is longer in cattle than in sheep. Thus, the formulation can reach in sufficient amounts the region of the predominant biting sites of the *Culicoides* species (feet, belly).

The results obtained from these experiments clearly show that Deltamethrin, when applied as a pour-on solution onto the back of the animals, has a significant killing effect on the *Culicoides* species, which are known vectors of the Bluetongue virus in Europe. Furthermore, even if the protection might not be 100%, any killed female *Culicoides* prevents its possible progeny and hinders the transmission of agents of diseases.

During the above described studies, the animals were provided with adequate shelter against the rain. The efficacy of deltamethrin on wet animals was, therefore, not tested. It is common that ruminants stay under the rain or exposed to water when they stay at pasture for grazing. Therefore, the same group of researchers in 2009, carried out the next step of the above study, i.e. the new task was to determine if thoroughly wetting the test subjects, twice a week, would affect the efficacy of Deltamethrin. Cattle and sheep were treated with Butox 7.5 along the neck or dorsal midline, as described earlier. Test animals were wet thoroughly with tap water twice weekly. Control animals remained dry. Hair was clipped off the legs, near the claws, at day7, 14, 21, and 28 after treatment of test and control animals. Recently caught *C. obsoletus* midges were then exposed to the hair for 15, 30, 60, and 120 seconds. The midges were then transferred to filter paper in plastic petri dishes and observed. The time needed for the midges to die after the exposure was recorded.

In both cattle and sheep, the product remained active for at least up to 4 weeks (28 days - end of the experiment), even in the animals wet with water twice weekly over the 4-week period. In sheep, the time between exposure and death of the midges was definitely lengthened in animals that were wet. In cattle, the results were different in that in some cases, time between exposure and death of the midges was shorter in wet animals than in dry animals, while in other cases the results were similar. Compared with the sheep, the time between exposure and death is generally quicker, probably, due to differences in hair structure. All midges exposed to hair from treated sheep or cattle, wet or dry, died even after only 15 seconds of exposure to the hair. In cases where the period between exposure and death of the midges was very long, it is likely that the midges were unable to bite as they showed signs of paralysis immediately after contact with treated hair. The fact that Butox 7.5 pour on remains effective in animals regularly exposed to rain is an important finding towards the protection of ruminants from the attack of midges and the risk of disease transmission.

3.2. Ticks

Another study of Mehlhorn and others took place recently in 2011 in order to investigate the efficacy of Deltamethrin (Butox® 7.5 pour on) against specimens of two important species (*Ixodes ricinus* and *Rhipicephalus sanguineus*). Ticks can transmit a broad spectrum of agents of diseases in cattle or sheep and the use of an effective long lasting acaricide is needed to protect livestock. Four sheep and four young cattle were treated along the vertebral column with 10ml Butox® (deltamethrin) per sheep or 30ml Butox® per cattle. Day 7, 14, 21, and 28 after the treatment, hair was shaved off from the head, ears, the back, belly, and the feet being collected in separate, suitable plastic bags, and transported to the institute, where these hair were brought into close contact with either adult and/or nymph stages of *I. ricinus* and *R. sanguineus*. As results, strong, acaricidal effects were seen, which varied according to the parasite species, the origin of the hair (e.g., head, leg, etc.) and according to the period after the treatment.

In sheep, the acaricidal effect was noted for the whole period of 28days along the whole body with respect to adults and nymphs of *I. ricinus*, while the acaricidal effects of Deltamethrin were reduced for *R. sanguineus* stages beginning at day 21 after treatment. In cattle, the full acaricidal effect was seen for 21 days in *I. ricinus* stages and for 14 days in *R. sanguineus*, while the acaricidal efficacy became reduced after these periods of full action—beginning at the hair taken from the legs. Only *R. sanguineus* adults did not show any reaction on day 28 after treatment. Besides these acaricidal effects, repellent effects were also noted. Full repellency for both species was seen during the first 14 days in sheep and cattle against *Ixodes* and *Rhipicephalus*, while the repellency was later reduced, especially in contact with hair from the legs. As conclusion, Deltamethrin, besides its very good effects against biting insects, brings acaricidal as well as repellent effects against ticks, thus protecting the sheep and cattle from transmission of agents of diseases.

3.3. Nuisance flies

Other researchers carried out experiments testing the efficacy of Deltamethrin against nuisance flies of ruminants. More precisely, Franc and Cadierques in 1994, applied the pour on formulation of 0.75 % Deltamethrin to cattle on 6 farms in southwest France (3 treated, 3 control). Ten ml per 100 Kg body weight were applied to the backs of 77 cattle (with a max. of 30 ml) and adequate control of hornfles (*Haematobia irritans* and *Hippobosca equina* (with a 95% or better) was achieved during 10 weeks. The protection against non biting flies *Musca autumnalis* was better than 75 % during 6 weeks and better than 50 % during the next 14 weeks in 2 farms. In the other farm the number of *Musca autumnalis* fell by 75.5% only during the first week.

3.4. Mange

Finally, in the international literature, in 2001, exists a publication of Khalaf-Allah and El-Bablly, who evaluated the effect of Deltamethrin for control of sarcoptic mange in naturally infested cattle. The infested calves (28 calves with 8-16 months of age) were randomly allocated into two groups each consisting of 14 animals. The first group was sprayed with Deltamethrin at the concentration recommended by the manufacturer using a motor sprayer, while the second group was left untreated and served as control. Besides this, 14 healthy calves at the same farm were used to compare between them and the infested calves for the haematological and biochemical parameters. Before application of the acaricide, mange lesions were carefully scraped so as to remove the scales and crusts under which sarcoptic mites are hidden. Skin scrapings were taken from the affected lesions and mites were identified at day (0) and at weekly intervals post-treatment.

The results revealed that Deltamethrin provided a high level of sarcoptic mange control which lasted up to 42 days post-treatment. The mean haematological values of RBCs, Hb, PCV were significantly lower in mange infested calves than that of control, whereas the mean WBCs was significantly higher in infested animals. As well, the mean biochemical parameters estimated in mange infested animals were significantly lower than that of controls. The mean values of the haematological and biochemical parameters in infested animals were restored and nearly returned to its normal levels one month post-treatment.

3.5. Fleas

Herein follow our results from a pilot study which was carried out in order to evaluate under field practise the effect of Deltamethrin (Butox®, MSD) against fleas infesting small ruminants in Greece.

Fleas pose a significant problem in dairy sheep and goat farms of the country, since they attack not only animals but farmers as well (Figure 2). There are several papers in the international literature, regarding flea infestation of livestock in many countries around the Mediterranean basin and elsewhere (Ethiopia, Greece, Israel, Libya, Morocco etc). In all the cases of severe infestation, fleas cause, additionally to nuisance, high mortality, morbidity and disease transfer.

Control is difficult, because fleas spend much time off the host. Furthermore, insecticide residues in milk, when treatment is applied during milk production, are a restraining factor. The great advantage of deltamethrin (Butox®, MSD) is the very short withdrawal period in milk (12 hours) making treatment against ectoparasites practically possible at any time of animal production. Very limited information, to our knowledge, exists in the scientific literature, regarding flea control using insecticides on livestock.

Twenty (15 goat and 5 sheep) farms were identified and Deltamethrin (Butox®, MSD) was applied to all animals at the recommended dose rate. Herds/flocks consisted of 100-200 head of local dairy breeds. Information was collected regarding the management system of the farms, particularly on manure handling (Figure 3).

Figure 2. Reaction on human leg after flea feeding

Figure 3. A typical farm environment favouring flea reproduction

Animals within each farm were randomly inspected and fleas, if present, were counted every week for a minimum period of one month (Figure 4). Controls, untreated animals, were not used (accepted by the WAAVP guidelines) for both ethical reasons and because the aim of the study was to eliminate fleas from the farm premises. Practically no fleas were found during the post-treatment period. In more details, the mean (±sd) number of fleas before and after the Deltamethrin treatment were 104.5 (±12.6) and 3.6 (±2.3 fleas), respectively. The overall success of flea control was >96.6%. The main flea species identified was *Ctenocephalides felis*, which is known to be very common and widespread. These results offer a sustainable approach to flea control in Greece due to the long protection period and if combined with hygienic treatment of the farm premises, may contribute significantly to flea control.

Figure 4. The presence of fleas was inspected using a comb

4. Concluding remarks

In conclusion, Deltamethrin (Butox®, MSD) can be successfully used for farm animal protection in control programmes against many arthropods with important vector-borne or nuisance capacity, including midges, ticks, flies and fleas. Effective control of ectoparasites is of major significance, not only for increased animal production and welfare, but for the public health protection as well.

Author details

Papadopoulos Elias

Laboratory of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, Greece

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Section 7

Biotechnology and Other Advances in Pest Control

Use of Biotechnology in the Control of Insects-Prague

Gleberson Guillen Piccinin, Alan Augusto Donel,
Alessandro de Lucca e Braccini,
Lilian Gomes de Morais Dan, Keila Regina Hossa,
Gabriel Loli Bazo and Fernanda Brunetta Godinho

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53302

1. Introduction

Productivity gains in agriculture are satisfactory with the use of genetically modified plants and the dependency of application of insecticides on crops becomes smaller over the years. The consequences of the development and marketing of corn genetically modified (GM) have been profound, and in 2011 the area planted in the United States of America (USA) with at least one GM trait corresponded to more than 88% of was over acreage.

In addition, the efficiency gains in the production chains were only possible thanks to the entrepreneurship and management of rural producers, who adopted the most modern technologies available for science. Among these, stand: the tillage, fertilization and soil correction, the techniques of integrated management of invasive plants, diseases and insect pest and the growing adoption of improved seeds with high productive capacity. It is observed that the simple hybrids corn came to dominate the market of seeds embedded in technologies and seeds are more easily adopted by producers. This is the case of transgenic seeds, which in the culture of corn were widely adopted, including the major world producers of this cereal USA and Argentina.

In crops of corn, the losses caused by pests are limiting factor to achieve high productivity. The fall armyworm (*Spodopterafrugiperda*) is the main plague of corn in Brazil culture, causing severe damage. The attack on the plant occurs since its emergence to the booting and the silking.However, the critical period is flourishing. Losses due to Caterpillar attack may reduce production in up to 34%. Survey conducted among some 1,100 farmers who produce more than 8,000 kg ha-1 showed that, among the crops sampled, 15% received from 4 to 5 applications of insecticides and 6% received 6 to 8 applications for pest control.

Other recent surveys, conducted by Embrapa maize and sorghum, have shown that in some regions the number of applications of insecticides for the control of Caterpillar-cartridge can reach 10. In addition, there is no efficient method of chemical control for at least two other important species: or the control of the corn earworm (*Helicoverpazea*) and the Maize stalk borer (*Diatraeasaccharalis*).

The insects have been one of the biggest causes of damage in food production being these losses of the order of 20 to 30% of world production in [1]. It is estimated that approximately 67,000 species of insects cause damage to plantations and tropical regions, usually the poorest in the world, those who suffer most from the high incidence of insects-Prague in [2].

The attack of any pest depends on the development of culture, as well as the intensity of the attack, which can significantly affect the performance of the same. Chemical control is the primary measure used to prevent the immediate damage that reach the level of economic damage. Many times, the insecticides do not have the desired effectiveness and have a high cost, because are usually required multiple applications.

The insecticides used in pest control as an example in corn culture are often of low selectivity, therefore can affect the population of natural enemies, favoring the proliferation of pests and even resurgence of others. Due to these factors, the search for alternatives that can minimize or even replace the conventional insecticides was intensified and, currently, the new tactics comprise a series of alternatives: resistant plants, selective insecticides, parasitoids and entomopathogenic microorganisms. Among the entomopathogenic *Bacillus thuringiensis* (Bt), notable for its wide use in the control of pest insects of the order Lepidoptera, especially in corn culture.

Currently, transgenic production is widespread in almost all agricultural regions of the planet and with the adoption of biotechnology by greater productivity with the producers reaches lower use of insecticides. An example of this is the use of gene technology (Cry) of bacteria (Bt) in control of the main pests of maize culture. The Bt gene technology diffusion aims to make the environment more sustainable, decreasing the concentration of inert products in foods using insecticides rationally. However, many challenges must still be overcome, with that biotechnology has as a fundamental role, seek new research, sustainable in modern agriculture.

2. Importance of biotechnology in the control of insects-prague

Biotechnology is defined as a set of techniques for manipulation of living beings or part thereof for economic purposes. This broad concept includes techniques that are used on a large scale in agriculture since the early 20th century, such as tissue culture, the biological fixation of nitrogen and organic pest control. But the concept includes also modern techniques of direct modification of the DNA of a plant or a living organism, in order to change precisely the characteristics of that organism in [3].

Agricultural biotechnology varieties are used as a tool for agricultural research characterized by gene transfer of agronomic interest (and, consequently, of desired characteristics) between a donor agency (which may be a plant, a bacterium, fungus, etc.) and plants, safely.

Studies dating this year in India and China show that the Bt cotton production increased 10% to 50%, respectively, and the use of insecticides has reduced in both countries by 50%. In India, the producers have increased the income up to $ 250 or more per hectare, the farmer's income increasing national $ 840 million to $ 1.7 billion last year. Chinese farmers saw similar gains with increasing yields on average $ 220 per hectare, or more than $ 800 million nationwide. It is important to emphasize the trust farmer in technology, with 9 of 10 Indian farmers reseeding the biotech cotton 100% year after year and Chinese farmers using the technology.

It is important to recognize the need for scientific research that point on the General mode of action of Bt toxins and also that their toxicity is influenced by several factors. However, it is known that, in General, the toxicity of the Bt toxin on target organisms depends on factors such as certain pH, proteases, and the receivers in [4]. On the other hand, and more specifically, the extrinsic factors can also influence and co-factors in specific efficacy of Bt toxin on target organisms resistant and/or can also have an impact on the selectivity and toxicity to non-target organisms in [5].

Therefore, the emergence of modern biotechnology marks the beginning of a new stage for agriculture and reserves a starring role to molecular genetics. The advances in the field of plant genetics have the effect of reducing the excessive reliance on agriculture mechanical and chemical innovations, which were the pillars of the green revolution. In addition to increased productivity, modern biotechnology can contribute to the reduction of production costs, better quality foods and for the development of less aggressive to the environment in [3].

3. The *Bacillus thuringiensis* (Bt)

The *Bacillus thuringiensis*, was discovered in 1901, by the occurrence of an epidemic of mortality of larvae of the silkworm in Japan. Researchers found that it was caused by a previously unknown bacterium.

The bacterium entomopathogenic (Bt) stands out on the world stage since 1938, when the first product formulated with this pathogen was released in France.

In 1911, in Germany, the Berliner managed to isolate and characterize this bacterium, baptizing it *Bacillus* (by its cylindrical shape*) thuringiensis* (named after the German region of Turíngea). In 1938, France formulations containing right-handed bacteria colonies were sold as insecticides and, in 1954, its mode of action was discovered and its use today.

Since then more than 100 products were launched on the market and currently constitute more than 90% of gross revenues with biopesticides in [6, 7].

In some studies, this bacterium was considered inefficient in controlling *S. frugiperda*in [8, 9]. However, with the advances provided by new laboratory techniques and greater interest of researchers' positive results were obtained in [10]

The Bt a soil bacteria present in various continents, Gram-positive, aerobic and family Bacillaceae, when environmental conditions become adverse can sporulate to survive these conditions in [11]. Are found in every terrestrial environments and also in dead insects, plants and debris in [12, 13, 14, 15, 16, 17, 18, 19]. The methods to isolate this pathogen are powerful and usually easy to perform in [20, 21, 22, 23]. The number of cells obtained from Bt varied between 102 and 104 colony-forming units (CFU) per gram of soil, while in plants this number varies between 0 and ufc 100 cm^{-2} in [24].

Produces sporangia containing a endospore and crystalline inclusions of proteins that are responsible for their action entomopathogenic, among which stands out the protein CRY. This crystal is composed of a protein polypeptide called endotoxin in [25]. When larval forms of insects feed on such proteins, initiates a series of reactions that culminate with the death of the same.

4. Biotechnology vs insecticide

The insects have been one of the major causes of damage to food production in [1] and, in world terms, the losses caused by pests and diseases are quite high. The same causing losses of the order of 38% in [26]. Withdrawals in Brazil indicate that pests can be liable for loss of 2.2 billion dollars for the main Brazilian crops in [27].

Control of harmful insects is done, most of the time, by agrochemicals and, on a much smaller scale, by the employment of biological insecticides. The indiscriminate use of pesticides in combating the causal agents causes, despite its efficiency, environmental problems severe, human health, reduces number of natural enemies, and provides an accelerated selection of resistant insects in [28]. In contrast, biopesticides, Bt based, used for over a century, retainers of features less impactful on the environment and less harmful to humans ever occupied a prominent place on the market for the sale of pesticides in [29].

From the Decade of 80, because of genetic advance, it became possible to develop a new pest control strategy, which consists of the genetically modified plants resistant to insects in [and with effectiveness similar to conventional insecticides in [31, 32].

The first experiments with genetically modified (GM) plants were made in 1986, in the United States and in France. The first variety marketed a vegetable species produced by genetic engineering was the "FlavrSavr Tomato ", developed by the American company Calgene and marketed from 1994.

Between 1987 and 2000 there were more than 11,000 field trials in 45 countries and cultures more frequently tested were corn, tomatoes, soybeans, canola, potatoes and cotton, and the genetic features introduced were herbicide tolerance, product quality, virus-resistance and resistance to insects in [33].

Rank	**Country**	**Area (million hectares)**	**Biotech Crops**
1	USA*	69.0	Maize, soybean, cotton, canola, sugarbeet, alfafa, papaya, squash
2	Brazil*	30.3	Soybean, cotton, maize
3	Argentina*	23.7	Soybean, cotton, maize
4	India*	10.6	Cotton
5	Canada*	10.4	Canola, maize, soybean, sugarbeet
6	China*	3.9	Cottton, papaya, poplar, tomato, sweet paper
7	Paraguay*	2.8	Soybean
8	Pakistan*	2.6	Cotton
9	South América*	2.3	Maize, soybean, cotton
10	Uruguay*	1.3	Soybean, maize
11	Bolivia*	0.9	Soybean
12	Australia*	0.7	Canola, cotton
13	Philippines*	0.6	Maize
14	Myanmar*	0.3	Cotton
15	Burkina Faso*	0.3	Cotton
16	Mexico*	0.2	Cotton, soybean
17	Spain*	0.1	Maize
18	Colombia	<0.1	Cotton
19	Chile	<0.1	Maize, soybean, canola
20	Honduras	<0.1	Maize
21	Portugal	<0.1	Maize
22	Czech Republic	<0.1	Maize
23	Poland	<0.1	Maize
24	Egypt	<0.1	Maize
25	Slovakia	<0.1	Maize
26	Romania	<0.1	Maize
27	Sweden	<0.1	Potato
28	Costa Rica	<0.1	Cotton, soybean
29	Germany	<0.1	Potato
Total		**160.0**	

* 17 biotech mega-countries growing 50,000 hectares, or more, of biotech crops.

** Rounded off to the nearest hundred thousand

Source: in [34].

Table 1. Global Area of Biotech Crops in 2011: by Country (Million Crops)**.

These days, according to the annual report of 2011 on the use of transgenic crops, the non-profit organization International Service for the acquisition of AgriBiotech Applications (ISAAA) observed an increase of 94 times in planted area of 1.7 million hectares in 1996 to 160 million hectares in 2011 (Table 1), allowing biotech crops become more agricultural technology adopted in the history of modern agriculture.

The endless search for alternative methods of insect control-Prague has been held strongly by several research groups worldwide, due to the need of a more sustainable agriculture and more committed to environmental preservation in [35].

In this way, farmers have adopted this technology Bt targeting an increasing effective production to sustainable agriculture in [36]. The benefits of this technology are: reduction of environmental effects on toxins, safety in use, efficiency, conservation of natural enemies and reduction of fungal diseases.

The first advantage is the production of protein *Cry*, by plants-Bt, which is not affected by environmental factors such as atmospheric fallout, light incidence, and high temperatures in [37]. In addition, the homogeneity of the protein, in plant tissues, allows a more efficient use of insecticide effect than the application (spraying) of biopesticides, Bt, based on plants. The second advantage is the possibility of a higher level of security in relation to insecticide formulated because the proteins and does not accumulate in fatty tissues, are not toxic to humans and pets. Tied to these characteristics, the protein Cry, has no activity by contact, being necessary, the ingestion of the toxin by the insect, to have the effect of insecticide. The third advantage is the *Heliothisvirescens*control significant and *Pectinophoragossypiella*, for example, in Bt cotton culture, between 95 and 99% efficiency in [38]. The fourth advantage is the preservation of natural enemies, therefore, secondary pests can become a problem if the population of beneficial insects is reduced by the use of chemical insecticides of low selectivity. The fifth benefit, no less important, is the reduction of fungal diseases. The lesions caused by insects, in the organs of plants, fungi infection, create opportunities mainly in the genus *Fusarium e Aspergillus*in [39]. The primary importance of these fungi is the presence of micotoxins, particularly fumosinsandaflatoxins produced by them. The fumosins can be fatal to horses and pigs in [40]. And aflatoxinis extremely toxic to animals and humans in [39]. The dramatic reduction of insect attack, leads to reduction of insect attack, and consequently, decreases the production of micotoxins.

5. Mode of action of protein Cry

Currently the insect resistant transgenic plants expressing genes, inductors, an insecticidal protein called Cry, derived from the bacterium *Bacillus thuringiensis*(Bt). The mechanisms by which proteins exert their effect are *Cry* elucidated by pore formation model discussed below:

The mode of action of Cry proteins, produced by the plant, it is accomplished, orally, by susceptible insect. The process begins by solubilization of crystals in alkaline pH around 9.5, in the gut of insects, releasing protoxin of 130 kDa to Cry1 and Cry2 to 79kDa.. After this breakdown, the protoxinare activated by digestive enzymes, forming toxic fragments of 60-65 kDa. These monomers bind to receptors specific primary, located in the apical membrane of the microvillus membranes of the columnar cells of the intestine of the larva. It is in this step that the affinity between the toxin and the receiver, for example, Cry1ae protein, lepdopteros is recognized as an important factor in determining the spectrum insecticidal Cry proteins. Later, the monomers bind to secondary receivers, which are proteins ancoradorasglicosil-phosphatidyl-inisitol (API), as phosphates and alkaline, to the lepidoptero*Heliothisvirences*. After this binding, the now oligômerose inserts into the membrane, where there are receptors for API, and leads to the formation of pores in the cell membrane of the intestinal epithelium and therefore destruction of microvilli membranes, hypertrophy of epithelial cells, vacuolization of cytoplasm, cell lyses and intestinal paralysis/death of the insect in [11, 41].

6. The safety of the use of Btplants

In relation to the safety of the use of Bt corn plants as an example, several tests are conducted to certify the safety of its use in the environment and in food and feed. Initially, the protein *Cry* is tested in animal models, such as rats and mice, for the verification of the toxic potential. One of these tests, called acute toxicity consists in forced ingestion of a pure animal protein by solution and on the observation of effects of this. The product only goes to the next steps of assessment if no effect is observed and diagnosed.

We can cite as an example the test performed with Safety, this protein in maize Herculex®. This protein was tested on mice to the level of 576 mg per kilogram of live weight and no side effect was observed. To be exhibited at similar level, a person weighing 70 kg would take almost 5 tons of raw corn grains. This without taking into consideration the aspect that the human digestive tract, not to have alkaline pH, would not be able to downgrade this protein crystal.

Other reviews include the potential to cause allergies as well as the corn grain consumption by other animals such as chicken and fish, and what is called substantial equivalence, which is comparing the nutritional profile of the genetically modified maize with conventional maize. The corn will only be released commercially and, therefore, will go to the market when, in these analyses, the nutritional content between the conventional and transgenic corn were exactly the same, except, of course, the presence of protein inserted.

In the analysis of environmental safety, non-target organisms, how insects from another order, class or species, natural enemies and beneficial insects like bees, for example, are exposed to proteins inserted or the pollen grains that express and are evaluated its effects.

If all tests present results within expected ranges and be proven that there is no risk of harm or damage to health and the environment, these damages are compiled and submitted to the competent authorities of the country where you intend to market the product for analysis and approval of use and consumption. In Brazil these analyses are made by the national technical Commission on Biosafety (CTNBio) and the approval of a product in one country does not guarantee that the same is approved in another. For example, the event MON810 (Yield Gard®) was approved in 1996 in the United States, in 1998 in Argentina and only in 2007 in Brazil.

For the use of Bt corn, just the producer, in addition to using the seeds of biotech corn, fulfill two rules: the coexistence, required by law, and the rule of Insect Resistance management (MRI), recommended by (CTNBio).

The coexistence rule requires the use of a 100 m isolating surround of transgenic maize plantations of corn to retain without transgenic contamination. Alternatively, you can use a surround of 20 m, provided they are sown maize transgenic not 10 ranks (equal-sized and transgenic maize cycle) isolating the area of transgenic maize.

The CTNBiorecommendation for Insect Resistance management is the use of the area of refuge. This recommendation is the result of consensus that the cultivation of Bt corn in large areas will result in the selection of biotypes of target pests resistant to Bt toxins. Obviously, the monitoring of the infestation of plants is also important because, depending on the used hybrid and intensity of infestation, the producer may need to adopt additional control measures.

The biggest concern with the use of Bt corn is on transgenic crops and coexistence of transgenic crops do not. Coexistence is the set of agricultural practices allowing farmers grain production from conventional transgenic and organic crops, according to standards of purity and to meet legal requirements for labeling. The adoption of the rules of coexistence is essential to preserve the freedom of choice of producers and consumers. Coexistence is also a topic particularly relevant when there is market incentive for the provision of non-transgenic maize. Evidence of their practical viability is the coexistence of a considerable number of different varieties of open pollination still in use.

Showed that companies in possession of this technology must guide growers on the rules of coexistence. The producer also held technical information, stick to them properly and conscious.

Information on packages of seed of Bt corn, there is a contract in which the producer, to open it, assumes the responsibility of following the rules of coexistence and the resistance management. Therefore, it is incumbent upon the producer responsibility of use of these rules. It is important to remember that the incorrect use of technology can take it to ineffectiveness in little time. If the producer is interested in paying more for Bt corn seed, is because he believes in the benefits that this technology is bringing to your production system. Therefore, it must be motivated to use this technology in a responsible way (using the area of refuge), to take ownership of this benefit for much longer.

In relation to Bt cotton to China is the leader in this technology. In 2006 6.3 million farmers, or more than 60% of the number of farmers who have sown transgenic in the world in [42].

China is one of the only exceptions in the world to require shelters, although this may be changing. Even though the refuges were a way to reduce the accumulation of resistance to Bt toxin, the large number of small properties makes this strategy is difficult to apply.

The use of refuge in a developing country such as China becomes a challenging activity. Studies on policies of refuge on a large scale, in extensive agricultural systems of the United States of America, show that monitoring and implementation costs are negligible. Although this practice is reasonable in extensive production systems and with a small number of farms, they may not be suitable in developing countries. In developing countries like China, the agriculture sector is fragmented into millions of smallholdings, where each family has a diverse set of cultures in [43].

As a result, it is likely that the implementation of the strategy of refuge to the style (IE, all farmers planting Bt cotton are forced to grow cotton non-Bt with refuge) would require a large implementation effort, making these types of strategies of refuge becomes unviable unless farmers received individual incentives to implement refuges based on self-interest. This is unlikely, since the build-up of resistance to Bt technology is a collective evil (compared to the more common public as well) that is unlikely to be accounted for by individuals in [43].

The area of refuge is the sowing of 10% of the area planted with Bt corn hybrids using Btnot equal size and cycle, preferably their isogenic hosts. The area of refuge should not be more than 800 m away from the transgenic plants. This is the average distance by dispersion of adults of LCM in the field in [44].

All these recommendations are in order to synchronize the intersections of potential adults surviving in area of Bt corn with which emergency in the area of refuge. The structured refuge must be drawn according to the acreage with the Bt corn to plots dimensions above 800 m in the shortest side (or Ray), cultured with Bt corn refuge will be needed in their tracks internal plots. Yet, according to the recommendation, in the area of refuge CTNBiois allowed the use of other methods of control, provided that they are not used Btbased bioinsecticide.

7. Future trends for the Bt technology

The worst drought in more than half a century in corn-producing region of the United States should reduce the crop in that country at the lowest level in five years, where their stocks will be reduced to the lowest level in 17 years. The initial productivity is bad in the few fields harvested in areas of the Midwest, which represents 75 percent of the area with corn and soybeans in the United States. With this the world, returned his eyes to the Brazilian corn crop this year had one of the largest capacities of the whole story.

Brazilian agriculture won in the early 1980, an important milestone and helped the country to assume the rank among the major food producers in the world. Called when the off-season summer pós-safra was used by the producers for the planting of corn seeds uncultivated, and subsequently became part of the farmer's strategy to increase your productivity.

In more than 30 years of history, the off-season if expanded, gained strength and hit record. According to the 9th Brazilian harvest survey of grain (2011/2012), released by the national supply company (Conab) in June, the area planted with corn in the off-season is estimated at 7.188 million hectares, number 22% higher than last year's off-season.

According to Conab, the number is explained by good price prospects for climate advantage provided with the anticipation of rains for planting, and by the good harvest of soybeans, which encouraged producers to extend their crops.

Most producers that have soy as flagship summer crop production, bet on corn cultivation off-season, with attractive price and the advent of biotechnology has been the increase of productivity and safety in pest management and, in addition, there were the intangible gain with the decrease of insecticide applied in the environment.

The good news for the cultivation of corn in Brazil, according to Conab, are linked to the main corn producing States off-season: MatoGrosso, Paraná and MatoGrosso do Sul, which added to the total cultivated in the past year, the areas of 732.7 thousand, 283.4 billion and 193.2 thousand hectares, respectively.

With the data of the survey, the company foresees a production of about 32.9 million tones for corn second crop, or 53.1% to 21.5 million achieved last season. The Brazilian farmer realizes, each year, the off-season is a good deal, that is, it is an opportunity to increase the profitability of farming, maximizing the use of resources already invested.

Logically that this increased production, requires a quick response companies to address the needs of new hybrids of corn, which led to the increase of releases in this area, where the market turned to the specific needs of each region. With this, the producers have the opportunity to plant the best genetic, associated with the best biotechnology.

According to the Brazilian Association of producers of corn (Abramilho), the winter harvest has been growing a lot for two reasons: first, because soy has open space for the cultivation of this crop and, second, because of the technology. The conventional corn planting and with few seeds has become the past, and today, the use of increasingly technology for these cycles has achieved nearly the same results of the summer harvest.

Between farmers, the assessment is that the off-season will consolidate its position as an important complement in income and must, year after year, to expand to areas that do not yet have this established planting. There is a very strong demand for producer hybrids with more technology. Therefore, in recent years, companies have expanded investment in research in Brazil to bring to market the best product for the features of each region.

With the events of biotechnology of the culture of corn producers expect to achieve greater productivity per area, plants tolerant to various events and reduce production costs, primarily related to less use of pesticides.

In the case of Bt crops (which produce a toxin in their cells), their adoption allowed a reduction of 56 million kg of insecticides between 1996 and 2011. In General, the transgenic seed calculation led to decrease of 183 million tonnes in the use of pesticides. In Brazil, the Bt seed companies are also newly tolerant to herbicides, thereby opening a new path of efficiency in the management of pests and weeds, factors that interfere with productivity and steal the producer's profit.

Due to various factors the area planted with genetically modified seeds should reach 36, 6milhões hectares in the next harvest, second 1° monitoring of adoption of agricultural crop 2012/13. The forecast points to a 12.3% higher adoption in comparison to the previous year and means 4 million new acres with transgenic varieties.

The leadership in adopting biotechnology continues with soybeans, which must have 88.1% of crops with genetically Modified seeds, an area estimated at 23.9 million hectares. And corn, which begins to cultivate the fourth crop with transgenic hybrids, already approaching that level. The winter crop represents the second highest rate of adoption, with 87.8%, or 6.9 million hectares of transgenic seeds. In the case of the summer harvest, the adoption must represent 62.6% of the total area or 5, 2 million hectares.

The cotton must have 50.1%, or 546 thousand hectares of the total area with transgenic seeds. The continuous growth of adoption of biotechnology should be attributed to the increase of new varieties available in the market and that, today, are adapted to the different agricultural areas of the country. The direct and indirect benefits arising from the use of these seeds have been singled out by farmers as one of the biggest reasons for choice. In relation to States, MatoGrosso follows in the lead, with 9, 6milhões hectares, followed by Paraná with 6.6 million hectares. Herbicide tolerance technology follows in the lead with 25, 3milhões hectares, followed by seeds with resistance to insects, with 5.7 million hectares, and the gene technology, combined with 5.6 million hectares.

All these good news coming from the field are a major impasse regarding the prices of maize, companies that buy corn in Brazil must face an even more difficult year in terms of price in 2013, compared with 2012, and projected smaller cereal availability in 2012/13.

In the first half of 2012 prices were behaved and there was even falling prices to the extent that it was becoming clear that we would have a great off-season.

Cereal prices in the international market began to rise and reached record levels in recent weeks on the Chicago Stock Exchange in function from the perspective of large us crop failure caused by the worst drought in more than 50 years. Prices in Brazil are now strongly tied to international prices due to a large demand from international buyers. Certainly we have a Brazilian corn buyer pressure by international customers, as strong or stronger than this year. Because the major supplier of the world's corn, which are the United States, will have less exportable surplus in history.

Corn futures in Chicago (CBOT) reached the highest value of all time before disclosure of the report of the United States Department of agriculture (USDA), which should cut forecasts for USA crops this year.

The contract of the new crop, basis December reached the peak of 8.2975 dollars a bushel, the highest ever recorded in the Chicago Stock Exchange and above the previous record of 8.2875 dollars per busheltested three weeks ago by September.

As we saw in this chapter to biotechnology is a very important tool for the development of Brazil as world agricultural power, but we must emphasize the importance of research. And to get an idea of its importance, the increase in productivity in the various cultures saved 60 million hectares to Brazil, but still in some cultures our average productivity is low. In corn, for example, our productivity is half of U.S.A productivity. Search is a technology and factor income generator to the field, but Brazil has employed little recourse in the area. In 2011 employed 1.3% of GDP in science and technology, and in 2012 must employ only 0.9%, which is a setback and lack of objectivity.

8. Perspectives and final considerations

The biggest challenge of this century is to feed the whole world's population; poverty and hunger are inextricably linked and are about 1 billion people, mainly rentals. So what we will do to overcome this in a sustainable way? What are the threats to the production, distribution and safety of these foods? And, mainly, what we will do to allow people to have access to them?

In 2011, approximately half of the world's poor were small resource-poor farmers, whilst another 20% were the rural landless who are completely dependent on agriculture for their livelihoods. Thus, 70% of the world's poor are dependent on agriculture – some view this as a problem, however it should be viewed as an opportunity, given the enormous potential of both conventional and the new biotechnology applications to make a significant contribution to the alleviation of poverty and hunger and to doubling food, feed and fiber production by 2050.

In the next fifty years will be nine billion people and the world will consume twice as much food as the world has consumed since the beginning of agriculture 10.000 years ago. The challenge of feeding the world and interest to increase the potential of biotechnology are intimacies trailers and, as biotech crops already occupy about 160 million hectares or 10% of the world's arable land, is significant and visible the ancestry of this market in today's society.

According to some international institutes, the world until 2020 will grow 20% in food production and Brazil will have a 40% growth in production with an increase of just 16% of the area. To see the responsibility, since the increment of production in traditional countries like the USA will be 15%, China 15%, and in the whole of Europe, 4%.

With all this challenge, it should be noted that the transgenic plants are not panacea to solve all the problems of agriculture. Transgenic agriculture is only a complement to conventional agriculture, organic and other modalities. However the endless search for improvement of this tool will provide future generations, guarantees of more sustainable living conditions and higher quality food.

Author details

Gleberson Guillen Piccinin, Alan Augusto Donel, Alessandro de Lucca e Braccini,
Lilian Gomes de Morais Dan, Keila Regina Hossa, Gabriel Loli Bazo and
Fernanda Brunetta Godinho

Department of Agronomy, State University of Maringá, Maringá, Paraná, Brazil

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Chapter 20

Polymeric Nanoparticle-Based Insecticides: A Controlled Release Purpose for Agrochemicals

Bruno Perlatti, Patrícia Luísa de Souza Bergo,
Maria Fátima das Graças Fernandes da Silva,
João Batista Fernandes and Moacir Rossi Forim

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53355

1. Introduction

Insects are one of the biggest animal populations with a very successful evolutive history, once they can be found chiefly in all possible environments all over the world, and the number of species and individuals. Their success can be attributed to several important evolutionary aspects like wings, malleable exoskeleton, high reproductive potential, habits diversification, desiccation-resistant eggs and metamorphosis, just to name a few. Some species are especially valuable for humans due to their ability in providing several important goods, such as honey, dyes, lac and silk. On the other hand, many insects are vectors of many diseases, and many others damages crop plantations or wood structures, causing serious health and economic issues.

Among all identified insects, over 500,000 species feed on green leaves. About 75% of them have a restrict diet, eating only a limited range of species, sometimes being even specie specific [1]. This kind of insect brings major concern to the agriculture. Their high selectivity implies in a closer insect attack on crops. It is estimated that about 10,000 insect species are plagues and, compromising the food production, either in the field or after the harvest [2]. It was estimated that somewhere around 14-25% of total agriculture production is lost to pests yet [3].

Agriculture is one of the main pillars of human population increase over the last millenniums, providing mankind with several important commodities such as food, fuel, healthcare and wood. This huge production should feed 7 billion people, and also generate several inputs for many industrial processes and commercial applications. In order to combat the nu-

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merous losses that are caused by insects on agriculture, several chemicals have been used to kill them or inhibit their reproduction and feeding habits. Those classes of compounds are collectivity known as insecticides. These molecules are able to interfere in the insect metabolism. They alter is in such a way that the plague cannot feeds on the crop or the harvest or even reproduce anymore. The use of insecticides is described since ancient times, with documents providing evidences as far as in the 16th century BC. The *Ebers Papyrus*, wrote by the Egyptians, reports several chemical and organic substances used against overcome fleas, gnats and biting flies among others [4]. Nowadays, the insecticides are widely employed around the world. Several known substances are extremely effective in controlling or even wiping out almost all important agricultural plagues. This multi-billion-dollar has an estimated production of 2 million metric tons of hundreds of chemical and biological different products, with a budget of a US$35 billion dollars worldwide [5].

Insecticides are used in different ways, based on the physical-chemical characteristics of the each chemical substance, the area that needs to be covered and the target. Typical application of insecticides in crops is made by spraying a solution, emulsion or colloidal suspension containing the active chemical compound, which is made by a vehicle which may be a hand pump, a tractor or even a plane. This mixture is prepared using a liquid as a carrier, usually water, to ensure a homogenous distribution. Other methods for applying insecticides are through foggers or granule baits embedded with the active compound, among others that are less used. However, due to several degradation processes, such as leaching or destruction by light, temperature, microorganism or even water (hydrolysis), only a small amount of these chemical products reaches the target site. In this case, the applied concentrations of these compounds have been much higher than the required. On the other hand, the concentration that reaches its target might be lower than the minimum effective one. In general, depending of the weather and method of application, the amount of applied agrochemicals, as much as 90%, may not reach the target and so do not produce the desired biological response. For this reason, repeated application of pesticides become hence necessary to efficient control of target plagues, which increase the cost and might cause undesirable and serious consequences to the ecosystems, affecting human health [6]. Due to the lack of selectivity, their unrestrained use can also lead to the elimination of the natural enemies, what implies in the fast growth of plague population. Moreover, it often makes the insects resistant to the pesticides.

Another important point that needs attention is the formulation for the application of the insecticide on the crops. There are several different classes of compounds, which sometimes do not match with a simple dilution in water and must be prepared by other means such as powders, emulsions or suspensions. Some kinds of formulations must be handled with more precaution, since it can severely contaminate workers on the field with small airborne solid particles that can be inhaled [7].

The advances in science and technology in the last decades were made in several areas of insecticide usage. It includes either the development of more effective and non-persistent pesticides and new ways of application, which includes controlled release formulations (CRFs). The endeavors are direct towards the successful application of those compounds on crops and their efficacy and availability improvement and reduction of environmental contamination and workers exposure [8]. In that line, new types of formulation were developed. One of the most promising is the use of micro and nanotechnology to promote a more efficient assembly of the active compound in a matrix.

2. Application of insecticides nanoformulations

2.1. Nanoemulsions

Casanova *et al.* [9] evaluated the production of a nicotine carboxylate nanoemulsion using a series of fatty acids (C10 – C18) and surfactant. The oil-in-water nanoemulsion showed a monomodal distribution of size, with mean particle sizes of 100nm. The bioactivity of the insecticide formulations was evaluated against adults of *Drosophila melanogaster* by assessing the lethal time 50 (LT_{50}). They observed that the encapsulation efficiency decreased with increasing size of the fatty acids tested. The bioactivity followed the same trend, with better bioactivity when the chain length decreased. This would be readily attributed to the higher amount of active compound inside the nanoemulsion. For the smallest fatty acid emulsion used, the capric acid (C10) one, the greatest encapsulation efficiency was observed, but it had the lowest bioactivity. The results were explained in terms of lesser bioavailability of the insecticide in its active form due to increased stability of the organic salt formed between the insecticide and the fatty acid. This experiment highlights the necessity of developing different kinds of possible assembles between the active compounds and matrix, and extensively studying the interactions in nanoscale formulations, where sometimes nontrivial effects might be unexpectedly observed.

Wang *et al.*[10] developed an assemble of oil-in-water nanoemulsion (O/W) with 30 nm droplets by careful control of experiment conditions, using the neutral surfactant poly(oxyethylene) lauryl ether and methyl decanoate to encapsulate highly insoluble β-cypermethrin. The dissolution of the insecticide was enhanced. The stability tests were performed by spraying nanoemulsion in a glass slide and observing under polarizing light microscopy. They showed no apparent precipitate in nanoemulsions samples. These results were different from the ones obtained using a commercial β-cypermethrin formulation, with apparent signs of solid residues after 24 hours. This enhanced stability may be used to decrease the concentration of insecticides in commercial spray applications, without losing efficiency.

2.2. Classical micro and nanoparticles

Allan *et al.* [11] published the first report on a controlled release system of an insecticide through a polymeric encapsulation. Even so, at first the encapsulated systems were not so effective. Problems associated with controlled release and particle stability hindered their practical field application for some decades. In one of the first successful works in the field of pesticides encapsulation, Greene *et al.* [12] used poly (n-alkyl acrylates) (Intelimer®) to produce temperature-sensitive microcapsules of the organophosphate insecticide diazinon.. The active chemical was controlled release by increasing the ambient temperature above

30ºC, which is the melting temperature of the polymer,. Experiments were performed with Banded cucumber beetle *Diabrotica balteata* and Western corn rootworm *Diabrotica virgifera* as target insects at 20ºC and 32ºC, under and above the polymer melting point respectively. Mortality was compared to commercial granular formulation. At lower temperatures, the commercial formulation showed the best mortality. At higher temperatures the activity of the encapsulated formulation was better, showing about 90% of mortality for over 8 weeks. The commercial formulation had indeed lost some of its activity, presumably due to heat degradation.

Latheef *et al.* [13] tested several different polymers such as poly (methyl methacrylate) (PMMA), ethyl cellulose, poly(α-methylstyrene) and cellulose acetate butyrate to produce microcapsules of the insecticide sulprofos. Ethyl cellulose formulations were the only ones that had shown good results against eggs and larvae of the tobacco budworm *Heliothis virescens* in cotton plants. The results were comparable to the ones obtained with the use of an emulsifable-concentrate (EC) commercial formulation of sulprofos.

In other to develop commercial formulation containing microencapsulated cyfluthrin, Arthur[14] evaluated its use against the rice weevil *Sitophilus oryzae* in stored wheat, for a period of 8 months. Survival of beetles was statistically correlated with the concentration of the pyrethroid insecticide in the formulation. The average survival rate was only 12% when 4ppm was used, with constant activity throughout the entire experiment. This evidenced the controlled release of the substance over a long period of time.

In the work carried out by Quaglia *et al.* [15], a hydrophobic waxy prepared through a mixture of di- and triglycerides of PEG esters was used to construct microspheres containing the insecticide carbaryl. Microparticles was obtained with particle size ranging from 16 to 20μm. Controllable release dynamics depended on the amount of gelucire used, Studies of release profiles from the encapsulated formulation showed a lower vertical mobility of the insecticide when compared to a commercial nonencapsulated formation. This suggested that the controlled release profile of the microcapsules may be useful to avoid or minimize groundwater contamination.

Cao *et al.* [16] produced diffusion-controlled microcapsules with diameter ranging from 2 to 20μm with encapsulated acetamiprid, an alkaline and high temperature-sensitive insecticide, using tapioca starch as matrix with urea and sodium borate as additives. The particle showed increased degradation resistance by heat for 60 days, and UV radiation over 48h, with no more than 3% of degradation. This represents less than one tenth when compared to the UV degradation of commercial emulsifable concentrate. Even in those conditions, it was also able to promote controlled liberation of the active compound for up to 10 weeks depending on the formulation used.

In another work with acetamiprid, Takei *et al.* [17]produced microparticles with diameter of 30-150μm using poly-lactide (PLA) as the polymeric matrix. Initial results showed that microspheres containing only PLA did not have a good release kinetic of the active chemical compound from its interior. It is presumably due to their tight structure and high hydrophobicity, which hinders water diffusion and therefore limits the insecticide liberation. The inclusion of poly(ε-caprolactone) (PCL) into the matrix in 50-80% weight were analyzed, with formation of microspheres of PLA/PCL blend with 20-120µm of the diameter, showing up to 88,5% of insecticide release in aqueous media over a 48h period.

In contrast to conventional desire to produce compounds with extended residual activity, quick-release microcapsules are demanded in certain areas of agriculture. However, sometimes it is also necessary a quick liberation of the active compound from the matrix after the application. The strong backbone might pose as a problem to effectively deliver. Studies performed by Tsuda *et al.* [18,19] have shown that is possible to assemble "self-bursting" microcapsules that retain its form in water suspension, but easily burst after solvent evaporation. They used the interfacial polymerization method to assemble spherical polyurethane microcapsules containing the insecticide pyriprofixen, obtaining particles with mean diameter of 23µm. The entrapment ratio was 99% for all formulations tested, greatly improving the solubility of the pesticide in water. According to the results, there is a correlation between the wall thickness of the microcapsules and the self-bursting phenomenon. Tuning this property a controlled released can be achieved.

The effectiveness of encapsulated formulations, it is not restricted to extend the residual activity of insecticides, but should also include the overcoming of problems associated with accumulation of recalcitrant organic pollutants that remains in ecosystems in amounts above the Maximum Residual Level (MRL). Therefore, it can be harmful to the environment and to people who might consume the treated crops. For instance, Guan *et al.* [20] encapsulated imidacloprid, a chloro-nicotinyl systemic and broad spectrum insecticide in a mixed sodium alginate/chitosan microparticle through self-assembly layer-by-layer (LbL) methodology. The capsules showed a mean diameter of 7µm. Particles were impregnated with a photocatalyst made of SDS/TiO2/Ag, and the photocatalytic property and the insecticidal activity of the microcapsule was evaluated. Prolonged residual activity of the encapsulated formulation was observed. The toxicity was higher in the *Martianus dermestoides* adult stage compared to the one of pure insecticide. In a field test with soybean [21], the nano-imidacloprid formulation prevented the accumulation of the pesticide on the soybean leaves and soil. The results showed pronounced degradation over 25 days of trials when compared to commercial concentrate formulations, even though the initial concentration of both formulations was equivalent. In this way, regardless the initial effectiveness of the insecticide, safer levels of agrochemicals can be obtained in less time, improving the safety of insecticide application.

2.3. Entomopathogenic microorganisms encapsulated

Besides the chemical compounds, the micro- and nanotechnology have also been developed and applied to microorganisms that need special protection or to improve their solubility in aqueous phase. For instance, Ramírez-Lepe *et al.* [22] developed an aluminium-carboxymethylcellulose microcapsule with photoprotective agents for holding a *Bacillus thuringiensis* serovar *israelensis* (B.t.i.) spore-toxin complex named δ-endotoxin. The protein produced by this gram-positive bacterium during sporulation is extremely toxic to larval stage of some mosquitoes and flies which are vectors for important tropical diseases such as malaria and dengue. The encapsulated formulation was tested for its UV irradiation protective efficiency in laboratory conditions. While the protein in its natural form had lost all of its activity after 24 hours of exposure, encapsulated formulations showed up to 88% of larvae mortality.

In their turn, Tamez-Guerra *et al.* [23] also tested the encapsulation of the spore-toxin of *Bacillus thuringiensis* Berliner, evaluating over 80 formulations of spray-dried microcapsules made of lignin and corn flour with and without photoprotective agents. The best formulations showed improved insecticidal activity in laboratory tests against neonates of European corn borer *Ostrinia nubilalis* when compared to nonencapsulated or commercial formulations of the same endotoxin. In a field test, the microcapsules showed increased residual insecticide activity in cabbage after 7 days against neonates of the cabbage looper *Trichoplusia ni* when compared to commercial formulations.

Very promising results have been obtained by the Agricultural Research Service of the USDA regarding the encapsulation of biopesticides made of species-specific nucleopolyhedroviruses (NPV) isolated from several insects, including celery looper *Anagrapha falcifera* (Tamez-Guerra *et al*., 2000 [24-26]), alfalfa looper *Autographa californica* [27], codling moth *Cydia pomonella* [28] and fall armyworm *Spodoptera frugiperda* [29]. In these works, formulations were developed using different mixtures of corn flour and lignin, through spray-drying technique to encapsulate the viruses. All results obtained in laboratory and field tests performed have shown improvements in insecticidal activity, resistance to environmental conditions, like rain and UV light exposure, and a prolonged residual activity against pests in field studies. Samples were kept in storage for up to 12 months and maintained their insecticidal activity.

2.4. Novel micro and nanoparticles for bioinseticides

Conventional protocols for encapsulation usually run under relatively high temperatures, which might be inadequate for preserving plant-derived essential oils integrity. Processes which use high pressure instead of temperature can be an alternative for encapsulating these sensible extracts. Varona *et al.* [30,31] developed new methods to produce stable particles of lavandin (*Lavandula hybrida*) essential oil, using polyethylene glycol 9000 (PEG9000) or n-octenyl succinic (OSA) modified starches as the shell material. The methods for preparing the microcapsules were based on PEG precipitation from a mixture of molten polymer and essential oil in supercritical CO_2, and PGSS-drying an oil-in-water emulsion of the essential oil with OSA starch. The difference between these processes is the presence of water on the latter, which needs to be removed by carefully tuning the equipment conditions to promote water evaporation. Microcapsules produced by these methods show a mean particle size of 10-500μm for PGSS, and 1-100μm for PGSS-drying. One important observation by scanning electron microscopy (SEM) images is that the experimental conditions can influence the shape of the microparticles. While PEG particles were only spherical (the best shape for controlled release mechanism), in PGSS-drying needle-like structures are formed,, depending on the pre-expansion temperatures of the mixtures, The last one, probably does not hold the active ingredient, presenting some limitations to this specific method without further improvements. Release kinetics were evaluated over a 20-day period. The amount of oil released was proportional to the initial oil concentration on particles, with less than 20% of liberation for low oil concentrations, and about 60% liberation for high oil concentration.

Yang *et al.* [32] assembled polyethylene glycol (PEG) nanoparticles loaded with garlic essential oil using a melt-dispersion method, reaching over 80% of encapsulation efficiency, with round shaped nanoparticles of lower 240nm of average diameter. The encapsulated formulations had their insecticidal activity evaluated against adult red flour beetle *Tribolium castaneum*. While the control experiment done with free garlic oil showed only 11% of efficiency over a five month period, the encapsulated formulation efficiency remained over 80% after five months. This was attributed to the slow and controlled release of the essential oil, and thus could be used as an effective pest control to stored products.

The basic structure of the polymer chitosan was used by Lao *et al.* [33] to build the amphiphilic-modified *N*-(octadecanol-1-glycidyl ether)-*O*-sulfate chitosan (NOSCS). Octadecanol glycidyl ether and sulfate were the hydrophobic and the hydrophilic groups sources respectively. They successfully entrap the herbal insecticide rotenone in the polymer. This chemical compound has been allowed for application in organic crop production due to its natural origin, short persistence in the environment, safety to non-target organisms and low resistance development. The encapsulation was necessary to defeat the problems of chemical stability of the substance to environmental effects and also to improve the solubility of this pesticide in water, which is usually quite low (2.0×10^{-6}g.L^{-1}). Using the reverse micelle method, the authors have assembled nanometric micelles with 167.7-214.0 nm of diameter, with values of critical micellar concentration (CMC) of those chitosan derivatives ranging from 3.55×10^{-3} to 5.50×10^{-3} g.L^{-1}. Although the entrapment efficiency was not very high, they also improved the aqueous solubility of the chemical compound in 13,000 fold, up to 0.026g.L^{-1}, favoring a controlled release of the substance in aqueous media. The complete controlled release took more than 230 hours, almost 10 times more when compared to the chemical compound without nanoencapsulation.

Chitosan derivatives were prepared [34]. They synthesized 6-*O*-carboxymethylated chitosan with anchorage of ricinoleic acid at the *N*-linkage, which further improve its solubility at neutral water (pH = 7.0), to encapsulate the herbal insecticide azadirachtin. Nanoparticles of 200-500nm were obtained by water dispersion with more than 50% of loading efficiency and tested for their stability in outdoor as controlled release systems. Results were compared against simple azadirachtin water dispersion and modified dispersion containing ricinoleic acid and azadirachtin. In 5 days of sun exposure, all content of control samples were lost, while the encapsulated formulation had a nearly constant residual concentration detected throughout the 12 days of the experiment, indicating that the nanoparticles produced were effective at controlling the degradation rate and the release mechanism of the botanical insecticide.

Extracts of Neem were prepared contend high concentration of azadirachtin being nanoencapsulated by Forim *et al.* [35]. Through the use of poly-(ε-caprolactone) polymer, they prepared nanocapsules and nanospheres with average diameter of 150.0 and 250.0nm, respectively. The morphological analysis revealed spherical nanoparticles (Figure 1). The azadirachtin was used as reference. The nanoformulations showed high entrapment efficiency (> 95%) for this compound and a UV stability at least of 30 times more when compared with commercial products.

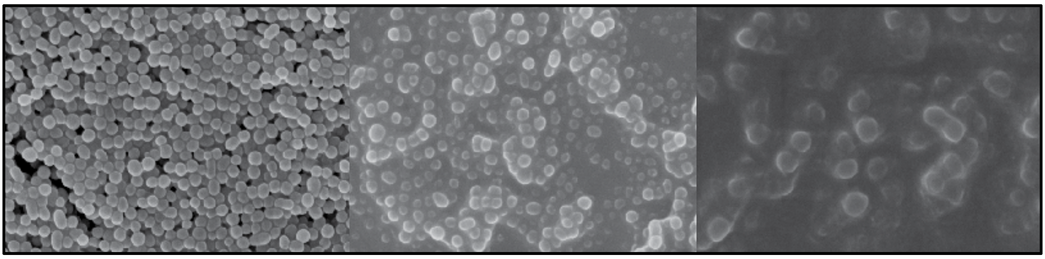

Figure 1. Scanning electron microscopy images of nanoparticles containing extracts of Neem.

2.5. Commercial products

The interesting results obtained in academic researches over the last few decades have been closely followed by several companies. Nevertheless, R&D in nano-based agrochemicals is led mainly by world's largest agroscience companies, further enhancing their market share and consolidating the market structure based on oligopoly that have been seen in late 20th century and early 21th century, when the 10 biggest companies hold around 80% of market [36].

Some companies over the last decade, such as Syngenta, Bayer, Monsanto, Sumitomo, BASF, and Dow Agrosciences have already deposited several different patents comprising a wide range of protocols for production and application of encapsulated formulations, which can be used to produce nanoinsecticides [37-46]. Despite the hard work and heavy investment, no commercial nano-insecticide formulation has been extensively commercialized up to 2012.

Along with those big industries, several other companies, as well as individual researches have been actively depositing patents in the area, thus promoting even more the research and investments in this new field of applied technology. However, as strongly reinforced throughout the world by dozens of organizations such as the ETC Group, the impact of nanotechnology is still unclear, and care should be taken to assure that its use will not bring more problems than solutions [47].

3. Developing new nanopesticides

Many attempts have been made to manage plague insects, for example, using biological control, which is very time consuming. Controlled release systems dawn in this scenario as a very attractive alternative in this battle field.

Controlled release formulations (CRFs) associate the active compound with inert materials. The last ones are responsible for protecting and managing the rate of compound release into the target site in a defined period of time. The main purpose of controlled release systems is ruling the (bio) availability of the active compound after the application [48]. They find the greatest applicabilities in two major agricultural fields: nutrition and protection. In the first one, CRFs are employed in the delivery of fertilizers [49-51]. In the second one, CRFs are mostly used to target plague insects in a sustainable way [52,53], but they can also be applied to block the growth of weeds [54]. Tomioka *et al.*, 2010. Controlled release formulations become especially interesting in cases of antagonist activity of biocides, what can naturally leads to a lower in effectiveness of one or both compounds. In this case the formulation should be "programmed" to release each one at different times [55,56]. Furthermore, still talking about protection, the application of CRFs in wood surfaces, like furniture or floor covering, helps to prevent the deterioration. Van Voris *et al.* [57] patented a formulation in which an insecticide is continually released in a minimum level for a long period of time and is absorbed by the wood. It thus creates a "chemical barrier", blocking the insect attacks.

Most of those controlled release biocides applications were and still are successfully made due to the advances in nanotechnology area.

Micro- and nanomaterials-based formulations are known for some decades. The first microcapsule-based formulation became commercially available in the 1970s [58]. Nanocapsules have been widely used in medicinal area as drug carrier in treatment of diverse diseases [59], from tropical ones [60] up to cancer [61].

Microencapsulation has been used as a versatile tool for hydrophobic pesticides, enhancing their dispersion in aqueous media and allowing a controlled release of the active compound. The use of nanotechnology is a recent approach, and has been a growing subject on several different areas of the science, with an overwhelming perspective. In general, materials that are assembled in nanometric scales (<1000nm) have distinct and almost always better characteristics when compared to the same material built in a conventional manner [62]. One nanometer is a billionth of a meter (1nm = 10^9m). In general, the chemical properties of materials in nanometric scale may be controlled to promote an efficient assemble of a structure which could present several advantages, such as the possibility to better interaction and mode of action at a target site of the plant or in a desired pest due to its tunable controlled release system and larger superficial area, acting as an artificial immune system for plants [34,63]. As smart delivery systems, they confer more selectivity, without hindering in the bioactive compounds towards the target pathogen [65]. Other advantages of the use of nanoparticle insecticides are the possibility of preparing formulations which contain insoluble compounds that can be more readily dispersed in solution. It reduces the problems associated with drifting and leaching, due to its solid nature, and leads to a more effective interaction with the target insect. These features enable the use of smaller amount of active compound per area, as long as the formulation may provide an optimal concentration delivery for the target insecticide for longer times. Since there is no need for re-applications, they also decrease the costs), reduce the irritation of the human mucous-membrane, the phytotoxicity, and the environmental damage to other untargeted organisms and even the crops themselves [65,66]. In a few words, nanotechnology can be applied in several ways in order to enhance efficacy of insecticides in crops.

3.1. Biopolymers

When a commercial formulation for a practical field application is desired, it is very important to employ materials that are compatible with the proposed applications: environment-friendly, readily biodegradable, not generating toxic degradation by-products and low-cost. The use of several biopolymers, i.e., polymers that are produced by natural sources, which at the same time have good physical and chemical properties and still present mild biodegradation conditions, are an interesting approach to avoid the use of petrochemical derivatives that might be another source of environmental contamination. The common polymers (synthetic and natural ones) used in CRFs for insecticides application are listed in Table 1.

3.2. The nanoparticles used in biocides controlled release formulations

The most popular shape of nanomaterials (Figure 2) that have been using in CRFs for biocides delivery are:

a. Nanospheres: aggregate in which the active compound is homogeneously distributed into the polymeric matrix;

b. Nanocapsules: aggregate in which the active compound is concentrated near the center core, lined by the matrix polymer;

c. Nanogels: hydrophilic (generally cross-linked) polymers which can absorb high volumes of water

d. Micelles: aggregate formed in aqueous solutions by molecules containing hydrophilic and hydrophobic moieties.

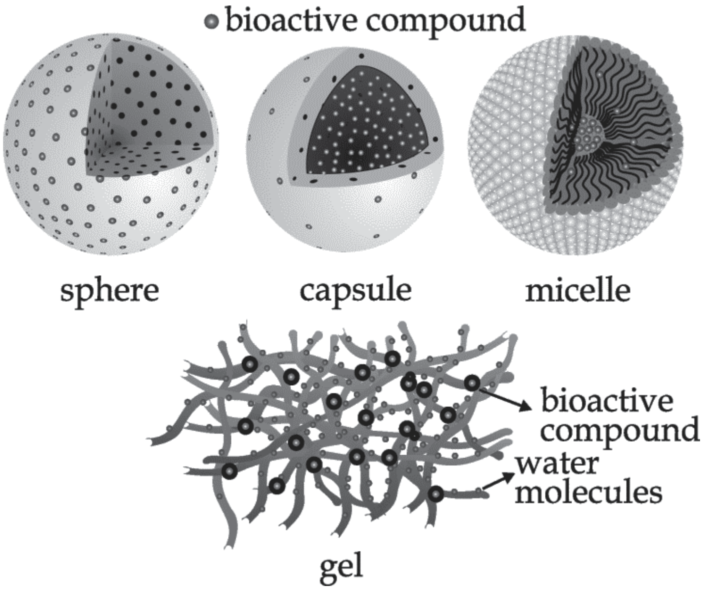

Figure 2. Morphological representation of different nanoparticles.

Polymer	Active compound	Nanomaterial	Ref.
Lignin-polyethylene glycol-ethylcellulose	Imidacloprid	Capsule	[67]
Polyethylene glycol	B-Cyfluthrin	Capsule	[68]
Chitosan	Etofenprox	Capsule	[69]
Polyethylene	Piperonyl Butoxide And Deltamethrin	Capsule	[70]
Polyethylene glycol	Garlic Essential Oil	Capsule	[32]
Poly(acrylic acid)-b-poly(butyl acrylate) Polyvinyl alcohol Polyvinylpyrrolidone	Bifenthrin	Capsule	[71]
Acrylic acid-Bu acrylate	Itraconazole	Capsule	[72]
Carboxymethylcellulose	Carbaryl	Capsule	[73]
Alginate-glutaraldehyde	Neen Seed Oil	Capsule	[74]
Alginate-bentonite	Imidacloprid or Cyromazine	Clay	[75]
Polyamide	Pheromones	Fiber	[76]
Starch-based polyethylene	Endosulfan	Film	[77]
Methyl methacrylate and methacrylic acid with and without 2-hydroxy ethyl methacrylate crosslinkage	Cypermethrin	Gel	[78]
Lignin	Aldicarb	Gel	[79]
Lignin	Imidacloprid Or Cyromazine	Granules	[75]
N-(octadecanol-1-glycidyl ether)-O-sulfate chitosan-octadecanol glycidyl ether	Rotenone	Micelle	[33]
Polyethyleneglycol-dimethyl esters	Carbofuran	Micelle	[80]
Carboxymethyl chitosan-ricinoleic acid	Azadirachtin	Particle[a]	[34]
Chitosan-poly(lactide)	Imidacloprid	Particle[a]	[81]
polyvinylchloride	Chlorpyrifos	Particle[a]	[82]
Cashew gum	*Moringa Oleifera* Extract	Particle[a]	[83]
Chitosan-angico gum	*Lippia Sidoides* Essentioan Oil	Particle[a]	[84]
Polyvinylpyrrolidone	Triclosan	Particle[a]	[85]
Anionic surfactants (sodium linear alkyl benzene sulfonate, naphthalene sulfonate condensate sodium salt and sodium dodecyl sulfate)	Novaluron	Powder	[86]
Vinylethylene and vinylacetate	Pheromones	Resin	[87]
Glyceryl ester of fatty acids	Carbaryl	Spheres	[15]
Poly(ε-caprolactone)	Active Ingredients[b]	Spheres	[88]
Poly(methyl methacrylate)-poly(ethylene glycol) Polyvinylpyrrolidone	Carbofuran	Suspension	[89]

[a] The authors do not mention which active compounds they encapsulated in the nanospheres; [b] The authors do not mention if the particles are spheres or capsules

Table 1. Several examples of polymers often used in the nanoparticle production.

Dendrimers, nanoclays, nanopowders and nanofibers are other possible formulations which might be used during nano or microparticle production[75, 76, 86, 90]. On the other hand, nanotubes are mostly applied in plants improvement. The polymeric nanoparticles and gels are by far the mostly used for insecticides application, because they have an extra advantage of being biodegradable.

3.3. Methods for preparation of nanomaterials based controlled-release formulations for biocides application

According to Wilkins [48], the methods for CRF preparation can be separated in chemical or physical ones (Figures 3 and 4, respectively).

The chemical methods are based on a chemical bond (usually a covalent one) formed between the active compound and the coating matrix, such as a polymer. This bound can be placed in two different sites: in the main polymeric chain or in a side chain. In the first one, the new "macromolecule" is also called a pro-biocide, because the compound will get its properties in fact when it is released. In the second one, the insecticide molecule can bind initially to the side-chain of one monomer and then the polymerization reaction takes place or the polymerization occurs first and only after that, the biocide binds to the side chain. There is still a third way, based on the intermolecular interactions. In this case, the biocide is "immobilized" in the net produced by the cross-linkages in the polymer.

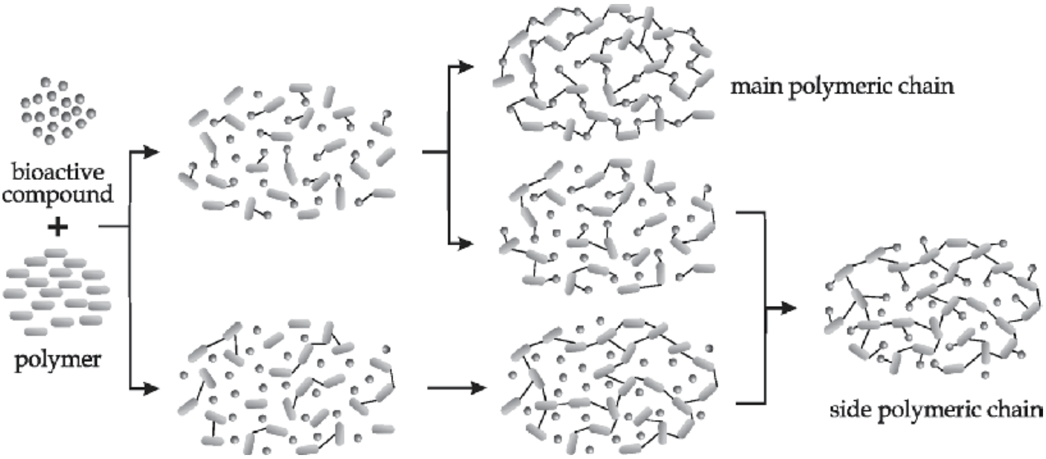

Figure 3. Chemical methods for CRF preparation

The physical methods can also be split in two distinct categories. In the first, a mixture of biocide and polymer is made. As the last has a higher energy density, it moves to a more external layer, forming a kind of monolithic structure. In the other one, the polymeric chain forms a "membrane" isolating the bioactive compound from the external environment. This is the method which will produce the nanocapsules themselves.

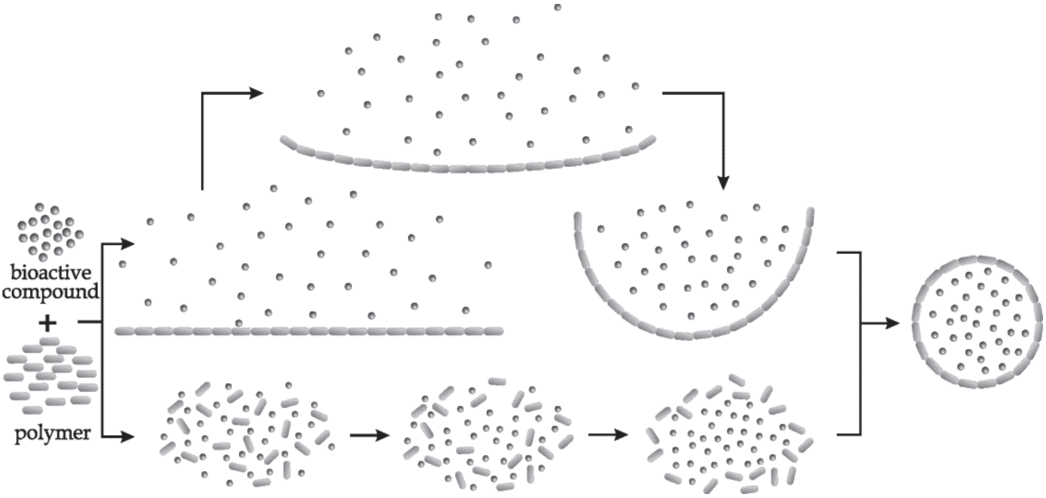

Figure 4. Physical methods for CRF preparation

Although there are some different kinds of nanomaterials that can be used in CR formulations, the micro- and nanocapsules are by far the most widely used for controlled release of biocides. For this reason, the techniques described here will be restricted to micro and nanoencapsulation process.

3.4. Micro and nanoencapsulation techniques

The first formulation containing polymeric-based nanocarriers for controlled release of biocides dates from the early 1970's [11,92]. Recently, John *et al.* [93] reviewed the most commonly techniques used to prepare micro- and nanocapsules containing microorganisms (for this kind of application, see section 2.3). However, the techniques they commented can be also utilized to prepare nanocapsules for insecticides application in general. Shahidi and Han [94] and Wilkins [48] classified them as physicochemical, chemical or physical process-based. Some are described below.

3.4.1. The physicochemical-based techniques

a. *Emulsion*: This technique is used to produce a system of two immiscible liquid phases (water and oil), where one (the dispersed phase) is dispersed into the other (continuous phase) in a controlled way (usually in a dropwise one). The bioactive compound (usually water-soluble) and the polymer are solubilized each one in a phase (water or oil). One of the solutions is gradually dripped into the other under vigorous stirring. After the homogenization, the emulsion is formed. If the oil is the dispersed phase, the emulsion is classified as O/W (oil/water). If it is water, the emulsion is called W/O (water/oil) [95].The emulsion itself also represents a crucial step for some other more complexes preparation ones.

b. *Coacervation*: This process is based on the reduction of polymer's solubility. According to Wilkins[48] the encapsulation goes through a separation of phases and can be simple or complex. In a simple coacervation, the addition of an external agent, like a salt or water-miscible solvent, to an aqueous solution containing a hydrophilic polymer-insecticide complex causes its precipitation. Complex coacervation involves opposite charges and electrostatic attraction. A solution containing different ionizable polymers is submitted to a pH change. The polymers turn positively or negatively charged. The electrostatic attractive forces between the opposite charges become much stronger than the particle-solvent intermolecular ones, leading to the copolymer precipitation.

c. *Emulsion-solvent evaporation*: According to Iwata and McGinity [96] this technique comprises two or three steps. In the first one, an O/W (or W/O) emulsification must be initially formed. The polymer is usually solubilized in the dispersed phase. If the emulsion has only two components like this one, it is called a single emulsion. For this type, the whole process has only two steps and the first one ends here. However, there is also other type, called double emulsion, represented as W/O/W′, where the emulsion already prepared in the first step is dispersed into an organic solvent, like acetonitrile. In this case, the aqueous solution containing the active compound is dripped in an oil phase (usually a vegetable oil), under stirring. This emulsion is then dispersed, under stirring, in an organic solvent solution containing the polymer. The last step, common for single and double emulsion, is the evaporation of the solvent, what can be performed at room temperature or under reduced pressure. After solvent removal, the particles are ready for use.

d. *Emulsion crystallization/ solidification*: According to the procedure published by Iqbal *et al*. [97], an emulsion is initially prepared as already described in this section. The only difference remains in the temperature in which it is made. The authors prepared the emulsion at 60°C. The next step is crucial for technique success. The warm emulsion is pumped through a capillary partially immersed in a coolant liquid (temperature: 10°C). At the capillary exit, the emulsion forms spherical drips which move to raise the cooling liquid's surface. The drop is cooled down during the course, solidifying and forming the particles which are collected at the top.

e. *Diffusion-controlled emulsion*: In this process, a monomer rich phase is laid over the aqueous solution containing the insecticide, under a smooth stir. The monomers then diffuse into the aqueous fase, "trapping" the bioactive molecules in a micellar structure [98].

f. *Liposome entrapment*: Some protocols to prepare liposomes are described by Mozafari *et al*. [99]. The standard one is resumed here.

In the first step, an organic solution (chloroform or methanol ones) containing hydrophobic molecules such phospholipids and cholesterol is prepared. The solvent then is evaporated forming a thin film. Next, an aqueous solution containing the bioactive compound is spread over this film. Some mechanical or thermal perturbation like ultrasound or heating is applied to the system to promote the formation of single or double layer sheet. The sheet will detach from the support, closing itself, forming the liposomes. During this closing process, the sheet traps the biocides molecules.

3.4.2. The chemical techniques

a. *Interfacial polymerization*: As the name says, this technique is based in a polymerization reaction which occurs in an interface of two immiscible liquids. According to Wilkins [48], polymerization can occur through an addition or condensation reaction. In the mostly addition-governed process, the polymerization starts in the oil phase, where the monomers and insecticide are dispersed. However, the reaction only takes place when it is catalyzed by free radicals, which are dissolved in the aqueous phase. In condensation-governed process (the most suitable route for biocides nanoencapsulation), the reactive monomers are dissolved each one in a different phase. As the dispersed phase is dripped into the continuous phase, the reaction occurs in the droplet interface, producing the polymer. When a solvent with a low boiling temperature is used as the oil phase (either in dispersed or continuous one) and contains the monomers dissolved, the process is a little different. After the dripping, the system is heated. The solvent thus evaporates, leaving the particules that, due to the water insolubility, precipitates. This particular technique variation can also be called interfacial polymer deposition [100].

b. *Molecular inclusion*: This technique is used to increase the solubility of water-insoluble compounds in aqueous solution. Macromolecules like cyclodextrins [101] have an inner hydrophobic face and an outer hydrophilic face. An oil phase containing the biocide is dripped, under continuous stirring, into the aqueous macromolecule solution. During the dripping, the macromolecule "traps" the insecticide molecules via intermolecular interactions.

3.4.3. The physical techniques

a. *Extrusion:* The bioactive compound is mixed with hydrocolloids and then, the colloid is squeezed out under pressure. The pressure during the process should be adjusted according to the viscosity of colloids.

b. *Spray drying*: This technique is based in solvent evaporation at high temperatures. The spray drying process has already been described in details by Ré [102]. The following text is only a brief resume. Initially, the active compound and the polymeric matrix are solubilized in their respective solvents, which should not be miscible. Then, they are mixed under vigorous stirring to form an emulsion (or dispersion whether one of the components is in the solid state). The emulsion undergoes an atomization to produce droplets. In the next step, the droplets are submitted to a hot air flow that forces the solvent (generally water) evaporation, leaving only a dry powder. The greatest advantage of this technique is that it can be easily scaled up for a large scale nanocapsules production.

c. *Freeze drying*: This technique is also known as liophilization. It is the opposite of the spray drying, because it uses a low temperature system. A suspension or emulsion is prepared to enable the polymer-insecticide formation. For emulsions, an additional step is required before the execution of the technique: the removal of the oil or organic solvent under reduced pressure. For both (emulsion and suspension), the aqueous phase is frozen and submitted to a low pressure system. When the pressure is drastically reduced, the water sublimes (goes from solid to vapor state), leaving only the particles.

3.5. Mechanism of biocide release

In the paper published by Kratz *et al.* [103] the text begins with the statement: "Nanoparticles only start working after they are placed in a desired location". In other words, an efficient CR formulation must remain inactive until the active compound is released.

The way how an inert material, such the nanopolymers, controlss the amount and rate a chemical is released is object of study since the late 1960's [104] and early 1970's [105].

How the release of the bioactive compound occurs depends basically on the chemical nature of the formulation. In various polymeric nanomaterials, the controlled release proceeds via diffusion. It does not matter if the bioactive compound is dissolved (micro- or nanospheres) or if it is encapsulated (micro or nanocapsules). The process does not depend on the chemical structure of the formulation constituents [11] neither on the intermolecular interactions. The rate control is made based on the interactions between the carrier and the biocide. The stronger the interaction will be slower the release rate. In the 1990's, the release dynamics was investigated via the use of ^{14}C-labelled molecules of herbicides [106,107]. Qi *et al.* [107] studied the dynamic of controlled release for herbicides. They used ^{14}C-labelled molecules of benthiocarb and butachlor and observed that the release is made by a diffusive process. Some years later and without any radiolabeled molecules, Fernandez-Perez et al. [108] found the same results. They prepared a granule-based CRF constituted by lignin and imidacloprid. They measured the amount of compound released in water under a dynamic flow condition during a defined period of time. The data fitted a diffusion curve based on the model proposed by Ritger and Papas [109,110]. Since then, other similar studies have been published [111-114].

Some other polymeric nanomatrixes, especially those formed by a carboxylic acid and a metallic cation, can be disassembled when in contact with water, releasing the bioactive compound [92]. The release rates depend on the physicochemical characteristic of both molecules. The more hydrophobic the polymer slower will be the bioactive compound release. The same applies to the last one: the higher water-solubility, faster it will be released. The formulation itself also affects directly the release rate. In water-based one, the rate control tends to disappear, due to the matrix (or support) degradation. If the particles are solubilized in an organic solvent, like acetone, the formulation becomes sticky and the release rate slows down. A granule-based formulation sounds more efficient. It can be applied direct to the soil and the bioactive compound will be released according to the soil moisturize (water content), leading to a long lasting control.

In other formulations, the bioactive compound is covalently bound to the polymeric matrix [115]. To the release takes place, a chemical interaction must be broken. It usually occurs via a hydrolysis reaction, what affects many polymer-insecticide bounds in a chain reaction. The release control depends on the strength of those chemical bounds, the chemical properties of both molecules and on the size and structure of the macromolecule formed [11]. The higher the biocompound solubility in water, faster the reaction occurs. Concerning the chemical properties of the polymer, Allan *et al.* [11] studied the differences in the release kinetics when 2-methyl-4-chlorophenoxy acetic is chemically bound to polyvinylalcohol (a water-soluble polymer) or when it is bound to cellulose or lignin (water-insoluble polymers). In the first situation, the level of the applied herbicide tends to go down, because the equilibrium

$$Polymer-insecticide \rightleftharpoons Polymer + Insecticide$$

will always exist. I the last situation, as the "free polymer" is water–insoluble, the equilibrium moves towards the right side and the level of the applied herbicide tends to go up (Figure 5).

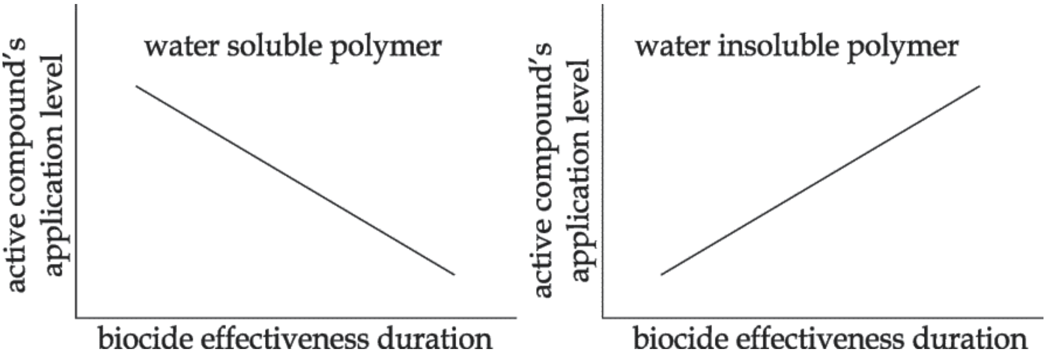

Figure 5. Trend in active compound's application rate (*Adapted* from [11]).

Whatever the mode of liberation, it should be kept in mind that controlled release formulations have a limited maximum amount for the release of the biocide[116]. This means that the total amount of released product may not be necessarily equal to the amount of chemicals incorporated to the formulation neither to the amount of free applied product[117]. This is the reason why the concentration of the active compound in a CRF is usually higher than in a conventional one. However it does not contradict what was said earlier about advantageous reduced amount if biocide applied, since the number of applications should be smaller.

Studies recently published suggested that the encapsulation of biocides reduces their toxicity [117,118]. However, many issues regarding the toxicity of the nanomaterial themselves towards the environmental and even the worker's health remains unclear [119].

4. Conclusion

The increasing worldwide demand for foods requires modern techniques of agricultural production minimizing losses in the crops, transportation and storage. Among the main causes of agricultural losses there are the plague insects. Insecticides are an important control tool. However, some collateral effects may be credited to their indiscriminate use such as environmental contamination, human poisoning, reduction in the number of natural enemies, insecticide resistance by plague insects, etc.

In this scenario, nano- and microparticles have been reaching a prominent position. Formulations containing insecticides have been prepared in colloidal suspensions or powder, in nano or micro scale, where they present several advantages such as increasing stability of the active organic compound (UV, thermal, hydrolysis, etc.), foliar settling, reduction in foliar leaching, systemic action, synergism, specificity, etc. As consequence, the amount of insecticide necessary (dosage), the number of applications, human exposure to insecticides and environmental impact are reduced. The nano- and microformulations have been employed not only for synthetic insecticides but also in alternative products to control plague insects such as natural products (herbal extracts) and entomopathogenic microorganisms.

In order to prepare nano- and microformulations, several chemical and physical techniques have been developed. In general, they should be prepared by using polymeric materials which are biocompatible and biodegradable. This practice has the aim to avoid the emergence of new environmental and toxicological problems. The biopolymers are produced by microorganisms, synthesis or even petroleum derivate products. In common, when exposed to the environment they are easily destroyed by UV radiation and/or microorganism enzymes generating CO_2 and H_2O as final product. The degradation processes of biopolymers may lead, or not, to the release mechanisms of active organic compounds of a nano- or microparticles. Processes such as swelling, hydrolysis, diffusion, erosion, etc., must be manipulated in a controlled way in order to obtain the desired characteristics of application and biological activity for the formulated products.

As a result of the application of these new nano- and micro- technologies, which have been quickly developed due to new sensitive analytical technologies of characterization, new ways to control plague insects are emerging, thinking not only in lethal action on the target insect, but also in all ecosystems, which include fishes, natural enemies, vegetation, microorganisms, animals, the man himself, etc.

Acknowledgments

The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), for the financial support.

Author details

Bruno Perlatti, Patrícia Luísa de Souza Bergo, Maria Fátima das Graças Fernandes da Silva, João Batista Fernandes and Moacir Rossi Forim

Federal University of São Carlos, Department of Chemistry, Brazil

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Edited by Stanislav Trdan

This book contains 20 chapters about the impact, environmental fate, modes of action, efficacy, and non-target effects of insecticides. The chapters are divided into 7 parts. Part 1 covers the non-target effects of insecticides, whereas part 2 is dedicated to integrated methods for pest control, in which insecticides are an important element for diminishing the populations of insect pests. Part 3 includes chapters about the non-chemical alternatives to insecticides, such as metabolic stress and plant extracts. Insecticides and human health are the main topic of part 4, and the interactions between insecticides and environment are discussed in part 5. Part 6 includes the chapters about insecticides against pests of urban areas, forests and farm animals, whereas biotechnology and other advances in pest control are discussed in part 7.

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ISBN 978-953-51-5348-1

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