

Pesticides

Advances in Chemical and Botanical Pesticides



Edited by R.P. Soundararajan

PESTICIDES – ADVANCES IN CHEMICAL AND BOTANICAL PESTICIDES

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Pesticides – Advances in Chemical and Botanical Pesticides

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Contributors

Malaya Ranjan Mahananda, Bidut Prava Mohanty, María Inés Maitre, Alba Rut Rodríguez, Carolina Elisabet Masin, Tamara Ricardo, Erin N. Wakeling, April P. Neal, William D. Atchison, Ahmed S. Abdel-Aty, Svetlana Hrouzková, Eva Matisová, Raymond A. Cloyd, Binata Nayak, Shantanu Bhattacharyya, Jayanta K. Sahu, Dipsikha Bora, Bulbuli Khanikor, Hiren Gogoi, Simon Koma Okwute, Rosdiyani Massaguni, Siti Noor Hajar Md Latip, Annick Tahiri, Jackie Stevens, Kerry Dunse, Jennifer Fox, Shelley Evans, Marilyn Anderson, Tatiana Baidyk, Oleksandr Makeyev, Ernst Kussul, Marco Antonio Rodríguez Flores, Rafael Vargas-Bernal, Esmeralda Rodríguez-Miranda, Gabriel Herrera-Pérez, Nédia de Castilhos Ghisi

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Janeza Trdine 9, 51000 Rijeka, Croatia

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Nédia de Castilhos Ghisi

Preface

Since the synthesis of DDT during 1874 several insecticide molecules have been identified and synthesized globally for the control of insect pests, pathogens, microbes, vectors of human and animal diseases, weeds and other obnoxious organisms. Currently, 1.8 billion kgs of pesticides are used annually worldwide in the form of herbicides, insecticides and fungicides. There are more than 1055 active ingredients registered as pesticides till date implying that there is no best alternate for the chemical pesticide. Pesticides are credited to save millions of lives by controlling diseases, such as malaria and yellow fever, which are insect-borne. However, pesticide exposure causes variety of adverse health effects and environmental pollution. Alternate methods and restricted use of pesticide can minimize the risk of pesticide usage. In agricultural pest management the use of plant based products and research works on identification of toxic principles in the plant parts are worthwhile.

This book volume comprises of three different sections of which first section is on Pesticide Toxicity with seven chapters. The section covers the mode of action of pyrethroid group compounds, toxic effects of malathion on Indian toads and status of farmers' friend 'earthworm' in soils of natural and agriculture-livestock fields. In addition, the toxicity of pesticides on cyanobacteria and natural enemies, some of non-traditional pesticide compounds are also elaborately described. The second section of the volume deals with botanical pesticides and pest management in six chapters. Recently the pest management packages for agricultural and horticultural crops are formulated with non-chemical approach by including botanical and microbial pesticides. Biotechnological and molecular approaches are recent advancement in pest management. This section is mainly focused on plants and plant products having pesticidal principles and biotechnological approaches for insect pest management. An interesting technique of LIRA to recognize insect larval density in the field as forecast for applying pesticide and other management tactics is also included in this section. The third section deals with biomarkers in the pesticide assay in two chapters. Recently biomarkers are used for pesticide assays. Biosensors are innovative components used to determine quantitative and qualitative parameters of pesticide compounds and the detection is fast, reliable and with high portability.

I hope that this volume comprising the current status of pesticides with relevance to pesticide toxicity, non-chemical pest management strategies and scope for biomarkers

for pesticides assays will provide a significant insight to the scientists involved in pesticide research. I appreciate all the authors for their valuable contribution.

I am indebted to Professor K.Gunathilagaraj, Tamil Nadu Agricultural University, India for his inspiration and eminent guidance to hone my skills in editing. I acknowledge Dr. N. Chitra my wife, for her support and encouragement during the book chapters review process.

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R.P. Soundararajan

Assistant Professor (Agricultural Entomology)

National Pulses Research Centre

Tamil Nadu Agricultural University

Tamil Nadu

India



Pesticide Toxicity

Toxicity on Biochemical and Hematological Parameters in *Bufo melanostictus* (Schneider) (Common Indian Toad) Exposed to Malathion

Malaya Ranjan Mahananda and Bidut Prava Mohanty

Additional information is available at the end of the chapter

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1. Introduction

The widespread application of pesticides has attracted the attention of ecologists to understand the impact of the chemical on natural communities. A large number of laboratory-based single species studies of pesticide, such studies can only examine direct effect. However in natural communities, species can experience both direct and indirect effect. Anthropogenic chemicals are pervasive in nature and biologists are faced with challenge of understanding how these chemical impact ecological community. A diversity of pesticides and their residues are present in a wide variety of aquatic habitats [1,2,3]. While pesticides have the potential to affect many aquatic taxa, the impacts on amphibians are of particular concern in the past decade because of the apparent global decline of many species [4,5,6]. The lists of possible causes of amphibian declines are numerous and pesticides have been implicated in at least some of these declines. Pesticides occur in amphibian habitats [7,2], amphibians living with insecticides in these habitats exhibit physiological signatures of these pesticides and declining population are correlated with greater amounts of upwind agriculture where pesticide use is common. While these correlative studies suggest that pesticides may affect amphibian communities, there are few rigorous experiments to confirm that pesticides are altering amphibian communities.

The widespread application of pesticides has attracted the attention of ecologists that struggle to understand the impact of the chemical on natural communities. A large number of laboratory-based single species studies of pesticides, such studies can only examine direct effect. However in natural communities, species can experience both direct and indirect effects.

World wide amphibian diversity and population numbers have been reported to be declining [8,9,10]. Pesticides are sometimes implicated yet few studies have been conducted

to determine if pesticides actually present a hazard to them [11]. In addition, most published studies on the effects of pesticides on amphibians have been conducted on embryo and tadpole life stages [12,13,14,15,16]. Only one study has been conducted on the effects of malathion (diethyl mercaptosuccinate, S-ester with O, O-dimethyl phosphorodithioate) on amphibians in a post-metamorphic life stage. Two woodland salamander species (*Plethodonglutinosus* and *P. cinereus*) to substrates which malathion had been applied to. *Plethodonglutinosus* showed significant inhibition of cholinesterase activity after 3 days of exposure to a 5.6 kg/ha application of malathion.[17]. *Plethodoncinereus* did not show this effect, thus indicating variations in species susceptibility to malathion. In the 1980's, malathion was applied annually to 4,486,000 ha in the United States [18]. It is used most commonly in the control of mosquitoes, flies, household insects, animal ectoparasites, and human lice. Malathion has been element labeled and applied to fields to study its potential translocation and bioaccumulation; and small rodents, insects and birds had detectable levels 1 yr after treatment [19]. Malathion is lipophilic and readily taken up through the skin, respiratory system, or gastrointestinal tract, with absorption enhanced if malathion is in the liquid form [20]. The predominant mechanism of organophosphate toxicity is inhibition of acetylcholinesterase in the nervous system causing accumulation of acetylcholine [21]. This causes hyper excitability and multiple postsynaptic impulses generated by single presynaptic stimuli. Minimal work has been conducted on effects of organophosphorus compounds on disease susceptibility. At intraperitoneally injected doses above 230 mg/kg the mice showed chromosomal abnormalities at 6 hr post-injection [22]. Humans occupationally exposed to organophosphorus compounds, including malathion, have marked impairment of neutrophil chemotaxis.[23] In addition, these workers had increased frequency of upper respiratory infections which increased with the number of years of exposure to organophosphorus compounds. Organophosphorus compounds can also affect immune function of macrophages and lymphocytes in culture [24,25].

The main objectives of the present investigation is to find out the toxic effect of malathion on total protein, total lipid and total carbohydrate content in brain and liver of *Bufo melanostictus* as well as to observe the changes in hematological parameters in Indian Toad exposed to Malathion.

2. Materials and methods

Both male and female toads (*B. melanostictus*) of various size (male body weight ranging from 21-65 gm and female body weight ranging from 13-100 gm) were collected during night time and the test samples were brought into the laboratory and immediately transferred to the glass container supplemented with mud and sand to provide a natural habitat to the Indian toad. The samples were feed with liver and earthworm along with adequate water. The samples were maintained at room temperature for a period of seven days for acclimation to the laboratory condition and then used for experimentation in the eighth day.

To study the effect of Malathion, ten toads were placed in each glass container irrespective of sex and size and sorted out in to two groups of each experiment i.e., one set is for control (without

Malathion) and another is for experiment (with Malathion). One ml of Malathion in concentrations of 25 ppm and 50 ppm (in acetone as solvent) each were injected subcutaneously in the abdominal region of the Test samples species with the help of an insulin syringe.

After which the samples were sacrificed by pitching and both liver and brain tissue were dissected out to estimate the protein, lipid and carbohydrate content. The blood was collected to estimate the Hb, WBCs and RBCs in both experimental set and control set. Total protein, [26], Lipid [27] and Carbohydrate [28] contents were estimated in the Brain and liver of *Bufo melanostictus* at 24 h, 48h, 72 hr and 96 hrs post-treatment with the test chemical. Sahli's haemoglobinometer was used to estimate of haemoglobin RBC count was done by Neubaur's improved double haemocytometer using Hayem's solution as diluting fluid whereas for WBC count instead of Hayem's solution, Turk's fluid (W.B.C. diluting fluid) was used. A batch of untreated (control) sample was also kept for comparison purposes.

The data obtained were analysed by using SPSS 10.0 package (SPSS INC, USA) and Two-way ANOVA test was applied to find out the significant difference between the exposure period and concentrations.

3. Results

Total protein content

In Malathion-treated samples after 24h exposure the reduction in protein content in liver was found to be 22.22% and 30.55%. In the Brain tissue the reduction was 75% and 44% in the malathion-treated samples at concentrations of 25 and 50 ppm respectively. At 48 hour of exposure the reduction in protein content was 31.42% and 40% in liver whereas in brain the reduction was 73.33% and 80%. Similarly during 72 hour of exposure the reduction in protein content was 34.28% and 42.85% in liver whereas in brain the reduction was 82.35%. During 96 h duration the reductions in protein content in the liver were recorded as 42.85% and 48.57%. In brain the decrease was 82.35% and 88.23% in the treated samples at the desired concentrations of malathion respectively (Table 1).

Exposure Duration in hour	Control		25 ppm		50 ppm	
	Liver	Brain	Liver	Brain	Liver	Brain
24	0.36±0.021	0.18±0.008	0.28±0.016 (22.22%)	0.05±0.021 (72.22%)	0.25±0.014 (30.55%)	0.04±0.014 (77.77%)
48	0.35±0.014	0.15±0.008	0.24±0.014 (31.42%)	0.04±0.08 (73.33%)	0.21±0.016 (40%)	0.03±0.024 (80%)
72	0.35±0.014	0.17±0.021	0.23±0.016 (34.28%)	0.03±0.094 (82.35%)	0.20±0.014 (42.85%)	0.03±0.007 (82.35%)
96	0.35±0.021	0.17±0.021	0.20±0.014 (42.85%)	0.03±0.014 (82.35%)	0.18±0.014 (48.57%)	0.02±0.008 (88.23%)

Table 1. Shows the protein content in both liver and brain tissue in *B. melanostictus* exposed to 25 ppm and 50 ppm of malathion. The data in parentheses reflects the percent decrease over control in the protein content

Subjected to two way ANOVA a significant difference was observed between the exposure period ($F_{1\ 0.05} = 6.02$) as well as between the concentrations ($F_{2\ 0.05} = 92.46$) in case of liver tissues whereas a non significant difference was observed between the exposure period ($F_{1\ 0.05} = 2.96$) in brain tissue. However, between concentration significant difference was observed ($F_{2\ 0.05} = 374.22$)

Total lipid content

Total lipid content was estimated in the liver and brain of the treated organisms. After 25 ppm and 50 ppm of Malathion treatment, for 24 h the lipid content was found to be 56.36% and 61.81% in Malathion treated liver respectively. In the brain of Malathion treated toad the reduction was 64% and 68 % respectively. At 48 h exposure the decrease in lipid content in liver was 58.18% and 63.63% where as in brain it was 65.21% and 69.56%. Simultaneously, during 72 h of treatment the percent reduction in total lipid content in Malathion treated liver was 60% and 65.45% and in brain 66.66% and 75% was observed respectively. At 96 hour of treatment with 25 ppm and 50 ppm of Malathion the lipid content was found to be 61.81% and 65.45% respectively. In case of Malathion treated brain of the test samples the reduction was found to be 69.56% and 78.26% (Table 2). Subjected to two way ANOVA test a non significant difference was observed between the exposure duration ($F_{1\ 0.05} = 3.47$) where as between the concentrations significant difference was noticed ($F_{2\ 0.01} = 3256.06$) in case of liver tissue. Simultaneously the data obtained from the treated brain a significant difference was found between exposure period and the concentrations. ($F_{1\ 0.05} = 11$ and $F_{2\ 0.01} = 1461$).

Exposure Duration in hour	Control		25 ppm		50ppm	
	Liver	Brain	Liver	Brain	Liver	Brain
24	55±0.81	25±1.42	24±1.41 (56.36%)	9±1.63 (64%)	21±1.41 (61.81%)	8±1.41 (68%)
48	55±0.41	23±0.81	23±2.82 (58.18%)	8±1.41 (65.21%)	20±1.41 (63.63%)	7±1.42 (69.56%)
72	55±0.71	24±0.82	22±0.81 (60%)	8±1.63 (66.66%)	19±1.41 (65.45%)	6±0.81 (75%)
96	55±1.63	23±0.85	21±0.021 (61.81%)	7±1.63 (69.56%)	19±1.41 (65.45%)	8±0.81 (78.26%)

Table 2. Reflect the Lipid content in both liver and brain tissue in *B. melanostictus* exposed to 25 ppm and 50 ppm of malathion. The data in parentheses reflects the percent decrease over control in the Lipid content.

Total carbohydrate content

In this present experiment, when the toads were exposed to the desired concentrations of the test chemical for different time interval a drastic reduction in total carbohydrate content in liver as well as in brain tissue was observed. After 25 ppm and 50 ppm of

Malathion treatment, for 24 h the carbohydrate content was found to be 45.58% and 54.41% in liver tissue respectively. In the brain tissue the reduction was 55.28% and 57.14 % respectively. At 48 h exposure the decrease in carbohydrate content in liver was 53.96% and 57.14% where as in brain it was 60.52% and 63.15%. Simultaneously, during 72 h of treatment the percent reduction in total carbohydrate content in liver was 60% and 61.53% and in brain 60.6% and 63.63% was observed respectively. At 96 hour the carbohydrate content in both liver and brain was found to be 60.93%, 64.06% and 66.66% respectively in both the concentrations (Table 3). When the data obtained in case of liver and were analyzed by two way ANOVA test a significant difference was observed between the exposure duration ($F_{1\ 0.05} = 11.67$) and between the concentrations ($F_{2\ 0.01} = 939.50$). Whereas, the data obtained from the treated brain non significant difference was found between exposure periods ($F_{1\ 0.05} = 1.37$) however, a significant difference was noticed between the concentrations $F_{2\ 0.01} = 781.25$).

Exposure Duration in hour	Control		25 ppm		50ppm	
	Liver	Brain	Liver	Brain	Liver	Brain
24	0.68±0.008	0.35±0.008	0.33±0.036 (45.58%)	0.16±0.021 (55.28%)	0.31±0.021 (54.41%)	0.15±0.014 (57.14%)
48	0.63±0.016	0.38±0.008	0.29±0.012 (53.96%)	0.15±0.008 (60.52%)	0.27±0.016 (57.14%)	0.14±0.014 (63.15%)
72	0.65±0.016	0.33±0.016	0.26±0.021 (60%)	0.13±0.094 (60.60%)	0.25±0.021 (61.53%)	0.12±0.008 (63.63%)
96	0.64±0.008	0.36±0.016	0.26±0.021 (60.93%)	0.12±0.014 (66.66%)	0.23±0.016 (64.06%)	0.12±0.008 (66.66%)

Table 3. Reflect the Carbohydrate content in both liver and brain tissue in *B. melanostictus* exposed to 25 ppm and 50 ppm of malathion. The data in parentheses reflects the percent decrease over control in the carbohydrate content

Hemoglobin content

After treatment with 25 ppm and 50 ppm of Malathion in different time interval the bloods from the test samples were collected and hemoglobin was measured. From the result it was observed that during 24 hr of exposure the percent reduction in hemoglobin content was 26% and 6.57 %. At 48 hr of treatment the percent reduction in hemoglobin in malathion treated blood was found to be 7.89% and 9.21%. After 72 hour of exposure a reduction of 7.89% and 10.52% in the hemoglobin content was observed for 25 ppm and 50 ppm concentration respectively. A decrease of 8% and 10.66 % was found after 96 hour of exposure (Table 4).

When the data were subjected to two-way ANOVA a significant difference was observed between the exposure periods ($F_{1\ 0.05} = 6.55$) as well as between the concentrations ($F_{2\ 0.05} = 97.80$)

Exposure Duration in hour	Control			25 ppm			50ppm		
	Hb	WBC	RBC	Hb	WBC	RBC	Hb	WBC	RBC
24	7.6±0.081	4.96±0.94	7.65±0.47	7.2±0.16 (5.26%)	4.76±0.47 (4.03%)	7.55±1.69 (1.30%)	7.1±0.16 (6.57%)	4.28±0.94 (13.70%)	7.54±1.88 (1.43%)
48	7.6±0.081	4.96±1.69	7.64±0.94	7±0.16 (7.89%)	4.68±1.88 (5.64%)	7.54±1.88 (1.30%)	6.9±0.14 (9.21%)	4.22±0.47 (14.91%)	7.43±0.47 (2.74%)
72	7.6±0.21	4.96±0.94	7.63±1.69	7±0.14 (7.89%)	4.42±0.94 (10.88%)	7.38±0.94 (3.27%)	6.8±0.21 (10.52%)	4.09±0.47 (18.54%)	7.29±0.47 (4.45%)
96	7.5±0.081	4.96±0.94	7.62±2.49	6.9±0.14 (8%)	4.09±0.47 (17.54%)	7.15±1.69 (6.16%)	6.7±0.17 (10.66%)	3.84±0.47 (22.58%)	7.08±0.47 (7.08%)

Table 4. Reflects the Hb, WBC and RBC content in *B. melanostictus* exposed to 25 ppm and 50 ppm of malathion. The data in parentheses reflects the percent decrease over control in the Hematological parameters.

WBC Content

From the experiment it was observed that the WBC content of *B. melanostictus* was also reduced drastically. After 24 hr of exposure to 25 ppm and 50 ppm of malathion the decrease in the WBC was found to be 4.03% and 13.70%. Similarly at 48 hr a drastic reduction of 5.64% and 14.91% in the WBC content of toad was found at 25 ppm and 50 ppm of malathion concentration. At 72 hour the percent inhibition of 10.88% and 18.54% was recorded respectively and after 96 hour of exposure to the desired concentrations of the test chemical the reduction in WBC content was found to be 17.54 % and 22.58%.

Subjected to two-way ANOVA, non-significant difference was observed between the exposure periods ($F_{1, 0.05} = 2.88$) whereas between concentrations a significant difference was observed ($F_{2, 0.05} = 31.43$).

RBC Content

From the experiment it was observed that the RBC content of *B. melanostictus* was also reduced drastically like that of WBC content. After 24 hr of exposure to 25 ppm and 50 ppm of malathion the decrease in the RBC was found to be 1.30% and 1.43%. Similarly at 48 hr a drastic reduction of 1.30% and 2.79% in the RBC content of toad was found at 25 ppm and 50 ppm of malathion concentration. At 72 hour the percent inhibition of 3.27% and 4.42% was recorded respectively and after 96 hour of exposure to the desired concentrations of the test chemical the reduction in RBC content was found to be 6.10 % and 7.08%.

Subjected to two-way ANOVA, non-significant difference was observed between the exposure periods ($F_{1, 0.05} = 4.68$) whereas between concentrations a significant difference was observed ($F_{2, 0.05} = 8.83$).

4. Discussion

The organophosphates are compounds widely used as insecticides and chemical warfare agents. Although extremely toxic in some cases, these materials are generally short lived in

the environment compared to halogenated organics and related compounds. The toxicity of an organophosphate is related to its leaving group, the double bonded atom, usually O or S and the phosphorous ligands, the groups surrounding the phosphate in the compound. The metabolic replacement of sulphur by oxygen in the liver or other detoxification organ activates the sulphur containing organophosphate into a much more potent form. The extreme toxicity of these compounds is due to their ability to bind to the amino acid serine, rendering it incapable of participating in a catalytic reaction within enzyme as the further blocking of the active site by the organophosphate residue.

The decrease of total protein content in both liver and brain is may be due to less incorporation of amino acids in the translation process i.e., a reduced incorporation into any kind of proteins and pesticides disturb the protein synthesis. In the present study the total protein content in both liver and brain in Indian Toad decreased after malathion (25 ppm and 50 ppm) treatment.

The reduction in total protein contents after pesticide application in different insects was reported by many workers. See [29, 30, 31, 32, 33, 34, 35]. The protein reduction the liver and kidney of reptiles was also reported [36,37]. The present investigations also appear to be in line with the earlier findings. The present results therefore confirm the findings in this respect.

Carbohydrates are less sensitive as compared to lipids. A reduction in the glycogen concentration in the treated groups could have happened due to activation of glycogenolytic enzymes like phosphorylase system leading to decrease in glucose concentration by malathion in the liver tissues of treated animals. The treated animals being under malathion stress, the stress hormone (epinephrine) released from the adrenal medulla possibly have acted on the liver tissues via circulation leading to glycogenesis, mediated by adenylatecyclase, cAMP, protein kinase and finally the activated phosphorylase system. From this present investigation it was observed that, malathion has a strong potential to reduce the carbohydrate content in liver and brain tissue of treated toad.

In the current study, the decreased total lipid content may possibly due to either decreased lipogenesis or suppressed translocation/transportation of lipid to plasma. The effect of the doses of whole body treated seems to have acted in same way to depress lipogenolysis possibly by denaturing or by inactivating some of the lytic enzymes, or by hampering the transportation of these molecules to other steroidogenesis tissues via the plasma pool due to alternation in membrane functions. Therefore the enhanced level of cholesterol concentration may have contributed to an overall decrease in the total lipid pool of the liver tissue of the treated animals.

The stress induced changes in the total leucocytes count and differential count in mammals have been reported [38,39]. The release of granulocytes from bone marrow as a result of stress induced stimulation mediated by corticosteroids a stress hormone may be the possibility [40]. The lymphocytes which constitute the dominant leucocytes type in toads appear to decrease. Such as decrease in the lymphocytes count may either be due to rupture or degradation of some of these aged circulating immuno competent cells.

The hemolysis of Red blood cells have been reported in various physical and chemical stress [41,42] Under such condition the total circulation red cell population is expected to show a decline in number. The observed decrease in the circulating red cell count can be accounted for the possible mechanisms such as decrease production of renal erythropoietin which stimulates the bone marrow and spleen to release more erythrocytes. From this experiment it was observed that malathion has a strong potential to reduce hemoglobin, WBC and RBC in *Bufo melanostictus*.

Author details

Malaya Ranjan Mahananda and Bidut Prava Mohanty

Department of Environmental Sciences, Sambalpur University, Jyoti-Vihar, Burla, Orissa, India

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Evaluation of Earthworms Present on Natural and Agricultural-Livestock Soils of the Center Northern Litoral Santafesino, República Argentina

María Inés Maitre, Alba Rut Rodríguez,
Carolina Elisabet Masin and Tamara Ricardo

Additional information is available at the end of the chapter

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1. Introduction

For a long time in Argentina, farmers had concentrated on a mixed system of livestock and crops, mainly wheat and corn. Soybean was not a traditional crop [1].

During the 1990's (20th century) the agricultural frontier reached a planted surface that rose 11.000.000ha with the adoption of technologies that includes transgenic seeds, limited tillage and application of pesticides and fertilizers [2, 3]. The main crops are soybean monocultures and soybean/wheat soybean double crops corn. This two crop sequences have different C dynamics response to management such as tillage or fertilization [4]. In the 2000-2009's pesticide market had triplicate [5] being the most popular endosulfan, lambda-cyhalothrin, cypermethrin, chlorpyrifos, metamidophos and the herbicides glyphosate, atrazine and 2,4D [6]. Importance of edaphic fauna to the soil fertility is well known, especially with oligochaeta that are being used as bioindicators of the soil health [7-13].

Earthworms spent their whole life cycle in the soil horizons and because of their feeding and burrowing behaviour they directly or indirectly help to improve every physical, chemical and biological process of the soil. Earthworms participate in the mixing of organic and inorganic fractions of soil, formation of stable clusters, dynamic and recycling of nutrients from the decomposition of organic matter, their burrows help to the aeration, infiltration and drainage of soil [14]. Earthworms represent over the 80% of the biomass invertebrate of the soil, therefore they are an useful group to evaluate the effect of pesticides either on field as on laboratory tests. The ecotoxicological impact of Argentina's current production system

is not well known and evaluated and requires the realization of deeper studies using fast and reliable bioindicators in order to understand the biological processes related with the anthropogenic alterations of the environment [15-28]. Therefore we performed ecotoxicological bioassays under laboratory conditions and field evaluations of parameters such as biomass, richness and density of earthworms.

2. Materials and methods

2.1. Field experiences

2.1.1. Study site description

In order to evaluate the influence of different production systems over the oligochaetofauna, five sites from different areas of the north and centre of Santa Fe province were sampled (Fig.1, Table 1).



Figure 1. Map of the sampling sites.

1. *Livestock in woodland (Ganadería en Monte Nativo: GMN)* located in Naré, is a native woodland, used for bovine livestock alternated with fallow periods. Characterized by the presence of *Prosopis nigra*, *Eucaliptus spp.*, *Acacia acaven*, *Erythrina crista-galli*, *Enterolobium contortisiliquum*, *Cynodon dactylon*, *Cynodon rotundus*, *Digitaria sanguinalis*.
2. *Fallow field (Lote en Descanso: LD)* located in Sarmiento, used 3 years ago as paddock for bovine livestock. At present without activities. Characterized by the presence of *Cynodon dactylon*, *Cirsium vulgare*, *Solanum sisymbriifolium*, *Digitaria sanguinalis*, *Melia azedarach L.*
3. *Non Tillage (Agrícola con Siembra Directa: ASD)* located in Isleta Norte, with over 30 years of agricultural practices (last 15 years with minimum tillage). Crops consisted in sunflower, soybean, corn and sorghum. Pesticides applied were glyphosate-coadyuvants (4.5 l.ha⁻¹), atrazine (3 l.ha⁻¹) and superphosphate triple calcium fertilizer (65 kg.ha⁻¹).
4. *Non Tillage with added organic amendments (Agrícola con Siembra Directa y Abono Orgánico: ASDAO)* located in Ocampo Norte, with over 20 years of agricultural practices with minimum tillage. Crops consisted in soybean, sunflower, cotton, corn and oat. Synthetic fertilizers superphosphate triple calcium (65 kg.ha⁻¹) and organic fertilizers (cow dung), glyphosate (4 l.ha⁻¹) and clorimuron (60g.ha⁻¹) are applied.
5. *Livestock in grassland (Ganadería en Pastizal Natural: GPN)* located in El Sombrerito, is a natural grassland used for the feeding of ovine cattle, rotating with fallow periods. Agrochemicals and mechanic weed control are not used. Vegetation consist of *Erythrina crista-galli*, *Enterolobium contortisiliquum*, *Brachiaria platyphilla*, *Digitaria sanguinalis*, *Paspalum quadrifarium*, *Cynodon dactylon*, *Sorghum halepense*, and others.

Sites	Geographical Coordinates		Location	Year/ Season
	South Latitude	West Longitude		
1. Livestock in woodland (GMN)	30° 53' 12.09"	60° 27' 01.92"	Naré (San Justo)	2009/Spring
2. Fallow field (LD)	31° 04' 25,11"	61° 10' 32,30"	Sarmiento (Las Colonias)	2011/Spring
3. Non tillage (ASD)	28° 27' 34.18"	59° 18' 57.17"	Isleta Norte (Gral. Obligado)	2011/Summer
4. Non tillage with added organic amendments (ASDAO)	28° 27' 21.84"	59° 22' 26.93"	Ocampo Norte (Gral. Obligado)	2011/ Summer
5. Livestock in grassland (GPN)	28° 38' 00.43"	59° 27' 59.79"	El Sombrerito (Gral. Obligado)	2011/ Summer

Table 1. Geographical coordinates of the sampling sites and sampling season.

2.1.2. Field sampling design

Twenty samples were taken for each site using a zigzag transect of random direction according to the TSBF standard method [29]. Each sample consisted of a soil block of 0,30 x 0,30 x 0,30 m with a distance between samples of 15 m, to assess the independence of data from each block. The collect of earthworms: adult, juveniles and cocoons took place at laboratory. Sexually mature earthworms (clitellated) were anesthetiated according to methodology described by Moreno & Borges [30]. The identification of earthworms was performed using a binocular stereoscopic microscope and the species diagnosis was performed according to Mischis taxonomy [31]. Abundance (total number of organisms on sampled site), species richness (number species on sampled site (S)) and density (org/m²) were recorded.

After the extraction and counting of earthworms, a soil sample of each site was taken to perform physical and chemical analysis at a soil specialized laboratory (IDICYT-Universidad Católica de Santa Fe). Pesticide residues determination were performed at Laboratorio de Medio Ambiente (INTEC-Universidad Nacional del Litoral). Insecticide residues were determinate by gas chromatography (GC) and herbicide residues by high performance liquid chromatography (HPLC) with specific detectors. Statistical analysis of the mean was performed using one-way ANOVA and Tukey's multiple test ($p < 0.05$).

2.2. Bioassay characterization

Our main goal was to assess under laboratory conditions, the effects of the pesticides endosulfan, glyphosate and lambda-cyhalothrin on the earthworms *Eisenia fetida* and *Aporrectodea trapezoides* (standard bioassay species and common species in the sampled sites respectively) from the family Lumbricidae (Oligochaeta). In all the experiences, the laboratory tests were validated according to ISO 11268-1 Guideline [32], with a room temperature of 23± 2°C and photoperiod of 16:8 (intensity~800 lux). Acute and chronic tests were performed in OECD artificial soil or reference soil according to the OECD protocol [33]. Due the arthropod-specific action of pyrethroids their acute toxicity to earthworms is low and is more suitable to perform an avoidance test as an alternative to rapid toxicity assessment based on behavioural responses [34].

Earthworm adults (clitellated) *Eisenia fetida* or *Aporrectodea trapezoides*, obtained from our laboratory stock culture were used. Before starting the bioassays, earthworms fasted 24 hours to clean their guts. Transparent polypropylene boxes of 20x10x15cm with perforated lids were filled with 500g of dry substrate. The moisture content was kept using distilled water for the control groups and pesticide solutions with the proper concentration for each treatment. Hydrophobic pesticides as lambda-cyhalothrin and endosulfan were mixed with hexane to obtain the desired concentrations and placed under hood for 24 hours to evaporate the solvent. For *E. fetida* bioassays organisms were fed with dry, triturated and sieved cow dung on a 7 days frequency. For the *A. trapezoides* bioassays organisms were fed with a mixture (1:3) of cow dung (same as described before) and domestic organic residues on a 20 day basis. Survival (number of living organisms), moist weight (expressed on

grams), cocoon production and juveniles number was recorded weekly or monthly for *E. fetida* and *A. trapezoides* bioassays respectively.

Statistical approximation of effective concentration (EC₅₀) was obtained graphically and lethal concentration (LC₅₀) by Probit analysis. For the biomass and reproduction parameters one-way Anova followed by a post hoc Dunnet Multiple Comparison test were performed.

2.2.1. Experiences with endosulfan

Even when their application and manufacture is banned or restricted in several countries, endosulfan (Class II) [35] is one of the most used organochlorinated insecticides and acaricides in Argentina, especially in Santa Fe province. Nevertheless the endosulfan importation is going to be banned in July 2012 and their formulation and use will be banned in July 2013 [36]. Endosulfan is applied to control several pests as *Rachiplusia un*, *Nezara viridula*, *Piezodorus guildinii*, *Spodoptera frugiperda*, *Colias lesbia*, *Heliothis zea*, *Spilosoma virginica* and *Anticarsia gemmatalis*. Due their high biocide action is applied both on extensive crops (soybean, corn, wheat, alfalfa, cotton, fruit crops and tea crops) as on intensive crops (floricultural and horticultural). Their physicochemical characteristics, frequency and application doses make endosulfan a highly available pesticide for earthworms who are either responsible of organic matter degradation and humification processes in the soil than an important prey for many predators (20; 10). Prior to the bioassay the percent of active ingredient of commercial endosulfan (Atanor® 35%) was determined by GC using VARIAN 3700 with electronic capture detector. Acute toxicity test consisted in a range of 5 concentrations and a control group. They were set in 4 replicates containing OECD soil and 10 *Eisenia fetida* earthworms clitellated and with a mean weight of 300mg (ea) per box. Exposure time was 14 days and at the end of the test the number of dead organisms was recorded. For the chronic toxicity test range was 2; 3; 4; 7; 10 mg.kg⁻¹dw, with an exposure time of 56 days to test survival and biomass. Cocoon and juveniles production was recorded at 56 and 84 days respectively and they were kept in boxes with the corresponding treatments until control juveniles developed a clitellum.

In order to perform degradation in soil analyses, samples of 10g of soil from the 2; 4; 10 mg.kg⁻¹dw treatments were taken and sent to the gas chromatography laboratory for further analysis. Samples were analyzed by triplicate according to Miller & Miller [37]. Detection limits were 0.003; 0.003 and 0.005 mg.kg⁻¹ for endosulfan α, β and endosulfan sulfate respectively.

In order to assess the kinetics of degradation of biocide studied, an exponential regression analysis was performed for each data group: concentration vs. time of exposure. The velocity constant was determined for each case through the equation:

$$C_t = C_0 * e^{-kt} \quad (1)$$

Where C_t=pesticide concentration at time *t* (mg.kg⁻¹); C₀=initial concentration (mg.kg⁻¹); k=days⁻¹; half-life time, t_{1/2} (days), was determined by Equation (1) replacing C_t by C₀/2 resulting in: t_{1/2} =ln 2/k.

2.2.2. Experiences with glyphosate

Transgenic soybean crops require the utilization of the herbicide glyphosate (Class III) [35]. Their persistence in the soil matrix could vary from days to months and depends of multiple edaphic and climatic factors. Prior to the bioassay the percent of active ingredient of commercial glyphosate was determined by HPLC with post column derivatization and Millennium³² data acquisition system.

Chronic bioassays at the sublethal concentration range: 7; 11; 18; 30; 50 mg.kg⁻¹dw of commercial glyphosate (Round up Monsanto® 48%) were performed. Each treatment and the untreated control group were set in 4 replicates containing reference soil and 10 adult clitellated *E. fetida* with a mean weight of 450mg (ea) per box. Glyphosate was mixed with distilled water in order to reach a 50% moist content. The same moist content was used for control groups by applying distilled water. Exposure time was 28 days. Detection limits were 0.05 µg.kg⁻¹ for glyphosate and their metabolite AMPA. Soil physicochemical parameters: humidity, C, N, C/N, texture, pH, CIC, P and soluble K were analyzed.

2.2.3. Experiences with Lambda-cyhalothrin

Lambda-cyhalothrin (Class II) [35] is a 4th generation pyrethroid insecticide widely used in Santa Fe province. It is highly active against a broad spectrum of pests in public and animal health and is also used in agriculture to control several pests such as hemiptera and lepidoptera in both extensive and intensive crops [38, 39]. Pyrethroids interfere with the normal function of nervous system of invertebrates. Their toxicity on non-target soil organisms is observed even at concentrations lower than the agricultural application rates [20, 21, 26, 40, 41].

2.2.3.1. *Eisenia fetida* assays

Bioassays were performed using commercial lambda-cyhalothrin (Cilambda Ciagro® 5%). The moist content was 25% for avoidance test and 50% for chronic test. For the chromatographic determination on both soil and organisms, 98% lambda-cyhalothrin isomers mix, Chem. Service® was used. Concentration range for the 48 hours avoidance behaviour test was: 1.25; 7.5; 16.25; 32.5; 65 mg.kg⁻¹dw, according to the ISO 17512-1 protocol [42]. Each treatment was set in 3 replicates. Plastic boxes were divided in two compartments using a piece of plastic fitted transversally in the box. One half of the box was filled with contaminated OECD soil and the other with OECD control soil. Then the separator was removed and 10 clitellated adults of *E. fetida* were placed in the separating line of each container test. The boxes were then covered with perforated plastic lids. At the end of the test period, the control and treatment soil were carefully separated and the number of earthworms in each section was determined. Individuals found between sections were considered as being in the soil as which the head was directed. Organisms found dead are considered as affected by the toxic [34, 38, 43].

For the statistical interpretation of avoidance test results the “Habitat Function” [44] was applied, considering toxic those soils where less than the 20% of the organisms were

found. The response of the organisms was measured using the equation proposed by Garcia [34]:

$$NR = ((C - T) / S) \times 100 \quad (2)$$

Where: NR=net response; C=sum of earthworms observed in control soil; T=sum of earthworms observed in treated soil; S=total number of earthworms per replicate

Concentration ranges for the chronic test were: 1; 2; 4; 8 mg.kg⁻¹dw. Each treatment and the control were set with 4 replicates containing 10 clitellated adult *E. fetida* with a mean weight of 272 ± 15 mg (ea). Exposure time was 56 days to test survival, biomass and cocoon production. After the weekly register of cocoons, they were placed in a separate plastic box containing test or control soil and allowed to hatch. Number of juveniles was recorded weekly for an exposure time of 63 days and the results were fitted with a logistic regression model [34]:

$$Y = \frac{c}{1 + e^{(b \cdot (x-a))}} \quad (3)$$

where: Y=number of juveniles; a=natural logarithm (Ln) of EC₅₀ (mg.kg⁻¹); b=slope; c=mean number of juveniles in control; x=Ln of concentration (mg.kg⁻¹)

Surviving organisms from the concentrations 2, 4 and 8 mg.kg⁻¹ were aconditionated to assess the bioaccumulation at the end of test using the equation according ASTM [45] protocol:

$$BAF = \frac{C_B}{C_S} \quad (4)$$

where: BAF=bioaccumulation factor; C_B=earthworm tissue concentration (mg.kg⁻¹); C_S=soil concentration (mg.kg⁻¹)

In order to perform lambda-cyhalothrin degradation in soil analyses, samples of 10g from concentration 2; 4 and 8 mg.kg⁻¹dw were taken at days 0, 56 and 86. Samples were dried at room temperature, extracted twice with proper solvent, cleaned-up, concentrated and analyzed by GC (d.l. 29ng.kg⁻¹).

2.2.3.2. *Aporrectodea trapezoides* assays

Concentration range for the 24 hours avoidance test were 1; 3; 9; 27 mg.kg⁻¹dw. Each treatment were set in 3 replicates containing a reference soil with moist content of 30% and 6 clitellated adults of *A. trapezoides*. At the end of the test period, the control and treatment soil were carefully separated and the number of earthworms in each section was determined as described for *E. fetida*. The response of organisms was measured using Equation 2 and the EC₅₀ value was determined. Concentration range for the chronic test were 4.7; 6; 8 mg.kg⁻¹. Each treatment and control were set with 3 replicates with a moist content of 35% and 5 clitellated adults *A. trapezoides* with a mean weight of 750±0,5 mg (ea). Exposure time was 70 days to test survival, behaviour and biomass.

3. Results and discussion

3.1. Field experiences

Sites ASD and ASDAO had the more acidophilus soils (Table 2). Those pH values could be explained by the exportation of bases that these soils suffers because of soybean crops and the application of nitrogenated fertilizers [46, 47]. Soils with pH between 6 and 7.5 as the GMN, LD and GPN from our research are consider optimums for the growth of the main cultures of this region [48].

Organic matter (OM) and the relation between Carbone (C) and Nitrogen (N) of the soil (C/N relation) are key indicators of the health and fertility of soil. Organic matter content was higher in LD site followed by GMN and GPN, this could be explained by the presence of weeds covering the soil which are incorporated as organic matter after the plant dies [49]. In the site ASDAO, even if the soil exploitation includes non tillage and addition of synthetic fertilizers and cow dung as organic fertilizer, the OM, C and N values were the lowest from all the tested sites (Table 2) indicating that the levels also depends on the intensity of the exploitation [48, 50-53].

	Site				
	GMN	LD	ASD	ASDAO	GPN
pH	6.05	6.10	5.80	5.80	6.00
OM	2.60	4.04	1.87	1.56	2.06
C (%g)	1.51	2.34	1.08	0.90	1.19
N (%g)	0.14	0.23	0.10	0.09	0.11
C/N (%G)	11.25	10.00	12.00	11.00	11.00
Conductivity (mmhos/cm)	0.86	0.89	0.24	0.327	0.31
Ca++ (me/100g)	9.30	6.60	11.00	7.80	8.80
Mg++ (me/100g)	1.15	1.00	0.40	0.40	1.00
Na+ (me/100g)	1.04	1.07	0.62	0.50	0.57
K+ (me/100g)	0.80	4.06	0.44	0.50	0.30
Species richness	<i>A. trapezoides</i>	<i>A. trapezoides</i>	<i>A. trapezoides</i>	<i>A. trapezoides</i>	<i>A. trapezoides</i>
	<i>A. rosea</i>	<i>A. rosea</i>		<i>A. rosea</i>	<i>A. rosea</i>
	<i>M. dubius</i>				<i>O. tyrtaeum</i>
Density (org./m²)	54	42	9	87	31

Table 2. Physicochemical and biological characteristics of the sampled sites.

When crops alternate within a short time period as in soybean crops, the amount of weeds and stubbles is low and leads to a progressive loss of fertility. About C/N relation, soils from the all sampled sites showed values between 10 and 12 which indicate an equilibrium between mineralization and humification process. According to López [49] the soil is considered as fertile when the relation C/N is near to 10 as found in LD soil.

Conductivity was high in the LD and GMN soils and the lowest value was for ASD (Table 2). Cationic exchange capacity (C.I.C.) is the ability of the soil to retain and exchange different ion (Ca^{++} , Mg^{++} , Na^+ , K^+) and it is influenced by organic matter and mineralization. Site ASD showed high values of Ca^{++} which can be explained by the use of synthetic fertilizers [47]. Sites GMN and LD showed high values of Mg^{++} , Na^+ y K^+ (Table 2) due to the non intensive exploitation [54-56]. Soils were analyzed to determinate the presence of organochlorated, organophosphorated, pyrethroids and herbicides (atrazine and phenoxiacetics) not finding residues over the detection limits (between 5 and 45 ng.g^{-1} for the first three groups; 0.05 $\mu\text{g.g}^{-1}$ for atrazine and metabolites and 0.5 $\mu\text{g.g}^{-1}$ for phenoxiacetics); glyphosate residues could not be determined. Sites were sampled at seasons where precipitations were scarce and temperatures moderate to high, factors that influence bioavailability and water solubility of pesticides. According to Caffarini & Della Penna [57] pesticides impact over non target invertebrates not only by immediate or long term exposition but also affecting factors like the susceptibility and recovering (biological factors). In the last two decades, a significant increase in earthworm casting activity has been observed across the world suggesting that highly toxic and persistent pesticides have been supplanted by less toxic and easily degradable pesticides [58].

Oligochateofauna varied significantly between sites (F 8,09 $p<0.05$), having the ASDAO the higher density followed by sites GMN, LD, GPN and least ASD (Table 2). Agricultural soils with non tillage had effects over the properties of the edaphic environment inducing changes that makes it less favorable for earthworms [59, 60]. Organisms collected from sites ASD and ASDAO were mainly juveniles that could be associated to the presence of pesticides [61-63]. However the highest earthworm density was found at ASDAO, this could be due to the presence of herbicides and organic supplies (cow dung) stimulating the development of microorganisms that are part of the earthworms diet [64]. This compensates the physical and chemical disturbances generated by the agricultural practices [65]. Sites GMN and GPN showed the highest species richness with 3 different species each (Table 2). *Microxcoles dubius* and *Octolasion tyrtaeum* are species from soils rich in OM and with low perturbation levels [9, 66, 67] as the sites as they were found. The genus *Aporrectodea* is generally found in sites with medium to deteriorated fertility [8-10, 68-74]. Even if ASDAO site showed the highest density, general results shows that species richness decrease in soils with higher exploitation.

3.2. Laboratory tests

3.2.1. *Endosulfan*

Lethal bioassay showed a LC_{50} value of 41 mg.kg^{-1} at 14 days. Although these LC_{50} value is greater than those of other authors [75, 76] dead organisms showed inflamed blisters and

sores all over their bodies, surviving earthworms showed either broad zones or small segments with inflammations. At sublethal exposure, survival of earthworms was not affected but the behaviour was affected in all tested concentrations. Control organisms remained vivacious, mobile and clitellated, T₁ organisms (2 mg.kg⁻¹dw) exuded abundant mucus when contacted the soil and remained mobile during the entire test. After the first week, the rest of the treatments (3; 4; 7 and 10 mg.kg⁻¹dw) showed a rigid aspect with abundant coelomic fluid excretion by dorsal pores and mucus through the body wall, symptom that accentuate during the exposure period. Some organisms presented ulcerated inflammations and yellow creamy exudates, they were rigid or moved by rolling either the whole body or the caudal portion. Furthermore Liu *et al.* [77] determined that endosulfan induce DNA damage in earthworms. During the test only control organism increased their weight, while exposed organisms accused significant weight loss (F 38.28 $p < 0,05$) with a notable change after day 14 (F 5.58 $p = 0.003$). Soil surface at the treatments showed traces of food. Stereomicroscope observation of the anterior region of the gut of the exposed earthworms showed no traces of food. The main weight loss (23.01%) corresponded to 10 mg.kg⁻¹dw treatment. In the remaining treatments weight loss oscillated between 14 and 21% (Fig. 2-A). Biomass changes can be a good indicator of chemical stress, which may link chemical effects to energy dynamics and ultimately inhibit growth [78]. Earthworms usually show a recovery a few weeks after being removed of treated soil, however in real life, earthworms cannot be removed from soil exposed to pesticides. Instead, they would continuously be exposed to chemicals until the chemicals degraded [79]. At the start of the experiment it is common that there was no significant difference between the mean biomass of the control group and test group. But at the end of the experience the mean biomass in exposed group is significantly lower because of earthworms are able to resist the toxicant in terms of diminishing energy necessary to support other processes [78-81].

After two week exposed organisms began to lose their clitellum, condition that increased with exposure time and concentration. At the end of the test only the control organisms remained clitellated while in treatments showed a loss of clitellum that ranged between 30 and 100%.

Cocoon production started at day 7 in all treatments decreasing with exposure time and concentration (F 21.49 $p \leq 0.05$). The number of juveniles hatching from treated cocoons was also lower (F 40.59 $p \leq 0.05$); immature organisms were less mobile, exuded coelomic fluid and mucus, being their size smaller than control groups. Survival of treated juveniles ranged from 90 to 75%, their growth experimented a significant delay (F 27.24 $p \leq 0.05$) and never reached sexual maturity (Fig. 3-A, Table 3).

Fecundity in earthworms is sensitive to pesticides even though the earthworms may be not immediately impacted, changes in the reduction of population in the longer term might occur [79]. The effects on the reproductive output can be interpreted either as a direct effect to an interaction with key mechanisms for reproduction, or as an indirect effect, via assimilation of nutrients, growth, and maintenance of the energetic balance [82].

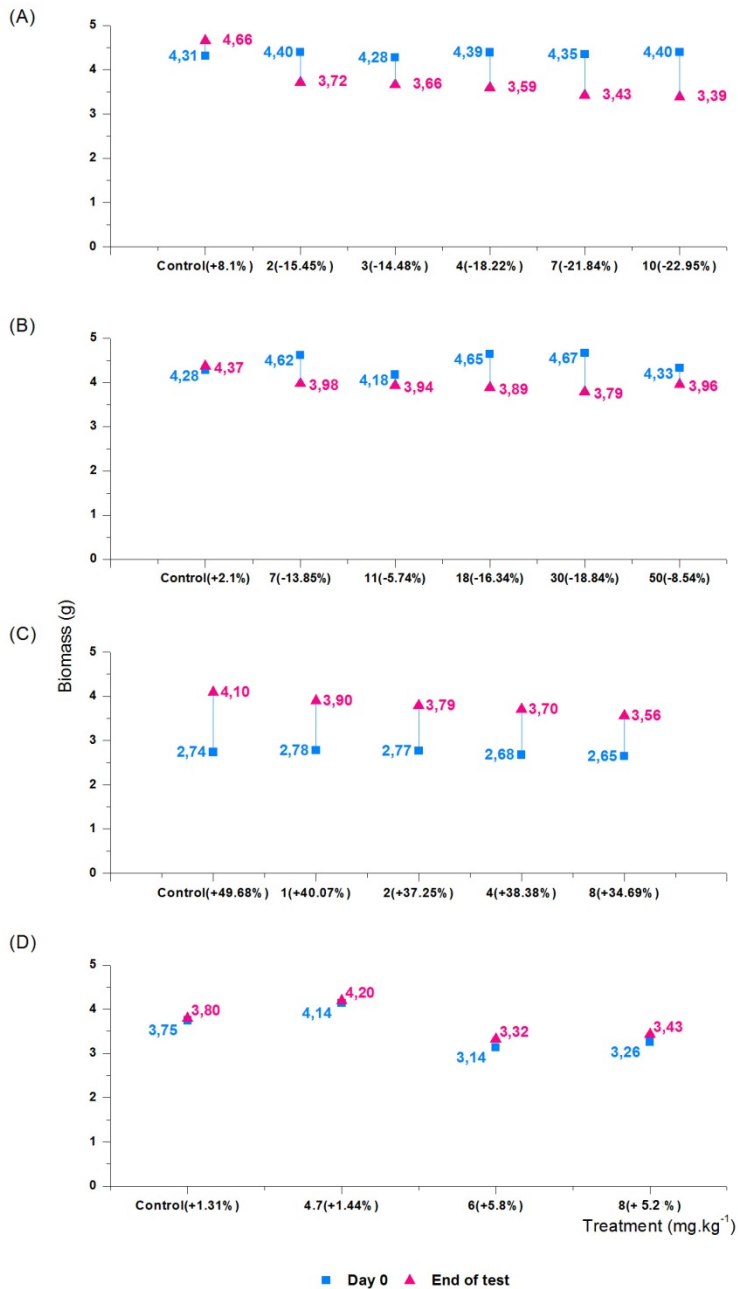


Figure 2. Biomass changes in the chronic toxicity test (percents in brackets). (A-C) experiences with *Eisenia fetida*: (A) Endosulfan (B) Glyphosate, (C) Lambda-cyhalothrin, (D) experiences with *Aporrectodea trapezoides* and lambda-cyhalothrin.

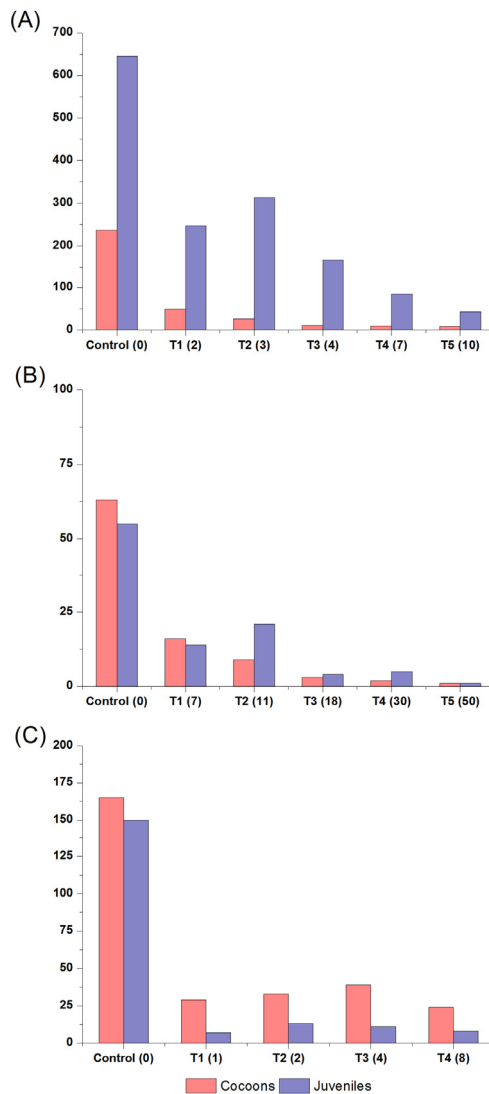


Figure 3. Results of reproduction bioassay: number of cocoons and juveniles. (A) Endosulfan, (B) Glyphosate, (C) Lambda-cyhalothrin. Test concentration mg.kg^{-1} are in brackets.

Degradation in soil assay with 2 mg.kg^{-1} dw endosulfan indicated half-life times of 40 and 60 days for isomers α and β , 47 and 87 days for 4 mg.kg^{-1} and 45 and 86 days for 10 mg.kg^{-1} . In all the treatments the toxic metabolite endosulfan sulphate was detected at days 57, 46 and 66 respectively. According to the bibliography half-life range from 1-2 months for endosulfan and 4-6 months for endosulfan sulphate, being a high threat for soil organisms because of their effect over reproduction.

Treatment (mg.kg ⁻¹ dw)	Increase of biomass (%)	Development of clitellum (days)	Cocoon production (days)
Control	97.35	112	155
2	92.54	-	-
3	90	-	-
4	81.90	-	-
7	66.79	-	-
10	52.27	-	-

Table 3. Biomass, sexual development of juveniles, cocoon production.

Volatilization is important for endosulfan α at soils with high humidity and their mobility is higher than isomer β , being the mobility of isomer β higher than endosulfan sulphate [83]. Tests performed in Australia and Brazil indicates that soil microorganisms degrade endosulfan. Fungi oxidized it to sulphate (high toxicity) and bacteria to diol (30 days), sulphate (60 days) and unknown metabolites (90 days). Microbial activity, humidity and temperature lead to a delay on the recovery of earthworm populations which impacts over the quality of the soil and the trophic relations [1, 83, 84].

3.2.2. Glyphosate

No mortality was registered. Organisms at control group and 7 mg.kg⁻¹ remained active (burrows all over the substrate) while at higher concentrations organisms exudated a high amount of mucus and their burrowing activities decreased with time and concentration corresponding with the observed by Correia & Moreira [85] over different concentrations of glyphosate (10-1000 mg.kg⁻¹). Morowati [86] found that glyphosate induce histochemical changes in the intestine of *Pheretima elongate* which affect survival, feeding and mobility. In the other hand, Pereira *et al.* [87] found that glyphosate (ai) within a range of 6-46 mg.kg⁻¹ and Spasor (commercial glyphosate) ranging from 4-162 mg.kg⁻¹ did not have a negative impact over *Eisenia andrei* behaviour. Pesticides enter to the organisms through ingestion and absorption [85] leading to alterations in the metabolism of earthworms which decrease appetite, biomass and growth [79, 80, 88]. Control organisms gained weight during the test and were fed weekly. Treated organisms showed a no significant decreased of their weight with a mean value of 390mg (F 48.47 $p=0.78$) and represent about a -12.6% from the initial weight (Fig. 2-B). It should be stated that undigested food traces were found in treatment boxes. Glyphosate interfere with the normal development and reproduction rates of *E. fetida* with direct impact over their poblational dynamics and indirectly over soil fertility [82, 85]. Treated earthworms began to lose their clitellum after 7 days of exposure, tendency that increased with time and concentration. At the end of chronic bioassay, 100% of the organisms from the highest concentration (50 mg.kg⁻¹) lost their clitellum. In the other treatments the percent of non clitellated organisms ranged from 75 to 97%. Control organisms showed a loss of clitellum of 7.5% due the normal reproductive cycle of the specie. Fecundity parameters also showed significant differences between contaminated and control soils. The highest cocoon production was found at control group representing a

67.02% of total cocoons (Fig.3-B). In treatments the number of cocoons decreased with exposure time and concentration but the inhibitory effect is already shown at the 7 mg.kg⁻¹dw, with a significant difference respect to the control (F 25.12 $p<0.05$). Cocoon size in the treatments was lower (F 45.72 $p<0.05$) with a mean of 3.87 mm x 1.95 mm, representing a decrease of the 25% respect to control (4.56 mm x 2.18 mm). Juveniles production was lower and showed a smaller size at all treatments (F 18 $p<0.05$).

3.2.3. Lambda-cyhalothrin

Exposure time at behavioural test should be between 24 and 48 hours. At longer exposure time the natural behaviour of the organisms to mix the soil, could cause a 'soft mixing' of both soils along time, inducing a decrease in the difference between soil from both sections of the test containers [89, 90]. Since the only difference between test soil and control is the presence of the investigated chemical, a statistical difference between the soils indicates an effect caused by the test chemical. Since avoidance test and reproduction test had a comparable sensitivity, avoidance tests can be used as suitable screening test [44]. Temperature, moist content and organic matter content of the soil modify the bioavailability of pesticides which impacts over poblational parameters of earthworms [82, 91, 92]. Survival was not affected at lambda-cyhalothrin exposure as observed in previous researchs [20, 41, 93, 94].

3.2.3.1. Eisenia fetida

Significant avoidance responses (F 21.35 $p<0.05$) were observed even at the lowest concentration (Fig. 4). Probit estimation EC₅₀ was 1.36 mg.kg⁻¹ (C.L.0.24-2.80 mg.kg⁻¹) for tested concentrations which indicates that lambda-cyhalothrin is easily detected by *E. fetida* sensorial organs even at concentrations that are close to agricultural application rates [34; 38].

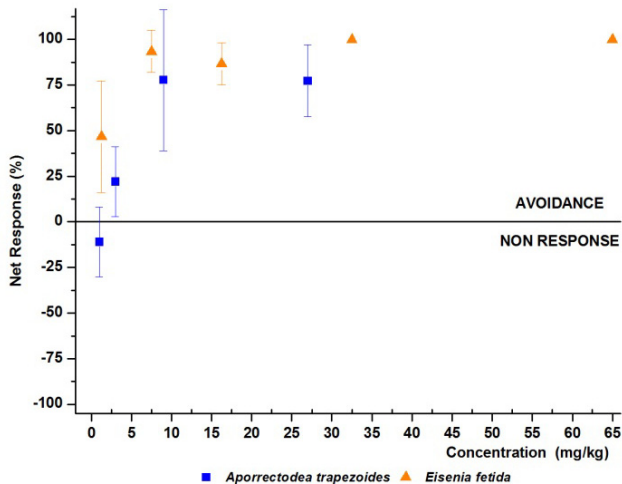


Figure 4. Avoidance or attraction response of *E. fetida* and *A. trapezoides* exposed to lambda-cyhalothrin concentrations in OECD and reference soil (mean net response and standard deviation bars).

Chronic test showed no significant differences in biomass ($F 2.37 p > 0.05$), even so the weight on the control group was higher (49.68%) than the treatments (34 -40%) (Fig.2-C). Organisms from the control group showed well developed clitellums at the end of the tests, some of the treated organisms lose their clitellum. Exposed organisms started to recover their clitellum from the 7th week of the test, tendency that could be related to a reduction of the concentration of pesticide on contaminated soils. Cocoons production was higher in control groups (Fig. 3-C), decreasing significantly in all the tested lambda-cyhalothrin concentrations ($F 11.94 p < 0.05$). Cocoons hatched after 21 days both in control group as in 1mg.kg. In the rest of treatments, a delay in the normal hatching period [92] was observed with a range of 28-35 days, which increased with concentration. The observed results indicate that lambda-cyhalothrin has a direct effect over fecundity on *E. fetida* affecting cocoon production and their viability. These effects are usually related to testicular malformations but due to the limitations of this study cannot be determined. Similar results were found by others authors [80, 82, 91, 92, 95]. The lowest observed effect concentration value (LOEC) for reproduction was estimated at 1 mg.kg⁻¹ corresponding with the lower tested concentration. Furthermore soils are considerate toxic when <50% of the number of juveniles determined for the control were counted [44]. Number of juveniles was significantly below the 50% threshold (4.67-8.67% of the control) in all treatments. Experimental points could not be fitted with the regression model from equation (3) making the EC₅₀ unable to be calculated (Fig. 5).

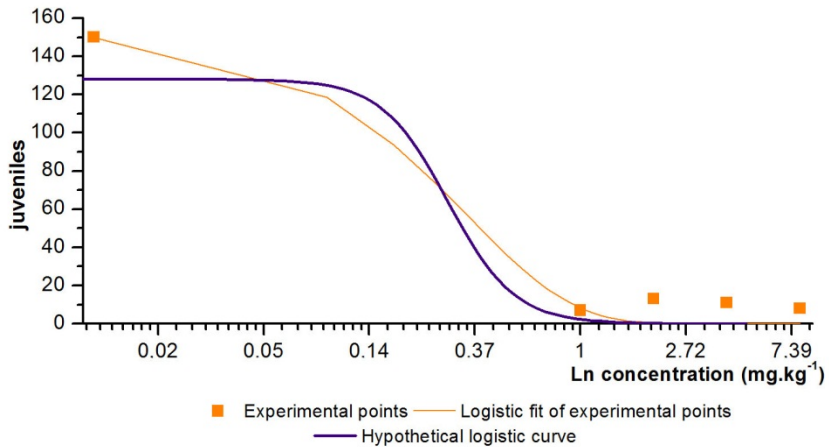


Figure 5. Graphic estimation of the EC₅₀ for juveniles production using a logistic regression model.

Bioaccumulation factors of lambda-cyhalothrin at the end of the test were 0.0076; 0.0056 and 0.0845 for the 2; 4 and 8 mg.kg⁻¹ treatments respectively. Soil lambda-cyhalothrin degradation was estimated at 86 days (99%).

Persistence of lambda-cyhalothrin in soil ranged from 4 to 12 weeks with a half-life value of 30 days in most soils [18] which matches our results. Some studies indicate that endogeic earthworms do not accumulate pyrethroids [96] but their lyphophilic characteristics made

them available to be absorbed by epigeic earthworms with preference by some isomers [1, 97, 98] which could be related to the traces of lambda-cyhalothrin found in *E. fetida* at the end of the test.

3.2.3.2. *Aporrectodea trapezoides*

Estimated EC_{50} at avoidance test was 7 mg.kg^{-1} being significantly above than the found for *E. fetida* [99]. Control and treated groups gained weight (Fig. 2-D) with significant differences in 6 and 8 mg.kg^{-1} ($F=11.67 \text{ } p<0.05$). Presence of organic clusters was recorded at control and 4.7 mg.kg^{-1} which indicate high mobility and intensive burrowing activity of the organisms, no traces of food were found. The burrowing behaviour from earthworms induce physicochemical and biochemical changes in the soil. Their depositions and mucus production increase the content of nutrients which are necessary to the growth of microbial biomass [100, 101].



Figure 6. (A) Production and distribution of organic clusters in control and treatments (B) General aspect and size of organism in control and treatments.

The mobility was reduced at 6 and 8 mg.kg⁻¹ where the burrows were restricted to the middle and lower fractions of the substrate and the soil surface showed food traces and few organic clusters. Pesticides affect the detoxification processes of earthworms leading to a decrease of feeding, biomass and mobility (diapauses or migrations to lower layers of soil) [102, 103].

Organisms exposed to 8 mg.kg⁻¹ curled their bodies to minimize the contact between pesticide and body wall. Stress generated from lambda-cyhalothrin exposure had a lower impact over *A. trapezoides* (endogeic) than in *E. fetida* (epigeic) that could be explained by the fast reaction associated to ingestion/egestion rate of epigeic earthworms as a consequence of their direct contact with the pesticide [7, 102, 104-111]. Researches performed by Cerón Rincón & Melgarejo Muñoz [112] and Renella *et al.* [113] registered stress symptoms at the microfloral, microbial and enzymatic activities which could explain the results of the higher exposure concentrations (6 and 8 mg.kg⁻¹).

As stated at field results, *A. trapezoides* is the dominant earthworm specie of agricultural soils from Santa Fe playing a crucial role in their fertility (68-72). According to Tripathi *et al.* [97] earthworm sensitivity is related to the characteristics of the organisms and their ecological category. Results of the bioassay indicate that *A. trapezoides* ecophysiology is severely affected by lambda-cyhalothrin with negative impact over soil fertility [75, 114].

4. Conclusions

Effects of soil exploitations on different environments influence the composition and taxocenosis structure of earthworms affecting their density and diversity.

Conventional production practices for agriculture (minimum tillage, non tillage, organic fertilizers) and non intensive livestock benefit the conservation and increase of edaphic fauna.

Endosulfan, glyphosate and lambda-cyhalothrin induced negative changes over feeding, reproduction and behaviour of *Aporrectodea trapezoides* and *Eisenia fetida* at tested concentrations and exposure times.

These changes lead to detrimental effects on the population dynamics of earthworms that affect trophic relationship

Author details

María Inés Maitre, Carolina Elisabet Masin and Tamara Ricardo
INTEC (CONICET-Univ. Nacional del Litoral), Santa Fe, Rpca. Argentina

Alba Rut Rodríguez
INTEC (CONICET-Univ. Nacional del Litoral), Santa Fe, Rpca. Argentina
Facultad de Humanidades y Ciencias (Univ. Nacional del Litoral), Santa Fe, Rpca. Argentina

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Pyrethroids and Their Effects on Ion Channels

Erin N. Wakeling, April P. Neal and William D. Atchison

Additional information is available at the end of the chapter

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1. Introduction

Ion channels are integral membrane proteins that are critical for neuronal function. They form pores in the plasma membrane that allow certain ions to travel with their concentration gradient across the membrane. Those that open in response to a change in membrane potential are called voltage-gated ion channels. Channels that open in response to binding by a chemical signal or molecule are ligand-gated ion channels. In neurons, ion channels are essential for chemical communication between cells, or synaptic transmission. Ion channels also function to maintain membrane potential and initiate and propagate electrical impulses.

Due to their importance in neurons, ion channels are often the molecular targets of neurotoxins. Pyrethrin compounds first identified in the pyrethrum extract of *Chrysanthemum cinerariaefolium* are neurotoxins that disrupt the normal function of voltage-gated sodium channels (VGSCs). Pyrethrum extract is a very effective natural insecticide [1], however, it rapidly degrades upon exposure to light, making it unsuitable for commercial agriculture use. Beginning in the mid 20th century, synthetic analogs of pyrethrins, pyrethroid insecticides, were developed by modifying the structures of pyrethrins to increase photostability and insecticidal activity. Pyrethroid insecticides target VGSCs, but also affect the function of voltage-gated calcium and voltage-gated chloride channels. This chapter provides an overview of pyrethroid structure and toxicological properties in insects and mammals concentrating on the effects of pyrethroid on various ion channels.

2. Pyrethroid structure and toxicity

Initially isolated in 1924, two pyrethrin compounds were determined to be responsible for the insecticidal action of pyrethrum. They were designated pyrethrin I and II; pyrethrin I had a monocarboxylic acid moiety (chrysanthemic acid) while pyrethrin II had a dicarboxylic acid (pyrethric acid) [2, 3]. The other main chemical moiety of interest in the

pyrethrins was an alcohol, a substituted cyclopentenolone. Of the two compounds, pyrethrin I was more potent in insecticidal assays with *Aphis rumicis* L. (Aphids) [4, 5], which concurred with earlier work on cockroaches. Together the early insecticidal assays of the two compounds indicated that the insecticidal action was likely driven by pyrethrin I. However, subsequent studies conflicted regarding the potency of pyrethrin I and II: some studies indicated that pyrethrin I was more potent whereas others indicated pyrethrin II was more potent [3, 5, 6]. These discrepancies were ascribed to the difficulties encountered by early researchers in isolating pure pyrethrin I and II, photodegradation of stored fractions, and differences in test species selection. Eventually it was determined that pyrethrin I was more effective for killing insects while pyrethrin II was more effective for knockdown [1]. This combination of knockdown and lethal effects was largely responsible for the effectiveness of pyrethrum extracts against biting insects and prompted investigations designed to improve insecticidal potency by modifying the chemical moieties of the pyrethrin compounds.

In the first generation of the synthetic pyrethroids, which included *allethrin* (*Allyl* analog), *tetramethrin* (*tetrahydrophthalimidomethyl* analog), and *resmethrin* (discovered at Rothamsted Experimental Station), the acid moiety was based on the chrysanthemic acid present in pyrethrin I. This acid has a cyclopropane ring which is not present in more recent synthetic pyrethroids, such as *fenvalerate* (*phenylisovalerate* pyrethroid). In terms of insecticidal potency, the major breakthrough was the introduction of a cyano (CN) residue at the α carbon of the 3-phenoxybenzyl-alcohol moiety. Addition of this CN group enhanced insecticidal activity roughly 3-6 fold compared to non-cyano pyrethroids.

This cyano group in synthetic pyrethroids allowed for the classification of pyrethroids into two distinct subclasses. Type II compounds have the cyano group while type I agents do not. For example, permethrin is a common type I pyrethroid while cypermethrin (named for the addition of the cyano group) is a type II (**Figure 1**). Pyrethroids are highly flexible with many theoretically attainable geometries; most have several potentially stereogenic carbons [7, 8]. Due to this complex geometry, pyrethroids can be present as mixtures of isomers; for example, allethrin can be used as a mixture of four isomers (4*RS*) or as a single isomer (S-bioallethrin). In contrast the synthetic pathway used to generate deltamethrin and esfenvalerate results in the enrichment of a single isomer [7]. In general *R* esters of type I pyrethroids and *S* esters of type II pyrethroids exhibit the highest insecticidal potency [1, 9-11]. The presence of the stereo-inactive isomers can attenuate the effects of the active isomers [12].

Although the potency increased, first and second generation pyrethroids were still highly photolabile [13]. The photodegradation of pyrethroids generally results in decomposition of the parent compound to less toxic products. The first photostable pyrethroids developed was permethrin in 1973 [14], and permethrin is still one of the most heavily used pyrethroids in agricultural settings. Permethrin and other halogen-containing pyrethroids such as decamethrin and fenvalerate can persist on plant cuticles and have prolonged insecticidal action compared to the more photolabile chemicals [1].

Compared to later generation chemicals, first generation pyrethroids have limited outdoor uses due to their photolability. However, they are still used prominently in household insecticide sprays for flying and crawling insects [15]. Indoor pyrethroids are also used in topical creams, sprays, and shampoos for controlling biting insects and head lice in both humans and their domestic pets. Recent studies have shown that sensitive populations such as children and pregnant women are exposed to pyrethroids via these indoor applications [16, 17]. The outdoor pyrethroids are used in agricultural settings and disease vector control. The two most commonly used pyrethroids in agriculture are cypermethrin and permethrin, which together account for an annual usage of 3 million pounds [18, 19]. Several pyrethroids are registered for use in the United States for control of the malaria-bearing mosquito species *Anopheles* [20], and pyrethroid-treated bed nets are used in tropical regions worldwide to prevent malaria. Pyrethroid use in agriculture and vector control has increased in recent years due to the reduced use of chlorinated, carbamate, and organophosphate pesticides [15], resulting in increased human exposure [21].

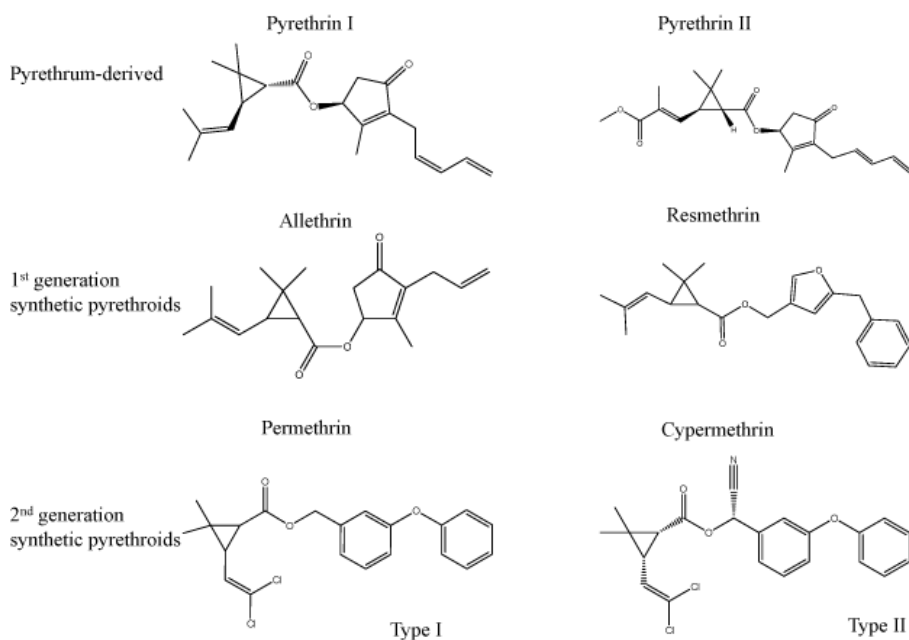


Figure 1. Pyrethrins, prototypes for the synthetic pyrethroids, were derived from the pyrethrum extract of *Chrysanthemum* species. Introduction of an α -cyano group determines whether pyrethroids are classified as type I (cyano lacking) or type II (cyano-containing).

Pyrethroid exposure in insects is predominately through the insect cuticle. Rapid absorption, particularly with the halogen-containing pyrethroids, causes disruption of insect neurotransmission causing knockdown and possibly death within seconds to minutes. In contrast to insects, human dermal absorption of pyrethroids is low relative to absorption via lung or gut [15, 22]. Humans absorb approximately 1% of pyrethroid exposure through skin

but up to 36% through ingestion [22]. However, sprays combining pyrethroid formulations with N,N-diethyl-m-toluamide (DEET) can increase dermal absorption of pyrethroids. After absorption, pyrethroids are distributed throughout the body and can cross the blood-brain barrier to access the nervous system. In occupational settings such as agriculture, inhalation of aerosolized pyrethroid formulations can result in toxicity, necessitating the use of personal protective equipment to prevent intoxication. Due to relatively low mammalian toxicity and the adequacy of personal protective gear in occupational settings of high exposure, there have been relatively few cases of human poisoning. The most commonly encountered human symptom of pyrethroids toxicity is paresthesia, which can be treated by decontamination of the exposed skin followed by bathing with oil [22]. Systemic poisonings in humans can be hard to control; central and peripheral nervous system effects in humans must be treated by a multisystem approach due to the numerous targets of pyrethroids in mammals [22].

Studies on pyrethrin I and II and first generation synthetic pyrethroids showed low mammalian toxicity. A large proportion of the administered dose was excreted in an unmetabolized form. Furthermore, the bioavailable dose is metabolized easily in mammals through cleavage by esterases and cytochrome P450 mixed function oxidases in liver or plasma [23-25]. The (+)-trans form of some pyrethroids, such as bioresmethrin, is much more toxic to mammals than the (+)-cis isomer. Mammalian systems metabolize the (+)-trans isomers much faster than the (+)-cis form, effectively removing the most toxic species [26]. Consequently, pyrethroids do not appear to accumulate in mammalian tissues. Clearance from plasma is relatively fast as pyrethroids are distributed throughout the body [27], with plasma half lives ($t_{1/2}$) of the pyrethroid class ranging from 9-15 hours (hrs) [28]. In animal models pyrethroids exhibit peak concentration in the brain within 2-3 hrs after oral administration and decrease rapidly [27, 29]. Brain pyrethroid levels are relatively low (0.1-0.3% of body burden), even at the time of peak symptom expression [28]. Clearance rates of pyrethroids from the brain can vary; for example in a study comparing clearance rates of several pyrethroids from the brain, deltamethrin was cleared quickest, followed by fenvalerate, cypermethrin, and permethrin [30]. Although the majority of the administered pyrethroid dose is metabolized quickly in plasma and liver, the unmetabolized fraction can partition into fat where pyrethroid metabolism and clearance is much slower [27, 30].

Although efficient metabolism in mammals reduces the likelihood of acute toxicity, high exposures can result in intoxication. Type I and type II pyrethroids produce different toxicological symptoms in mammals, likely due to differences in the effects of the classes on mammalian neurons (**Table 1**) [31, 32]. Mammalian intoxication by type I pyrethroids causes symptoms characterized by tremor (T-class), exaggerated startle response, and hyperexcitability [7, 11, 33]. Intoxication by type II pyrethroids results in burrowing and pawing behavior, followed by salivation and coarse tremor, which evolves into choreoathetosis (involuntary movement and writhing) [34]. This second type of poisoning is referred to as CS-class. Some pyrethroids, such as fenpropathrin and cyphenothrin can result in symptoms from both T and CS-class [9, 31], and have been designated by some to belong to a third class (TS: tremor and salivation) [35]. One hypothesis for the difference in toxicity symptoms is that the cyano group in the type II pyrethroids results in a prolonged effect on neurons, causing a different spectrum of toxicological symptoms [7, 11].

T-Type	CS-Type
Severe fine tremor	Coarse tremor
Marked reflex hyperexcitability	Moderate reflex hyperexcitability
Sympathetic activation	Sympathetic activation
Paresthesia (dermal exposure)	Paresthesia (dermal exposure)
Clonic seizures [†]	Tonic seizures
Hyperthermia [‡]	Choreoathetosis
Uncoordinated twitches [‡]	Increased extensor tone
Aggressive sparring [‡]	Profuse watery salivation
	Sinuuous writhing [†]
	Rolling gait [‡]
	Chewing; nosing; pawing; burrowing [‡]

Table 1. Pyrethroid toxicity syndromes in mammals. Species-specific effects are indicated †= mouse, ‡= rat. Modified from [21, 34].

The T-, CS-, and TS-syndromes are dose-dependent, acute responses to overt pyrethroid toxicity. They exhibit a fast onset, occurring from within a few minutes to over 1 hr but take about 2-8 hrs to peak [28]. Recovery is generally complete within 24-48 hours after cessation of exposure. The appearance of neurobehavioral symptoms follows this time course; symptoms begin around 1 hr, peak around 4-8 hrs and are resolved 12-48 hrs after oral exposure [34].

The safety of the pyrethrins and early generation pyrethroids is largely due to their instability. By producing more stable pyrethroids, the potential for mammalian toxicity increased. The halogen-containing pyrethroids lack many of the characteristics that originally deemed the earlier pyrethroids and pyrethrins as safe. For example, the halogen-containing pyrethroids are much more photostable. This allows them to persist on the surface of plants much longer than the earlier pyrethroids. They are also highly lipophilic, which promotes higher bioavailability in mammals [1]. In fact, fluorinated pyrethroids induce toxicity symptoms with a much faster onset than non-halogenated compounds [35] and halogenation coupled with the cyano group increases toxic potency in mammals by an order of magnitude [34].

Despite low incidence of acute poisoning in humans, there is substantial interest in the effect of chronic exposure to pyrethroids. The early studies on pyrethroid toxicity focused on their effects on neuronal targets in acute paradigms [35]. However, the identification of other endpoints of concern suggests that there are prolonged effects of pyrethroid exposure, perhaps mediated by active metabolites in addition to the parent compound [7]. Several laboratories have independently identified that chronic pyrethroid exposure causes alterations to the dopaminergic system in rodents [36-40]. This is particularly interesting in the context of motor activity changes during the acute toxicity syndromes [34]. Induction of apoptosis by deltamethrin in dopaminergic neurons was demonstrated *in vitro* [41] and others have shown that sub-chronic exposure to permethrin in rats resulted in neuronal loss in the hippocampus and motor cortex [42]. Repeated exposure to pyrethroids resulted in

altered dopamine transporter function, changes in the number of dopaminergic binding sites in rat brain [36], and possibly increased dopamine turnover resulting in decreased dopaminergic function [38, 49]. Decreased dopamine levels were also observed during chronic exposure to the type II pyrethroid cypermethrin, an effect accompanied by dopaminergic neurodegeneration [40]. The underlying mechanism for dopaminergic sensitivity to pyrethroids is unknown, but the effects observed across laboratories illustrate a consistent picture of pyrethroid effects in the dopamine neurotransmitter system.

The effects of cypermethrin on dopaminergic neurons listed above only occurred in animals exposed to the pyrethroid during both developmental and adult exposure and did not occur in animals exposed only during adulthood [40]. This may indicate that developmental exposure predisposes adult animals to pyrethroid sensitivity. Several studies have suggested that neonatal mammals are more sensitive to pyrethroids than adult animals. Brain levels of pyrethroids in neonatal rats are higher than for adult animals [43]. Further, neonatal rats have less capacity to metabolize pyrethroids than adults, leading to 4-17 fold increased sensitivity to pyrethroids [44, 45]. An additional factor in the susceptibility of developing animals to pyrethroids is the differential expression of pyrethroid targets during development. For example, some developmentally expressed isoforms of the sodium (Na^+) channel are much more sensitive to pyrethroids than isoforms expressed in the adult brain [33, 46]. The differences in sensitivity between neonates and adults are greatest with the type II pyrethroids; type I pyrethroids in neonates produce LD_{50}^1 values roughly 2-3 fold lower than in adults, but type II pyrethroids had 16-24 fold lower LD_{50} s in pups. Besides acute lethality, rats exposed to pyrethroids during postnatal days 10-16 exhibited increased motor activity and lack of habituation. These effects were accompanied by changes in neurotransmitter receptor levels [47], which persisted for up to 5 months after cessation of exposure. Together, results from these studies suggest that neonatal mammals are more sensitive than adult animals, due to a combination of differential expression of pyrethroid targets, reduced metabolic capacity, and increased pyrethroid levels in neuronal tissues.

3. Pyrethroid effects on nerve impulses

Action potentials are the electrical impulses that travel along the axons of neurons and result from the movement of Na^+ and potassium (K^+) ions across the membrane [48] (**Figure 2**). Binding of excitatory neurotransmitters to their receptors opens cation-permeable ion channels causing the membrane to depolarize or become more positive. This depolarization activates (opens) VGSCs allowing Na^+ to enter the neuron further depolarizing the membrane. This increase in membrane permeability to Na^+ is responsible for the rising phase of the action potential, eventually causing the membrane polarity to reverse (overshoot phase). The falling phase of the action potential is caused by the inactivation of the VGSCs and the opening of voltage-gated potassium channels allowing K^+ to leave the cell. The efflux of K^+ ions results in hyperpolarization (undershoot phase) of the membrane.

¹ LD_{50} : The median lethal dose, or the dose required to kill 50 percent of the exposed animals.

Ultimately the voltage-gated K^+ channels close and the membrane potential returns to its resting state.

In microelectrode recordings² of action potentials in cockroaches, treatment with pyrethrum extract resulted in repetitive discharges followed by a block in nerve conduction [49, 50]. Allethrin, the first synthetic pyrethroid, also caused repetitive discharges and a subsequent conduction block. This effect was concentration and temperature-dependent [51-53]. Higher concentrations of pyrethrum and allethrin induced conduction block more rapidly and exposure at lower temperatures resulted in greater insecticidal activity. Allethrin treatment also slowed the rising and falling phases of the action potential and resulted in a more positive resting membrane potential. Increasing or decreasing the extracellular K^+ concentration did not alter the effects of allethrin, indicating that pyrethroids alter Na^+ conductance through action on VGSCs. Electrophysiological experiments in frog, squid and crayfish confirmed that type I pyrethroids cause repetitive discharges upon a single stimulus similar to allethrin and pyrethrins, whereas type II pyrethroids cause stimulus-dependent membrane depolarization and conduction block [54-57, 32].

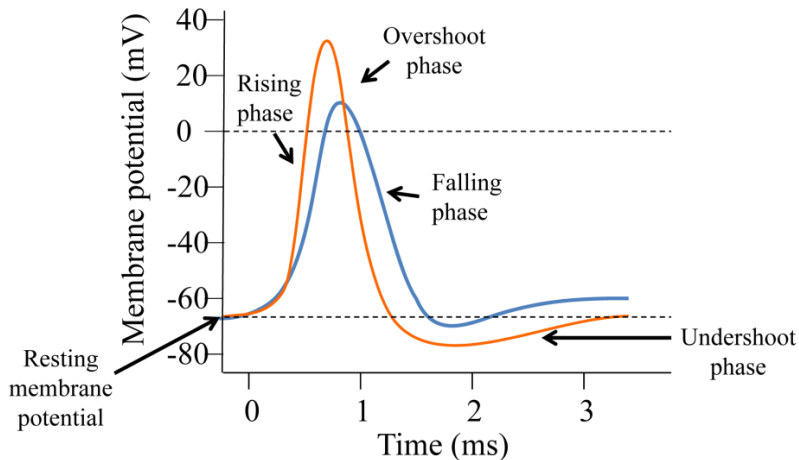


Figure 2. Phases of an action potential. Orange- normal action potential. Blue- action potential in the presence of allethrin. Modified from Motifolio Biomedical PowerPoint Toolkit Suite.

4. Pyrethroid effects on insect voltage-gated sodium channels

VGSCs consist of a pore forming subunit (α subunit) and an auxiliary subunit [58]. The α subunit contains four domains (I-IV) each with 6 transmembrane segments (**Figure 3A**). The amino and carboxy termini of the protein are intracellular. The fifth and sixth transmembrane segments (S5 and S6) and the loop between them form the channel pore and

² Microelectrodes are typically made of a glass pipette pulled to a very fine tip and filled with an electrical conductor, such as a high salt solution. When the electrode is connected to a voltmeter and inserted into a cell it can detect changes in transmembrane voltage.

confer selectivity for Na^+ . Positively charged amino acids in S4 serve as the voltage sensor initiating a conformation change that opens the channel upon membrane depolarization. The cytoplasmic linker between domains III and IV serves as the inactivation gate. VGSCs can exist in 4 states controlled by the opening and closing of two distinct “gates,” known as the activation and inactivation gate, respectively (**Figure 3B**) [48]. At the resting membrane potential the channel is closed and the inactivation gate is open. Upon membrane depolarization, the channel opens allowing Na^+ to enter the cell. During inactivation, the inactivation gate closes occluding the pore. Finally the channel closes during a state called deactivation. Removal of deactivation is needed to restore the “closed” state of the channel and prepare it for another activation (opening).

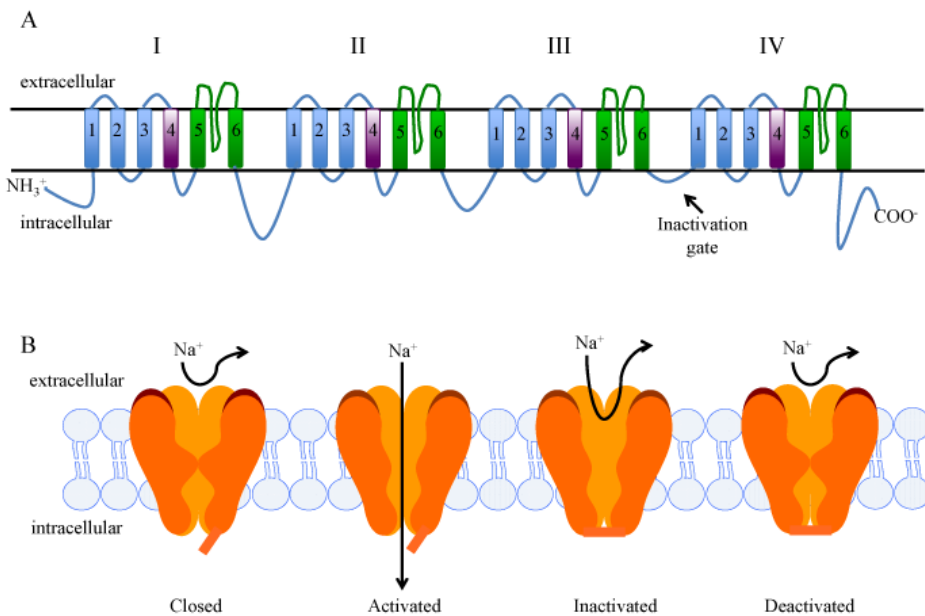


Figure 3. The α subunit of the voltage-gated sodium channel. A. Schematic of VGSC α subunit indicating the four domains (I-IV) and their six transmembrane segments (1-6). The transmembrane segments and loop that form the channel pore are shown in green. The 4th transmembrane segment, shown in purple, acts as the voltage sensor. B. Four states of the VGSC. At resting membrane potentials the channel is closed. During the rising phase of an action potential the channel activates or opens. Channel inactivation contributes to the falling phase. During the undershoot phase the channel deactivates prior to returning to the closed phase once resting membrane resting potential has been restored. Modified from Motifolio Biomedical PowerPoint Toolkit Suite.

In insects, a single gene encodes the VGSC α subunit. In *Drosophila melanogaster* this gene is called *para* [48]. Messenger RNA transcripts from this gene are alternatively spliced resulting in mature transcripts containing different combinations of exons. The transcripts also undergo RNA editing in which some nucleotides are converted from one base to another often changing the amino acid that is encoded. The combination of alternative

splicing and RNA editing results in VGSCs with distinct gating properties. The auxiliary subunit, TipE in *D. melanogaster*, increases cell surface expression of *Para*, enhances the peak Na⁺ current and alters the kinetics of channel inactivation.

Advancements in electrophysiological techniques, particularly the development of the voltage and patch-clamp techniques³, enabled researchers to study the effect of pyrethroids on Na⁺ current. Pyrethroids slowed VGSC activation leading to a decrease in peak Na⁺ current [53, 55, 59-61]. Pyrethroids slowed VGSC inactivation and deactivation leading to a prolonged VGSC open time. Type II pyrethroids prolonged channel open time more than type I pyrethroids. The longer channel open time results in more Na⁺ entering the cell leading to hyperexcitability with type I pyrethroids, membrane depolarization and conduction block with type II pyrethroids. Even though not every VGSC is altered by pyrethroids, modification of a small percentage of VGSCs can increase Na⁺ current substantially [91, 95].

In addition to the electrophysiology data, there is genetic evidence that pyrethroids target the VGSC. Pyrethroid resistant, or knockdown-resistant (*kdr*), houseflies were first reported in the 1950s [62, 63]. Genetic mapping of the *kdr* and super-*kdr* trait in houseflies revealed that pyrethroid resistance was tightly linked to the housefly VGSC gene, *Vssc1* [64]. Similar genetic mapping experiments in tobacco budworms, German cockroaches and mosquitoes also linked *kdr* and super-*kdr* traits in those species with their respective VGSC genes [65-67]. Comparative sequence analysis of *kdr* and super-*kdr* housefly *Vssc1* with the wild-type gene identified two point mutations in domain II [68]. The first mutation, a leucine to phenylalanine change in transmembrane segment 6, was found in two *kdr* and six super-*kdr* housefly strains. The second mutation, a methionine to threonine change in the intracellular loop between transmembrane segments 4 and 5, was only found in super-*kdr* strains. Sequencing of the VGSC genes from other species with *kdr* resistance identified several other mutations (**Table 2**) [58, 69-73].

The most common *kdr* mutation is a leucine (L) to phenylalanine (F), histidine (H), or serine (S) change in domain II segment 6 (DIIS6). As this mutation does not alter expression or localization of the VGSC, it was suspected to alter the affinity of the channel for pyrethroids [74]. Expression of this L to F mutant channel in *Xenopus laevis* oocytes resulted in VGSCs that were 10 fold less sensitive to cismethrin as assessed using voltage-clamp experiments [75]. The L to F mutation is found in combination with a methionine (M) to threonine (T) change in highly resistant houseflies. When expressed in *X. laevis* oocytes, the double mutant channels were 200x more resistant to deltamethrin and almost insensitive to permethrin [76]. Several other *kdr* mutations have been assayed in *X. laevis* oocytes and in general mutant channels required much higher concentrations of pyrethroid to produce Na⁺ currents that were similar to those produced by pyrethroid treated wild-type channels [77-80].

³ The voltage-clamp technique typically uses two microelectrodes, allowing the experimenter to control the membrane potential and record transmembrane currents that result from ion channels opening and closing. Patch-clamp is a highly sensitive version of the voltage-clamp technique in which currents flowing through a single ion channel can be measured. A single electrode serves both to measure voltage and pass current.

The clustering of *kdr* and six super-*kdr* mutations in DIIS4-S5 linker, DIIS5 and DIIS6 suggest that these regions are part of the pyrethroid-binding site. This is supported by computer modeling in which fenvalerate is predicted to interact with amino acids in DIIS4-S5 linker, the DIIS5 and the cytoplasmic end of DIIS6 [81]. The computer model is supported by competitive binding assays using active and inactive permethrin [82]. The L to F mutation in DIIS6 was found to reduce binding of the inactive pyrethroid by 16 fold. Alternatively, the *kdr* and six super-*kdr* mutations could alter VGSC gating properties in a way that counteracts pyrethroid action. In support of this second hypothesis, the L to F mutation in DIIS6 and the M to T mutation in DIIS4-S5 linker enhanced inactivation by shifting the voltage-dependence of inactivation to more negative membrane potentials and by increasing the rate of inactivation [76]. Further study of naturally occurring *kdr* mutations and splicing isoforms of VGSCs will provide a greater understanding of the molecular interactions between pyrethroids and VGSCs.

Mutation	Location in VGSC	Species
Leucine to Phenylalanine	DIIS6	<i>Musca domestica</i> , <i>Blattella germanica</i> , <i>Plutella xylostella</i> , <i>Myzus persicae</i> , <i>Anopheles gambiae</i> , <i>Culex pipiens</i> , <i>Culex quinquefasciatus</i> , <i>Haematobia irritans</i> , <i>Leptinotarsa decemlineata</i> , <i>Frankliniella occidentalis</i> , <i>Cydia pomonella</i> , <i>Ctenocephalides felis</i>
Leucine to Serine	DIIS6	<i>Culex pipiens</i> , <i>Anopheles gambiae</i>
Leucine to Histidine	DIIS6	<i>Heliothis virescens</i>
Methionine to Threonine	DIIS4-S5 linker	<i>Musca domestica</i> , <i>Haematobia irritans</i> , <i>Heliothis virescens</i>
Aspartate to Glycine	Amino terminus	<i>Blattella germanica</i>
Glutamate to Lysine	DI and DII linker	<i>Blattella germanica</i>
Cysteine to Arginine	DI and DII linker	<i>Blattella germanica</i>
Proline to Leucine	Carboxy terminus	<i>Blattella germanica</i>
Valine to Methionine	DIIS6	<i>Heliothis virescens</i>
Methionine to Isoleucine	DIIS1-S2 linker	<i>Pediculus capitis</i>
Leucine to Phenylalanine	DIIS5	<i>Pediculus capitis</i>
Threonine to Isoleucine	DIIS5	<i>Plutella xylostella</i> , <i>Pediculus capitis</i>
Threonine to Cytosine	DIIS5	<i>Frankliniella occidentalis</i>
Threonine to Valine	DIIS5	<i>Ctenocephalides felis</i>
Phenylalanine to Isoleucine	DIIS6	<i>Boophilus microplus</i>
Leucine to Proline	DIII-DIV linker	<i>Varroa destructor</i>

Table 2. List of some of the species with *kdr* mutations. Mutations in red occur at the same leucine residue. Mutations in blue occur at the same threonine residue.

5. Pyrethroid effects on mammalian voltage-gated sodium channels

The α subunit of VGSCs in mammals is very similar both in structure and amino acid sequence to the insect VGSC [83]. In contrast to insects, mammals have nine VGSC genes (*Nav1.1-1.9*) that differ in channel properties and tissue expression patterns (Table 3). Transcripts produced from these genes also undergo alternative splicing to produce more functionally diverse channels. The auxiliary subunit in mammals, or β subunit, is required for proper localization of the α subunit to the plasma membrane. Interaction of the β subunit with the α subunits also modifies the voltage-dependence and kinetics of gating. Mammals have four β subunit genes ($\beta 1$ - $\beta 4$); all four β subunits are expressed in the central nervous system and heart, but skeletal muscle only expresses $\beta 1$.

Similar to insects, exposure to pyrethroids increases excitability in mammalian neurons by slowing the action potential falling phase [84-87]. Type I and type II pyrethroids delay mammalian VGSC inactivation resulting in a prolonged Na^+ current [88, 89]. The length of channel open time is dependent on the pyrethroid type, with type II pyrethroids holding VGSCs open much longer than type I. For example, tetramethrin increased the open time of mouse neuroblastoma⁴ VGSCs ten-fold, whereas deltamethrin increased the open time 200-fold [89, 89]. The ability of pyrethroid to delay mammalian VGSC inactivation was also temperature-dependent, with lower temperatures resulting in slower Na^+ current decay [89-91]. Electrophysiology studies demonstrating mammalian VGSC sensitivity to pyrethroids were also supported by *in vitro* assays. Pyrethroids enhanced $^{22}\text{Na}^+$ uptake by toxin activated VGSCs in cultured mouse neuroblastoma cells and rat brain synaptosomes⁵ [84, 92]. Binding studies using radioactive pyrethroid demonstrated specific binding of the pyrethroid to rat brain VGSC α subunits [93].

Channel Name	Former Names	Expression Pattern
Nav 1.1	brain I	Central and peripheral nervous system
Nav 1.2	brain II/IIa	Central nervous system
Nav1.3	brain III	Embryonic/neonatal nervous system
Nav1.4	$\mu 1/\text{SkM1}$	Skeletal muscle
Nav1.5	H1/SkM2	Cardiac muscle
Nav1.6	NaCh6/PN4	Central and peripheral nervous system
Nav1.7	PN1	Central and peripheral nervous system
Nav1.8	SNS/PN3	Peripheral nervous system
Nav1.9	NaN	Peripheral nervous system

Table 3. Mammalian sodium channels and tissue expression pattern. Channels in boldface are resistant to TTX, a voltage-gated sodium channel blocker, but are more sensitive to pyrethroids.

Although, mammalian VGSCs are less sensitive to pyrethroids than insect VGSCs, there is variability in pyrethroid sensitivity among the channel isoforms. Dorsal root ganglion cells

⁴ Mouse neuroblastoma cells are an immortal cell-line derived from a spontaneous tumor of neural crest origin.

⁵ In this model, neurons are harvested from rat brains and processed via subcellular fractionation to isolate the nerve terminals.

are sensory neurons that express two classes of VGSCs, tetrodotoxin (TTX)-sensitive and TTX resistant channels. TTX is a potent VGSC inhibitor that prevents action potential generation. Nav1.8 and Nav1.9, which are resistant to TTX, are highly sensitive to pyrethroids [94-98]. Allethrin (at 10 μM) was found to have significant effect on TTX-resistant channels but no effect on TTX-sensitive channels. Similar results were obtained with tetramethrin and deltamethrin. Expression and voltage clamp studies in *X. laevis* oocytes demonstrated that Nav1.2 was less susceptible to pyrethroid modification than other sub-types [99-101]. Rat Nav1.3, which is expressed in the developing central nervous system, is more sensitive to type II pyrethroids than Nav1.2 [46,100]. The high pyrethroid sensitivity of Nav1.3, may underlie the increased neurotoxicity of pyrethroid to developing mammals. Nav1.6, which is highly expressed in the adult brain, is fifteen times more sensitive to tefluthrin and deltamethrin than Nav1.2 [101]. VGSCs are also critical for normal cardiac function. Tefluthrin, fenpropathrin and cypermethrin prolonged Na⁺ current in rat cardiomyocytes and increased the intervals between heartbeats in perfused hearts [102, 103]. Further investigation into structural differences between mammalian VGSC isoforms and how those differences affect pyrethroid sensitivity will be required to understand the molecular basis of pyrethroid toxicity in mammals. Such studies will be essential to the development of new pyrethroid insecticides with lower mammalian toxicity.

6. Pyrethroid effects on mammalian voltage-gated calcium channels

Voltage gated calcium channels (VGCCs) play essential roles in diverse cellular functions. They mediate Ca²⁺ influx into the cell from the extracellular environment following membrane depolarization. This can alter cell signaling, neurotransmission, and gene expression. VGCCs contain one transmembrane pore-forming and voltage-sensing subunit designated α_1 . The association of the transmembrane segments with a water-filled cavity forms the pore, through which Ca²⁺ traverses. A ring of glutamate residues, which line the pore, imparts Ca²⁺ selectivity. Changes in membrane potential cause a conformational shift resulting in the movement of a voltage-sensing domain to open the ionic pore. VGCCs are classified in three groups (Cav1-3)⁶. Cav1 consists of the L-type high-voltage-activated VGCCs, Cav2 consists of P/Q-, N-, and R-type high-voltage activated VGCCs, and Cav3 consists of T-type low-voltage activated VGCCs. The terms “high-voltage activated” and “low-voltage activated” derived from the characteristics associated with the voltage-dependence of the two classes. “High-voltage activated” channels require strong levels of depolarization from the resting membrane potential to open, whereas “low-voltage activated” channels need only minimal depolarization from the resting potential to become activated. The α_1 subunit forms the major structural and functional unit of the channel, but VGCC activity can be modulated by accessory subunits α_2 , β , δ , and γ . The α_2 and δ

⁶ The nomenclature of VGCCs genes is complex since the original naming system, based on the type of Ca²⁺ current observed during electrophysiological recording, was in place before the more systematic HUGO Human Gene Nomenclature was adopted. We will use the nomenclature developed by ion channel researchers throughout this review but refer the reader to (Ertel et al., 2000) for further discussion of VGCC naming conventions.

subunits are the product of a single gene and are covalently linked by a disulfide bond. The four VGCC β subunits ($\beta 1-4$) are cytoplasmic and play an instrumental role in cellular trafficking, intracellular signaling, and channel activity modulation. The γ subunit (eight isoforms: $\text{Ca}_v\gamma 1- \gamma 8$) is only expressed in skeletal muscle and may also play a role in cell trafficking [104]. The ratio of subunits exists in a 1:1:1:1 $\alpha_1\alpha_2\delta\beta\gamma$ ratio, but diversity among subunit isoforms allows for isoform-specific effects.

L-type VGCCs (Ca_v1) are essential to somatodendritic Ca^{2+} influx in mammalian central neurons. They mediate influx in response to back-propagating action potentials, synaptic plasticity, and excitatory activity-dependent modulation of gene transcription. The Ca_v2 family members are all expressed in central neurons, where they are localized to axons, soma, and dendrites. In peripheral neurons, they are expressed differentially. Their localization was determined using specific pharmacological inhibition of isoform-specific currents (**Table 4**). $\text{Ca}_v2.1$ (P/Q-type) and $\text{Ca}_v2.2$ (N-type) are the predominate VGCCs involved in presynaptic voltage-dependent Ca^{2+} influx which triggers vesicular release of neurotransmitters. However, at some synapses either or both L-type and R-type channels also participate in this essential function. The final family member, Ca_v3 , consists of T-type VGCCs that activate at sub-threshold membrane potentials and are critical for regulation of plasma membrane Ca^{2+} permeability near resting membrane potentials and during action potentials. Depending on the brain region, T-type VGCCs are localized to soma, dendrites, or intracellular targets.

The primary target of pyrethroids is widely accepted as the VGSC [105]; however, pyrethroids can interact with other targets. The ciliate protozoa *Paramecium tetraurelia* do not express VGSCs and yet exhibit high sensitivity to type II pyrethroids, via a mechanism involving disrupted Ca^{2+} homeostasis [106]. Though unique in subunit structure, there are fundamental similarities between VGCCs and VGSCs. Both contain a pore-forming α subunit, intracellular β subunits, and similar gating mechanisms, although the VGCC has additional $\alpha\delta$ and γ subunits [107]. Several studies have noted effects of pyrethroids on mammalian neuronal VGCCs in the same concentration range as their effects on neuronal VGSCs [11, 98, 108, 109].

Neurons in culture develop spontaneous networks of interconnected neurons that exhibit electrical activity in response to neuronal signaling. This activity can be measured as either excitatory or inhibitory post-synaptic currents (EPSC or IPSC, respectively) using electrophysiological methods. EPSCs can be either action potential-dependent or action potential-independent; in the presence of TTX, EPSCs are present as miniature events and are termed miniature EPSCs (mEPSCs). In contrast, in the absence of TTX, EPSCs have a much larger magnitude. Both type I and type II pyrethroids inhibit spontaneous activity in neurons [110, 111]. Permethrin (type I) and deltamethrin (type II) both decreased the number of EPSCs in hippocampal neuron networks in a concentration-dependent manner; deltamethrin was more potent. In contrast, only the type I permethrin increased the frequency of mEPSCs in hippocampal neurons [112].

The stimulatory effect of permethrin on mEPSCs was dependent on the presence of extracellular Ca^{2+} [112], showing that the effects observed on EPSCs by type I pyrethroids are downstream of Ca^{2+} entry through VGCCs. Effects of type I pyrethroids on VGCCs have been described in several electrophysiological studies. The type I pyrethroid allethrin potently blocked recombinant rat L-, P/Q-, and T-type VGCCs with IC_{50} in the low micromolar range in human embryonic kidney (HEK) 293 cells [108], which is lower than or in the same magnitude of allethrin concentrations that affect insect VGSCs [61, 98]. However, another study found allethrin differentially modulated endogenous VGCC subtypes in rat pheochromocytoma (PC12) cells, with marked differences in sensitivity, stimulating L-type and inhibiting N-type VGCCs. L-type VGCCs exhibited an EC_{50}^7 in the mid-pM range while N-type VGCCs exhibited an IC_{50} in the low μM range [109].

Subtype	Calcium Current	α subunit	Expression Pattern	Pharmacological Inhibitors
Ca _v 1.1	L-type	α_{1S}	Skeletal muscle	Dihydropyridines (DHPs)
Ca _v 1.2	L-type	α_{1C}	Heart, smooth muscle, brain, pituitary, adrenal gland	DHPs
Ca _v 1.3	L-type	α_{1D}	Brain, pancreas, kidney, ovary, cochlea	DHPs
Ca _v 1.4	L-type	α_{1F}	Retina	DHPs
Ca _v 2.1	P/Q-type	α_{1A}	Central and peripheral nervous system	ω -agatoxin-IVA/IIIA, ω -grammotoxin SIVA, ω -conotoxin MVIIC
Ca _v 2.2	N- type	α_{1B}	Central and peripheral nervous system	ω -conotoxin MVIIC/GVIA/CVID, ω -grammotoxin SIVA, farnesol, petidylamines
Ca _v 2.3	R-type	α_{1E}	Brain, cochlea, pituitary, retina, heart	Tarantula toxin derived peptide SNX-482
Ca _v 3.1	T-type	α_{1G}	Central and peripheral nervous system	Kurtoxin, ethosuximide, nickel
Ca _v 3.2	T-type	α_{1H}	Brain, heart, kidney, liver	Kurtoxin, ethosuximide, nickel
Ca _v 3.3	T-type	α_{1I}	Brain	Kurtoxin, nickel

Table 4. Naming system, tissue localization, and pharmacological agents of VGCC subtypes.

The VGCC subtype-specific effects seen in PC12 cells [109] but not in recombinant assays [108] may be due to differences in VGCC subunit expression. A combination of rat α_{1A} (P/Q), α_{1C} (L) or α_{1G} (T) with the β_{1B} and $\alpha_{2\delta}$ subunits was expressed in HEK293 cells [108]. While the α subunit composition would remain the same, PC12 cells express β_1 , β_2 , and β_3 mRNA,

⁷ Half maximal effective concentration (EC_{50}) refers to the concentration at which 50% of the maximum response is observed during a stimulatory effect.

and different β subunits associate in the native N-type channel [113]. Additionally, the β subunit can undergo extensive post-translational modification, resulting in numerous splice variants [114]. The β subunit in VGCCs has direct effects on VGCC inactivation, with VGCCs containing the β_{2a} subunit exhibiting slower inactivation than those containing the β_{1b} or β_3 subunit [114-116]. Furthermore, the identity of the β subunit can shift the voltage dependence of VGCC currents, P/Q-type VGCCs containing β_4 exhibit peak current at more hyperpolarized potentials than β_{1b} , β_{2a} , or β_3 , [116]. Effects of pyrethroids on VGSCs can be modulated by the identity of the VGSC β subunit [46], so perhaps pyrethroid effects on VGCCs may also be modulated by auxiliary subunit composition.

VGCC sensitivity to pyrethroids may also be modulated by the channel phosphorylation state. N-type VGCCs in particular are heavily influenced by phosphorylation by protein kinase C (PKC). PKC phosphorylates several sites in the N-type VGCC; phosphorylation at threonine-422 (T422) is stimulatory while at serine-425 (S425) it is inhibitory [117, 118]. Effects of PKC phosphorylation are dependent on β subunit expression [119]. In recombinant systems, application of phorbol-12-myristate, 13-acetate (PMA) potentiates current through N-type VGCC [120]. Inhibition of the PKC isoforms β II and ϵ block this potentiation. Deltamethrin differentially modulated recombinant N-type VGCCs based on channel phosphorylation status at the T422 residue [121]. Deltamethrin exposure of a mutant N-type VGCC that mimics phosphorylation at residue T422 resulted in channel activation. Wild-type channels and mutant channels incapable of phosphorylation are inhibited by deltamethrin. Although effects of type I pyrethroids on this mutant channel have not yet been investigated, this study raises the interesting possibility that VGCCs exhibit phosphorylation-specific sensitivity to pyrethroids

In addition to direct measurement of the effects of pyrethroids on Ca^{2+} current and VGCC properties, there have been many studies on pyrethroids effects on Ca^{2+} influx and neurotransmitter release. These endpoints serve as measures of functional outcomes of pyrethroid modulation of VGCCs. Alternatively, as pyrethroids are lipophilic and may independently partition into cells, results from these assays could indicate pyrethroid action on intracellular Ca^{2+} channels. Several studies were performed using rat synaptosomal preparations. Deltamethrin caused a significant increase in Ca^{2+} influx in rat synaptosomes in a stereospecific manner, with 1R-deltamethrin causing a roughly 1.7 fold increase in Ca^{2+} influx and 1S-deltamethrin having no effect on influx [122]. TTX had no effect on the stimulation of Ca^{2+} influx by deltamethrin, indicating that the influx was independent of VGSCs. However, incubation with ω -conotoxin MVIIC abolished the stimulatory effect of deltamethrin on Ca^{2+} influx, suggesting that the Ca^{2+} influx was via N- or P/Q- type VGCCs. In a follow up study, the deltamethrin-stimulated influx was reduced by 63% in the presence of ω -conotoxin GVIA, suggesting that more than half of the deltamethrin-stimulated influx was via N-type VGCCs.

Cismethrin had a lesser stimulatory effect on Ca^{2+} influx than deltamethrin but only with high (>20 mM) levels of potassium-induced depolarization [121, 123]. The cismethrin-mediated influx was unaffected by ω -conotoxin GVIA but was reduced by 50% with TTX

[123]. In the presence of nimodipine, cismethrin increased Ca^{2+} influx [121]. A similar stimulatory effect on Ca^{2+} influx by cismethrin was observed in the presence of ω -conotoxin GVIA [121]. The results with cismethrin suggested that the pyrethroid is primarily increasing Ca^{2+} via a TTX-sensitive mechanism whereas the mechanism with deltamethrin is likely TTX-insensitive. This may suggest that the Ca^{2+} influx observed with cismethrin is a result of increased Na^+ influx through VGSCs followed by replacement of Na^+ with Ca^{2+} via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger [121].

Deltamethrin caused a modest, yet significant, increase in glutamate release from rat synaptosomes in a stereospecific manner, with 1R-deltamethrin stimulating glutamate release and 1S-deltamethrin having no effect [122]. TTX had no effect on the stimulation of glutamate release by deltamethrin, indicating that the effect was independent of VGSCs. However, incubation with ω -conotoxin MVIIC abolished the stimulatory effect, suggesting that the release was mediated by activation of N- or P/Q- type VGCCs. In a subsequent study, ω -conotoxin GVIA reduced this deltamethrin-mediated increase in glutamate release by 70%, but nimodipine (L-type VGCC blocker) had no effect, suggesting that the enhanced glutamate release was predominately through stimulation of N-type VGCCs. Two other pyrethroids, cismethrin and bioresmethrin, had no effect on glutamate release in this model system [121, 122].

The studies described above suggested that while both type I and type II pyrethroids stimulate Ca^{2+} influx, type II pyrethroids are more potent modulators of glutamate release. However, as only a limited number of chemicals were tested, further studies are needed to determine if there were inter-class differences in these effects. A study of 11 pyrethroids, showed that they could be separated into 3 groups based on a cluster analysis of their behavior in Ca^{2+} influx and glutamate release assays. Group 1 consisted of cismethrin (type I) and bifenthrin (type I); group 2 consisted of bioallethrin (type I), tefluthrin (type I), and fenpropathrin (type II); and group 3 consisted of permethrin (type I), cyhalothrin (type II), cyfluthrin (type II), deltamethrin (type II), cypermethrin (type II), and esfenvalerate (type II). Thus, Group 1 and 2 were largely composed of type I pyrethroids while Group 3 consisted of mainly type II pyrethroids. Only Groups 1 and 3 elicited a concentration-dependent effect on Ca^{2+} influx into rat synaptosomes, while all three groups elicited a concentration-dependent effect on glutamate release, with the highest potency observed with Group 3 [124]. Thus, in this study the group with the most type II pyrethroids exhibited the highest potency in Ca^{2+} influx and neurotransmitter release assays. These findings were confirmed in another study on 11 pyrethroids. A cluster of 6 pyrethroids (λ -cyhalothrin, cypermethrin, permethrin, deltamethrin, cyfluthrin, and esfenvalerate) had the highest potency in both the Ca^{2+} influx and glutamate release assays. These 6 pyrethroids overlap with the pyrethroids in Group 3 of [124]. Another cluster of pyrethroids (tefluthrin, bio-allethrin, and fenpropathrin) affect glutamate release but not Ca^{2+} influx. These pyrethroids are identical to those in Group 2 of [124] and performed similarly in the Ca^{2+} influx and glutamate release assays in both studies. Finally, bifenthrin and cismethrin had modest effects on Ca^{2+} influx only at the highest concentrations tested and had no effect on glutamate release. These

pyrethroids were in Group 1 [124], and while neither study observed pronounced effects of these pyrethroids on Ca^{2+} influx, [124] did observe effects of these pyrethroids on glutamate release. Thus, there is good agreement between two independent studies that type II pyrethroids generally are more potent against Ca^{2+} influx and glutamate release than are type I agents.

Most pyrethroid formulations used by humans consist of mixtures of various pyrethroids. Thus, an active area of investigation is the way pyrethroid mixtures interact with the pyrethroid targets. Binary mixtures of deltamethrin with the type II pyrethroids λ -cyhalothrin, cypermethrin, and esfenvalerate increased intracellular Ca^{2+} levels in an additive manner [125]. The type I pyrethroids permethrin and cismethrin also produced additive effects on Ca^{2+} influx when combined with deltamethrin in binary combinations. In contrast, S-bioallethrin and tefluthrin (type I) and fenpropathrin (type II) in binary mixtures with deltamethrin caused less-than-additive effects on Ca^{2+} influx [125]. These studies suggested that pyrethroids which cause increased Ca^{2+} influx work together in mixtures to stimulate Ca^{2+} influx in an additive manner. However, those pyrethroids with weak stimulatory activity or inhibitory activity on influx do not synergistically modulate Ca^{2+} influx. The additive effects on Ca^{2+} influx did not predict the effects of the binary combinations of pyrethroids on glutamate release. A more-than-additive effect on glutamate release was observed when a subset of type I or type II pyrethroids was combined with deltamethrin, but none of these mixtures had produced more-than additive effects on Ca^{2+} influx [125].

7. Pyrethroid effects on GABA_A receptors

GABA (γ -aminobutyric acid) is a small molecule inhibitory neurotransmitter utilized by nearly 1/3 of the mammalian brain's synapses. In contrast to excitatory neurotransmitters, inhibitory neurotransmitters prevent a neuron from firing an action potential [48]. There are three types of GABA receptors, A, B and C. GABA type A (GABA_A) and GABA type C (GABA_C) receptors are ligand-gated chloride ion (Cl^-) channels. They exist as pentamers containing various combinations of α , β , γ , δ , ρ , π , and ϵ subunits (**Figure 4**). The subunit composition of the GABA receptors determines the pharmacological and electrophysiological properties of the receptors [126]. Thus far 6 α , 3 β , 3 γ , 1 δ , 1 π , 1 ϵ , and 3 ρ subunits have been identified in the mammalian brain. They have distinct regional and cellular distributions, resulting in a diverse array of GABA receptors. However, the majority of GABA_A receptors have 2 α , 2 β and 1 γ subunit. GABA binds GABA_A receptors at the interface of the α and β subunits stimulating an influx of Cl^- that holds the neuron's membrane potential more negative than the threshold required to initiate an action potential.

In the 1980s, GABA_A receptors were postulated to play a role in type II pyrethroid poisoning signs. Diazepam, a benzodiazepine drug, delayed type II poisoning signs and reduced mortality in mice and cockroaches injected with deltamethrin or fenvalerate [127]. Benzodiazepines bind to GABA_A receptors and enhance the inhibitory effects of GABA-induced activation of the receptor. Diazepam offered no protection from treatment with the

type I pyrethroids, allethrin and permethrin. Type II, but not Type I pyrethroids also inhibit binding of picrotoxin and t-butylbicyclophosphorothionate (TBPS) to rat brain membranes *in vitro* [128, 129]. Picrotoxin and its derivative TBPS antagonize⁸ GABA-induced Cl⁻ flux by blocking the GABA_A ion channel. Picrotoxin and TBPS binding were effectively inhibited with 5 μM deltamethrin, cypermethrin and fenvalerate, whereas 50 μM of the type I pyrethroids cismethrin and permethrin had no effect. Pyrethroids had no effect on the binding of benzodiazepines or muscimol (binds at the GABA site), suggesting that type II pyrethroids bind the GABA_A receptor at the same site as picrotoxin/TBPS.

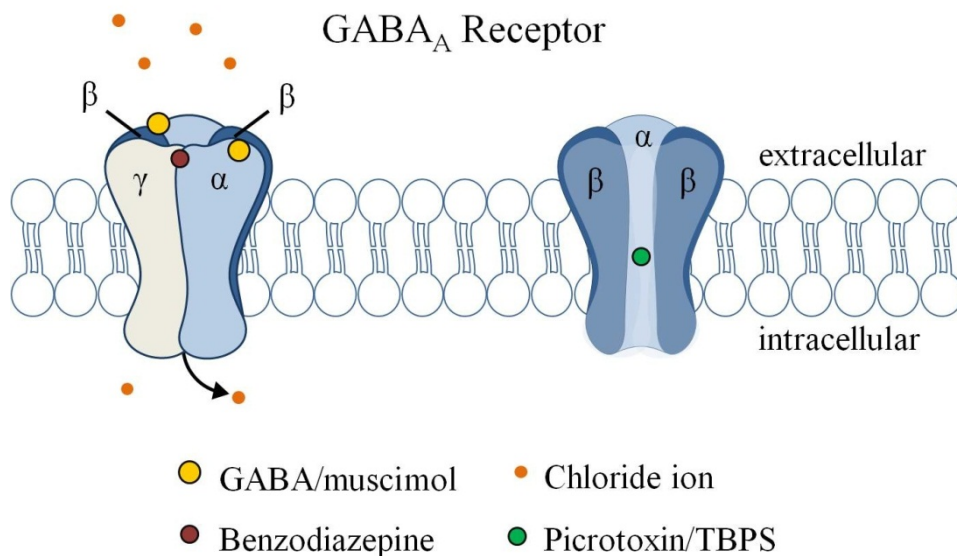


Figure 4. The GABA_A receptor. This schematic depicts the most common GABA_A receptor subunit composition and the binding sites of its ligand, GABA, and various drugs that influence the receptor's function. Modified from Motifolio Biomedical PowerPoint Toolkit Suite.

Functional evidence of type II pyrethroid inhibition of GABA_A receptors arose from electrophysiology experiments using crayfish claw opener muscles [130]. Bathing crayfish claw opener muscles in a GABA bath causes a decrease in input resistance, indicating an activation of GABA receptors. GABA_A receptor antagonists, such as picrotoxin, increase input resistance by blocking the ion pore. Several type II pyrethroids increased the input resistance of the claw opener muscle in a manner similar to picrotoxin. Furthermore, cypermethrin inhibited GABA-stimulated influx of radioactive ³⁶Cl⁻ into rat brain synaptosomes [131]. However, none of these experiments were conducted in the presence of TTX to block the action of pyrethroids on VGSCs. In the presence of TTX, the GABA induced inward Cl⁻ current of cultured rat sensory neurons was not affected by

⁸ Antagonists are chemicals that bind a receptor and blocks action of the receptor. Agonists are chemicals that bind the receptor triggering a response.

deltamethrin [132]. Antagonism of GABA_A receptors should decrease inhibition in the brain. However, cismethrin, fenvalerate or deltamethrin increased function of inhibitory neurons in the rat hippocampus [133]. Further evidence against a direct effect of pyrethroids on GABA_A receptors came from trout brain synaptosome ³⁶Cl⁻ influx assays [134]. Deltamethrin, cypermethrin and permethrin caused a concentration-dependent decrease in GABA-dependent ³⁶Cl⁻ influx. This effect was completely inhibited by TTX, indicating that previously observed effects of type II pyrethroids on GABA_A receptors are indirect and downstream of the effects on VGSCs.

8. Pyrethroid effects on voltage-gated Cl⁻ channels

Cl⁻ is the most abundant extracellular anion and its movement across cell membranes is involved in cell volume regulation, and acidification of intracellular compartments such as lysosomes [135-137]. In excitable cells, voltage-gated Cl⁻ channels (CICs) act to maintain resting membrane potential. Comparatively little is known about the CICs, but the channels are thought to function as dimers with two identical ion pores. In [134] an increase in basal uptake (GABA independent) of ³⁶Cl⁻ occurred upon pyrethroid administration. This pyrethroid-enhanced uptake was completely sensitive to TTX but only partially inhibited by a GABA_A channel blocker, providing the first evidence that CICs may be involved on pyrethroid action.

Deltamethrin decreased the probability of CIC opening in cultured mouse neuroblastoma cells in a concentration-dependent manner [138, 139]. This effect was initially thought to be type II specific as cypermethrin also decreased open channel probability. However, bioallethrin (type I) also decreased the probability of CIC opening, whereas esfenvalerate and cyhalothrin (type II) had no effect [140]. Therefore the ability of a pyrethroid to modify CIC is not solely determined by the presence of an α cyano group. The Cl⁻ channel agonist, ivermectin, increased the probability of CIC opening in deltamethrin-treated cultured mouse neuroblastoma cells [141]. *In vivo* experiments demonstrated that ivermectin decreased salivation and muscle twitching. Thus, CICs are biologically relevant sites of action for certain pyrethroids that contribute to some of the intoxication signs.

9. Conclusion

Pyrethroid insecticides are synthetic analogs of pyrethrin, the natural insecticides produced by *Chrysanthemum* species. Pyrethroid insecticides are divided into two groups based on the absence (type I) or presence (type II) of a cyano group. Both types disrupt action potentials in insects by prolonging the open time of VGSCs in a concentration and temperature dependent manner by delaying channel inactivation and deactivation. As the type II pyrethroids hold VGSCs open longer, they allow greater influx of Na⁺ resulting in neuron depolarization and conduction block. Pyrethroids similarly affect mammalian VGSCs and those effects are thought to underlie the signs associated with pyrethroid intoxication. Developing mammals are more sensitive to pyrethroids than adults, but the molecular mechanisms for this increased sensitivity is unknown. The developmental neurotoxicity of

pyrethroids may be due to effects on VGSC isoforms that are only expressed during development. There is also evidence that pyrethroid insecticides target mammalian VGCCs and CICs. Pyrethroid appear to cause VGCC sub-type specific effects, although there are conflicting data present in the literature. Some pyrethroids are capable of antagonizing CICs, and this action appears to be partly responsible for the hypersalivation and motor dysfunction seen in pyrethroid intoxication. There is currently little understanding as to how or if pyrethroid modification of VGCCs and CICs contribute to mammalian toxicity both in adult and developing animals. Furthermore, as most mammals are exposed continually to a mixture of pyrethroids it remains to be seen how the combined effects of different pyrethroids affect neuronal function long-term.

Author details

Erin N. Wakeling and William D. Atchison*

Department of Pharmacology & Toxicology, Michigan State University, East Lansing, MI, USA

April P. Neal

Food and Drug Administration, College Park, MD, USA

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* Corresponding Author

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Non-Traditional Pesticidally Active Compounds

Ahmed S. Abdel-Aty

Additional information is available at the end of the chapter

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1. Introduction

Several organic compounds have not been approved as applied pesticides showed some useful actions against different pests. They may be considered as cores of new pesticides. Some compounds were prepared and assessed for their pesticidal activities. They showed persuasive effects as fungicides, herbicides (phytotoxic effects), nematicides, molluscicides, insecticides as well as rodenticides comparing with commercial pesticides.

2. Materials and methods

2.1. Tested chemicals

Both indol-3-acetic acid GRG, El-Gomhouria Drug Company; indole-3-butyric acid, Sisco Research Laboratories, Mumbai, India, and other chemicals and solvents were purchased from El-Gomhouria Drug Company, Egypt. Standards of used herbicide, metribuzin (senecor), (4-amino-6-tert.butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one) and used fungicide metalaxyl, N-(2,6-dimethylphenyl-N-methoxyacetyl)-DL-alaninemethylester were donated by Kafr El-Zayat Company for pesticides, Egypt. Based on [1-2] with modification, some benzotriazole, benzylidene, coumarin, imidazolidine, indole, oxazolone and pyrazole, derivatives were prepared and identified [3-7].

2.2. Instruments

Structural confirmation was carried out by determination of melting points on kofler block; elemental micro analysis (C, H, N, X); IR, UV, NMR and Mass spectroscopy measurements in Microanalytical Center, Cairo University, Giza, Egypt. NMR spectra were recorded on Varian Mercury-VX-300 NMR Spectrometer using tetramethylsilane (TMS) as a standard. Mass spectra were recorded on a Shimadzu MS5988-mass spectrometer at 70 eV. Determination of soluble sugars, chlorophyll contents and total soluble phenols (TSP) were done on Unico-1200 Spectrophotometer. Both enzymatic activity and nucleic acids

contents were measured using Nicolet 100 UV-VIS Spectrophotometer, Thermo Electron Corporation.

2.3. Tested fungi

Wood decay fungi, *Coriolus versicolor* (Linnaeus) Quélet, strain CTB 863 and *Gloeophyllum tarbeum* (Persoo ex Fries) Murrill, strain BAM Ebw. 109 were provided from Laboratory of Wood Technology, Ghent University, Belgium. *Alternaria alternata*, *Fusarium calmorum*, *F. oxysporum*, *Helmintho-sporium* sp, *Macroformina phaseoli*, *Pythium debarianum*, and *Rhizoctonia solani* were provided by Plant Pathology Department, Faculty of Agriculture, Alexandria University, Egypt.

2.4. Tested animals

Albino norway rats strain (*Rattus norvegicus* var. *albus*) were taken from the Laboratory of Rodents, Department of Pesticide Chemistry and Technology, Faculty of Agriculture, Alexandria University, Egypt. *Spodoptera littoralis* Boisd strain was grown in the breeding sector of Pesticide Chemistry Department, Faculty of agriculture, Alexandria University, Egypt. *Thepa pisana* and *Eobania vermiculata* Muller snails, family Helicidae were collected from gardens of Faculty of Agriculture, Alexandria University.

Through these studies, *In vitro* antifungal assessment of the tested compounds was conducted using a mycelial radial growth technique [8-9]. Inhibition percent and IC₅₀ (the concentration caused 50% inhibition) values of the hyphal growth were calculated [10-11]. Significance was elucidated through three-way ANOVA completely randomized Student-Newman-Keuls Test. *In vivo* determination of polyphenoloxidase [12], Peroxidase [13] activities and DNA and RNA contents [14] were conducted. Protein content (mg) [15] and the specific activities of all treatments were calculated. Insecticidal activity was tested on both the 4th and 6th larval instars of *S. littoralis* Boisd. The tested larvae were reared on a semi artificial growing medium [16-17]. Mortality percents were calculated.

Seed treatment was carried out according to [18]. Toxic effects on the seedling stage (after germination) of both root and shoot systems using the plain agar was done according to [19]. In dried wheat seedlings, total soluble sugars (T.S.S), reducing sugars (R.S) and non-reducing sugars (non-R.S) expressed as µg/g dried plant were determined [20]. Chlorophyll (a and b) contents were calculated in µg/g tissue fresh weight [21]. Total soluble phenolics were determined as mg gallic acid equivalent (mg GAE)/g fresh weight [22-23]. Mortality test was carried out on Albino norway rats strain (*Rattus norvegicus* var. *albus*) by (No-choice test) [24]. Haemoglobin concentration (Hb%) was determined according to [25], using Boehring Mannheim Gm bH Diagonestic Kit. Haematocrit value (Hc%), white blood cells (WBCs) and red blood cells (RBCs) were counted [26]. *In vivo* determination of alanine transaminase (sALT) and aspartate transaminase (sAST) activities were carried out based on [27] using Boehring Mannheim Gm bH Diagonestic Kits. Effects of the prepared compounds could be summarized in the following points:

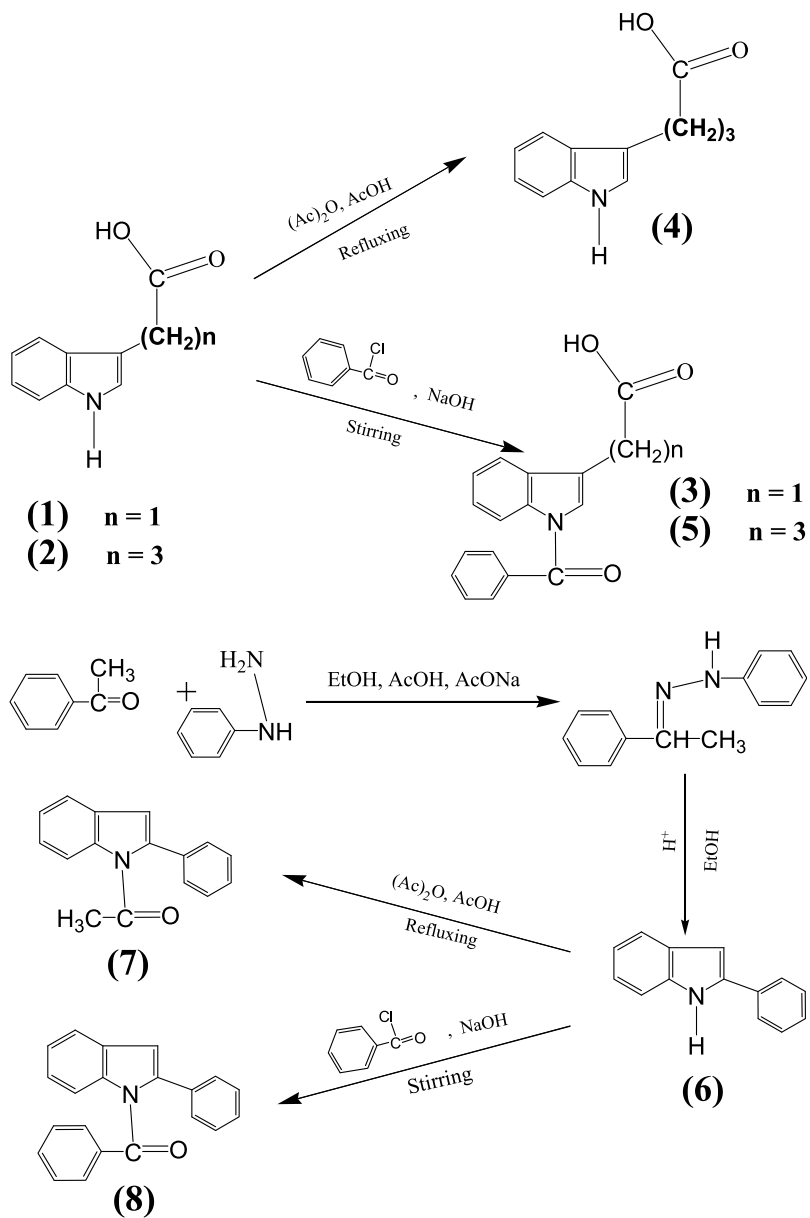
3. Results and discussion

3.1. Fungicidal activity of some indole derivatives [7]

In the vast heterocyclic structural space, the indole nucleus occupies a position of major importance as antimicrobial agents. Combination of IAA (at 100 µg/ml) with *Cryptococcus laurentii* suppressed blue and gray mold infections on pear fruit more than *C. laurentii* alone [28]. It exhibited antifungal activity against *Gibberella pulicaris* suppressing the dry rot infection of wounded potatoes optimally when combined with phenylacetic acid and tyrosol [29]. Its 5-methoxy- derivative and 1H-indole-4,7-diones showed antifungal and antibacterial activities against several species [30-31]. So, besides, both indol-3-acetic acid and indol-3-butyric acid, purchased from El-Gomhouria Drug Company, Egypt, Six indole derivatives: 1-benzoyl indole-3-acetic acid, 1-acetylindole-3-butyric acid, 1-benzoylindole-3-butyric acid, 2-phenylindole, 1-acetyl-2-phenylindole and 1-benzoyl-2-phenylindole were prepared and structurally confirmed. Their fungicidal effects against the damping off fungi as a very important economical group threatening several crops like *Fusarium calmorum*, *Rhizoctonia solani*, *Pythium debarianum* and *Macrofomina phaseoli* that causes post harvest fruits rotting were compared with the technical grade of metalaxyl (Radomil). As shown in Table (1), against *F. calmorum*, derivatives of 2-phenylindole were more effective than the standard fungicide. 1-Acetylindole-3-butyric acid appeared the most active. The other derivatives were less effective than metalaxyl. *M. phaseoli* was affected with less toxicity degree. 1-Benzoyl-2-phenylindole slackened in its effect to less than the standard. Fungicidal activity was increased against *P. debarianum* in all cases in comparison to *F. calmorum*. 2-Phenylindole, 1-acetyl-2-phenylindole and 1-benzoyl-2-phenylindole inhibited its hyphal growth with IC₅₀ values equaled 17.7, 15 and 81 µg/ml, respectively in comparison to 211 µg/ml of the standard fungicide. 1-Acetylindole-3-butyric acid was very active with IC₅₀ value equaled 19 µg/ml. *R. solani* appeared tolerant than other fungi for all compounds including the standard. From the mentioned results, fungicidal activity proved to be a function of both treated fungus and the structure. *P. debarianum* was the most sensitive, followed by *F. calmorum*, *R. solani* and *M. phaseoli*. Their hyphal growth was inhibited with Mean ± SE equaled 35.52 ± 2.16, 30.02 ± 1.99, 28.02 ± 1.66 and 25.31 ± 1.49 µg/ml, respectively with significant differences. Regarding the structure activity relationship, acylation of the natural auxin enhanced its fungicidal activity. Substitution with a 1-benzoyl moiety in indole-3-acetic acid (IAA) slightly increased the activity although in case of indole-3-butyric acid (IBA) it showed no significant effect. Acetylation of IBA strongly multiplied the activity against all tested fungi. Replacing the 3-aliphatic chain with 2-phenyl moiety firmly improved the toxicity against all the treated fungi. While benzoylation of 2-phenylindole decreased its activity, acetylation maintained its toxicity high. Based on statistical analysis, 1-acetylindole-3-butyric acid, 2-phenylindole, 1-acetyl-2-phenylindole and 1-benzoyl-2-phenylindole exhibited their inhibition with Mean ± SE equaled 44.65 ± 3.91, 43.07 ± 3.32, 42.36 ± 3.38 and 31.02 ± 2.76 µg/ml, respectively surpassing the standard fungicide with 29.05 ± 2.46 µg/ml. The other structures were less effective than the standard fungicide. The effect on hypha growth agreed with [32] who referred the reduction of mycelial dry weight and protein content of *F. oxysporum lycopersici* to IAA. It also inhibited *M. phaseolina* mycelial growth *in vitro* and reduced

the charcoal rot disease both in field and greenhouse [33]. Polyphenoloxidase in *R. solani* systematically responded to 2-phenylindole with IC_{50} equaled 80.27 $\mu\text{g/ml}$. 1-Acetylindole-3-butyric acid inhibited it with 39% at the lowest concentration, followed by activation with 78.9 and 84% of control at 1.0 and 2.0 IC_{50} values, respectively. 1-Acetylindole-3-butyric acid was more effective than 2-phenylindole inhibiting it with 41.5 and 80.2 $\mu\text{g/ml}$ IC_{50} values comparing with 87.6 and 117.2 $\mu\text{g/ml}$, respectively in case of *F. calmorum* and *M. phaseoli*. While *P. debarianum* enzyme activity was inhibited by 1-acetylindole-3-butyric acid with IC_{50} equaled 45.6 $\mu\text{g/ml}$, 2-phenylindole enhanced it with activating concentration of 50% (AC_{50}) equaled 35.1 $\mu\text{g/ml}$. Regarding peroxidase, in *R. solani* it was activated with AC_{50} equaled 14.5 and <11.7 $\mu\text{g/ml}$ in case of 2-phenylindole and 1-acetylindole-3-butyric acid, respectively. Both the two compounds exhibited narrow ranged inhibitory effects against the enzyme from *P. debarianum*. While in *M. phaseoli* treatment, the enzyme was activated systematically with 1-acetyl indole-3-butyric acid with $AC_{50} < 5.9$ $\mu\text{g/ml}$, 2-phenylindole affected it from -39 to 54.3 % inhibition regularly with increasing its concentration. It affected the activity of *F. calmorum* enzyme from 85.0 to -115.3 % inhibitions within its concentration range. This activity was inhibited with 2-phenylindole with IC_{50} equaled 49.9 $\mu\text{g/ml}$. On the other sight, both RNA and DNA contents were affected. Both RNA and DNA molecules are related to each other, so the results obtained were exhibited in systematic response. In *F. calmorum*, RNA and DNA contents as compared with control (51.5 and 49.5 mg/liter) were found to be reduced at the tested IC_{50} rates of 2-phenylindole. This reduction was increased with increasing the tested concentration to 0.5 IC_{50} then RNA content was dramatically increased to 26.3 and 24.7 mg/liter and DNA content was increased to 25.3 and 23.8 mg/liter at 1 and 2 IC_{50} . RNA and DNA contents in *R. solani* highly increased and reached to the maximum peak of increase at 1.0 IC_{50} of 2-phenylindole. RNA and DNA contents in *M. phaseoli* were reduced to less than 50% of control at the tested rates. They changed from 8.3 to 5.9 and from 8.0 to 5.7 mg/liter comparing with 16.1 and 15.5 mg/liter of control. These contents of *P. deparianum* behaved the same trend of these in *M. phaseoli* changing from 30.6 to 11.4 and from 29.4 to 10.9 mg/liter comparing with 32.2 and 31 mg/liter of control.

1-Acetylindole-3-butyric acid affected both RNA and DNA contents differently according to the tested fungus and concentration. It reduced them in *F. calmorum* in systematic arrangement at all the tested IC_{50} rates comparing with control. While RNA and DNA contents in *M. phaseoli* were decreased by increasing the tested rate with systematical arrangement. This decreasing effect was noticed in all fungi. Their contents were reduced from 45.4 to 20.3 and 43.6 to 19.5 mg/L in case of *F. calmorum*, they were reduced from 12.9 to 6.7 and from 11.7 to 6.5 in case of *M. phaseoli* comparing with 51.5, 49.5, 16.1 and 15.5 of their control, respectively. While the contents from *P. debarianum* were decreased until 0.5 IC_{50} and increased again at the two highest concentration rates, they were systematically increased with increasing the concentration in case of *R. solani*. General descriptive analysis proved that 2-phenylindole affected *M. phaseoli* significantly greater than *P. debarianum* with



- 1 Indole-3-acetic acid
- 2 Indole-3-butyric acid
- 3 1-Benzoyl indole-3-acetic acid
- 4 1-Acetyl indole-3-butyric acid

- 5 1-Benzoyl indole-3-butyric acid
- 6 2-Phenylindole
- 7 1-Acetyl-2-phenylindole
- 8 1-Benzoyl-2-phenylindole

Scheme 1. Preparation of Compounds 1-8

Treated fungus	Treatment	IC ₅₀ (95% C L) µg/ml	Slope ± S.E	χ ²	TF
<i>F. calmorum</i>	Indole-3-acetic acid ^d *	420 (222 – 823)	0.6 ± 0.005	4.75	2.19
	1-Benzoyl indole-3-acetic acid ^c	523 (322 – 859)	0.87 ± 0.011	7.04	2.72
	Indole-3-butyric acid ^a	576 (388 – 858)	1.28 ± 0.025	2.78	3.00
	1-Acetyl indole-3-butyric acid ⁱ	26.6 (21.3 – 33.3)	1.41 ± 0.01	4.54	0.14
	1-Benzoyl indole-3-butyric acid ^b	513 (335 – 793)	1.03 ± 0.015	1.29	2.67
	2-Phenylindole ^h	67.4 (53.0 – 85.8)	1.11 ± 0.008	8.57	0.35
	1-Acetyl-2-phenylindole ^g	86.7 (66.4 – 113)	0.98 ± 0.007	5.33	0.45
	1-Benzoyl-2-phenylindole ^f	99.9 (77 – 129.9)	1.02 ± 0.008	0.63	0.52
	Metalaxyl ^e	192 (126 – 296)	0.69 ± 0.006	3.6	1.0
<i>M. phaseoli</i>	Indole-3-acetic acid ^a	807 (440 – 1514)	0.81 ± 0.011	2.83	4.66
	1-Benzoyl indole-3-acetic acid ^d	572 (359 – 923)	0.97 ± 0.014	2.99	3.30
	Indole-3-butyric acid ^b	699 (458 – 1073)	1.38 ± 0.003	1.28	4.03
	1-Acetyl indole-3-butyric acid ^f	59.0 (47.0 – 74)	1.21 ± 0.009	3.87	0.34
	1-Benzoyl indole-3-butyric acid ^c	448 (325 – 622)	1.38 ± 0.026	2.62	2.59
	2-Phenylindole ⁱ	96 (74.6 – 123.4)	1.06 ± 0.008	8.1	0.55
	1-Acetyl-2-phenylindole ^h	93 (71.7 – 120.0)	1.03 ± 0.008	7.78	0.54
	1-Benzoyl-2-phenylindole ^g	355 (247 – 514)	1.02 ± 0.001	2.18	2.05
	Metalaxyl ^e	173 (127 – 237.6)	0.93 ± 0.008	4.78	1.00
<i>P. debarianum</i>	Indole-3-acetic acid ^b	301 (207.9 – 438)	0.93 ± 0.001	3.76	1.43
	1-Benzoyl indole-3-acetic acid ^b	171 (125 – 236)	0.91 ± 0.008	3.96	0.81
	Indole-3-butyric acid ^d	249 (179 – 349)	0.98 ± 0.001	9.33	1.18
	1-Acetyl indole-3-butyric acid ^g	19 (14.4 – 24.8)	1.1 ± 0.006	1.72	0.09
	1-Benzoyl indole-3-butyric acid ^a	488.4 (319 – 753)	1.0 ± 0.14	0.46	2.31
	2-Phenylindole ^f	17.7 (11.8 – 26.4)	0.67 ± 0.004	0.48	0.08
	1-Acetyl-2-phenylindole ^g	15.0 (9.5 – 23.2)	0.61 ± 0.004	2.15	0.07
	1-Benzoyl-2-phenylindole ^e	81 (57.2 – 115)	0.73 ± 0.005	3.99	0.38
	Metalaxyl ^c	211 (145 – 310)	0.80 ± 0.007	1.03	1.00
<i>R. solani</i>	Indole-3-acetic acid ^c	1009 (539 – 1923)	0.94 ± 0.016	4.42	4.36
	1-Benzoyl indole-3-acetic acid ^c	1244 (633 – 2515)	0.71 ± 0.008	9.08	5.38
	Indole-3-butyric acid ^a	644 (368 – 1151)	0.79 ± 0.01	2.38	2.78
	1-Acetyl indole-3-butyric acid ⁱ	117 (97.6 – 141)	1.57 ± 0.018	6.1	0.51
	1-Benzoyl indole-3-butyric acid ^b	663 (377 – 1192)	0.79 ± 0.01	2.64	2.87
	2-Phenylindole ^h	34.6 (25.1 – 47.5)	0.81 ± 0.005	7.14	0.15
	1-Acetyl-2-phenylindole ^f	37.5 (27.6 – 50.7)	0.85 ± 0.005	1.79	0.16
	1-Benzoyl-2-phenylindole ^d	122.2 (93 – 161)	1.0 ± 0.008	3.31	0.53
	Metalaxyl ^e	231 (167.7 – 321)	0.98 ± 0.01	3.21	1.00

TF: Toxicity factor related to Metalaxyl * Significance at 0.05 level against each fungus DF = 4

Table 1. *In Vitro* fungicidal activity of indole derivatives

(8.42 ± 0.86 and 25.05 ± 1.84) and (8.08 ± 0.83 and 24.1 ± 1.78) mg/liter means \pm SE of RNA and DNA contents. Although there was no significant difference between *R. solani* and *F. calmorum*, they differed significantly from the other tested fungi with (29.2 ± 2.55 and 29.23 ± 0.55) and (28.12 ± 2.45 and 28.16 ± 0.42) mg/liter of RNA and DNA contents. The same arrangement was exhibited in treatment with 1-acetylintole-3-butyric acid except achieving a significant difference among all the tested fungi. RNA contents were 10.57 ± 0.78 , 23.01 ± 1.61 , 28.57 ± 1.07 and 34.31 ± 2.61 mg/liter, while DNA contents were 10.09 ± 0.73 , 22.07 ± 1.53 , 27.52 ± 1.01 and 33.0 ± 2.51 mg/liter in case of *M. phasoli*, *P. debarianum*, *R. solani* and *F. calmorum*, respectively. Comparing with the untreated fungus, all sugar types in *M. phasoli* were reduced at 2-phenylindole concentrations with non-systematic arrangement. *R. solani* sugars contents were strongly multiplied at 0.1 and 0.25 IC₅₀ concentrations, followed by a firm decrease at 0.5 IC₅₀ and this reduction was increased at 1.0 IC₅₀. This effect was differed from the effect of 1-acetylintole-3-butyric acid as both reduced and non-reduced sugars were systematically decreased with increasing the concentration. Reduced, non-reduced and total soluble sugars were *in vivo* affected with the two studied compounds in a treated fungus and concentration dependent effect.

It could be concluded that 2-phenylindole and 1-acetylintole-3-butyric acid affected both RNA and DNA contents in the tested fungi, which may develop deformed and dead cells. These effects of indole acetic acid and some derivatives are due to formation of 3-methylene-2-oxindole, which may conjugate with DNA bases and protein thiols [34]. There were highly effective against polyphenoloxidase and peroxidase activities that means disturbance in the cell physiology as [28] revealed that IAA alone or with *C. laurentii* stimulated catalase, peroxidase and polyphenol oxidase activities of pear fruit. The studied indole derivatives may affect the treated fungi in another site of action as [35] found that IAA and IBA greatly increase somatic segregation in *Aspergillus nidulans* and increasing their concentrations increased mitotic segregation of the fungus.

3.2. Insecticidal activity of the prepared indole derivatives [36]

The Egyptian cotton leaf-worm, *S. littoralis* (Boisd.) is an important polyphagous insect attacking several crops and ornamentals worldwide. Persuasive effects against it were referred to plant alkaloids [37-39]. So, this study aimed to examine the indole derivatives against its stages.

Lethal effects

The tested compounds were more effective against the 4th larval instar than the 6th instar after 5 days except 1-acetylintole-3-butyric acid (3) and 1-acetyl-2-phenylindole (7). The effect was increased after nine days in all cases. 1-Benzoyl-2-phenylindole (8) was less effective on the 6th instar. 2-Phenyl indole (6) and its 1-acetyl derivative (7) were more effective on the 6th instar. Lethal effects were increased in all tested compounds against 6th instar except for compounds 2 and 5. It was also found that substitution of compound 3 raised the toxicity on the 6th instar. The increase due to its acetylation was greater than

benzylation. Substitution of 2-phenyl moiety on the indole ring in stead of side aliphatic carboxylic group increased the larval mortality in case of compound 6 more than in indole-3-acetic acid (1). Substitution with 1-acetyl on 2-phenylindole multiplied the lethality against the two tested larval instars, while substitution with 1-benzoyl in compound 8 enhanced the toxicity only against the 4th larval instar. The most effective compound was indole-3-butyric acid (2) with 70.9 and 39.7 µg/gm LC₅₀ values on the 4th instar after 9 and 13 days, while 1-acetylindole-3-butyric acid (3) and 1-acetyl-2-phenylindole (7) were more effective with 151.4 and 80.6 µg/gm LC₅₀ values against the 6th instar. So, compounds 2, 3 and 7 were chosen for egg treatment.

Sub-lethal effects (Fresh body weight)

The larval weight of the 4th instar (after 7 days) was differently affected with the applied derivatives. Benzylation of indole-3-acetic acid in compound 4 affected the larval weight in non systematic arrangement with concentrations. Acetylation of indole-3-butyric acid in compound 3 reduced the larval weight at 50 and 100 µg/gm, followed by an increase at higher concentrations. On the contrary, its benzoyl derivative (compound 5) increased the larval weight at lower concentration, followed by reduction at the two higher concentrations. Light reduction occurred at low concentrations, followed by gradual activation with increasing the concentration, which was exhibited by compound 6. Substitution with 1-acetyl moiety in compound 7 increased the larval weight at low concentration followed by inhibition percents ranging from 3.2 to 18.5% of control at 100-1000 µg/gm. Benzylation of 2-phenylindole in compound 8 decreased the reduction effect more than compound 7. Comparing with the untreated 6th larval weight (0.77 gm) after two days, all the tested compounds reduced the treated larval weight at all concentrations with different degrees and arrested their development to 7 days after treatment. Compounds 1 and 2 showed narrow differences among their concentrations with less reducing effect, followed by compounds 8, 6, 5 and 7. Compounds 3 and 4 were the most active derivatives in weight reduction. From these results, the hormonal effect was obviously clear through the activation of larval weight in most cases when applied earlier at the 4th instar more than at the 6th instar (Figure 1).

Development

Untrated 4th instar larvae developed to pupal and adult stages after 6-7 and 9-10 days, respectively. Indole-3-acetic acid (1) at 10 µg/gm delayed this development to 29 and 45 days, respectively. However, the other compounds were less effective causing developing of 50, 10, 75, 92, 13, 63, and 83% of the treated larvae to pupae in case of compounds 2, 3, 4, 5, 6, 7 and 8, respectively after 21 days. While, compounds 5 and 7 caused complete transformation of the treated population to adults, compounds 2, 4, 6 and 8 caused developing of 75, 75, 55 and 67 % of pupae to adults. Compound 3 (1-acetylindole-3-butyric acid) was the most effective structure blocking adult emergence to 25% of the treated population after 45 days. Regarding 6th larval instar, its control completely developed to the pupal and adult stages after 2-3 and 7-8 days, respectively. All compounds arrested the

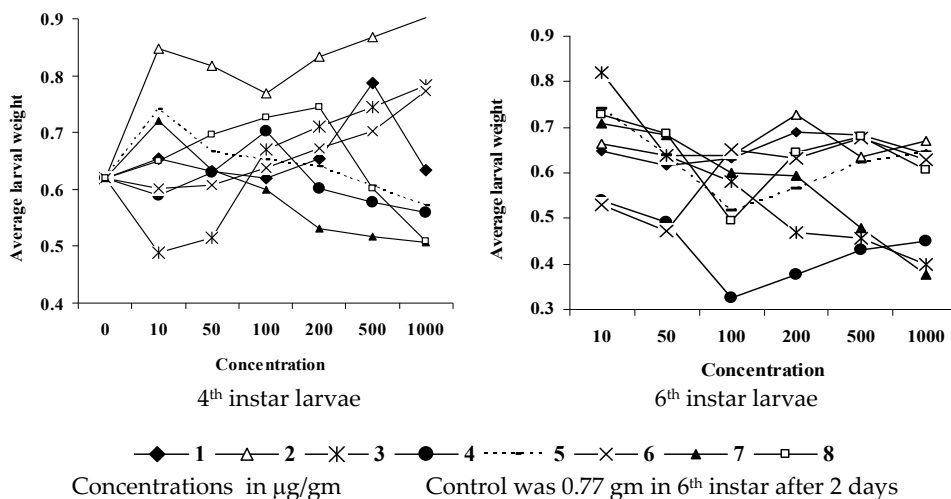


Figure 1. Effect of the tested compounds on fresh larval weight of *S. littoralis*; shown as average weight (gm) after 7 days of treatment. 1: Indole-3-acetic acid; 2: Indol-3-butyric acid; 3: 1-Acetylindole-3-butyric acid; 4: 1-Benzoylindole-3-acetic acid; 5: 1-Benzoylindole-3-butyric acid; 6: 2-Phenylindole; 7: 1-Acetyl-2-phenyl indole; 8: 1-Benzoyl-2-phenyl indole

larval development except compounds 3 and 5, which caused 25 and 18% pupation after 13 days. Compound 1 was the most effective inhibiting the adult emergence, followed by compound (4), 1-benzoylindole-3-butyric acid (5), indole-3-butyric acid (2), 2-phenylindole (6), 1-acetylindole-3-butyric acid (3), 1-benzoyl-2-phenylindole (8) and 1-acetyl-2-phenylindole (7). They blocked the adult emergence to 7, 14, 31, 33, 39, 46, 48 and 50% of the treated population after 35 days. From the results, the duration of *S. littoralis* larval stage was significantly affected. It required a longer time to reach next stadium differing from control (Figure 2).

Malformations

Comparing with the untreated larvae, compounds 1 and 2 exhibited 14.6% and 16.7% malformation in the intermediates of the treated 4th larval instar at 200 µg/ml, with no effect on 6th larvae. Acetylation of indole-3-butyric acid in compound 3 affected the intermediates at lower concentrations in the 6th larval instar, while its benzoylation increased this effect against the 4th instar only. Acetylation of 2-phenylindole caused 32.6 and 61.1% intermediate malformation at 100 and 200 µg/ml in treated 4th instar larvae. 1-Benzoyl-2-phenylindole affected 4th larvae at 10 µg/ml with 7.6% malformation. However, its effect was as high as 10.1% at the higher concentrations against 6th instar intermediates. These malformation symptoms appeared as larval-pupal intermediates in which the posterior portion of the body only exhibited the pupal shape, while the anterior portion had larval head capsule and thoracic legs (Figure 3). Malformation of the produced pupa (forming abnormal pupa without wings or that failed to shed the larval cuticle) resulted from the 4th

larval instar, which was more sensitive than that from 6th larval instar to treatment with compounds 1-3, 2-phenylindole (6) and 1-benzoyl-2-phenylindole (8). The effects of compounds 4 and 5 depended on the applied concentration. Benzoylation of 2-phenylindole increased the pupae malformation. Adult malformation (adult failed to shed the pupal cuticle or adult with dwarf wings) was affected with the tested compounds, concentration and larval instar. Adult emergence from both treated instars was affected. Compounds 1, 2, 3 and 5 blocked the adult emergence to 10.3 - 47.4%, 16.7 - 50%, 20.2 - 50.6% and 10.6 - 55.7% in systematic arrangement, respectively from 4th larval populations comparing with 100% of control. The blocking effect was reduced with increasing the concentration. They blocked adult emergence to 25.9-43.7%, 36.8-57.0%, 31.9-40.9% and 32.5-66.9%, respectively in non systematic arrangement in case of the 6th larval population. Compound 4 caused 9.5-20.8% and 22.9-69.5% adult emergence in case of the treated 4th and 6th larval instars, respectively. Although 2-phenylindole and its 1-acetyl derivative affected the adult emergence from both treated instars in non systematic arrangement, its 1-benzoyl derivative blocked the adult emergence with increasing the concentration. Adult emergence was more inhibited from 4th larval instar treatment indicating that treatment of the lower larval instars gave good results of control (Figure 4).

Effect on eggs

Egg hatchability was inhibited increasingly in systematic arrangement with concentrations. Both 1-acetylindole-3-butyric acid (3) and 1-acetyl-2-phenyl-indole (7) completely stopped hatching when mixed at 100 µg/gm with the medium. As the untreated egg mass hatched completely within 24 hours, treated eggs took 48-96 hours and 6-7 days at high concentrations of compound 2 and compounds 3 & 7, respectively. After 48 hours, only dipping the egg masses in solutions of compound 2 inhibited hatching with IC₅₀ value equaled 29.1 µg/ml and killed the produced larvae with LC₅₀ value equaled 26.2 µg/ml. Transferring treated eggs to the poisoned medium enhanced the toxicity to IC₅₀ equaled 13.2 µg/gm and LC₅₀ equaled 15.2 µg/gm. Although acetylation of compound 3 decreased larval mortality in dipping technique with or without transferring the eggs to the poisoned medium, it enhanced egg-hatching inhibition when dipped only in the toxic solutions. Although compound 7 was less effective when egg masses were dipped in it, its mixing with the used medium greatly enhanced the effect with IC₅₀ value equaled 15.3 µg/gm on egg-hatching and LC₅₀ value equaled 7.5 µg/gm on larval mortality. In conclusion, mortality of 4th instar larvae was increased with increasing the aliphatic side chain. Substitution of N-H of 2-phenylindole raised the toxicity, vice versa in case of indole-3-butyric acid against the same instar. The tested compounds affected larval weight, pupation and adult emergence indicating that treatment induced an effect typical to juvenile hormone excess. These effects varied according to the tested compound. These delayed effects are expressed as developmental abnormalities in the adult stage. These effects may be due to oxidative decarboxylation forming 3-methylene-2-oxindole, which may conjugate with DNA bases and protein thiols [34]. It may be also due to inhibition of

cholinesterase [40]. Its effect is associated with cell phenoloxidase (PO) and peroxidase activities [6, 41]. Phenoloxidase (PO) is believed to be a key mediator of immune function in insects.

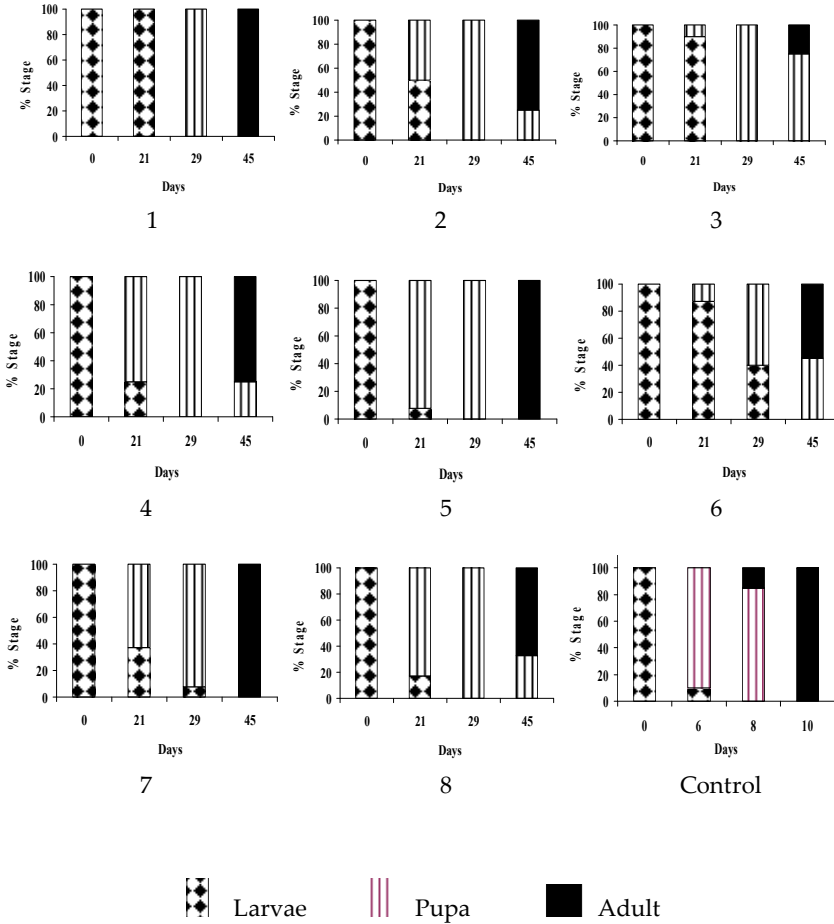
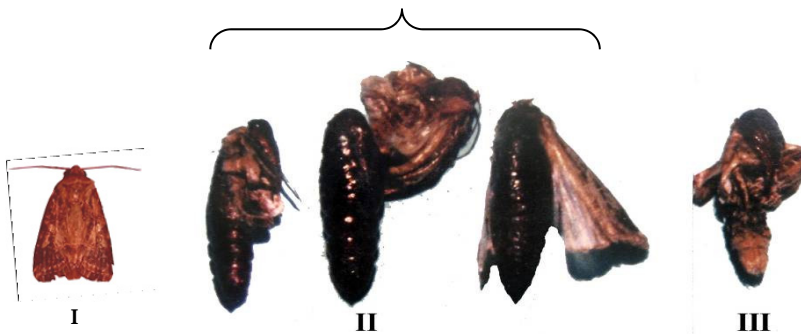


Figure 2. Effect on *S. littoralis* 4th larval development at 10 µg/gm; **1:** Indole-3-acetic acid; **2:** Indol-3-butyric acid; **3:** 1-Acetylindole-3-butyric acid; **4:** 1-Benzoylindole-3-acetic acid; **5:** 1-Benzoylindole-3-butyric acid; **6:** 2-Phenylindole; **7:** 1-Acetyl-2-phenyl indole; **8:** 1-Benzoyl-2-phenyl indole



Malformations in the produced pupae comparing with control

I Normal pupa (control), II, juvenilized larval-pupal intermediates, III, abnormal pupae failed to shed the larval cuticle



Different abnormal forms of the produced adults comparing with control

I, Normal adult (control), II, abnormal adults failed to shed the cuticle, III, adult with dwarf wings

Figure 3. Maleformations effects of the tested indole derivatives

This notice may clarify the effect of the tested compounds on adult emergence and pupation. N-H and N-substituted indole-2- and 3-carboxamide showed a strong inhibitory (95-100%) effect on superoxide anion (SOD). Substitution on 1-position of the indole ring caused significant differences between the activity results regarding lipid peroxidation inhibition [42] emphasizing the differences in effects due to the derivative structure.

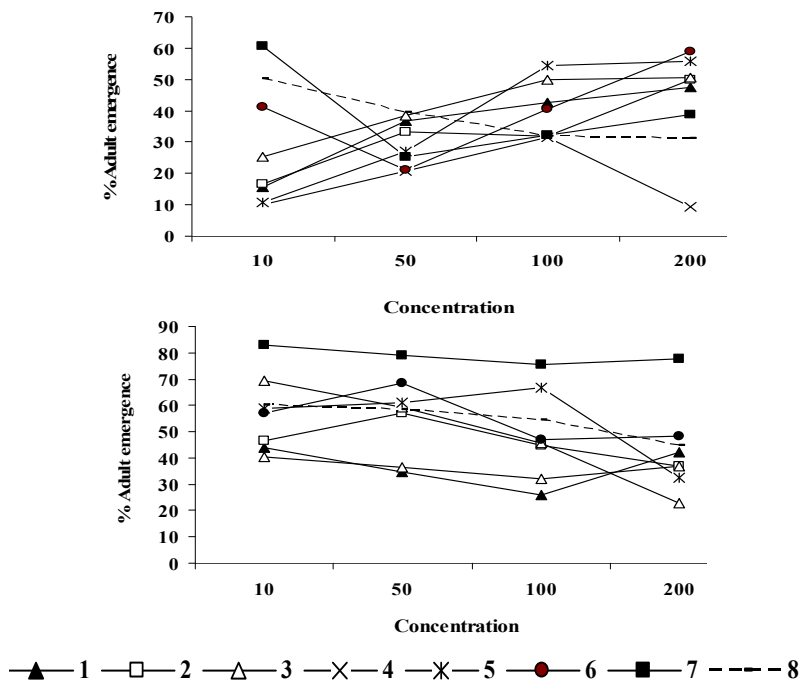
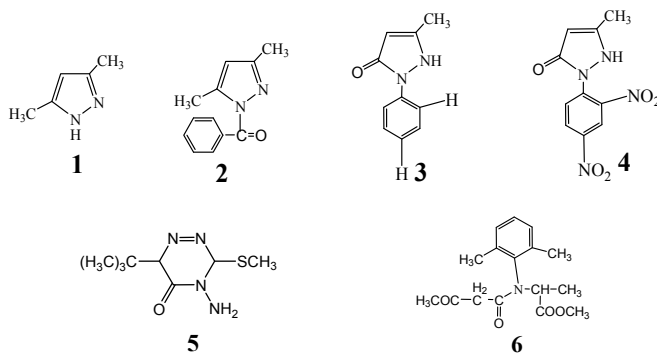


Figure 4. Emergence percents of *S. littoralis* adults produced from treated larvae. **Upper**, from treated 4th instar; **Lower**, from treated 6th larval instar; Concentrations (µg/gm). 1: Indole-3-acetic acid; 2: Indol-3-butyric acid; 3: 1-Acetylindole-3-butyric acid; 4: 1-Benzoylindole-3-acetic acid; 5: 1-Benzoylindole-3-butyric acid; 6: 2-Phenylindole; 7: 1-Acetyl-2-phenyl indole; 8: 1-Benzoyl-2-phenyl indole

3.3. Pesticidal activities of some pyrazole derivatives [5]

Due to antimicrobial activity of some 3,5-dimethylpyrazole derivatives [43], 3,5-dimethylpyrazole (1), 1-Benzoyl-3,5-dimethylpyrazole (2), 3-methyl-1-phenylpyrazol-5-one (3) and 3-methyl-1-(2,4-dinitrophenyl)-pyrazol-5-one (4) were prepared, structurally confirmed and studied for their effects against *Fusarium oxysporum*; *Pythium debarianum* *Rhizoctonia solani* and *Macrofomina phaseoli*. Metalaxyl (Radomil), methyl- N-(2,6-dimethylphenyl-N-methoxyacetyl)-DL-alaninate (6) was used as a standard fungicide. Their phytocidal effects were determined on both wheat (*Triticum aestivum*) and squash (*Cucurbita pepo*) seedlings comparing with metribuzin (sencor), 4-amino-6-tert.butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one (5). Insecticidal effects were evaluated on the 4th instar of cotton leaf worm, *S. littoralis* Boisid. Their fungitoxic effects as IC₅₀ values illustrated that comparing with metalaxyl, *R. solani* was less affected than the other fungi. 3,5-Dimethylpyrazole (1) proved to be moderately toxic with 470, 380 and 330 µg/ml IC₅₀ values against *P. debarianum*, *F. calmorum* and *M. phaseoli*, respectively after 6 days exposure, whereas 1-benzoyl-3,5-dimethylpyrazole (2) reduced the activity against all the tested fungi but 3-methyl-1-phenylpyrazol-5-one (3) enhanced the activity against *R. solani* with IC₅₀

value of 155 $\mu\text{g/ml}$ after 4 days exposure, *P. debarianum* and *M. phaseoli* with IC_{50} values of 68 and 170 $\mu\text{g/ml}$ after 6 days exposure, respectively whereas it was inactive on *F. calmorum* as its IC_{50} was $>500 \mu\text{g/ml}$. On the other hand, 3-methyl-1-(2,4-dinitrophenyl)pyrazol-5-one (**4**) caused the toxic effect against *R. solani*, *F. calmorum* and *M. phaseoli* with 100, 440 and 140 $\mu\text{g/ml}$ IC_{50} values, respectively after the same exposure time. From these results, some of the prepared compounds exceeded the standard fungicide in their effects against the tested fungi under the used experimental conditions.



Chemical structures of pyrazole derivatives and used standard pesticides

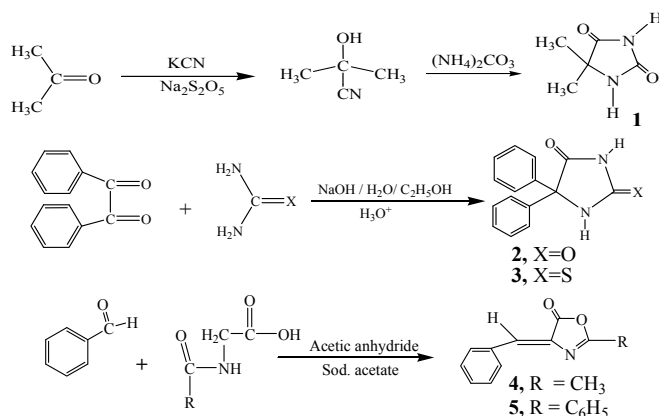
Pyrazole derivatives inhibited the growth of root and shoot systems of wheat and squash seedlings differently. Benzoylation of 3,5-dimethylpyrazole (**1**) decreased its inhibition on wheat shoot system growth, vice versa against its root system. Introducing the 2,4-dinitro- moiety enhanced the toxicity of 3-methyl-1-phenyl pyrazol-5-one (**3**) on wheat shoot and root systems. Compounds 1, 2, 3 and 4 inhibited cucumber seedlings root system with 95, 109, 95 and 58 $\mu\text{g/ml}$ and its shoot system with 38, 90, 60 and 78 $\mu\text{g/ml}$, comparing with 115 and 86 $\mu\text{g/ml}$ of metribuzin, respectively. It gave 81 and 52 $\mu\text{g/ml}$ on wheat shoot and root systems. The standard herbicide was less effective than the tested compounds on quash shoot system. Compound **1** was inactive against *S. littoralis* (Boisid.), its activity slightly increased to 10% mortality by substitution with 1-benzoyl- moiety. The effect became 23% mortality with reduction of palatability to 8.5-50 % of control in case of phenylpyrazol-5-one in. Substitution with 2,4-dinitrophenyl- moiety decreased the activity to 14 % mortality and 50-67 % palatability.

3.4. Pesticidal effects of some imidazolidine and oxazolone derivatives [6]

Actually we were interested to evaluate pesticidal actions of some imidazolidine and oxazolone derivatives as some of them are insecticides, herbicides and fungicides [44]. So three other derivatives of imidazolidine: 5,5-dimethylimidazolidin-2,4-dione, 5,5-diphenylimidazolidin-2,4-dione and 5,5-diphenylimidazolidin-2-thione-4-one and two oxazolone derivatives: 4-Benzylidene-2-methyloxazol-5-one and 4-Benzylidene-2-phenyloxazol-5-one were prepared and checked for their structure. Their fungicidal, phytocidal and insecticidal effects were carried out as in case of pyrazole derivatives.

Fungicidal activity

Imidazolidine derivatives appeared more effective than the oxazol-5-one derivatives on *R. solani* depending on the substituent on position 5. 5,5-Dimethyl moiety increased the toxicity of compound **1**, 5,5-dimethylimidazol-idin-2,4-dione than 5,5-diphenyl moiety in compound **2**, 5,5-diphenylimidazol-idin-2,4-dione with IC₅₀ values of 191.8 and 447.6 µg/ml, respectively. Replacing sulfur in compound **3**, 5,5-diphenylimidazolidin-2-thione-4-one instead of oxygen at position 2 increased its toxicity with 148.4 IC₅₀ value exceeding the standard fungicide (233.8 µg/ml). 2-Phenyl moiety enhanced the toxicity of compound **5**, 4-benzylidene-2-phenyl oxazol-5-one more than 2-methyl moiety in compound **4**, 4-benzylidene-2-methyl oxazol-5-one with 542.0 and 785.3 µg/ml, respectively. Vice versa against *P. debarianum*, compound **5** was the most effective among the other tested compounds with IC₅₀ of 76.9 µg/ml. Imidazolidine derivatives were nearly similar or more active than standard in its effect. Compound **3** was more toxic than compounds **1**, **2**, **4** and the standard fungicide against *P. debarianum* with IC₅₀ values 156.4, 357.1, 318.7, 516.5 and 334.3 µg/ml, respectively. Compounds **1** and **3** were more effective against *F. calmorum* than others with 306.7 and 314.1 µg/ml IC₅₀ values. The standard fungicide exceeded all compounds against *F. calmorum*. Compound **3** was the most effective against *M. phaseoli* with 139.0 IC₅₀ value surpassing all compounds including the standard fungicide.



Preparation scheme of compounds 1-5

From the obtained results, fungitoxic activities proved to be a function of both the tested compound and the used fungus. In general, through analysis of variance (ANOVA) of hyphal growth inhibition percents, compound **3**, 5,5-diphenylimidazolidin-2-thione-4-one was the most active against the tested fungi with Mean \pm SE of growth inhibition equaled 34.69^a. The other tested compounds were arranged as Mean \pm SE was 32.74 \pm 2.53^d, 28.67 \pm 2.79^c, 25.08 \pm 2.44^b and 24.65 \pm 2.29^b and 19.93 \pm 2.00^a, respectively in case of standard fungicide, compound **5**, compounds **1** and **2**, compound **4**. *P. debarianum* was more sensitive than *R. solani*, *F. calmorum* and *M. phaseoli* with Mean inhibition% \pm SE of 29.55 \pm 2.23^d, 27.88 \pm 2.07^c, 27.30 \pm 1.98^b and 25.77 \pm 2.01^a, respectively.

Phytocidal activity

The tested compounds inhibited germination and shoot growth of treated *T. aestivum* seeds. Compound 5 inhibited shoot growth exceeding the other prepared compounds with EC₅₀ value equaled 98.6 µg/ml. Compound 2 surpassed compounds 1, 3 and 4 with EC₅₀ values equaled 154.1, 177.9, 282.6 and 703.4 µg/ml, respectively. The tested compounds inhibited germination of treated seeds with EC₅₀ values ranged from 517.3 to 726.8 µg/ml. Metribuzin as a standard herbicide was the most effective inhibiting germination and shoot growth with EC₅₀ values of 92.4 and 54.8 µg/ml. As a result of being these compounds more effective on seedling shoot growth than on germination process, they were tested against both the root and shoot systems of pregerminated seeds of wheat (*T. aestivum*) as a narrow leaf plant and squash (*C. pepo*) as a broad leaf plant. Compound 1 showed the lowest effect, followed by compound 4 against both the root and shoot systems of *T. aestivum*. Compound 3 exhibited the strongest effect with EC₅₀ values equaled 25.2 and 35.6 µg/ml on root and shoot comparing with 53.6 and 60.6 µg/ml of the used standard herbicide. Differences in the tested compounds controlled their effect on the broad leaf plant. The standard herbicide proved to be the most active with EC₅₀ values equaled 104.1 and 113.7 µg/ml and compound 5 was the next with 274.1 and 203.6 µg/ml EC₅₀ values on its root and shoot systems growth. While compound 4 was less effective with 886.9 and 613.7 EC₅₀ values against the root and shoot systems, the other tested compounds affected this plant with EC₅₀ values ranged from 320.2 to 437.7 µg/ml.

Insecticidal activity

The tested compounds exhibited low mortality on 24 hours treated *S. littoralis* larvae. Among the studied imidazolidine derivatives, compound 2 affected it with LC₅₀ value equaled 867.3 µg/ml inhibiting the feeding activity with effective concentration on 50% (EC₅₀) equaled 31.78 µg/ml. The other two derivatives caused very weak mortal effects and inhibited feeding with EC₅₀ values equaled 3200 and 3489.9 µg/ml in case of compounds 1 and 3, respectively. Regarding the oxazolone derivatives, although compound 4 exhibited mortality percent as high as 24%, it reduced the feeding with EC₅₀ value equaled 376.8 µg/ml. The other oxazolone derivative (compound 5) caused LC₅₀ value equaled 659.7 µg/ml and reduced the feeding activity with EC₅₀ equaled 982.5 µg/ml. So these compounds affected as antifeedants more than as killers against the studied insect and compound 2 was the most effective structure among them (Figure 5).

The tested compounds exhibited phytocidal and fungicidal activities higher than their insecticidal effects. Differences in these compounds could be referred to chemical structure as in imidazolidine derivatives, presence of the 2-thione in compound 3 increased its fungitoxic effect nearly against all of the tested fungi. Substitution of 5,5-dimethyl moiety in compound 1 increased the toxicity more than 5,5-diphenyl moiety in compound 2 against *R. solani* and *F. calmorum* fungi. On contrary against *P. debarianum* and *M. phaseoli*, they showed almost the same effect. Their insecticidal effects were changed against the treated larvae as compound 2 exceeded the effects of the two other imidazolidine derivatives. Regarding the

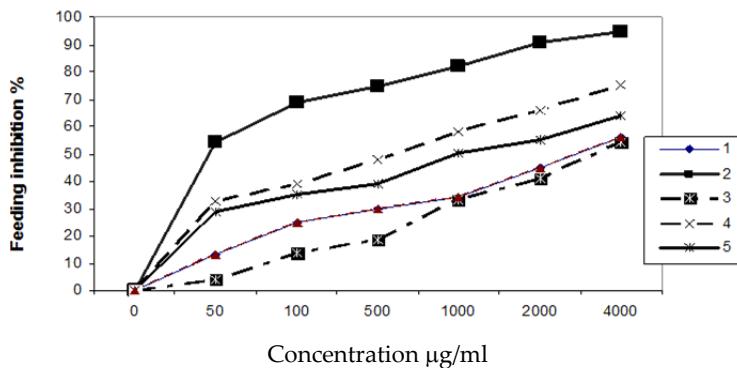


Figure 5. Feeding inhibition on *S. littoralis* Boisd. 1, 5,5-Dimethylimidazolidin-2,4-dione; 2, 5,5-diphenylimidazolidin-2,4-dione; 3, 5,5-di-phenylimidazolidin-2-thione-4-one; 4, 4-Benzylidene-2-methyloxazol-5-one; 5, 4-benzyl-idine-2-phenyloxazol-5-one.

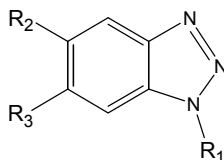
prepared oxazolones, compound 5 appeared more effective than compound 4 against the tested fungi and larvae. This difference between oxazolone derivatives could be due to the substituted moiety on C-2 position [45] as they revealed that substitution of functional group (s) at C-4 and C-2 positions plays a vital role in oxazolone series activity. They also revealed that oxazolone derivatives demonstrated excellent *in vitro* tyrosinase inhibitory. Fungitoxic effects of oxazolone derivatives maybe due to their mutagenic potential during *in vitro* DNA synthesis inducing mainly dAMP insertion [46]. The effect may referred to inhibition of fungal RNA synthetase [47]. In conclusion, compound 3, 5,5-diphenylimidazolidin-2-thione-4-one was the most useful fungitoxic structure among the prepared compounds and so, it might be useful in controlling plant pathogenic fungi after suitable formulation and helping in integrated management programmes. It also proved to be the most suitable structure for phytotoxicity, especially for the narrow leaf weeds.

3.5. Fungicidal effects of certain benzotriazole and coumarin derivatives [48]

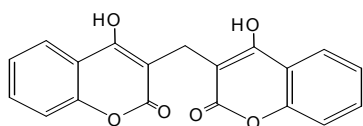
To extend the spectrum of newly discovered antifungal compounds facing continuous fungal infections, six benzotriazole derivatives as well as two coumarin derivatives were synthesized, confirmed for their structure and evaluated on *F. oxysporum*; *R. solani*; *M. phasoli*; *Helminthosporium sp* and *Alternaria alternata*. Triazole ring was chosen due to discovering some effective fungicidal triazole derivatives [49-51].

In vitro fungitoxicity effects

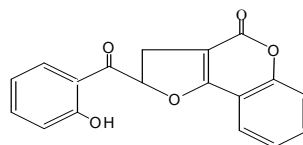
Effect of the tested 1,2,3-triazole and coumarin derivatives on three soil fungi and two foliar fungi based on their structure differences. 5,6- Dichlorobenzotriazole proved to be highly toxic against *R. solani*, *A. alternata* and *Helminthosporium sp* with IC_{50} values of 12, 20 and 27 µg/ml and moderately fungitoxic against both *M. phasoli* and *F. oxysporum* with IC_{50} values equaled 53 and 56 µg/ml. Toxicity categories are devised by [52]. However benzotriazole



Compound	R1	R2	R3
1,2,3-Benzotriazole	H	H	H
5,6-Dimethyl benzotriazole	H	CH ₃	CH ₃
5,6-Dichloro benzotriazole	H	Cl	Cl
1-Acetyl-5,6-dimethyl benzotriazole	CH ₃ CO	CH ₃	CH ₃
1-Benzoyl-5,6-dimethyl benzotriazole	C ₆ H ₅ CO	CH ₃	CH ₃
1-benzoylbenzotriazole	C ₆ H ₅ CO	H	H



3, 3'-Methelene bis-4-hydroxy coumarin,
(Dicoumarol)



2,3-Dihydro-2-(2-hydroxybenzoyl)-
-4H Furo[3,2 -C] [1] benzopyran-4- one,
(Furoprone)

Scheme 2. Chemical structure of tested triazole and coumarin derivatives

and 1-acetyl-5,6-dimethylbenzotriazole caused moderate effects against *M. phasoli* and *F. oxysporum* as soil fungi, respectively with equal IC₅₀ values (155 µg/ml). 5,6-Dimethyl-, 1-benzoyl-5,6-dimethyl- and 1-benzoyl- benzotriazoles as well as dicoumarol and furoprone (coumarin derivatives) needed increasing their highest concentration (200 µg/ml) to get 50% inhibition against hyphal growth of all fungi, and in case of 1,2,3-benzotriazole against *F. oxysporum* and *R. solani*; and 1-acetyl-5,6-dimethylbenzotriazole against *R. solani* and *M. phasoli*. *Helminthosporium sp* (foliar fungus) was moderately affected by 1-acetyl-5,6-dimethyl- and 1-benzoyl-5,6-dimethyl- benzotriazoles with IC₅₀ values of 150 and 165 µg/ml, respectively. The other compounds could not reach 50% fungitoxicity against it at the concentration range. *A. alternata* was also moderately affected by benzotriazole; 1-benzoyl-5,6-dimethyl-; 1-acetyl-5,6-dimethyl- and 5,6-di-methylbenzotriazoles with IC₅₀ values equal 150; 155; 165 and 170 µg/ml, respectively. This fungus was less sensitive to 1-benzoylbenzotriazole and both coumarin derivatives. However, 5,6-dichloro substituent highly improved the fungitoxic effect of benzotriazole against all tested fungi, comparing with other benzotriazoles in addition to dicoumarol and furoprone (coumarin derivatives). 1-Benzoylbenzotriazole and coumarin derivatives were less effective against all tested fungi. The weak effects of coumarin derivatives may be due to their classification as mammalian poisons. So, it could be concluded that 5,6-dichlorobenzotriazole was very good fungicide against all tested fungi. On the other hand, *A. alternata* (as a foliar fungus) was moderately affected by the other tested benzotriazoles except 1-benzoylbenzotriazole and coumarin derivatives.

In Vivo biochemical effects

a. Effect on polyphenoloxidase and peroxidase activities

5,6-Dichlorobenzotriazole at 0.1, 0.25, 0.5, 1 and 2 IC₅₀ rates in µg/ml affected both polyphenoloxidase (PPO) and peroxidase (PO) enzymes for each tested fungi. Their activities were in non-systematic response depending on the type of fungus and concentration. The activity of polyphenoloxidase was highly increased in *F. oxysporum*; slightly increased in *R. solani*; weakly increased in *M. phasoli* with increasing the tested concentration; whereas, in *Helmintho-sporium sp* its activity weakly increased until 0.5 IC₅₀ then weakly inhibited by IC₅₀ and 2 IC₅₀ rates. In case of *A. alternata* this enzyme weakly inhibited by 33.2% inhibition at 0.5 IC₅₀ value, then the enzyme was slightly activated with increasing the tested rates to IC₅₀ and 2 IC₅₀. Concerning peroxidase, its activity weakly increased in *F. oxysporum* at all the tested rates, weakly inhibited in *R. solani* at the lower two rates then weakly increased. In *M. phasoli* and *A. alternata*, peroxidase enzyme was highly inhibited with I₅₀ values equal 39.64 and 5.78 µg/ml, respectively. 5,6-Dichlorobenzotriazole was very effective to inhibit peroxidase enzyme in *A. alternata* through all the tested rates.

b. Effect on DNA and RNA contents

5,6-Dichlorobenzotriazole at several rates of its IC₅₀ values affected DNA and RNA contents in each tested fungus. In *F. oxysporum*, DNA and RNA contents as compared with control were reduced at the two lower rates (0.1 and 0.25 of IC₅₀) of 5,6-di-chlorobenzotriazole then increased with increasing the tested rates. On the other hand, their contents in *R. solani* highly increased more than control reaching the maximum peak at 0.5 IC₅₀ then decreased but still more than control. DNA and RNA contents in both *M. phasolina* and *Helminthosporium sp* were decreased with increasing the tested rates of IC₅₀ values. The contents in *A. alternata* were highly increased with increasing the tested rates of IC₅₀ values. In conclusion, 5,6-dichlorobenzotriazole may be useful as a good fungi-cide against all the tested fungi. The 5,6-dichloro- substituent was required to improve benzotriazoles effects against all treated fungi. So, it was highly effective against the activities of polyphenoloxidase, peroxidase and DNA and RNA contents.

3.6. Rodenticidal activity of certain benzotriazole and coumarin derivatives [53]

The previously explained benzotriazole and coumarin derivatives were studied also for their rodenticidal effects against the white Noway rat. In fact the two coumarin derivatives might be expected in their effects, while the benzotriazole derivatives were tested to stand on their toxicity related to studied coumarin comparing with Coumachlor, 3-(α -acetyl-4-chlorobenzyl)-4-hydroxy-coumarin as standard anticoagulant rodenticide.

During the baiting of the tested rats (*Rattus norvegicus* var. *albus*), the illness symptoms were observed as inactivity, ceasing sounds, closed eyes, bloody face, bleeding and paralysis followed by death. The internal symptoms were also observed as change the colour of liver, kidney, swelling of stomach and lungs with obvious changes, bloody bladder and intestines and the body cavity was intensively bloody. Mortality percents caused by synthesized

dicoumarol, furopyrone and 1-acetyl-5,6-dimethylbenzotriazole increased with increasing the dosages; their LD₅₀ values were 64, 400 and 580 mg/kg body weight, respectively. So, both dicoumarol and furopyrone were moderately toxic, whereas 1-acetyl-5,6-dimethylbenzotriazole was slightly toxic [52]. The average times to death were ranged between 6.3 and 5.5 days. However, EP₅₀ and EP₉₈ (Effective periods of 50% and 98% mortalities) of 100 mg/kg were 4.5 and 11.5 days, respectively. These compounds exhibited good rodenticidal properties on three consecutive dosages in a week when compared with coumachlor with LD₅₀ equal to 50-100 mg/kg if applied on five consecutive days [54].

Biochemical effects

Benzotriazole derivatives weakly affected the haemoglobin and haematocrit of both males and females within the tested doses (10-300 mg/kg) with ED₅₀ of >300 mg/kg. While, dicoumarol and furopyrone were highly and moderately toxic against haemoglobin of male and female rats with ED₅₀ values of 24 & 27 mg/kg and 90 & 130 mg/kg body weight, respectively. Furopyrone and dicoumarol were moderately active on haematocrit of males with ED₅₀ values of 53 and 65 mg/kg but on females with 135 and 195 mg/kg respectively. Red blood cells (RBC's) of females were found to be more sensitive to coumachlor, dicoumarol, furopyrone, 5,6-dimethylbenzotriazole, benzotriazole followed by 1-benzoyl-5,6-dimethylbenzotriazole as highly toxic compounds reducing RBC's of males with ED₅₀ values of 7, 12, 19, 28, 40 and 44 mg/kg, respectively (Loomis, 1976). Coumachlor, dicoumarol, furopyrone and 5,6-dimethylbenzotriazole were also highly toxic against RBC's of females with ED₅₀ value of 10.5, 25, 32 and 40 mg/kg, respectively. However the other compounds moderately reduced the RBC's counts of males and females. White blood cells (WBC's) of males were highly sensitive to 5,6-dichlorobenzotriazole, coumachlor and dicoumarol with ED₅₀ values of 12, 13, and 37 mg/kg, whereas dicoumarol and 5,6-dichlorobenzotriazole were highly toxic in reducing it in females with ED₅₀ values of 32 and 42 mg/kg, respectively. The other compounds proved to be moderately toxic in both males and females except benzotriazole, 1-acetylbenzotriazole and furopyrone. 5,6-Dichlorobenzotriazole was nearly equal to coumachlor in reducing males WBC's, whereas dicoumarol and 5,6-dichlorobenzotriazole were more effective than coumachlor against female WBC's.

Benzotriazole derivatives as well as furopyrone weakly affected sALT enzyme in both males and females. 5,6-Dimethylbenzotriazole was more potent reducing sAST enzyme activity in both males and females with ED₅₀ values of 8.8 and 13.5 mg/kg, respectively. Dicoumarol was also highly toxic compound against sAST in males and females with ED₅₀ of 24 and 32 mg/kg, respectively whereas coumachlor was highly toxic against females and moderately against males with 24 and 54 mg/kg ED₅₀ values, respectively. 5,6-Dimethylbenzotriazole was more potent reducing sAST activity in both males and females with ED₅₀ values of 8.8 and 13.5 mg/kg, respectively. Dicoumarol was also categorized as highly toxic against sAST in males and females with ED₅₀ of 24 and 32 mg/kg, respectively whereas coumachlor was highly toxic against females and moderately against males with 24 and 54 mg/kg ED₅₀ values, respectively. 1-Acetylbenzotriazole was moderate reducing sAST activity in males

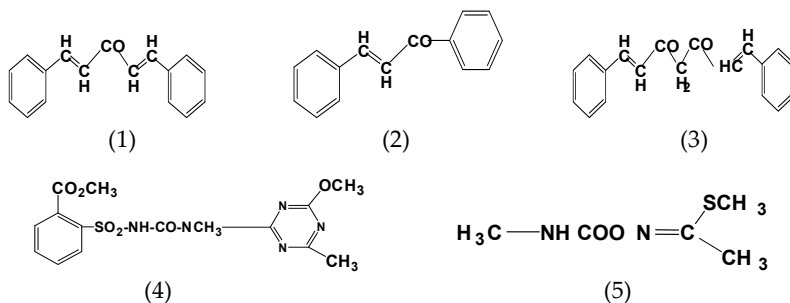
with ED₅₀ equalled 160 mg/kg. The other derivatives and furopyrone weakly affected it in both sexes.

3.7. Pesticidal activity of some benzylidene derivatives [4]

Actually α,β -unsaturated ketones were prepared according to Claisen Schmidt reaction (CSR) mechanism for searching their potency controlling some pests based on their biological history [55-56]. These biological activities of chalcone derivatives (as benzylidene derivatives) directed the attention to prepare some chalcone derivatives; dibenzylideneacetone, dibenzylideneacetylacetone and benzylideneacetophenone (chalcone).

Phytocidal Effects

Benzylideneacetophenone (chalcone), dibenzylideneacetone and dibenzylideneacetylacetone were active against the root system of wheat seedlings (*Triticum aestivum*) with EC₅₀ values equal 54, 55 and 68 $\mu\text{g/ml}$, respectively. On the other hand, their shoot systems were less sensitive, since benzylideneacetophenone (chalcone) was less effective, followed by dibenzylideneacetylacetone and dibenzylideneacetone. It could be concluded that, the three prepared compounds proved to be highly toxic on root system whereas dibenzylideneacetone and dibenzylideneacetylacetone proved to be moderately toxic against the shoot system of wheat seedlings. On the other hand, root system of squash seedlings (*Cucurbita pepo*) was highly affected by dibenzylideneacetylacetone, benzylideneacetophenone and dibenzylideneacetone. These prepared compounds also affected the shoot system of squash seedlings. However the standard herbicide, Granstar was more effective against both wheat and squash seedlings than the prepared compounds. Although the obtained results revealed that the prepared compounds could be considered as a moderate phytotoxicants against wheat and squash seedlings but they were more specific on root system of wheat seedlings.



Chemical structures of the tested compounds and standard pesticides

1, dibenzylideneacetone (1,5-diphenylpenta-1,4-dien-3-one); 2, benzylidene acetophenone (1,3-diphenyl propen-3-one) (Chalcone); 3, dibenzylidene acetylacetone (1,7-diphenyl hepta-1,6-dien-3,5-dione); 4, tribenuron methyl, (2-[4-methoxy-6-methyl-1,2,3-triazin-2-yl] methyl carbamoyl sulfamoyl benzoic acid) (Granstar); 5, methomyl, S-methyl-N-(methyl carbamoyl-oxy) thioacetimidate

Insecticidal and molluscicidal effects

Comparing with methomyl (Lannate), Dibenzylideneacetone and lannate proved to be highly toxic against the cotton leaf worm (*S. littoralis*) with LC₅₀ values < 10 µg/ml. Dibenzylideneacetylacetone and benzylideneacetophenone slightly affected it with LC₅₀ values equalled 510 and > 2000 µg/ml, respectively. Dibenzylideneacetone weakly affected the tested snails, whereas benzylideneacetophenone was very weak against *E. Vermiculata* but it was not mortal on *T. pisana*. Dibenzylideneacetylacetone showed no lethal effects against the two terrestrial snails.

Generally, the prepared compounds caused moderately phytotoxic effects on both wheat and squash seedlings but they were specific on root system of wheat seedlings. Dibenzylideneacetone caused nearly the same effects as methomyl against cotton leaf worm. So, it could be concluded that dibenzylideneacetone after different biological tests may be safe as an insecticide against cotton leaf worm as it was previously prepared as a sun protection cream [57].

3.8. Evaluation of certain benzylidene and pyrazole derivatives against wood decay fungi [58]

Wood decay fungi are destructive agents of wood industry. They degraded the used fungicides [59,60]. Due to their importance and the activities of benzylidene and pyrazole derivatives, their toxic effects were evaluated on the white rot fungus *Coriolus versicolor* and the brown rot fungus *Gloeophyllum trabeum*.

In Vitro fungitoxic effects were dependent on their concentrations, chemical structures and the treated fungus. 1,5-Diphenylpenta-1,4-dien-3-one (compound **1**) exhibited its fungitoxicity with IC₅₀ of 295.4 and 976.9 µg/ml against *C. versicolor* and *G. trabeum*, respectively. While, the toxicity was diminished because of the CH₂CO- moiety in 1,7-diphenylhepta-1,6-dien-3,5-dione (compound **2**) with IC₅₀ of 317.1 and 1995.4 µg/ml in case of the two studied fungi. Fungitoxicity was more than three nine times against *C. versicolor* and *G. trabeum*, respectively without -CH=CH- moiety in 1,3-diphenylpropen-3-one (Chalcone) (compound **3**). 3,5-Dimethylpyrazole (compound **4**) was less effective against the tested fungi with IC₅₀ values of 867.7 and 944.8 µg/ml against *C. versicolor* and *G. trabeum*, respectively. Substitution with 1-phenyl moiety changing to pyrazol-5-one ring in 3-methyl-1-phenylpyrazol-5-one (compound **5**) increased the effects with IC₅₀ values of 744.2 and 632.4 µg/ml against the treated fungi. Higher enhancement was achieved by replacing the substituted 1-phenyl ring with 2,4-dinitrophenyl moiety in 3-Methyl-1-(2,4-dinitro-phenyl)-pyrazol-5-one (compound **6**), the most active with IC₅₀ of 19.6 and 112.7 µg/ml against the white and brown rot fungi. In general, significantly *C. versicolor* appeared more sensitive than *G. trabeum* with general mean ± SE of mycelium growth inhibition percents of 39.18 ± 3.12 and 32.7 ± 2.58, respectively. Additionally, compound **6** was the most effective followed by compound **3**, exceeding boric acid as a standard compound with mean mycelium growth inhibition percents of 61.0, 46.9 and 40.9%, respectively. While, the other tested compounds were less effective than the standard (Table 2).

Tested Compound	<i>Coriulus versicolor</i>			<i>Gloeophyllum trabeum</i>		
	IC ₅₀ µg/ml (95% C L)	Slope ± S.E	χ ²	IC ₅₀ µg/ml (95% C L)	Slope ± S.E	χ ²
1,5-Diphenylpenta-1,4-dien -3-one (1)	295.4 ^c (250-350)	1.51 ± 0.019	5.9	976.9 ^b (796-1198)	1.17 ± 0.02	6.8
1,7-Diphenyl hepta-1,6-dien-3,5-dione (2)	317.1 ^b (263-383)	1.35 ± 0.02	0.4	1995.4 ^a (1452-2747)	0.93 ± 0.02	6.4
1,3-Diphenylpropen-3-one (Chalcone) (3)	84.5 ^e (57.1-124.4)	0.86 ± 0.01	4.3	103.9 ^g (66.6-160.8)	0.73 ± 0.01	2.5
3,5-Dimethylpyrazole (4)	867.7 ^a (759-992)	1.96 ± 0.026	3.0	944.8 ^c (784-1138)	1.3 ± 0.021	2.6
3-Methyl-1-phenylpyrazol-5-one (5)	744.2 ^b (655-846)	2.18 ± 0.028	0.5	632.4 ^d (549.6-728)	2.06 ± 0.026	3.9
3-Methyl-1-(2,4-dinitro-phenyl)-pyrazol-5-one (6)	19.6 ^f (16.7-22.9)	2.16 ± 0.028	9.1	112.7 ^f (88.9-142.7)	1.17 ± 0.01	3.9
Boric acid	252.5 ^d (226-282.3)	2.38 ± 0.033	2.5	189.1 ^e (166.3-215)	2.0 ± 0.026	7.9

Results in the same column with the same superscript are not significantly different ($p < 0.05$), DF = 4

Table 2. Fungicidal effects of certain benzylidine and pyrazole compounds on *Coriulus versicolor* and *Gloeophyllum trabeum* fungi

In vivo antifungal activity

After six weeks exposure to fungal attack, the average mass loss in control was 41.27 and 41.53% for poplar (*Populus nigra*) and Scots pine sapwood (*Pinus sylvestris*), respectively. Regarding poplar, compounds **3** and **6** reduced the mass loss to 30.43% and 29.23% (75% and 71% of control) at the lowest concentration. This effect was significantly increased reaching 23.87% (57.7% of control) and 13.67 % (33.1% of control) mass losses in systematic arrangement in un-leached samples in case of compound **3** and **6**, respectively at the highest concentration (10 IC₅₀ value). Leaching reduced antifungal effects of the two compounds to 28.10 % and 28.63% mass loss at the highest concentration with a narrow range of difference with their lowest concentration (0.5 IC₅₀) as the mass loss was 33.03% and 31.13% in compound **3** and **6**, respectively. The tested compounds protected the Scots pine sapwood samples in the same manner as compound **3** reduced its mass loss to (30.83% - 24.80%) while compound **6** reduced its mass loss to (30.0% - 14.47%) at concentration used in comparison to 41.53% of control samples. Leaching of the used blocks decreased the effect to (34.87% - 29.0%) and (33.30% - 27.5%), respectively (Table 3).

Treatment	Conc (% values)	<i>Populus nigra</i>				<i>Pinus sylvestris</i>			
		Retention Kg/m ³	Mass loss % ± SE		Retention Kg/m ³	Mass loss % ± SE			
			Un-Leached	Leached		Un-Leached	Leached		
Control	0.0	0.0	41.27 ^g ± 0.43	41.27 ^e ± 0.43	0.0	41.53 ^g ± 0.42	41.53 ^g ± 0.42		
1,3-Diphenyl-propen-3-one Chalcone (3)	0.5	0.022	30.43 ^f ± 0.77	33.03 ^d ± 0.37	0.021	30.83 ^f ± 0.92	34.87 ^f ± 0.37		
	1.0	0.044	28.10 ^e ± 0.61	31.07 ^c ± 0.58	0.043	28.10 ^e ± 0.35	32.30 ^d ± 0.15		
	5.0	0.194	26.23 ^d ± 0.55	29.33 ^b ± 0.26	0.210	26.4 ^d ± 0.49	31.10 ^c ± 0.51		
	10.0	0.447	23.87 ^c ± 0.61	28.10 ^a ± 0.42	0.463	24.80 ^c ± 0.68	29.0 ^b ± 0.21		
3-Methyl-1-(2,4-dinitro-phenyl)-pyr-azol-5-one (6)	0.5	0.004	29.23 ^f ± 0.82	31.13 ^c ± 0.52	0.019	30.0 ^f ± 0.32	33.30 ^e ± 0.38		
	1.0	0.009	23.40 ^c ± 0.67	30.13 ^c ± 0.55	0.039	25.57 ^d ± 0.43	31.17 ^c ± 0.15		
	5.0	0.042	18.07 ^b ± 0.45	29.40 ^b ± 0.35	0.199	20.87 ^b ± 0.20	29.47 ^b ± 0.55		
	10.0	0.089	13.67 ^a ± 0.54	28.63 ^b ± 0.37	0.394	14.47 ^a ± 0.34	27.5 ^a ± 0.58		

Results in the same column with the same superscript are not significantly different ($p < 0.05$).

Table 3. Average of retention (kg/m³) and mass losses (%) of Poplar (*P. nigra*) and Scots pine sapwood (*P. sylvestris*) mini-blocks treated with compounds 3 and 6 and exposed to *C. versicolor* and *G. trabeum*, respectively.

The effect of compound **6** was **reduced by leaching** samples more than compound **3** ensuring that the former is easily leached due to its hygroscopic nature. Descriptive analysis proved compound **6** more significantly effective with general mean of mass loss ± SE of 25.12% ± 2.58 in comparison to 29.98% ± 1.63 of compound **3** in case of un-leached poplar samples, while no significant differences between them in leached samples were observed. In Scots pine sapwood, significance appeared in both cases as compound **6** achieved general mean of mass loss ± SE of 26.49% ± 2.44 and 32.59% ± 1.31 comparing with 30.33 % ± 1.61 and 33.76 % ± 1.16 of compound **3** in un-leached and leached samples. Differences between the benzylidene derivatives in their fungicidal activity could be referred to the conjugation among carbonyl groups, phenyl rings and double bonds, so compound **2** was less effective due to lack of this conjugation because of CH₂ moiety. Compound **3** was more effective than compound **1** may be due to the lipophilicity [61]. The effect of benzylidene derivatives (benzaldehyde derived compounds) was greatly inhibited against *G. trabeum* than *C. versicolor*, which may be due to degradation as benzaldehyde and its metabolic intermediates were effectively degraded by *G. trabeum* to 3,4-dihydroxybenzoic acid. This was further metabolized via the decarboxylation reaction to yield 1,2,4-trihydroxybenzene, which is susceptible to the ring-fission reaction [62]. Compound **3** was retained approximately in the same amount in both wood specimens. The retained amount of compound **6** in *P. sylvestris* was four times more than in *P. nigra*. On the other hand, compound **6** was retained in about one fifth of compound **3** in *P. nigra*, although it was more effective. So it could be concluded that compound **6** was found to be more effective than compound **3** in all cases and it was more toxic against *C. versicolor* than on *G. trabeum*. Moreover, these compounds need to be applied at higher concentrations to enter wood preservatives clique.

3.9. Phytocidal effects of some azole derivatives [63]

Phytocidal effects of five-membered heterocyclic derivatives were studied on monocotyledonous (*Triticum aestivum* L.) and dicotyledonous (*Cucurbita pepo*) plants. Some activities of nitrogen heterocycles as herbicides [64-65] helped growing this idea.

In seed treatment, wheat seedlings growth was more sensitive to the tested compounds than seed germination. Pyrazole derivatives were less effective than both indole and benzotriazole derivatives against seed germination, while their effects against vegetation depended on the structure. 5,6-Dichlorobenzotriazole was more potent than the standard herbicide, metribuzin against both seed germination and growth of seedlings. 1-Acetylindole-3-butyric acid caused nearly the same effect of metribuzin on seed germination, whereas its effect on seedling growth was less than it. However, the other benzotriazole, indole and pyrazole derivatives were less effective than it against both seed germination and seedling growth. Screening effects of the tested compounds on root and shoot systems of squash (*C. pepo*) and wheat (*T. aestivum*) proved 5,6-dichlorobenzotriazole the most effective inhibiting squash root and shoot systems with EC_{50} values equaled 8.6 and 16.8 $\mu\text{g/ml}$ exceeding the standard herbicides with 86.2 and 97.2 $\mu\text{g/ml}$, respectively. 1-Acetyl-5,6-dimethylbenzotriazole and 3-methyl-1-(2,4-dinitrophenyl)pyrazol-5-one were also more potent than metribuzin, with EC_{50} values equaled (26.2 and 47.2) and (72.2 and 77.1) $\mu\text{g/ml}$ against root and shoot systems of squash seedlings. 5,6-Dimethylbenzotriazole, 1-benzoylbenzotriazole and 1-acetylindole-3-butyric acid were more effective than it against squash root system with 63.1, 71.2, and 80.1 $\mu\text{g/ml}$ EC_{50} values. Indole-3-butyric acid inhibited squash shoot system with EC_{50} value equaled 63.7 $\mu\text{g/ml}$. The other tested benzotriazoles, indole and pyrazole derivatives were less effective than the standard herbicide. Comparing with 55.8 and 68.2 $\mu\text{g/ml}$ EC_{50} values of the standard herbicide against root and shoot systems of wheat, 5,6-dichlorobenzotriazole, indole-3-butyric acid, indole-3-acetic acid, 1-acetylindole-3-butyric acid, benzotriazole, 1-benzoylindole-3-acetic acid and 3-methyl-1-(2,4-dinitrophenyl)pyrazol-5-one were more potent with 16.9, 6.0, 3.3, 2.86, 1.82, 1.8 and 1.45 times against root system of wheat. While 5,6-dichlorobenzotriazole was 5.3 times more effective than metribuzin on wheat seedlings shoot growth, the other benzotriazoles, indole and pyrazole derivatives were less effective than it. All the tested derivatives were more effective inhibiting growth of root system than shoot growth in squash (*C. pepo*) seedlings except indole-3-acetic acid with EC_{50} values equaled 311.4 and 201.4 $\mu\text{g/ml}$ and 3-methyl-1-(2,4-dinitrophenyl) pyrazol-5-one with EC_{50} values equaled 47.2 and 77.1 $\mu\text{g/ml}$. The same trend was obtained in case of wheat seedlings by benzotriazole and indole derivatives except 1-benzoylindole-3-butyric acid with EC_{50} values equaled 611 and 544 $\mu\text{g/ml}$ and 1-benzoyl-2-phenylindole with EC_{50} values equaled 453 and 503 $\mu\text{g/ml}$, respectively. However pyrazole derivatives proved to be more effective against root than shoot depending inhibition degree on the chemical structure differences among the applied derivatives except 3,5-dimethylpyrazole with EC_{50} values equaled 208 and 172 $\mu\text{g/ml}$, respectively. Due to high effects of both 5,6-dichlorobenzotriazole and 3-methyl-1-(2,4-dinitrophenyl)pyrazol-5-one, they were applied in post emergence treatment to study their effects against some plant active sites of action. Pre emergence treatment with 5,6-

dichlorobenzotriazole inhibited both fresh and dry weights of wheat seedlings. Fresh weight of the emerged wheat seedlings was reduced with 45.1 - 94.7% at a concentration of 2 - 30 $\mu\text{g/ml}$ with EC_{50} values equaled 2.9 $\mu\text{g/ml}$, while their dry weight was reduced increasingly with increasing the concentration with 30.6 - 94.2% reduction with EC_{50} value equaled 3.6 $\mu\text{g/ml}$. At 50 $\mu\text{g/ml}$, it completely prevented seeds emergence. Post-emergence treatment of wheat seedlings with 5,6-dichlorobenzotriazole and 3-methyl-1-(2,4-dinitrophenyl)pyrazol-5-one affected their dry weight increase depending on concentration and time after treatment. Both two compounds reduced this increasing rate in comparison to control at all times. The highest effect was obtained during the first three days after treatment at all concentrations. 3-Methyl-1-(2,4-dinitrophenyl)pyrazol-5-one highly affected it during seven days after treatment. It was more potent than 5,6-dichlorobenzotriazole nearly at all the tested concentrations (Figure 6).

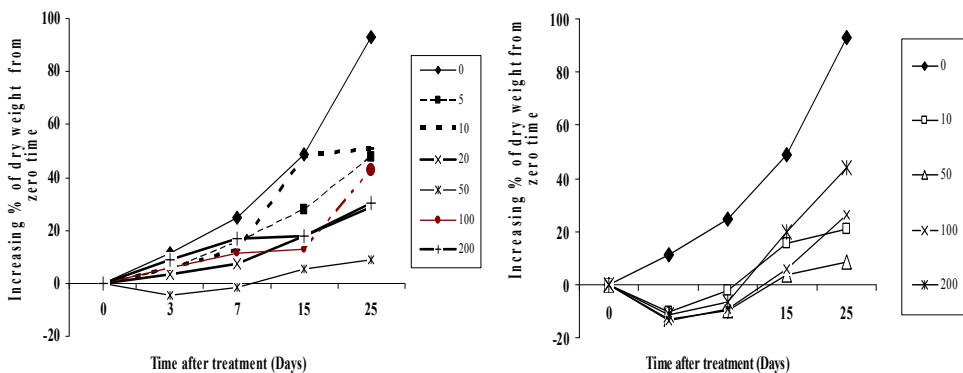


Figure 6. Effect of post emergence treatment on wheat seedlings dry weight. **Left,** 5,6-dichlorobenzotriazole; **Right,** 3-methyl-1-(2,4-dinitrophenyl)pyrazol-5-one

Concentrations in $\mu\text{g/ml}$

Single post-emergence treatment with both 5,6-dichlorobenzotriazole and 3-methyl-1-(2,4-dinitrophenyl)pyrazol-5-one affected the total soluble sugars contents in a function of concentration and time after treatment (Figure 7). Both reduced and non-reduced sugars alternatively changed regarding the time after treatment. 100 $\mu\text{g/ml}$ was the most effective concentration reducing TSS increasingly with time after treatment. Low activity at 200 $\mu\text{g/ml}$ might be referred to its difficult penetration. However 3-methyl-1-(2,4-dinitrophenyl)pyrazol-5-one showed its maximum activity after 7 days at 100 $\mu\text{g/ml}$. The highest effect of 5,6-dichlorobenzotriazole on chlorophyll was after 3 days. At 15 days after treatment, chlorophyll contents were enhanced at all concentrations. Chlorophyll **a** was more sensitive than **b**. Vice versa, 3-methyl-1-(2,4-dinitrophenyl)pyrazol-5-one affected chlorophyll **a** less than **b**. Enhancement was noticed at low concentrations at all the tested periods. The most reducing concentration was 100 $\mu\text{g/ml}$. Treatment with 5,6-dichlorobenzotriazole reduced the soluble phenolics content mostly until 3 days after treatment at all concentrations systematically with increasing the tested concentration. This effect was fluctuated

according to the applied concentration at 7 days after treatment. The most effective concentration was 100 $\mu\text{g/ml}$. At 15 days after treatment, it was too long to keep its effectiveness in reducing their content. While 3-methyl-1-(2,4-dinitrophenyl) pyrazol-5-one caused reduction of their content up to 15 days after treatment. Effects on chlorophyll content disturb several physiological processes in plants. The effect on soluble phenolics interferes in the protective compounds [66]. Fluctuated results of chlorophyll and soluble phenolics may be due to the interactive effects of temperature and the accumulated soluble phenolics [67]. 5,6-dichlorobenzotriazole may inhibit cell division and protoporphyrinogen oxidase leading to membrane disruption and inhibiting photosynthesis [64]. Benzotriazoles are effective in blocking photosynthetic electron transfer [68], slowing down the growth and decreasing plant size emphasizing our results on fresh and dry weight [69]. They may affect through inhibition of protein kinases [70]. Indole derivatives effects varied based on structure and concentrations inducing growth abnormalities leading to desiccation, tissue necrosis, and decay. They also increased H_2O_2 levels, which contributes to the induction of cell death, deoxyribonuclease (DNase) activity and chlorophyll loss as sensitive indicators for tissue damage [65]. Pyrazole derivatives are considered as branched chain amino acid synthesis (ALS or AHAS) inhibitor stopping cell division and plant growth.

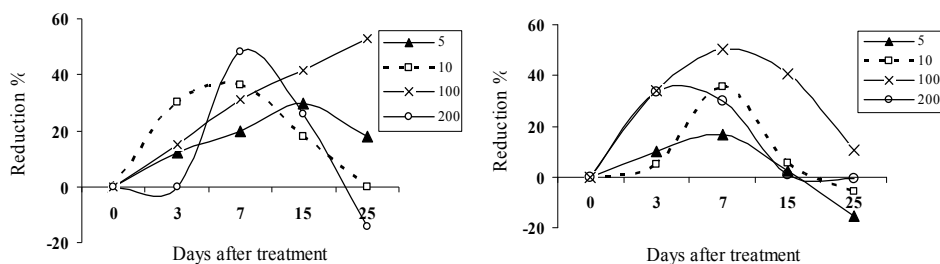


Figure 7. Effect of post emergence treatment on wheat seedlings sugars. **Left**, 5,6-dichlorobenzotriazole; **Right**, 3-methyl-1-(2,4-dinitro-phenyl) pyrazol-5-one; Concentrations in $\mu\text{g/ml}$

4. Conclusion

Several prepared organic compounds are tested for their pesticidal actions. Indole derivatives inhibited hyphal growth of several plant pathogenic fungi based on treated fungus and structure affecting sugars, RNA and DNA contents as well as enzymes disturbing cell physiology. They caused lethality, larval weight reduction, inhibition of pupation and adult emergence with inhibiting egg hatchability of *S. littoralis* Boisid. Tested pyrazole, imidazole and oxazole derivatives exhibited weak lethality with inhibition of insect palatability and moderate to high fungitoxic and phytotoxic effects according to structure, fungus and plant seedlings. Imidazolidine and oxazolone derivatives were antifeedants more than killers against *S. littoralis* and 5,5-diphenylimidazolidin-2,4-dione was the most effective structure. Their phytocidal and fungicidal activities were higher than insecticidal effects and 5,5-diphenylimidazolidin-2-thione-4-one was the most useful structure. Benzotriazoles changed in their fungicidal effects and 5,6-dichlorobenzotriazole was highly to moderately toxic against the treated fungi affecting both polyphenoloxidase, peroxidase and DNA & RNA contents. They caused lower effects on hae-

moglobin and haematocrit of rats, whereas dicoumarol and furofuroprone highly reduced them. However dimethyl- and dichloro- substituent increased the activity of non-substituted benzotriazole on RBC's, WBC's and sAST, acylation of 5,6-dimethylbenzotriazole decreased its effect on both male and female RBC's, sAST. Benzylidene derivatives caused moderately phytotoxic effects. Dibenzylideneacetone caused nearly the same effect as methomyl against cotton leaf worm. They differed in their mortality on *E. vermiculata* and *T. pisana* snails. Significantly *C. versicolor* was more sensitive than *G. trabeum* to benzylidene and pyrazole derivatives. 3-Methyl-1-(2,4-dinitro-phenyl)-pyrazol-5-one was the most effective followed by 1,3-diphenylpropen-3-one, exceeding boric acid, as a standard in case of un-leached poplar samples, while no differences were observed between them in leached samples. In Scots pine sapwood, significance appeared in both samples. 1,3-Diphenylpropen-3-one was approximately retained in the same amount in both wood specimens. Although 3-methyl-1-(2,4-dinitro-phenyl)-pyrazol-5-one was retained in one fifth of 1,3-diphenylpropen-3-one in *P. nigra*, it was more effective. Benzotriazole, indole and pyrazole derivatives inhibited wheat seedlings growth more than seed germination process. Pyrazoles were less than others inhibiting seed germination, effects on vegetation depended on structure. Some of derivatives exceeded the standard herbicides in their effects. 5,6-dichlorobenzotriazole was the most effective inhibiting monocotyledons and dicotyledonous seedlings growth. Its pre emergence treatment inhibited wheat seedlings fresh and dry weights. They also affected the total soluble sugars, chlorophyll and soluble phenolic contents in plants. The configuration of 5,6-dichlorobenzotriazole and 5,6-dichloro- substituent may be required to get good results. These results may exhibit 5,6-dichlorobenzotriazole as pre-emergent phytocidal compound, while 3-methyl-1-(2,4-dinitro-phenyl)pyrazol-5-one as post-emergent. These results proved 5,6-dichlorobenzotriazole to inhibit the chlorophyll content, cell division leading to membrane disruption, inhibiting photosynthesis, growth abnormalities leading to desiccation, tissue necrosis and decay and decrease the plant size emphasizing our obtained results on fresh and dry weight. In conclusion, this research might help finding active molecules are not famous as pesticides to be useful in integrated management programs.

Author details

Ahmed S. Abdel-Aty

Department of Pesticide Chemistry & Technology, Faculty of Agriculture, El-Shatby, Alexandria University, Alexandria, Egypt

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Endocrine Disrupting Pesticides

Svetlana Hrouzková and Eva Matisová

Additional information is available at the end of the chapter

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1. Introduction

Food and environmental samples represent nowadays an enormous challenge to analytical chemists in their efforts to determine residues of pesticides at trace levels, as pesticides can represent a risk for consumer and also safeguard the biodiversity in the environment. The concern has increased as certain pesticides and other synthetic chemicals may act as pseudo hormones which disrupt the normal function of the endocrine system in humans and wildlife (Colborn et al., 1993; Lintelmann et al., 2003). This specific category of pollutants comprises the compounds that may affect the normal hormonal function or possess endocrine-related functions, known as endocrine disrupting chemicals (EDCs) or endocrine disrupters. During the last decades the interest and concern related to endocrine disrupters among scientists, regulators and public has increased. In the last years a great deal of concern has been expressed worldwide over the increasing levels of EDCs found in the environment. This anxiety is caused by the adverse effects of these pollutants on the hormone systems of humans and wildlife even when present at levels under ppb (Jobling, 2004).

Known and potential EDCs in food and the environment originate from many different sources. Endocrine disrupting pesticides (EDPs) are the largest group of EDCs in numbers compared to other chemical groups. They are active at low concentrations in food daily consuming by adult population and in agricultural commodities consumed in large quantities especially by infants and children. Organisms under development are very sensitive to negative effects of EDPs. Understanding in which and how much biologically active compounds are in the environmental samples or products of human consumption is important not just to scientists and environmentalists, but also to governments, pediatricians, genetics, and the general public (LaFleur & Schug, 2011).

This contribution is devoted to pesticides that exhibit or are supposed to exhibit endocrine disrupting properties. First, the terms, definition and current state of EDCs list creation are discussed. Then the selected EDPs and their categories are presented. Next the common

analytical methods including sample preparation for the identification and quantification of EDPs by chromatographic analytical methods at ultratrace concentration level are briefly covered. The combinations of fast and effective sample preparation methods with conventional and fast capillary gas chromatography (GC) are presented. Selective mass spectrometric (MS) detection with negative chemical ionization (NCI) is discussed and compared to electron ionization (EI). The results leading to selectivity enhancement and decrease of the limits of quantification of selected EDPs using mass spectrometer operated in NCI mode are shown. Real-life analysis demonstrates the potential of studied sample preparation followed by fast gas chromatography.

2. Terms, definitions and background

The endocrine system is a complex integrative network of glands, hormones and receptors. It provides the key communication and control link between the nervous system and bodily functions such as reproduction, immunity, metabolism and behaviour. The endocrine system uses hormones to act as messengers that regulate reproduction, metabolism, growth, development, natural defences to stress, as well as water, electrolyte, and nutritional balance of the blood. Homeostasis is the balance of functions or levels in the body, returning biological variables to their biochemical baseline when perturbed and keeping them there. Maintaining homeostasis is one of the most important functions of the endocrine system. Therefore, the endocrine system includes number of central nervous system-pituitary-target organ feedback mechanisms that enable the body to react very flexibly on internal or external changes of hormone status (Lintelmann et al., 2003). This complex system is very sensitive toward disturbing influences that can severely impair the whole development of the organisms. A number of naturally occurring and synthetic chemicals have been shown to exert these adverse effects upon the endocrine system across animal classes including mammals. Concern for these chemicals initially focused on chemicals with estrogenic activity, and thus they were commonly referred to as environmental estrogens, or xenoestrogens (Rhomberg & Seeley, 2005). The initial focus has expanded to include compounds with androgenic activity, as well as thyroid-active chemicals (Rhomberg & Seeley, 2005). Consequently, also different variable terms appeared, e. g. endocrine disrupter (mainly used in Europe)/endocrine disruptor (in America), hormone mimics, hormone inhibitors, hormonally active chemicals, endocrine modulators (Jobling, 1998). Today, these compounds are commonly referred to as endocrine disrupting chemicals.

By definition adopted by European Commission (EC), “an endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations” (European Commission [EC], Endocrine disrupters website, 2011). EDCs were defined by Unites States Environmental Protection Agency (US EPA) as “an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process”. According to Diamanti-Kandarakis et al., 2009, it is necessary to broaden the term - the EDCs is a

compound, which through environmental or inappropriate developmental exposures, alters the hormonal and homeostatic systems that enable the organism to communicate and respond to environment.

Exposure to EDCs may cause disorder of endocrine system in a number of ways (Mendes, 2002). Many disrupters interact directly with hormone receptors, whereas some cause indirect activation of hormone receptors. They interfere by mimicking the action of a naturally-produced hormone, such as estrogen or testosterone, and thereby setting off similar chemical reactions in the body. EDCs can interfere by blocking the receptors in cells receiving the hormones (hormone receptors), thereby preventing the action of normal hormones. Some receptors interact with each other, such as through “cross-talk” between the estrogen and the growth factor receptors (Dybing, 2006). In other situations, EDCs may interact with multiple receptors. It is well-known, that inhibition of hormone synthesis and hormone transport, as well as alteration in hormone metabolism can affect endocrine system as the concentration of natural hormones alters. An example of how EDCs can interfere with receptor sites is shown in Fig. 1. The important role of well-working endocrine system functioning is the proper hormone-receptor binding at the appropriate level and time (Fig. 1. A). EDCs can give a weaker or stronger than normal response (Fig. 1. B) at inappropriate times compared to natural body’s hormones (LaFleur & Schug, 2011). At the environmental level, wildlife is particularly vulnerable to the endocrine disrupting effects of pesticides, effects noted in invertebrates, reptiles, fish, birds and mammals were reviewed by Mnif et al., 2007. Many pesticides and industrial chemicals are capable of interfering with the proper function of estrogen, androgen and thyroid hormones at the human level. For example, during pregnancy, lipophilic xenobiotics stored in maternal adipose tissue can be mobilized and enter the blood circulation and reach the placenta. As it was searched by Lopez-Espinosa et al., 2007, the presence of more pesticides in placenta was significantly associated with lower birth weight.

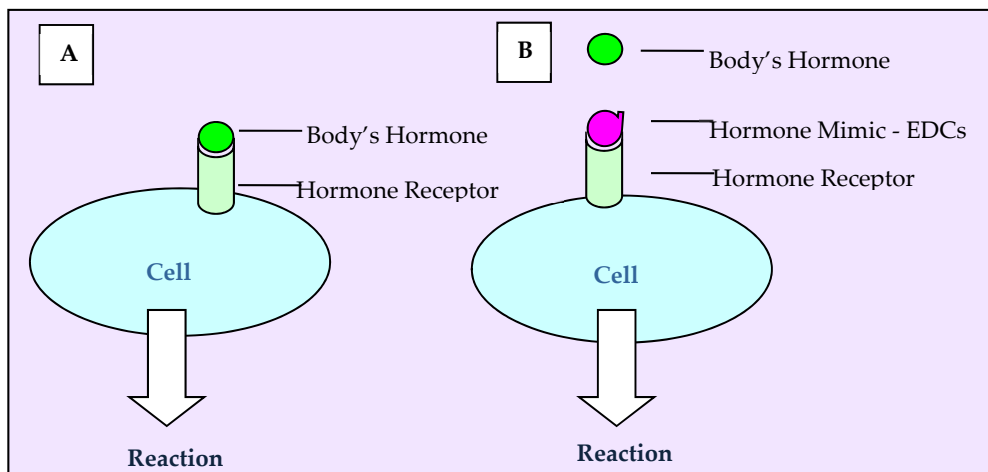


Figure 1. Outline of normal hormonal response (A) and EDCs interference with hormone receptors (B).

Changes in hormone levels affect developing organisms more than adults and can result in abnormalities in reproduction, growth, development and can disorder the immune system, as it was discussed by Mnif et al., 2011. A central feature of endocrine disruption is that may cause detrimental effects on organisms at very low chemical concentrations (Fang et al., 2001). Effects of EDCs at very low concentrations can be different from effects of the same chemical at higher concentrations (Colborn, 2012). Traditional approaches to determining safe exposure levels (for example, chemical risk assessments) do not work with EDCs.

3. Compounds of interest

The groups of molecules identified as EDCs are highly heterogeneous and include natural chemicals found in human and animal food (phytoestrogens), synthetic chemicals used as industrial solvents/lubricants and their by-products, plastics, plasticizers, pesticides, pharmaceuticals, etc. The scope of EDCs here has been narrowed specifically to known and potential endocrine disrupting pesticides.

The first list of suspected EDCs was published in scientific literature in 1993 by Theo Colborn (Colborn et al., 1993), followed by popular book for the layperson “Our stolen future” (Colborn et al., 1996). This book was instrumental in public awareness of the need to find out more.

In United States, the US EPA has been authorized to screen all manufacturing or processing chemicals and formulations for potential endocrine activity. The Endocrine Disruption Screening Program (EDSP) of EPA is mandated to use validated methods for screening and testing chemicals to identify potential endocrine disruptors, determine adverse effects, dose-response, assess risk and ultimately manage risk under laws. It is realized in two-tiered screening and testing process. In Tier 1, EPA hopes to identify chemicals that have potential to interact with the endocrine system. In Tier 2, EPA determines the specific effect caused by each disruptor and establishes the dose at which the effect occurs. In 2009, EPA released the Final list of Chemicals for Tier 1 Screening in the EDSP (United States Environmental Protection Agency [US EPA] Document, 2009), which is an update of Initial list from 2007 (some chemicals were removed). On November 2010 the US EPA published the second list of chemicals for further testing. This list of 134 chemicals includes a large number of pesticides (US EPA Document, 2010). The selection showing pesticides for EDSP screening is summarized in Table 1.

The European Union (EU) has done extensive work towards official designation of endocrine disrupting substances, collecting literature studies on many chemicals. In December 1999, the European Commission adopted a document entitled „Community Strategy for Endocrine Disrupters” to address the problem of EDCs. A part of this strategy was to establish a priority list that are presumably responsible for damaging human health by interference with hormones and to require the further evaluation of their role in endocrine disruption (EC document, 1999). The creation of the list was based on the published studies of these chemicals and was divided into categories according to

Final List (Initial) of Chemicals for Tier 1 Screening in the EDSP	Abamectin / 71751412; Acephate / 30560191; Atrazine / 1912249; Benfluralin / 1861401; Bifenthrin / 82657043; Captan / 133062; Carbamothioic acid, dipropyl-, s-ethyl ester / 759944; Carbaryl / 63252; Carbofuran / 1563662; Chlorothalonil / 1897456; Chlorpyrifos / 2921882; Cyfluthrin / 68359375; Cypermethrin / 52315078; 2,4-D (2,4-dichlorophenoxy acetic acid) / 94757; DCPA (chlorthal-dimethyl) / 1861321; Diazinon / 333415; Dichlobenil / 1194656; Dicofol / 115322; Dimethoate / 60515; Disulfoton / 298044; Endosulfan / 115297; Esfenvalerate / 66230044; Ethoprop / 13194484; Fenbutatin oxide / 13356086; Flutolanil / 66332965; Folpet / 133073; Gardona (cis-isomer) / 22248799; Glyphosate / 1071836; Imidacloprid / 138261413; Iprodione / 36734197; Linuron / 330552; Malathion / 121755; Metalaxyl / 57837191; Methamidophos / 10265926; 4,7-Methano-1H-isoindole-1,3(2H)-dione,2-(2-ethylhexyl)-3a,4,7,7a-tetrahydro- / 113484; Methidathion / 950378; Methomyl / 16752775; Methyl parathion / 298000; Metolachlor / 51218452; Metribuzin / 21087649; Myclobutanil / 88671890; Norflurazon / 27314132; o-Phenylphenol / 90437; Oxamyl / 23135220; Permethrin / 52645531; Phosmet / 732116; Piperonyl butoxide / 51036; Propachlor / 1918167; Propargite / 2312358; Propiconazole / 60207901; Propyzamide / 23950585; Pyridine, 2-(1-methyl-2-(4-phenoxyphenoxy) ethoxy)- / 95737681; Quintozene / 82688; Resmethrin / 10453868; Simazine / 122349; Tebuconazole / 107534963; Triadimefon / 43121433; Trifluralin / 1582098;
Removed from Initial list	Aldicarb / 116063; ; Allethrin / 584792; Azinphos-Methyl / 86500; Dichlorvos / 62737; Fenvalerate / 51630581; Methiocarb / 2032657;
Second List of Chemicals for Tier 1 Screening in the EDSP	Acetochlor / 34256-82-1; Acrolein / 107-02-8; Alachlor / 15972-60-8; Bensulide / 741-58-2; Clethodim / 99129-21-2; Clofentezine / 74115-24-5; Clomazone / 81777-89-1; Coumaphos / 56-72-4; Cyanamide / 420-04-2; Cyromazine / 66215-27-8; Denatonium saccharide / 90823-38-4; Dicrotophos / 141-66-2; Dimethipin / 55290-64-7; Diuron / 330-54-1; Endothal / 145-73-3; Etofenprox / 80844-07-1; Fenarimol / 60168-88-9; Fenoxaprop-p-ethyl / 71283-80-2; Fenoxycarb / 72490-01-8; Flumetsulam / 98967-40-9; Fomesafen sodium / 108731-70-0; Fosetyl-Al (Alette) / 39148-24-8; Glufosinate ammonium / 77182-82-2; Hexythiazox / 78587-05-0; Isoxaben / 82558-50-7; Lactofen / 77501-63-4; Molinate / 2212-67-1; Oxydemeton-methyl / 301-12-2; Oxyfluorfen / 42874-03-3; Paclbutrazol / 76738-62-0; p-Dichlorobenzene / 106-46-7; Pentachlorophenol / 87-86-5; Picloram / 1918-02-1; Profenofos / 41198-08-7; Propetamphos / 31218-83-4; Propionic acid / 79-09-4; Pyridate / 55512-33-9; Quinclorac / 84087-01-4; Quizalofop-p-ethyl / 100646-51-3; Sodium tetrathiocarbonate / 7345-69-9; Sulfosate / 81591-81-3; Temephos / 3383-96-8; Terbufos / 13071-79-9; Thiophanate-methyl / 23564-05-8; Triflumizole / 68694-11-1; Trinexapac-ethyl / 95266-40-3; Triphenyltin hydroxide (TPTH) / 76-87-9; Vinclozolin / 50471-44-8; Xylenes / 1330-20-7; Ziram / 137-30-4;

Table 1. The selection of pesticide active ingredients (compound name/ chemical abstract number CAS) from Initial and Second List of Chemicals according to US EPA studied in Tier 1 in the frame of US EDSP.

documented/potential endocrinal effect. This list of chemicals divides compounds into the following categories according to their impact on endocrine system:

- Category 1 – endocrinal effect recorded at least on one type of animal;
- Category 2 – a record of biological activity in vitro leading to disruption;
- Category 3 – not enough evidence or no evidence data to confirm/ disconfirm endocrinal effect of tested chemicals.

Category 1	<p>Acetochlor / 34256-82-1; Alachlor / 15972-60-8; Amitrol = Aminotriazol / 61-82-5; Atrazine / 1912-24-9; Beta-HCH / 319-85-7; Bifenthrin / 82657-04-3; Bis-OH-Methoxychlor = 1,1,1-Trichloro-2,2-bis(4-hydroxyphenyl)ethane (HTPE) / 63-25-2; Carbaryl / 63-25-2; Cis-Nonachlor / 5103-73-1; Cyhalothrin / 91465-08-6; DDT (technical) = clofenotane / 50-29-3; Deltamethrin / 52918-63-5; Dibromoethane (EDB) / 106-93-4; Dibromochloropropane (DBCP) / 96-12-8; 1,3-Dichloro-2,2-bis(4-methoxy-3-methylphenyl)propane / 30668-06-5; 2,4-Dichlorophenoxybutyric acid = 2,4-DB / 326354-18-7; Ethyl-4-hydroxybenzoate / 120-47-8; Ethylene thiourea (ETU) / 96-45-7; Fenarimol / 60168-88-9; Fenitrothion / 122-14-5; Fentin acetate = triphenyltin acetate / 900-95-8; Gamma-HCH (Lindane) / 58-89-9; Hexachlorobenzene (HCB) / 118-74-1; Hexachlorocyclohexane / 608-73-1; Chlordane (technical) / 12789-03-6; Chlordane (cis- and trans-) / 57-74-9; Chlordimeform / 6164-98-3; Ioxynil / 1689-83-4; Kepone (Chlordecone) / 143-50-0; Ketoconazol / 65277-42-1; Linuron (Lorox) / 330-55-2; 4-MeO-o,p'-DDE / 65148-81-4; 4-MeO-o,p'-DDT / 65148-72-3; 5-MeO-o,p'-DDD / 65148-75-6; 5-MeO-o,p'-DDE / 65148-82-5; 5-MeO-o,p'-DDT / 65148-74-5; m,p'-DDD / 4329-12-8; Mancozeb / 8018-01-7; Maneb / 12427-38-2; 3-MeO-o,p'-DDE / 65148-80-3; Metam Natrium / 137-42-8; Methoxychlor / 72-43-5; Methyl p-Hydroxybenzoate / 99-76-3; Metiram (Metiram-complex) / 9006-42-2; Metribuzin / 21087-64-9; Mirex / 2385-85-5; Nitrofen / 1836-75-5; Propylparaben (n-propyl p-hydroxybenzoate) / 94-13-3; 3-OH-o,p'-DDT / 43216-70-2; 5-OH-o,p'-DDT / 65148-73-4; o,p'-DDA-glycinat = N-[(2-chlorophenyl)(4-chlorophenyl) acetyl]glycin / 65148-83-6; o,p'-DDD / 53-19-0; o,p'-DDE / 3424-82-6; o,p'-DDMU / 14835-94-0; o,p'-DDT / 789-02-6; Omethoate / 1113-02-6; p,p'-DDD / 72-54-8; p,p'-DDE / 72-55-9; p,p'-DDT = clofenotane / 50-29-3; p,p'-Methoxychlor / 72-43-5; Pentachlorophenol (PCP) / 87-86-5; Phenol, 2-[[tributylstannyloxy]carbonyl]- / 4342-30-7; Picloram / 1918-02-1; Procymidon / 32809-16-8; 2-Propenoic acid, 2-methyl-, methyl ester = Stannane, tributylmeacrylate / 326354-18-7; Quinalphos = Chinalphos / 13593-03-8; Resmethrin / 10453-86-8; Stannane, (benzoyloxy)tributyl- / 4342-36-3; Stannane, tributyl[[1-oxo-9,12-octadecadienyl)oxy]-, (Z,Z)- / 24124-25-2; Stannane, tributyl[[[1,2,3,4,4a,4b,5,6,10,10a-decahydro-1,4a-dimethyl-7-(1-methylethyl)-1-phenanthrenyl]carbonyl]oxy]-, [1R-(1a,4ab,4ba,10aa)]- / 26239-64-5; Stannane, tributylfluoro- / 1983-10-4; Terbutryn / 886-50-0; Thiram / 137-26-8; 1,1,1,2-Tetrachloro-2,2-bis(4-chlorophenyl) ethane (tetrachloro DDT) / 3563-45-9; Toxaphene = Camphechlor / 8001-35-2; Trans-Nonachlor / 39765-80-5; Tributyl[[2-methyl-1-oxo-2-propenyl)oxy]stannane / 2155-70-6; 1,1,1-Trichloro-2,2-bis(4-chlorophenyl) ethane / 2971-22-4; Trifluralin / 1582-09-8; Vinclozolin / 50471-44-8; Zineb / 12122-67-7;</p>
Category 2	<p>Acephate / 30560-19-1; Aldicarb / 116-06-3; Aldrin / 309-00-2; Allethrin (d- trans allethrin) / 584-79-2; Bromoxynil / 1689-84-5; Carbenazodim / 10605-21-7; Carbofuran / 1563-66-2; 4-Chloro-2-methylphenol / 1570-64-5; 4-Chloro-3-methylphenol / 59-50-7; p-Cresol / 106-44-5; Cyanazine / 21725-46-2; Cypermethrin / 52315-07-8; Delta-HCH / 319-86-8; p,p'-DDA / 83-05-6; Diazinon / 333-41-5; 2,4-Dichlorophenoxy acetic acid (2,4-D) / 94-75-7; Dicolof = Kelthane / 115-32-2; Dieldrin / 60-57-1; Diisobutylphthalate / 84-69-5; Dimethoate / 60-51-5; Diuron / 330-54-1; Elsan = Dimephenthoate / 2597-03-7; Endosulfan / 115-29-7; Endosulfan (alpha) / 959-98-8; Endosulfan (beta) / 33213-65-9; Endrin / 72-20-8; Etridiazole / 2593-15-9; Fenothrin = sumithrin / 26002-80-2; Fenoxycarb / 72490-01-8; Fenvalerate / 51630-58-1; Fluralinate / 69409-94-5; Heptachlor / 76-44-8; Chlorfenvinphos / 470-90-6; Iprodione / 36734-19-7; Malathion / 121-75-5; Methomyl / 16752-77-5; Methylbromide (bromomethane) / 74-83-9; Methylparathion / 298-00-0; Mevinphos = Phosdrin / 7786-34-7; 4-Nitrophenol / 100-02-7; Oxychlorodane / 27304-13-8; Parathion = Parathion(ethyl) / 56-38-2; Permethrin / 52645-53-1; o-Phenylphenol / 90-43-7; Phosphamidon / 13171-21-6; Photomirex / 39801-14-4; Piperonyl butoxide / 51-03-6; Prochloraz / 67747-09-5; Prometryn / 7287-19-6; Propanil / 709-98-8; Pyrethrin / 121-29-9; Simazine / 122-34-9; Triadimefon / 43121-43-3; Triadimenol / 123-88-6; Trichlorfon = Diptex / 52-68-6; 2,4,5-Trichlorophenoxy acetic acid (2,4,5-T) / 93-76-5; Ziram / 137-30-4;</p>
Category 3	<p>Abamectin / 17151-41-2; Amitraz / 33089-61-1; Azadirachtin / 11141-17-6; Benomyl / 17804-35-2; Bitertanol / 55179-31-2; Bromacil / 314-40-9; Clofentezine = chlorfentezine / 74115-24-5; Cyproconazole / 94361-07-6; Demefion / 682-80-4; Demeton-s-methyl / 919-86-8; Difenoconazole / 119446-68-3; Dichlorvos / 62-73-7; Dimethylformamide (DMFA) / 68-12-2; Dinitrophenol / 25550-58-7; Dinoseb / 88-85-7; Diphenyl / 92-52-4; Epiconazole / 133855-98-8; Epoxiconazole / 135319-73-2; Esfenvalerate / 66230-04-4; Ethofenprox / 80844-07-1; Fenbuconazole / 114369-43-6; Fipronil / 120068-37-3; Fluzafip-butyl / 69806-50-4; Flutriafol / 76674-21-0; Formothion / 682-80-4; Glufosinate / 51276-47-2; Glufosinate-ammonium / 70393-85-0; Glyphosate / 1071-83-6; Heptachlor-epoxide / 1024-57-3; Chlordene / 3734-48-3; Chlorpyrifos / 2921-88-2; Imazalil / 3554-44-0; Molinate / 2212-67-1; Myclobutanil / 88671-89-0; Nabam / 142-59-6; Octachlorostyrene / 29082-74-4; Oryzalin / 19044-88-3; Oxydemeton-methyl / 301-12-2; Paraquat / 4685-14-7; Penconazole / 66246-88-6; Fenidimethalin / 40487-42-1; Pentachloronitrobenzene (Quintozene) / 82-68-8; Prodiamine / 29091-21-2; Pronamide / 23950-58-5; Propiconazole / 60207-90-1; Ronnel = Fenchlorfos / 299-84-3; Tebuconazole / 107534-96-3; Tetrachlorvinphos = Gardona / 22248-79-9; Thiazopyr / 117718-60-2;</p>

Table 2. The selection of endocrine disrupting pesticides (compound name/ chemical abstract number CAS) according to EU prioritization of EDCs into 3 categories.

For some chemicals the production and usage was already forbidden and others are still under testing. Majority of substances registered in this list of chemicals are pesticides (EC document, 2007). The selection of pesticides is summarized in Table. 2 according to their Category. From a total of 564 chemicals that had been suggested by various organizations or in published papers or reports as being suspected EDCs, 147 were considered likely to be either persistent in the environment or produced at high volumes. Of these, however, in a first assessment clear evidence of endocrine disrupting activity was noted for only 66 (assigned Category 1 using the criteria adopted in the study). A further 52 chemicals showed some evidence suggesting potential activity (Category 2). In total 118 substances were categorized in the first exercise of priority setting. Of the 66 chemicals in Category 1, humans were considered likely to be exposed to 60.

Selected substances have been included as persistent organic pollutants in the Stockholm Convention, which is a global treaty to protect human health and the environment from these compounds (EC document, 2006).

In June 2007, the new EU policy on chemicals, REACH - Registration, Evaluation, Authorization and Restriction of Chemicals, entered into force. The goal of REACH is a prompt, effective process for identifying the most hazardous chemicals on the European market and replacing them with safer alternatives. At the heart of the Authorization process is a "candidate list" of chemicals that meet the criteria of "Substances of Very High Concern" (SVHC) defined in the legislation, such as those that may cause cancer or persist in our bodies and the environment for long periods of time. Under REACH, SVHC are subject to the greatest scrutiny. The EU creates a specific list of these undesirable substances which will oblige importers, producers and downstream users to seek special authorization for continued use. Authorization may be denied, because REACH contains a provision that could replace some of these dangerous substances with safer alternatives. Under this activity, the International Chemical Secretariat (ChemSec) is a non-profit organization working for a toxic free world and publishing the SIN List (Substitute It Now). The SIN List applies REACH's own criteria to identify SVHC, and with the SIN 2.0 List update encompasses 378 chemicals. It contains 22 substances identified solely due to their endocrine disrupting properties. The following pesticides are included: Thiram (CAS 137-26-8), Zineb (CA S 12122-67-7).

4. EDPs analysis

Detection of EDPs and subsequent screening require sensitive and selective instrumental analytical techniques with sufficiently low limits of detection and quantification. Analyzing the EDPs at low concentration levels requires multistep sample preparation including cleaning and preconcentration of the resulting extract. As EDPs represent structurally diverse classes of substances, plentiful analytical methods could be applied for the identification and quantification of these compounds (Lagana et al., 2004; Petrovič et al., 2002). The most efficient approach to EDPs residues analysis involves the use of chromatographic methods (Comerton et al., 2009). Recently, methods based on biosensors have also been used (Bezbaruah & Kalita, 2010; Dostálek et al., 2007). Analytical techniques

as GC and liquid chromatography (LC) combined with MS or tandem MS are the techniques most frequently used and can reach satisfactory selectivity and sensitivity analyzing EDPs in complex food matrices mainly of food and environmental origin (Alder et al., 2006). Comparing mass-based methods with other analytical methods, such as Estrogen responsive chemically activated luciferase expression, Yeast estrogen screen, Enzyme-linked immunosorbent assay, it was shown that methods with MS detection (GC-MS, LC-MS, GC-MS/MS, LC-MS/MS) show lower detection limits (Chang et al., 2009). Comparing detection limits of enzymatic methods for the detection of organochlorine, organophosphate and carbamate pesticides with chromatographic methods it was concluded, that enzymatic methods achieve limits of detection in $\mu\text{g/l}$, whereas traditional chromatographic methods are often able to detect pesticides in ng/l (Van Dyk & Pletschke, 2011).

In the next part we will focus on analytical methods with limit of detection/quantification in the trace concentration level or ultratrace region and we will cite methods well-suited for analytical tests of low-level EDPs in food, environmental and biological samples.

Capillary gas chromatography coupled to MS detection has developed into a primary technique for identification and quantification of many EDCs using small bench-top instruments with sophisticated data systems (Holland, 2003). Electron ionization is the ionization technique of the first choice. In cases requiring enhanced sensitivity and selectivity the negative/positive chemical ionization is employed (Húšková et al., 2009a, 2009b, 2010a).

Within gas chromatographic techniques, fast GC technique satisfies the present day demands on faster and cost-effective analysis. Nowadays, fast GC can be performed on commercial gas chromatographs, which are standardly equipped with high-speed injection systems, electronic gas pressure control, rapid oven heating/cooling and fast detection (Dömötörová & Matisová, 2008). Advances in LC-MS interfacing, namely introduction of electrospray (ESI) and atmospheric pressure chemical ionization (APCI) have enabled sensitivity and reliability that are suitable for routine determinations of EDCs, particularly for more polar compounds that would require derivatization for GC-MS. LC-MS can reduce clean-up requirements over HPLC-UV (high performance liquid chromatography with ultraviolet detection), although care must be taken with matrix effects on ESI responses that may affect quantitation (Holland, 2003).

Signal enhancement and suppression due to matrix effects are reduced by the use of isotope-labelled internal standards or by application of matrix-matched standards. Tandem MS available on triple quadrupole, ion trap and hybrid analyzers are valuable for confirmation of identity, reduction of high background signals. They provide low limits of detection without the need for derivatization and sometimes also without the need of complicated sample preparation.

The overview of latest analytical methods combining preconcentration and chromatographic analytical methods for analysis of EDPs in food, environmental, and biological samples are summarized in Table 3. Various groups of EDPs were investigated by GC as carbamates, organochlorines, organophosphorous, organothiophosphates, organotin, triazines and others. Analysed samples varied from indoor air, water, sediments, food, to biological

Analytes	Matrix	Sample preparation	Injection technique	LOD	Separation and detection technique	References
GC						
23 pesticides	apples	QuEChERS	PTV, SVV	EI: 0.09-3.12 µg/kg NCI: 1.9-935 ng/kg	GC-MS (SIM) quadrup., NCI, EI	Húšková et al., 2009a
25 pesticides	apples	QuEChERS	PTV, SVV	EI: 0.02-6.32 µg/kg NCI: 0.15-619.3 ng/kg	fast GC-MS (SIM) quadrup., NCI, EI	Húšková et al., 2009b
20 OCPs	9 vegetable matrices	SBSE (PDMS 47 µl)	LVI - PTV, SVV	< 10 µg/kg	GC-MS (SIM) quadrup., EI	Barrida-Pereira et al., 2010
29 pesticides	fruit and vegetables	QuEChERS	PTV, SVV	≤ 5 µg/kg	fast GC-MS (SIM) quadrup., EI	Húšková et al., 2010b Hercegová et al., 2010
35 pesticides	fruit and vegetables	QuEChERS	PTV, SVV	EI: ≤ 5 µg/kg, NCI: ≤ 1 µg/kg	fast GC-MS (SIM) quadrup., EI, NCI	Hrouzková et al., 2011
9 pesticides, phthalates, 1 PAH	water	on-line SPE	on-column, retaining precolumn, SVV	0.1-20 ng/l	GC-MS (FS) quadrup., EI	Brossa et al., 2002
11 pesticides, phthalates	water	on-line SPE	LVI-PTV, SVV	1-36 ng/l	GC-MS (FS) quadrup., EI	Brossa et al., 2003
HCB, atrazine, lindane, vinclozolin, malathion, aldrin, α-endosulfan, 4,4'-DDE, dieldrin, endrin, 4,4'-DDT	river water	SBSE (PDMS 63 µl)	split/splitless, LVI - PTV, SVV	0.01-0.24 µg/l	GC-MS (FS), quadrup., EI	Peñalver et al., 2003
15 herbicides, 7 OPPs, 17 OCPs	water	SBSE (PDMS 47 µl)	PTV, SVV	0.025-0.400 µg/l	GC-MS (SIM) quadrup., EI	Seródio & Nogueira, 2004
32 EDCs and pesticides	water	SPE (LiChrolut EN/RP-18, Strata X)	splitless	5.3-95.9 ng/l	GC-MS/MS (MRM), EI, quad.,	Mansilha et al., 2010
15 OCPs	(i): water (ii): sediments	(i): LLE (ii): Soxhlet extraction, MAE	splitless	(i): 5.5-20.6 ng/l (ii): 0.6-2.1 µg/kg	GC-ECD	Fatoki & Awofolu, 2003
58 potential EDCs and PPCPs (18 pesticides)	drinking water, surface, ground, waste water (raw and treated)	SPE (HLB), LLE	splitless	1-10 ng/l	GC-MS/MS, EI, IT;	Trenholm et al., 2006
6 EDC herbicides and 3 degrade. products	natural surface water	SPE (Bond Elut-ENV)	splitless	2.3-115 ng/l	GC-MS (SIM) EI, quadrup.	Nevado et al., 2007

Analytes	Matrix	Sample preparation	Injection technique	LOD	Separation and detection technique	References
OPPs, OCPs, herbicides, PAHs, PCBs, phenols, organotins	estuarine and coastal water, sediments	SPE (Supelclean ENVI-18)	LVI-PTV, SVV	10-250 µg/l	GC-MS (SIM, FS) quadrup., EI	Almeida et al., 2007
33 multi-class pollutants	wastewaters, surface and ground waters	SPE	PTV, SVV; valve	0.2 and 88.9 ng/l	GC-MS LC-MS/MS	Baugros et al., 2008
EDCs (1 pesticide), carbamazepine, pharmaceuticals	wastewater irrigated soil	ASE, isolation SPE (Oasis HLB)	splitless	0.25 – 2.5 ng/g	GC-MS (SIM, FS) EI, quadrup.	Durán-Alvarez et al., 2009
PBDEs, PCBs, insecticides, phthalates	indoor dust from vacuum cleaner	Soxhlet extraction, alumina cleaning	n. r.	3-10 ng/g	GC-MS (SIM) EI, quadrup.	Hwang et al., 2008
18 OCPs	placenta samples from woman	SLE (Alumine), purification - preparative LC	n.r.	n.r.	GC-ECD GC-MS	Lopez-Espinosa et al., 2007
HPLC						
58 potential EDCs and PPCPs (18 pesticides)	drinking, surface, ground, waste water	SPE (HLB), LLE	valve	1-10 ng/l	LC-MS/MS, ESI+, ESI-, APCI, triplequad. (MRM)	Trenholm et al., 2006
9 EDCs (3 herbicides), 19 PPCPs	water, wastewater irrigated soils	SPE, ultrasonic extraction, silica gel cleaning	valve	water: 0.15-14.08 ng/l; soil: 0.06-10.64 ng/g	RRLC-MS/MS ESI	Chen et al., 2010
21 selected pesticides, phenols and phthalates	water	SPE, progr. field extraction system and Prospect	on-line SPE-LC	< 100 ng/l	LC-MS, APCI	López-Roldán et al., 2004

APCI – atmospheric pressure chemical ionization, ASE – accelerated solvent extraction, ECD – electron capture detector, ESI – electrospray, FS – full scan, HLB – hydrophilic-lipophilic balance, IT – ion trap, LLE – liquid-liquid extraction, LOD – limit of detection, LVI-large volume injection, MAE – microwave assisted extraction, MRM – multiple reaction mode, MS – mass spectrometry, MS/MS – tandem mass spectrometry, n.r. – not reported, OCPs – organochlorine pesticides, OPPs – organophosphorous pesticides, PAH – polycyclic aromatic hydrocarbon, PBDEs – Polybrominated Diphenyl Ethers, PCBs – polychlorinated biphenols, PDMS – polydimethylsiloxane, PPCPs – pharmaceuticals and personal care products, PTV – programmed-temperature vaporization (injector), QuEChERS – quick, easy, cheap, effective, rugged and safe, SIM – selected ion monitoring, RRLC – rapid resolution liquid chromatography, SBSE – stir bar sorptive extraction, SLE – solid-liquid extraction, SVV – solvent vent valve.

Table 3. An overview of analytical methods for analysis of EDPs with other groups of EDCs

samples. It is surprising, that indoor environment can be a significant source of exposure to some EDCs. Longer residence times and elevated contaminant concentrations in the indoor environment may increase chance of exposure to these contaminants by 1000-fold compared to outdoor exposure (Hwang et al., 2008).

In GC analysis, the most common injection systems are splitless and mainly PTV (programmed temperature vaporization) injector in solvent vent mode. Helium and exceptionally hydrogen were the most frequently used carrier gases. MS detector in SIM mode is used preferably. Specific and selective detectors as ECD (electron capture detector)

are also used. LC analysis is usually connected to MS detector with electrospray (ESI), atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI).

For sample preparation, liquid-liquid extraction, solid-liquid extraction and solid-phase extraction (SPE) are the most commonly used techniques. In the present era of “green chemistry”, the sampling preparation methods with large amounts of toxic solvents are difficult to justify for multiresidue determinations of EDCs (Serôdio & Nogueira, 2004). On the other hand, SPE is in some cases tedious, time-consuming and can present some disadvantages, i.e. the breakthrough of large sample volumes or the organic breakdown products that can interfere with the elucidation of unknowns, essentially at the ultra-trace level. The modern approaches are devoted to the development of a single comprehensive method utilizable for a wide variety of compounds with a single extraction in various matrices (Trenholm et al., 2006) or a solventless extraction technique at microscale level (Barrida-Pereira et al., 2010; Peñalver et al., 2003). Solid-phase microextraction (SPME) and stir bar sportive extraction (SBSE) are the often employed representative of microextraction techniques (Barrida-Pereira et al., 2010; Peñalver et al., 2003). The sample preparation approach known as QuEChERS, which stands for “quick, easy, cheap, effective, rugged and safe”, firstly introduced by Anastassiades et al., 2003a represents a widely used method of food sample preparation. QuEChERS approach uses acetonitrile for extraction of a 10-15 g homogenized sample followed by salt-out partitioning of the water from the sample using anhydrous $MgSO_4$, NaCl, and/or buffering agents, and further clean-up using dispersive solid-phase extraction (d-SPE) or disposable pipette extraction (DPX) with anhydrous $MgSO_4$, primary secondary amine (PSA) and/or in combination with C_{18} , graphitized carbon black (GCB) sorbents. It was used for extraction of EDPs from fruit and vegetable matrices (Hrouzková et al., 2011; Hůšková et al., 2009a, 2009b, 2010b).

5. Conventional capillary GC-MS for EDPs analysis

The contribution of our research group to the EDPs method development was as the first approach focused to the development of the conventional GC-MS method for separation, detection and quantification of EDPs belonging to different chemical classes – organochlorines, organophosphates, pyrethroids, dicarboximides, phtalamides, dinitroanilines, pyrazoles and triazinones in apple matrix (Hůšková et al., 2009a). The developed method involves the QuEChERS sample preparation method (Anastassiades et al., 2003a) modified according to our needs and resources. Subsequent analysis by conventional capillary GC-MS equipped with a PTV injector and quadrupole bench top mass selective detector. To obtain the low limits of detection (LODs) and limits of quantification (LOQs) required for regulation purposes or lower, selected ion monitoring (SIM) was used. EDPs were separated in 37.8 min.

Two ionization techniques, EI and NCI (with methane as reagent gas) were utilized and compared. Calibration in the NCI mode was performed at the concentration levels from 0.1 to 500 $\mu g/kg$ (coefficient of determination, $R^2 > 0.999$) and for EI in the range of 5 - 500 $\mu g/kg$

($R^2 > 0.99$). From the lowest calibration levels (LCLs) the LODs and LOQs were calculated and are summarized in Table 4. The LODs for all pesticides varied from 0.0019 to 0.94 $\mu\text{g}/\text{kg}$ for NCI and from 0.09 to 3.12 $\mu\text{g}/\text{kg}$ for EI mode. Repeatability of all measurements, expressed as relative standard deviations of absolute peak areas, met the EU criterion of relative standard deviation, $\text{RSD} \leq 20\%$.

No.	Pesticide	LCL ^a	RSD	LOD ^b	LOQ ^c	LCL ^a	RSD	LOD ^b	LOQ ^c
		(ng/mL)	(%)	(pg/mL)	(pg/mL)	(ng/mL)	(%)	(ng/mL)	(ng/mL)
		NCI				EI			
1.	trifluralin	0.1	1.7	1.90	6.32	5.0	2.0	0.10	0.33
2.	hexachlorobenzene	0.1	1.4	5.64	18.2	5.0	2.7	0.15	0.52
3.	dimethoate	0.1	5.9	42.3	140	5.0	7.2	0.41	1.38
4.	lindane	0.1	6.5	7.52	25.2	5.0	8.5	0.75	2.50
5.	metribuzin	0.1	2.3	14.1	47.4	5.0	6.9	0.28	0.94
6.	chlorpyrifos-methyl	0.1	2.9	37.2	120	5.0	6.7	0.25	0.83
7.	vinclozolin	0.1	5.0	7.71	25.2	5.0	5.5	0.68	2.27
8.	heptachlor	0.5	8.5	103	330	5.0	5.7	0.53	1.78
9.	fenitrothion	0.1	2.6	6.80	23.4	5.0	6.4	0.22	0.74
10.	malathion	0.1	8.1	42.4	140	5.0	9.3	0.78	2.63
11.	chlorpyrifos	0.1	4.4	5.91	19.1	10.0	6.0	0.96	3.22
12.	pendimethalin	0.1	2.4	21.4	71.2	5.0	6.9	0.30	1.02
13.	captan	1.0	11.1	935	3114	25.0	11.2	3.12	10.4
14.	folpet	1.0	10.1	754	2501	25.0	13.9	1.82	6.09
15.	fipronil	0.1	1.7	11.4	38.7	5.0	6.1	0.13	0.45
16.	methidation	0.1	5.6	50.5	160	5.0	6.0	0.50	1.66
17.	diazinon	0.5	1.7	113	351	5.0	7.5	0.14	0.36
18.	endosulfan-alfa	0.1	3.3	4.87	16.6	5.0	4.3	1.00	3.33
19.	endosulfan-beta	0.1	4.1	6.41	21.2	5.0	4.2	0.42	1.40
20.	iprodione	0.1	7.3	30.5	100	5.0	8.1	0.41	1.38
21.	bifenthrin	0.1	1.1	20.3	66.9	5.0	4.7	0.09	0.30
22.	mirex	0.5	2.4	162	550	5.0	6.0	0.27	0.92
23.	deltamethrin	0.5	2.5	211	711	25.0	6.2	2.34	7.81

Notes:^aLCLs - for some compounds with the highest response it would be possible to go to the lower LCLs; at 0.1 ng/mL for NCI and 5 ng/mL for EI the majority of compounds could be quantified; ^bLOD (limit of detection) - calculated as 3:1 S/N (signal to noise ratio) from calibration measurements; ^cLOQ (limit of quantification) - calculated as 10:1 S/N from calibration measurements; RSD – relative standard deviation, other abbreviations - in Tab. 3.

Table 4. The list of the studied endocrine disrupting pesticides in two detection modes (NCI, EI), instrumental LODs^b and LOQs^c and RSDs calculated from absolute peak areas of pesticides at the lowest calibration levels (LCLs) (Húšková et al., 2009a).

To illustrate the matrix phenomena, chromatograms of the target ions of the EDPs analyzed in the real apple sample extract at the concentration level 10 ng/mL (corresponding to 10 µg/kg in fruit sample) using both MS ionization techniques in the SIM mode are presented in Fig. 2. In the NCI mode, the influence of sample matrix is not relevant (Schulz, 2004) and

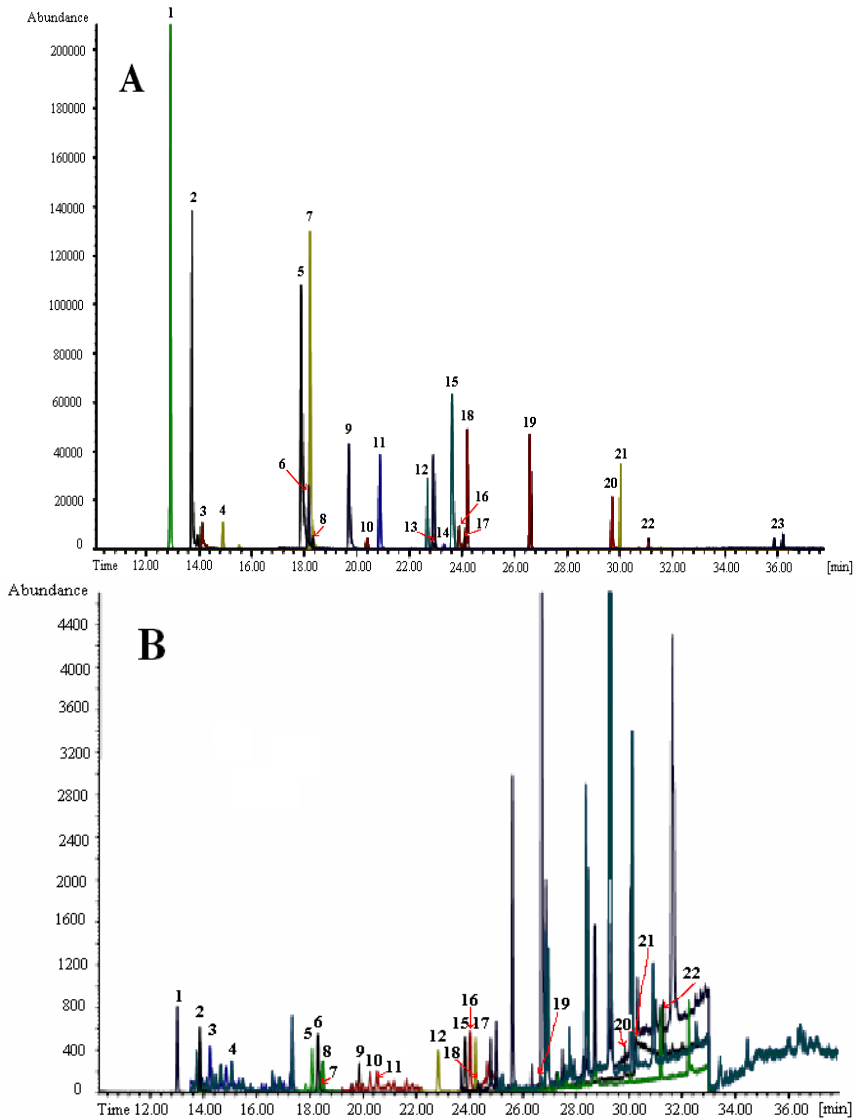


Figure 2. Chromatograms of target ions of endocrine disrupting pesticides analyzed by capillary GC–MS in SIM mode in matrix–matched standard solutions at the concentration level 10 ng/mL (corresponding to 10 µg/kg): A – NCI mode; B - EI mode (Hůšková et al., 2009a). Number of peaks is identical with the number of compounds given in Table 4.

this fact is evident from Fig. 2A, as a clean chromatogram of the target ions of EDPs without interfering peaks from matrix can be seen. In the EI mode, the pesticides peaks shapes are complicated due to interfering peaks of matrix which creates problems in evaluation of chromatograms. Important negative consequence of interfering peaks from the matrix compounds is a decreased signal to noise ratio in the EI mode. In general, a decreased response (decreased sensitivity) of the pesticides was observed in comparison to the NCI mode at the same concentration (Fig. 2B).

6. Fast capillary GC-MS for EDPs analysis

Numerous ways exist for speeding up the capillary GC separation as it was summarized in reviews (Dömötörövá & Matisová, 2008; Maštovská & Lehotay, 2003; Matisová & Dömötörövá, 2003). An approach utilizing narrow-bore columns for pesticide residues analysis was elaborated in our research group. Fast separation with narrow-bore capillary columns as a way to reduce the run times provides separation efficiency comparable or even higher than conventional capillary columns (Dömötörövá & Matisová, 2008; Hrouzková & Matisová, 2011; Matisová & Dömötörövá, 2003).

The benefits of the developed fast GC methods for selected EDPs by our group (Hrouzková et al., 2011; Húšková et al., 2009b, 2010b) provide higher laboratory throughput, reduced GC operating costs, and better analytical precision through replicate analyses compared to conventional GC (Húšková et al., 2009a).

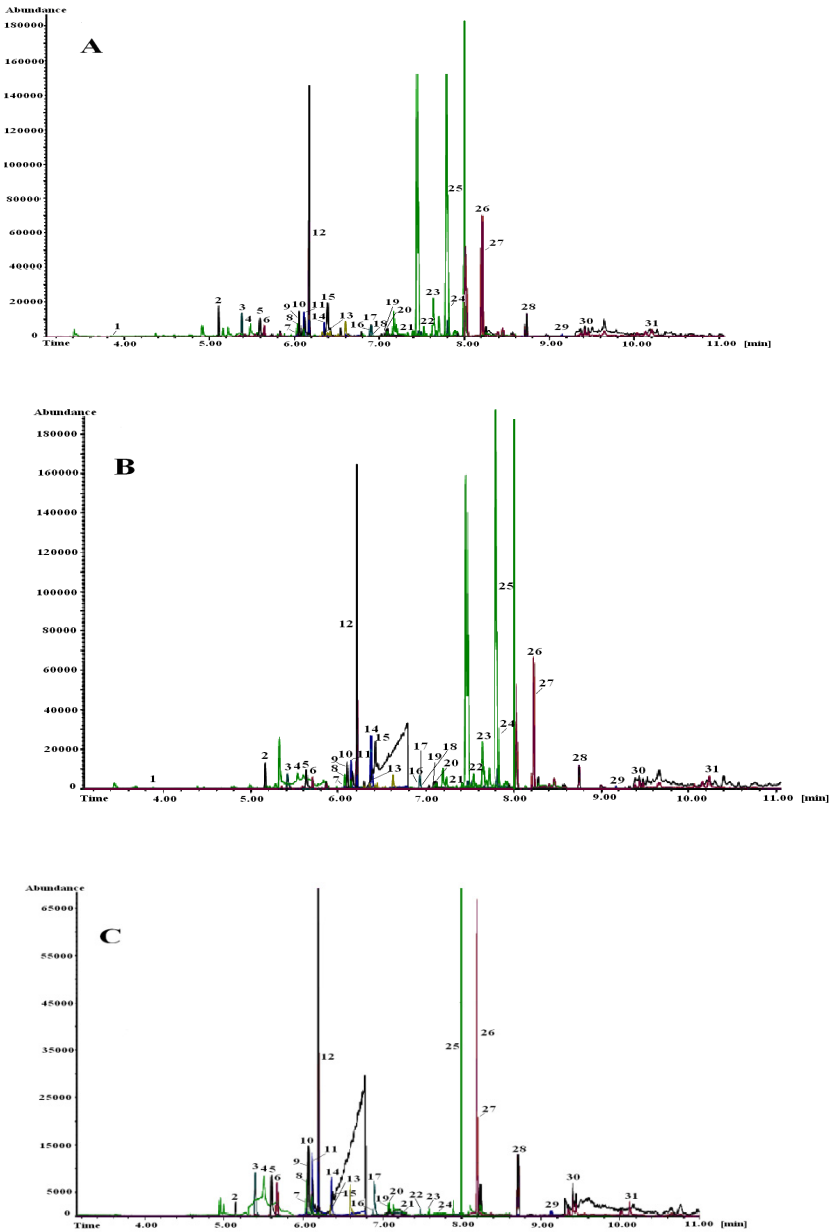
The fast GC-MS method for the determination of 29 pesticides proved or suspected to be endocrine disrupting chemicals (Table 5) was developed and validated by Húšková et al., 2010b. LOQs in the range of 0.04 to 10 µg/kg for the majority of pesticides were obtained, dicofol, linuron and prochloraz gave LOQs ≤ 21 µg/kg using matrix-matched standards for calibration. The search on different calibration approaches was elaborated. Despite of great efforts in the research of GC amenable pesticide residues analysis the issues are matrix effects and mainly matrix-induced chromatographic response enhancement (Kirchner et al., 2008). Injecting a real sample, the matrix components tend to block active sites in the GC inlet and column, thus reducing losses of susceptible analytes caused by adsorption or degradation on these active sites (Hajšlová & Zrostlíková, 2003). This phenomenon results in higher analyte signals in matrix-containing, versus matrix-free solutions. Ways to compensate matrix effects include: (i) method of standard addition; (ii) use of isotopically labelled internal standards; (iii) use of matrix-matched standards; and (iv) use of analyte protectants. The most widely used method in laboratories nowadays is the use of matrix-matched standards. This approach is, however, complicated by the fact, that the composition of matrix-matched standard should be as close as possible to the composition of real sample matrix in order to provide good compensation for matrix effects. However, it is difficult to obtain pesticide free matrix for less common commodities and this approach is also laborious. Analyte protectants protect co-injected analytes against degradation, adsorption, or both in the GC system. The novel concept idea was to add analyte protectants (APs) to sample extracts as well as to matrix-free (solvent) standards to induce an even

response enhancement in both instances (Anastassiades et al., 2003b). Main advantages of using APs should be easier preparation of calibration standards, improvement of trueness of analysis. Húšková et al., 2010b evaluated different calibration approaches based on matrix-matched standardization and application of analyte protectants (3-ethoxy-1,2-propanediol, D-sorbitol, L-gulonic acid γ -lactone) in apple samples. For illustration, chromatograms of target ions of EDCs pesticides in variety of standard solutions (matrix-matched standard solution, matrix-matched standard solution with APs, neat solvent with APs) analyzed by fast GC-MS in SIM mode at the concentration level of 50 ng/mL (corresponding to 50 μ g/kg) are presented in Fig. 3. Utilization of pesticide standards in a neat solvent (MeCN) with addition of APs was the simplest approach for routine use. However, it provided higher values of LODs and LOQs, particularly for the most volatile and problematic analytes. Calibration with matrix-matched standards provided the best results compared to other calibration approaches under study in terms of linearity of measurements expressed as R^2 , instrumental LODs, LOQs and the repeatability of absolute peak area measurements at LCLs expressed as RSDs. Selected validation parameters, LODs, LOQs and LCLs for three types of calibration standards are summarized in Table 5.

Analysis of synthetic sample spiked by EDPs at concentration of 50 μ g/kg yielded overestimation and/or underestimation of a number of EDPs using matrix matched standards without/with APs and MeCN with APs with maximal errors up to 22 % (Fig. 4). The degree of overestimation depends on a compound and its concentration and also on the number of injections and the GC system maintenance (periodicity of liner and precolumn change).

Performance of APs as additives for preparation of calibration standards in MeCN and matrix-matched standards was evaluated by comparison with currently widespread used matrix-matched calibration in fruit and vegetables extracts with the set of selected pesticides utilizing fast GC-MS with narrow-bore columns and QuEChERS sample preparation method (Hercegová et al., 2010). Extracts of fruit and vegetable samples representing different matrix type (apple, pear, cucumber, cauliflower) were subjected to estimation of extract solids to compare amount of co-extracted sample material.

The weight of matrix components was similar for apples and pears. Extract solids of cauliflower had the highest amount of matrix components and the lowest amount of co-extractants in cucumber compared to fruit extracts was obtained. To search the matrix effects intensity, the measurements of MeCN extracts in full scan mode and SIM monitoring for all matrices with the known concentration of pesticide residues (50 μ g/kg) were performed. An acceptable agreement of quantified pesticide residues concentrations with spiked fortified concentration (50 μ g/kg) was obtained utilizing matrix-matched calibration standards and matrix-matched standards with addition of APs in all studied matrices. Standards in a neat solvent (MeCN) with the addition of APs yielded overestimation for a number of pesticides under study. The overestimation was shown to be matrix dependent and influenced by the number of injections performed. In the case of MeCN standards with APs and quantification using absolute peak areas and normalized areas to internal standards (triphenylphosphate, heptachlor), overestimation of the results for majority the



Note: numbering of peaks is identical with the number of compounds given in Table 5, as well as abbreviations.

Figure 3. Chromatograms of target ions of EDCs pesticide in various standard solutions (50 ng/mL) analyzed by fast GC-MS in SIM mode: A - matrix-matched standard solution without APs; B - matrix-matched standard solution with APs; C - MeCN standard solution with APs (Húšková et al., 2010b).

No	Pesticide	Matrix			Matrix + APs			MeCN + APs		
		LCL ng/mL	LOD ng/mL	LOQ ng/mL	LCL ng/mL	LOD ng/mL	LOQ ng/mL	LCL ng/mL	LOD ng/mL	LOQ ng/mL
1	diuron	10	2.63	8.77	10	3.59	11.97	100	14.37	57.86
2	trifluralin	1	0.07	0.24	1	0.10	0.32	1	0.12	0.39
3	hexachlorbenzen	1	0.02	0.08	1	0.03	0.09	1	0.03	0.11
4	dimethoate	1	0.16	0.53	1	0.22	0.73	5	2.27	7.49
5	atrazine	1	0.85	2.83	1	1.16	3.87	1	1.41	4.70
6	lindan	1	0.52	1.73	1	0.71	2.37	1	0.80	2.67
7	acetochlor	1	0.77	2.57	1	1.05	3.51	1	1.19	3.96
8	chlorpyrifos-methyl	1	0.08	0.27	1	0.06	0.20	1	0.12	0.41
9	vinclozolin	1	0.29	0.97	1	0.22	0.73	5	3.45	11.38
10	alachlor	1	0.09	0.30	1	0.07	0.23	1	0.13	0.44
11	metribuzin	1	0.10	0.33	1	0.08	0.25	1	0.15	0.49
12	heptachlor	<i>Internal standard</i>								
13	dicofol	10	3.56	11.87	10	4.80	16.01	10	5.21	17.37
14	malathion	1	0.34	1.13	1	0.27	0.89	1	0.60	2.00
15	linuron	10	6.32	21.07	10	9.20	30.68	50	18.15	66.07
16	diazinon	1	0.09	0.28	1	0.13	0.44	1	0.16	0.53
17	procymidone	1	0.27	0.90	1	0.39	1.31	1	0.48	1.59
18	folpet	10	1.05	3.50	10	1.53	5.10	100	21.54	75.17
19	chlordane	1	0.10	0.32	1	0.15	0.49	1	0.25	0.87
20	endosulfan-alfa	1	0.46	1.53	1	0.32	1.07	5	2.72	9.41
21	myclobutanil	1	0.07	0.22	1	0.05	0.16	1	0.11	0.37
22	nitrofen	5	1.09	3.62	5	0.76	2.54	10	1.92	6.42
23	endosulfan-beta	1	0.01	0.04	1	0.02	0.05	5	1.02	3.69
24	chlordecone	10	0.91	3.03	10	0.61	2.02	50	3.61	12.82
25	TPP	<i>Internal standard</i>								
26	bifenthrin	1	0.02	0.08	1	0.03	0.11	1	0.04	0.12
27	iprodione	1	0.10	0.33	1	0.17	0.55	1	0.16	0.53
28	mirex	1	0.11	0.32	1	0.18	0.61	1	0.26	0.87
29	prochloraz	10	5.37	17.90	10	3.21	10.70	50	7.83	26.12
30	cypermethrin	5	2.05	6.83	5	2.87	9.56	5	3.24	10.79
31	deltamethrin	1	0.07	0.24	1	0.06	0.20	5	1.11	3.88

Notes. ^a calculated as 3:1 S/N ratio, ^b calculated as 10:1 S/N ratio, APs – analyte protectants; MeCN – acetonitrile; TPP – triphenylphosphate; other abbreviations in Tab. 3, 4.

Table 5. Instrumental LODs, LOQs / LODs^a, LOQs^b for all types of calibration standards (Hůšková et al., 2010b).

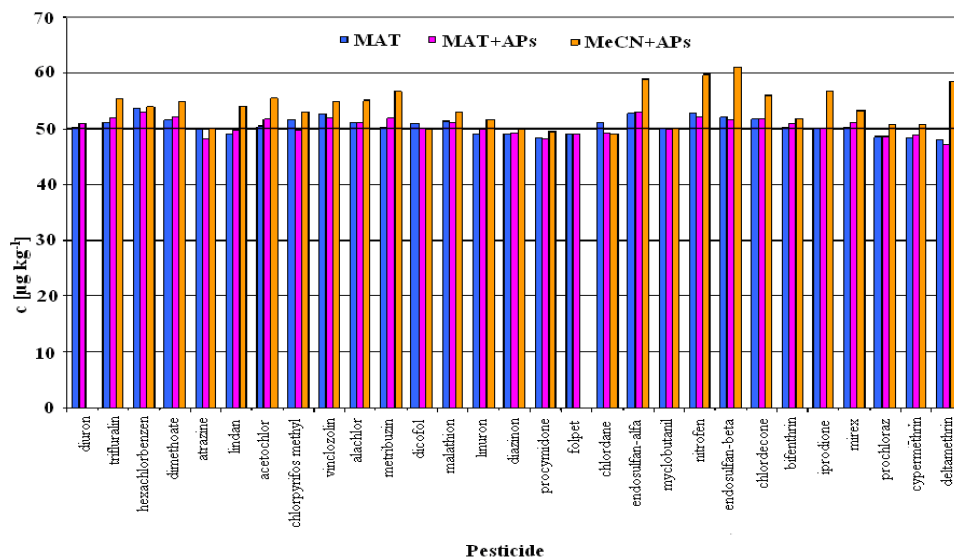


Figure 4. Graph of calculated concentrations of endocrine disrupting pesticides in synthetic sample using matrix-matched standards without/with APs and MeCN + APs vs. the expected 50 µg/kg concentration (Húšková et al., 2010b). For each of matrix-matched types of calibration standards the QuEChERS extract of apples was used. Six GC-MS measurements were performed for synthetic sample and relevant calibration standards. Abbreviations in Table 5.

tested pesticides in all matrices was observed. The maximal value of error of determination of average concentration was found to be 39.8 %. In some cases also underestimation of quantity was observed.

The fast GC set-up using narrow-bore column (0.15 mm I.D.) in combination with MS detector in NCI mode was introduced by Húšková et al., 2009b and compared to fast GC-MS with EI. Multi-residue method of 25 EDPs belonging to different groups (organochlorines, organophosphates, pyrethroids, dicarboximides, 2,6-dinitroanilines, triazinones, substituted ureas, phthalamides, cyclodienes, triazoles, imidazoles), varying in polarity, volatility and other physicochemical properties from non-fatty fruit and vegetable matrices based on fast GC with quadrupole NCI-MS was developed. The method LOQ was found to be 5 µg/kg (except for folpet, chlordecone, endosulfan-alfa and endosulfan-beta) in EI mode, 1 µg/kg in NCI mode for 12 compounds under study and 0.1 µg/kg for 13 compounds. The EU criterion concerning recovery rates was fulfilled at these concentration levels. The harmful effect of EDPs is relevant at very low concentrations, so the use of NCI-MS was shown to be an effective tool to decrease LOQs 5-50 times compared to EI mode. Changing the universal MS detection in EI mode by NCI, the selectivity was increased, and the measured sensitivity of the selected analytes was enhanced for a variety of active EDPs with the adverse effect on wildlife or human system. Comparison of relevant validation parameters is given in Tab. 6.

Method / Results	GC-NCI-MS	GC-EI-MS
LCLs	0.01, 0.05 µg/kg	1 µg/kg
R ²	0.9936 – 1.0000	0.9882 – 0.9999
LODs	0.15 – 88.82 ng/kg	0.01 – 6.32 µg/kg
LOQs	0.52 – 291.35 ng/kg	0.04 – 21.07 µg/kg

Notes: R² – coefficient of determination, other abbreviations in Tab. 3, 4.

Table 6. Comparison of validation parameters for NCI vs. EI mode of GC-MS analysis of pesticide residues in apple extract.

7. Chemmometric approach

Chemmometric study of pesticide signals in two MS modes answers two basic questions on NCI and EI signals proportionality and on the possibility of simultaneous evaluation of signals (Húšková et al., 2009a).

The mutual proportionality was searched by regression analysis. At first the regression coefficients were calculated for all 23 EDPs (Table 4) under calibration conditions described by Húšková et al., 2009a for two MS modes using linear models defined as signal vs. concentration of standards. For each pesticide the measurement sensitivity was found by the slopes b_{NCI} and b_{EI} concerning NCI and EI mode, respectively, including their corresponding standard deviations. Then further regression model was set: $b_{EI} = a + b \cdot b_{NCI}$; in this case b_{EI} and b_{NCI} were used as the regression variables. The resulting dependence is plotted in Fig.5.

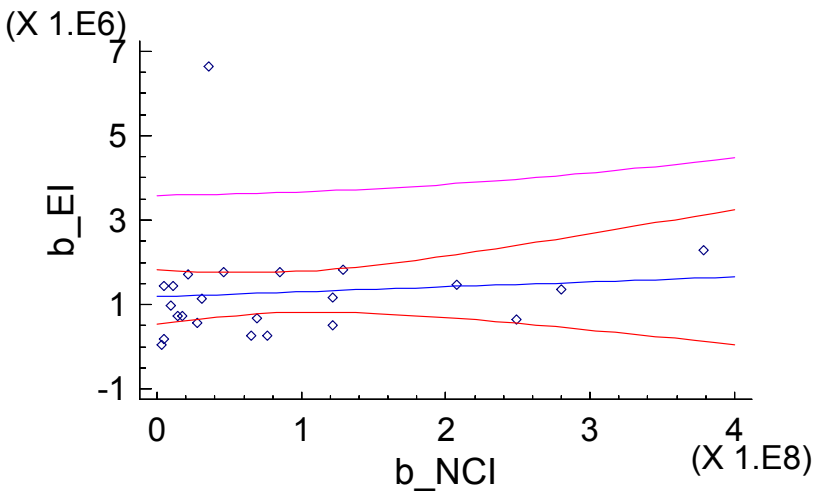


Figure 5. Ordinary least squares linear regression for the model $b_{EI} = a + b \cdot b_{NCI}$ and 23 endocrine disrupting pesticides numbered in Table 4. The points ranked by the increased value along the b_{NCI} axis (b_{NCI} in figure) correspond to the pesticides: 14, 13, 22, 8, 17, 23, 4, 16, 20, 3, 21, 6, 19, 11, 18, 12, 10, 9, 15, 2, 7, 5, and 1. In addition to regression straight-line the regression and prediction confidence bands (90 % probability) are plotted (the lower prediction band was cut off by the choice of the values on vertical axis).

Two influential points were observed: pesticide number 21 (bifenthrin) exhibited extraordinary large sensitivity in EI mode and can be considered as outlier and pesticide number 1 (trifluralin), which exhibits extreme NCI sensitivity. Found slope value a_1 was relatively small compared to the corresponding standard deviation s_{a1} , therefore mutual dependence of the EI and NCI signals appeared insignificant.

When pesticide number 21 was excluded from regression it was found for the slope $a_1 = 0.00249$ and $s_{a1} = 0.00125$, which means an insignificant dependence at 95 % probability level but significant one at 90 % probability. However, a fully correct way of regression is performed when the error of both regression variables, b_{EI} and b_{NCI} , are considered, since both the EI as well as NCI signals are random variables. Such a calculation provides bivariate least squares method (Mocák et al., 2003), which is a variant of weighted Deming regression, where each regression point is computed from four values - two variables and their standard deviations. The found regression equation for all 23 EDPs was $b_{NCI} = 1.330 \times 10^5 + 0.00593 b_{EI}$ with $s_{a1} = 0.00195$ and $s_{a0} = 1.012 \times 10^5$, which signifies a significant slope and an insignificant intercept at 95 % probability. The correlation coefficient was $r = 0.5197$, which is significant when compared to the critical value $r_{crit} = 0.4132$. The same final results concerning significance of the slope and intercept were found when 22 pesticides were studied (without number 21) with a slightly larger correlation coefficient, $r = 0.5566$. It can be concluded that the sensitivities of the EI and NCI signals are significantly mutually dependent despite the imperfect proportionality in case of some pesticides.

The question on the possibility of simultaneous evaluation of signals was studied by the principal component analysis, PCA, which is a multivariate data analysis method (Sharma, 1996) capable to express the collective effect of the EI and NCI signal sensitivities. In this method, new variables, the principal components are calculated by optimal linear combination of original variables. As it is obvious in this method, the original variables b_{NCI} and b_{EI} were standardized by the corresponding mean subtraction and division by the corresponding standard deviation. The calculated PCA plot PC2 vs. PC1 is depicted in Fig. 6. The first principal component, PC1, generally expresses the conjoint effect of all original variables, which means the common sensitivity in this study since it was found to be a positive linear combination of b_{NCI} and b_{EI} . The second principal component, PC2, expresses here the relative magnitude of the sensitivities in the EI mode (positive PC2 values) and the NCI mode (negative PC2 values). From the position of the pesticide samples in the PC2 - PC1 plane it is possible to understand several observed effects. The lowest PC1 values mean the smallest sensitivities, which exhibit pesticides 14 and 13; on the contrary, the highest PC1 values mean the largest sensitivities, exhibited by pesticides 1 and 21, then by 5, 2 and 7 in a smaller extent. A high PC2 value means extraordinary large EI sensitivity, a low (negative) PC2 value means extraordinary large NCI sensitivity. The occurrence of negative PC2 as well as PC1 values follows from the PCA data processing since the original variable values less than the mean are negative after performed standardization. It is clearly seen from Fig. 6 that relatively high EI signals (in decreasing order) have pesticides 21, 16, 22, 17, 6, 8, 3, 12, 23, and 4 (all with $PC2 > 0.15$); relatively high NCI signals (in decreasing order) have pesticides 1, 7, 5, 2, 9, 10, 18, 19, and 11 (all with $PC2 < 0.15$). Balanced (but low) NCI

and EI sensitivities exhibit pesticides 14, 13, 15, and 20. In general, the found LOD and LOQ values are inversely proportional to the observed sensitivities, e.g. the lowest LOD in the NCI mode has pesticide 1 and pesticide 21 in the EI mode.

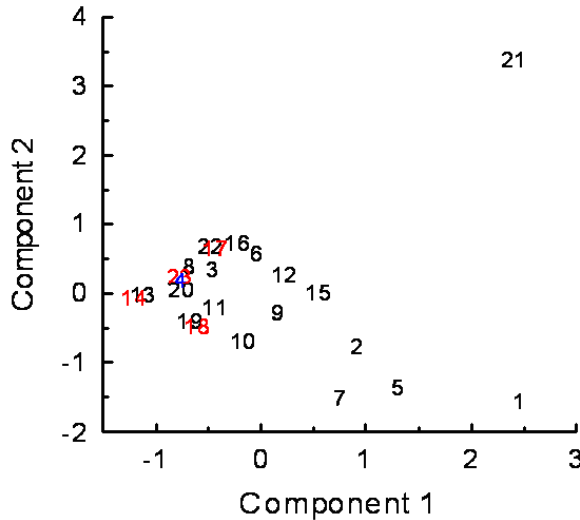


Figure 6. Dependence of principal components (PC) in principal component analysis. Component 1 = PC1, component 2 = PC2. Numbers in the figure denote endocrine disrupting pesticides listed in Table 4. First and second component contain 54.5 % and 45.5 % of the total variability of data.

8. Real-life EDPs analysis

The applicability of the developed and validated methods was demonstrated by real-life samples analyses showing that developed GC-MS methods in both, conventional and fast, arrangements are suitable for the analysis of EDPs at low concentration levels in a variety of fruit and vegetable samples.

Positive findings of EDPs in real samples determined by fast GC-MS were reported by Húšková et al., 2010b, particularly malathion in orange sample and iprodione in lettuce, strawberry, and plum. Matrix-matched standards (apple matrix) without/with APs and MeCN standards with APs were used for quantification. Concentration of quantified EDPs was in the range of 41-246 µg/kg.

Utilization of APs and its comparison with matrix-matched calibration standards was performed in the analysis of real samples with pesticide residues (Hercegová et al., 2010). Quantified concentrations of pesticide residues were lower than the MRLs for the corresponding matrix. Good match between results obtained using both calibration approaches was reached.

To show the potential of fast GC for the utilization in the ultratrace analysis of pesticide residues with endocrine disruption behaviour, the survey of EDPs in non-fatty food was

published by Hrouzková et al., 2011. An important objective was to assess the occurrence of pesticides from different chemical classes suspected or known to act as endocrine disrupters in fruit and vegetable samples available on the market in Slovakia. Thirty-four samples of 20 different commodities were analyzed. Twenty-one compounds at concentrations in the range of 0.003 – 2.14 mg/kg were detected in 28 positive samples. The MRL value was exceeded in the case of dimethoate (peach_A). In the case of fenitrothion (peach_B) the determined concentration was at the MRL level. Seven samples contained residues of three or more pesticides.

9. Conclusions

EDPs are known as a class of EDCs which have xenobiotic origin. They mimic or inhibit the natural action of the endocrine system in wildlife and humans, such as synthesis, secretion, transport, and binding. The chapter was devoted to the significance and importance of endocrine disrupters investigation, to the evolution and current state of EDPs list creation. The approach of regulatory agencies in European Union, in United States and further to the EDCs/EDPs problem solutions was discussed.

For the identification and quantification chromatographic methods hyphenated with mass-spectrometric detection provide the excellent sensitivity and precision. These methods generally comprise also preconcentration step based on the extraction of EDPs.

The main part of the chapter was devoted to the contribution in GC-MS methods development for EDPs with the utilization of conventional and fast GC. The search on the different calibration approaches based on the matrix-matched standardization, the application of analyte protectants and the influence of different matrices with differing amounts of co-extractants was studied with the aim to eliminate the adverse effects caused by matrix interferences. The combination of fast GC separation and selective MS detection with NCI resulted to selectivity enhancement and decrease of the limits of quantification.

For EDPs residues analysis ultrasensitive analytical methods are required and there is still the need to improve the performance and ruggedness of analyses. Despite the progress in the analytical instrumentation development, for most of substances there is continuous need to employ the extraction and preconcentration.

Identification and determination of endocrine disrupting pesticides is a relevant research trend and a progress of analytical methods as a base for necessary changes in regulations of the quality of food and environment in the future is expected.

Author details

Svetlana Hrouzková and Eva Matisová

*Institute of Analytical Chemistry, Faculty of Chemical and Food Technology,
Slovak University of Technology in Bratislava, Slovak Republic*

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Indirect Effects of Pesticides on Natural Enemies

Raymond A. Cloyd

Additional information is available at the end of the chapter

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1. Introduction

Pesticides including insecticides and miticides are primarily used to regulate arthropod (insect and mite) pest populations in agricultural and horticultural crop production systems. However, continual reliance on pesticides may eventually result in a number of potential ecological problems including resistance, secondary pest outbreaks, and/or target pest resurgence [1,2]. Therefore, implementation of alternative management strategies is justified in order to preserve existing pesticides and produce crops with minimal damage from arthropod pests. One option that has gained interest by producers is integrating pesticides with biological control agents or natural enemies including parasitoids and predators [3]. This is often referred to as ‘compatibility,’ which is the ability to integrate or combine natural enemies with pesticides so as to regulate arthropod pest populations without directly or indirectly affecting the life history parameters or population dynamics of natural enemies [2,4]. This may also refer to pesticides being effective against targeted arthropod pests but relatively non-harmful to natural enemies [5,6].

Pesticides vary in their activity, which not only impacts how they kill arthropod pests but also how they may indirectly influence natural enemy populations. Pesticides may be classified as contact, stomach poison, systemic, and/or translaminar [7,8]. In addition, the application method—foliar vs. drench or granular—may determine the extent of any indirect effects on natural enemies [9] as well as the pesticide mode of action. The type of natural enemy—parasitoid or predator—may be influenced differently based on the factors mentioned above. Furthermore, the type of pesticide may substantially contribute to any indirect effects on natural enemies. For example, broad-spectrum, nerve toxin pesticides such as most of the older pesticides in the chemical classes, organophosphate (acephate and chlorpyrifos), carbamate (carbaryl and methiocarb), and pyrethroid (bifenthrin and cyfluthrin) may be both directly and indirectly more harmful to natural enemies than non-nerve toxin type pesticides (often referred to a “selective pesticides”) including insect growth regulators (kinoprene and pyriproxyfen), insecticidal soaps (potassium salts of fatty

acids), horticultural oils (petroleum or neem-based), selective feeding blockers (flonicamid and pymetrozine), and microbials (entomopathogenic fungi and bacteria, and other micro-organisms) [10]. The non-nerve toxin pesticides are generally more specific or selective in regards to arthropod pest activity with broader modes of action than nerve toxin pesticides [3].

The effects of pesticides on natural enemies are typically associated with determining direct effects such as mortality or survival over a given time period (24 to 96 hours) [11]. While evaluations associated with the direct effects of pesticides on natural enemies are important, what are actually more relevant are the indirect or delayed effects of pesticides because this provides information on the long-term stability and overall success of a biological control program when attempting to integrate the use of pesticides with natural enemies [12-16].

Any indirect effects, which are sometimes referred to as sub-lethal, latent, or cumulative adverse effects may be associated with interfering with the physiology and behavior of natural enemies by inhibiting longevity, fecundity, reproduction (based on the number of progeny produced or eggs laid by females), development time, mobility, searching (foraging) and feeding behavior, predation and/or parasitism, prey consumption, emergence rates, and/or sex ratio [2,13,16,17-22].

2. Indirect effects of pesticides on natural enemies

In this book chapter, the term ‘indirect’ will be used for consistency. The indirect effects of pesticides on natural enemies (Table 1) have not been studied as extensively compared to direct effects, and those studies associated with indirect effects of pesticides have primarily involved evaluating fecundity and longevity [23-27].

* Longevity	* Reproduction
* Fecundity and/or fertility	* Development time (egg to adult or specific instars)
* Mobility	* Prey searching efficiency and feeding behavior
* Predation and/or parasitism	* Sex ratio
* Emergence rates	* Prey consumption
* Population growth/reduction	* Repellency
* Orientation behavior	* Prey acceptance (for oviposition by female parasitoids)

Modified from [2, 18].

Table 1. List of potential indirect effects of pesticides (insecticides, miticides, and fungicides) on the physiology and behavioral parameters of natural enemies (parasitoids and predators).

Although indirect effects may be more subtle or chronic compared to direct effects [14, 28-29] any indirect effects may inhibit the ability of natural enemies to establish populations; suppress the capacity of natural enemies to utilize prey; impact parasitism (for parasitoids) or consumption (for predators) rates; decrease female reproduction; reduce prey

availability; inhibit ability of natural enemies to recognize prey; influence the sex ratio (females: males); and reduce mobility, which could impact prey-finding [3, 27, 30-31]. In addition, more than one physiological and/or behavioral parameter may be indirectly affected after exposure to a pesticide. Furthermore, understanding the indirect effects of different concentrations of pesticides on fecundity, fertility, reproduction, adult and larva longevity, and prey consumption is important in successfully integrating natural enemies with pesticides and avoiding any indirect consequences on population dynamics [16,32].

The important physiological and behavioral parameters presented above are responsible for allowing natural enemies to regulate arthropod pest populations. Some factors affiliated with natural enemies that may influence the indirect effects of pesticides include natural enemy age, type of natural enemy (parasitoid vs. predator), life stages (immature vs. adult) exposed to pesticides, and sex (male vs. female) [9,33]. In addition, the type of pesticide (nerve toxin vs. non-nerve toxin) as well as the pesticide application method (foliar vs. systemic) may have significant consequences and thus impact the extent of any indirect effects on natural enemies based on exposure (immediate vs. chronic). For example, foliar applications of pesticides, which in most cases, represents immediate exposure, that do not directly harm natural enemies may have indirect effects. Another indirect effect may be related to residues remaining after a foliar application, which could inhibit the emission of volatile cues from plants that are utilized by certain natural enemies to detect prey location (prey patches) from long distances within plant communities, thus impacting foraging behavior and searching efficiency [34-37]. Moreover, any residues remaining after application may indirectly affect parasitoids by inhibiting adult emergence [38].

Furthermore, natural enemies, particularly parasitoids, may be indirectly affected by feeding on contaminated honeydew excreted by phloem-feeding insect prey [39,40], which could significantly affect their performance. Certain pesticides (insecticides and fungicides) may also exhibit repellent activity [16,41-46] or alter host plant physiology [13,47] thus indirectly affecting the ability of natural enemies to regulate existing arthropod pest populations [48].

This book chapter will now focus specifically on the indirect effects on natural enemies associated with different categories of pesticides including systemic insecticides, insect growth regulators, selective feeding blockers, microbials, miticides, and fungicides.

3. Systemic insecticides

Systemic insecticides, when applied as drenches or granules to the soil/growing medium, have been promoted to be relatively non-toxic to natural enemies due to lack of any direct exposure [49-51]. However, this may not be the case as systemic insecticides may exhibit indirect effects on natural enemies via several mechanisms including elimination of prey, contamination of floral parts by the active ingredient, consumption of the active ingredient while ingesting plant fluids, and contamination of prey ingesting either lethal or sub-lethal concentrations of the active ingredient [52-54]. Systemic insecticides, when applied to the soil or growing medium, may have minimal direct effects on aboveground natural enemies

(both parasitoids and predators); however, they may indirectly influence natural enemies if mortality of prey populations is high (>90%). This results in a reduction or potential elimination of available prey that serve as a food source for natural enemies [55-57], making it difficult for natural enemies to locate any remaining individuals. This would then lead to a decline in natural enemy populations either through starvation or dispersal thus suppressing establishment [1,55,58-59]. However, this effect is dependent on the foraging efficiency of the specific natural enemy. Furthermore, this may reduce the quantity or density of available prey or reduce their quality such that they are unacceptable as a food source for predators (both larvae and adults) or female parasitoids may not lay eggs. As such, reproduction, foraging behavior, fecundity, and longevity may all be indirectly affected [3].

The distribution of the systemic insecticide active ingredient into flower parts (petals and sepals) may indirectly impact natural enemies that feed on plant pollen or nectar as a nutritional food source including several species of predators such as minute pirate bug, *Orius* spp., which may feed on plants sometime during their life cycle [60-61] and certain parasitoids [62]. For example, adults of the parasitoid, *Anagyrus pseudococci* were indirectly affected after feeding on nectar of buckwheat (*Fagopyrum esculentum*) plants that had been treated with a soil application of a systemic insecticide [53]. Stapel et al. (2000) [11] found that foraging ability and longevity of the parasitoid, *Microplitis croceipes* was reduced after feeding on the extrafloral nectaries of cotton (*Gossypium hirsutum*) plants that had been treated with systemic insecticides. It was also noted that the application method (soil vs. foliar) and possibly timing of application (spatially and temporally) may influence any indirect effects on parasitoids that feed on flower pollen and nectar as a food source. In addition, foraging behavior may be altered depending on the exposure time and concentration of active ingredient present in floral portions of plants. As such, indirect effects associated with systemic insecticides may reduce the overall success of parasitoids in regulating arthropod pest populations under field conditions [11]. Translocation of systemic insecticides into flowers may indirectly affect natural enemies by altering foraging behavior as has been shown with the pink lady beetle, *Colemegeilla maculata*, the green lacewing, *Chrysoperla carnea*, and the parasitoid, *A. pseudococci* [53,63-64]. Nevertheless, the ability of systemic insecticides, when applied to the soil or growing medium as a drench or granule, to move into floral parts may be contingent on water solubility, application rate, and plant type [9,65].

In addition, the metabolites of certain systemic insecticides, which in general, may be more water soluble and toxic to arthropod pests, could be more concentrated in pollen and nectar than the actual active ingredient [66]. This might have a significant indirect effect on natural enemies. In fact, the metabolites associated with certain systemic insecticides have been implicated to indirectly affect natural enemies, primarily by contaminating flower pollen or extrafloral nectaries as the active ingredient is translocated and distributed throughout plant parts [9]. Furthermore, any natural enemies feeding on prey that have fed upon plants and have ingested concentrations of the systemic insecticide active ingredient may be indirectly affected [67-68]. This is associated with prey contamination, which can lead to subtle and long-term indirect effects on parasitoids and/or predators [5,69].

Any indirect effects of systemic insecticides may also be associated with alterations in prey quality or induced changes in host plants [1,70-71], which may reduce the attractiveness of plants to parasitoids [13]; thus impacting the foraging behavior and searching efficiency of natural enemies [13,72]. The indirect effects of systemic insecticides, particularly on predators, may vary depending on feeding habits. For example, hemipteran predators, which may feed on plants as a supplemental food source, would likely be more indirectly affected than coccinellid predators that only feed on prey [2,5,73-76]. Furthermore, any odors associated with treated plants, may result in an avoidance response, which could inhibit the performance and thus effectiveness of natural enemies [11].

Exposure via both contact and oral-ingestion to systemic insecticides at variable concentrations indirectly affected both foraging ability and parasitization (parasitizing ability) of the parasitoid, *Anagrus nilaparvatae* [72]. Another indirect effect was a decrease in the ability of the parasitoid to perceive host-plant volatiles after being exposed to various concentrations of a systemic insecticide [72]. In addition, applications of certain systemic insecticides have been demonstrated to reduce reproduction of vedalia beetle, *Rodolia cardinalis* females and inhibit development from larvae to adult [67]. However, in a study in which nymphs and adults of the plant bug, *Deraeocoris brevis* were exposed to a systemic insecticide, there were no indirect effects on development or reproduction [72], which indicates variability associated with any indirect effects due to natural enemy type and species.

4. Insect growth regulators

Insect growth regulators are compounds that are active directly on the immature stages (larvae or nymphs) of certain insect pests, and there are three distinct categories of insect growth regulators: juvenile hormone mimics, chitin synthesis inhibitors, and ecdysone antagonists [78-79]. Insect growth regulators have been presumed to be compatible, with minimal indirect affects on natural enemies [80-83], and numerous studies have evaluated the indirect effects of insect growth regulators on natural enemies, both parasitoids and predators, under laboratory and field conditions. However, there is distinct variability regarding the indirect effects of insect growth regulators on natural enemies, which is primarily associated with natural enemy type (parasitoid or predator), kind of insect growth regulator, life stage evaluated, and timing of application (spatially and temporally).

4.1. Pyriproxyfen

The insect growth regulator pyriproxyfen, a juvenile hormone mimic [84-85] was demonstrated to have no indirect harmful effects on adult female oviposition and egg viability of the green lacewing, *C. carnea* [86]. Similarly, pyriproxyfen exhibited no indirect effects on development time, female longevity, and fertility of an *Orius* sp. after exposure under laboratory conditions [86]; however, these results may be inconclusive as control mortality was nearly 70%. Pyriproxyfen did not negatively affect parasitism capacity of the

parasitoid, *Aphytis melinus* and there were no indirect effects on the sex ratio of the progeny whereas female *Coccophagus lycimnia* failed to produce any progeny [87]. However, exposure to pyriproxyfen delayed development and decreased the parasitization rate of the parasitoid, *Hyposoter didymator* [88]. In addition, pyriproxyfen has been demonstrated to substantially alter the development time of *Chrysoperla rufilabris* immatures [89] whereas pyriproxyfen did not indirectly impact *Delphastus catalinae* female fecundity after adults had fed upon treated eggs of the sweet potato whitefly, *Bemisia tabaci* [83]. In another study, exposure of *Podisus maculiventris* fifth instars to pyriproxyfen did not result any indirect effects on reproduction [5]. The parasitoid species may influence any indirect effects as both *Encarsia pergandiella* and *Encarsia transvena* were not indirectly affected after exposure to pyriproxyfen whereas *Encarsia formosa* exhibited reduced emergence rates, increased development time, and decreased parasitization when exposed to different concentrations of pyriproxyfen [90]. This demonstrates that the parasitoid species, natural enemy type, and developmental life stage may influence the extent of any indirect effects of insect growth regulators.

4.2. Kinoprene

Another juvenile hormone mimic insect growth regulator, kinoprene [7], has been shown to be indirectly harmful to natural enemies by inhibiting adult emergence of the leafminer parasitoid, *Opius dimidiatus* [91] and the aphid parasitoid, *Aphidius nigripes* [92]. Although directly harmful to the parasitoid, *Leptomastix dactylopii*, kinoprene did not indirectly affect percent parasitoid emergence from citrus mealybug (*Planococcus citri*) mummies [93]. Nevertheless, kinoprene may inhibit adult emergence when applied to prey parasitized with larval or pupal stages of certain parasitoids [92].

4.3. Fenoxycarb

Fenoxycarb is a juvenile hormone analog [79,94-95] that has shown to be indirectly harmful to certain natural enemies. For example, different concentrations of fenoxycarb delayed the development time from pupae to adult of *C. rufilabris* [95], and significantly delayed development of third instar larvae but not first instar larvae. In addition, female reproduction was inhibited when second and third instars were initially exposed to fenoxycarb [96]. Grenier and Plantevin (1990) [97] demonstrated that fenoxycarb (at various concentrations) increased duration of larval development of the tachinid parasitoid, *Pseudoperichaeta nigrolineata*, and Bortolotti et al (2005) [98] observed a similar response (increased longevity) for the third instar larvae of *C. carnea*. In addition, exposure to fenoxycarb indirectly affected female longevity and fecundity of the predator, *Micromus tasmaniae* [99].

4.4. Cyromazine

Cyromazine is an insect growth regulator that disrupts molting by affecting cuticle sclerotization through increasing cuticle stiffness in insects [79], and has been shown to

exhibit indirect effects on the reproduction of *Phytoseiulus persimilis* females [100] whereas no indirect effects, associated with adult emergence rates, were exhibited after the parasitoid, *Chrysocharis parksi* was exposed to cyromazine [101]. Furthermore, exposure to cyromazine did not indirectly affect longevity and reproduction of the leafminer parasitoids, *Hemiptarsenus varicornis* and *Diglyphus isaea* [57].

4.5. Diflubenzuron

Another insect growth regulator, diflubenzuron, which is a chitin synthesis inhibitor [79], has been shown, in general, to have minimal indirect impact on natural enemies—both parasitoids and predators—under laboratory and field conditions [10,102]. However, exposure to diflubenzuron decreased female longevity and reduced the parasitization rate of the endoparasitoid, *Hyposoter didymator* [88] and reproduction of the parasitoid, *Eulophus pennicornis* [103]. It was reported by [99] that *M. tasmaniae*, when exposed to diflubenzuron, resulted in indirect affects on reproduction, sex ratio (female bias), and longevity. In contrast, diflubenzuron exhibited no indirect effects on the reproduction of *Podisus maculiventris* adults [5]. Additionally, diflubenzuron displayed minimal indirect effects on the parasitoid, *Macrocentrus ancylivorus* [104]. Similar to other insect growth regulators, any indirect effects of diflubenzuron are likely associated with the natural enemy type, timing of application (spatially and temporally), and exposure time.

4.6. Buprofezin

Buprofezin, a chitin synthesis inhibitor [79,105], has been shown to sterilize certain natural enemies [106], and reduce the number of progeny produced per female and alter sex ratios [87]. In addition, feeding on buprofezin-treated sweet potato whitefly (*B. tabaci*) eggs resulted in a decrease in female fertility and fecundity, and sterilized the males of the predatory coccinellid, *Delphastus catalinae* [83] indicating no compatibility with this insect growth regulator. However, buprofezin did not negatively affect development (nymph to adult) of the predatory bug, *Orius tristicolor* [107] or inhibit female reproduction of the predatory mite, *P. persimilis* [100]. In addition, buprofezin demonstrated no indirect affects on oviposition and foraging behavior of certain parasitoids including *Eretmocerus* sp., and *Encarsia luteola* [108]. Buprofezin, when applied at three different concentrations (100, 500, and 1,000 mg active ingredient per liter), did not indirectly affect egg viability and subsequent development of *C. rufilabris*. However, the higher rates (500 and 1,000 mg active ingredient per liter) when applied to first instars did prolong overall development to adult whereas second and third instars and pupae were not affected [109]. This indicates that the specific life stage exposed to insect growth regulators may vary in susceptibility with early instars tending to be more susceptible than later instars and adults to chitin synthesis inhibiting insect growth regulators [108,110-111]. In addition, the concentration in which natural enemies are exposed to may influence any indirect effects to these types of insect growth regulators. Furthermore, any indirect effects on natural enemies associated with buprofezin may be due to volatility of the compound as buprofezin is known to be volatile and display vapor activity against certain insect pests [112].

4.7. Azadirachtin

Azadirachtin is an ecdysone antagonist [78,113-114], which may exhibit variability regarding any indirect effects on natural enemies [115]. It was reported by [116], for example, that azadirachtin inhibits oviposition of the green lacewing, *C. carnea* and indirectly affected both fertility and fecundity [117]. In addition, exposure to azadirachtin decreased longevity and predation rates, and inhibited prey finding. Furthermore, the sex ratio was male biased ([88]. Three different formulations (0.3%, 4.5%, and 1.6%) of azadirachtin were reported to indirectly affect the fecundity of *Macrolophus caliginosus* females [118]. Reproduction of the aphid predator, *Aphidoletes aphidimyza* was not indirectly affected after exposure to azadirachtin [119], and azadirachtin did not indirectly affect the fecundity of the parasitoid, *Aphidius colemani* [120]; longevity and foraging ability of the parasitoids, *Cotesia plutellae* and *Diadromus collaris* and sex ratio of progeny [6]; nor reproduction of the predatory mite, *Neoseiulus californicus* [121]. Cloyd et al. (2009) [122] found, under laboratory conditions, that exposure to azadirachtin did not inhibit prey consumption (fungus gnat larvae) of rove beetle, *Atheta coriaria* adults. However, it was reported by [123] that first instar larvae of *Harmonia axyridis*, when exposed to azadirachtin, exhibited increased development time whereas there was no indirect affect on adult female fecundity.

Similar to buprofezin, this demonstrates that any indirect effects of insect growth regulators such as azadirachtin may be more prevalent on the early instars than the later instars of certain natural enemies [123]. Likewise, as also demonstrated by [124], development time of *Coccinella septempunctata* larvae was indirectly affected in a dose-dependent manner with fourth instar larvae more sensitive to azadirachtin than first instar larvae, which suggests that any indirect effects may be stage and age specific. As such, azadirachtin may be more indirectly harmful to nymphs and larval instars than adults under laboratory conditions whereas under semi-field or field conditions any indirect effects associated with these life stages are nullified [115].

5. Selective feeding blockers

Selective feeding blockers, which include flonicamid and pymetrozine, inhibit the feeding activity of piercing-sucking insects (aphids and whiteflies) after initial insertion of their stylets into plant tissues and interfere with neural regulation of fluid intake through the mouthparts resulting in starvation [125-130]. It was reported by [130] that both flonicamid and pymetrozine, did not negatively affect the development time, fertility, and parasitism of a variety of natural enemies including the hoverfly, *Episyrphus balteatus*; the carabid beetle, *Bembidion lampros*; the parasitoid, *Aphidius rhopalosiphi*; the ladybird beetle, *Adalia bipunctata*; and the rove beetle, *Aleochara bilineata* under laboratory conditions. In general, pymetrozine exhibited minimal indirect effects on the reproduction of *N. californicus* under laboratory conditions [121]. Cloyd and Dickinson (2006) [131] found that flonicamid did not indirectly affect parasitism, the sex ratio, and adult emergence of the parasitoid, *L. dactylopii*. Overall, minimal research has been conducted to determine the indirect effects of these types of

pesticides on natural enemies; however, in general, they appear to be compatible, which is likely associated with their mode of action.

6. Microbials

Although entomopathogenic fungi and bacteria (*Bacillus thuringiensis*) are, in general, not indirectly harmful to natural enemies, this may vary depending on concentration, natural enemy type, life stage exposed, timing of application (spatially and temporally), and environmental conditions (temperature and relative humidity) [3,132]. Furthermore, any indirect effects may take longer to be expressed compared to other types of pesticides [133] as well as the fact that indirect effects may not be immediately associated with either the entomopathogenic fungi or bacteria, but may be due to altering the availability of the food source or killing prey before parasitoid immatures have completed development [134]. The bacterium, *B. thuringiensis* has been shown to have indirect effects on certain parasitoids although this is dependent on the formulation [135].

Natural enemies may ingest fungal conidia when grooming (cleaning themselves) or when feeding on contaminated hosts [10,104]; however, the extent of any indirect effects primarily depends on the concentration of spores present [136]. In addition, entomopathogenic fungi may indirectly affect certain natural enemies when feeding on prey that have been sprayed (contaminated prey). For example, larvae of the mealybug destroyer, *Cryptolaemus montrouzieri* were killed (50% mortality) after consuming mealybugs that had been sprayed with *Beauveria bassiana* [133]. Moreover, exposure to *B. bassiana* reduced the fecundity of *N. californicus* females [121] whereas the fungus *Cephalosporium lecanii* exhibited no indirect effects on longevity of the leafminer parasitoid, *Diglyphus begini* [137]. In another study, conducted under laboratory conditions, [122] reported that exposure to *Metarhizium anisopliae* had no indirect effect on prey consumption (fungus gnat larvae) of rove beetle, *A. coriaria* adults. It was shown by [132] that exposure to *Isaria* (= *Paecilomyces*) *fumosoroseus* at a low relative humidity (55%) resulted in no indirect effects on foraging behavior and longevity of the aphid parasitoid, *Aphelinus asychis* whereas both parameters were significantly reduced when exposed to a high ($\geq 95\%$) relative humidity, which could impact the ability of the parasitoid to regulate aphid populations. In addition, ovipositing females may avoid prey that are infected by entomopathogenic fungi [132].

The micro-organism spinosad has been demonstrated to be indirectly harmful to a variety of predatory insects including the green lacewing, *C. carnea* [138]; ladybird beetle, *Hippodamia convergens*; minute pirate bug, *Orius laevigatus*; big-eyed bug, *Geocoris punctipes*; and the damsel bug, *Nabis* sp. [139-140]. For example, it was determined by [141] that exposure to spinosad extended development time from first instar to adult and decreased fertility of *Harmonia axyridis* females. Nevertheless, exposure to spinosad did not inhibit foraging behavior and reproduction of *P. persimilis* females [142]. It has been shown by [143-144] that parasitoids may be indirectly affected by spinosad based on decreased reproduction and reduced longevity. However, exposure to spinosad did not indirectly affect the sex ratio of

the parasitoids, *Aphytis melinus* and *L. dactylopii*, and there was no significant effect on reproduction and longevity of *L. dactylopii* females [87].

7. Miticides

Miticides, similar to other pesticides, may demonstrate variability in regards to any indirect effects on natural enemies depending on the type of miticide and predatory mite species [145]. It was reported by [145] that the miticide fenpyroximate did not negatively affect prey consumption of *Neoseiulus* (= *Amblyseius*) *womersleyi* on twospotted spider mite (*Tetranychus urticae*) eggs compared to the miticide pyridaben. However, both miticides indirectly affected reproduction of *N. womersleyi* and *P. persimilis* females. Egg viability of *P. persimilis* was not affected by either miticide but was for *N. womersleyi*. Furthermore, the population growth, based on reproduction and egg viability, of *N. womersleyi* was indirectly affected more so by pyridaben than fenpyroximate. Overall, fenpyroximate appeared to be more compatible with both predatory mite species. Similarly, [146] found that exposure to different concentrations of fenpyroximate did not indirectly affect female reproduction, immature development time, fecundity, and the sex ratio of progeny associated with *N. womersleyi*. Nevertheless, exposure to variable concentrations of fenpyroximate indirectly affected longevity and fecundity of *P. plumifer* females [147]. In another study, pyridaben inhibited reproduction of *Galendromus occidentalis* [148] whereas [149] reported no indirect effects associated with sex ratio and prey consumption of *P. persimilis*.

The miticides bifenazate, etoxazole, acequinocyl, chlorfenapyr, and fenbutatin oxide were shown to exhibit no indirect effects on the reproduction of *P. persimilis* females under laboratory conditions, and adult females that fed upon prey treated with the miticides were not indirectly affected based on sex ratio of progeny, prey consumption, and female reproduction [150]. This indicates that these miticides are in fact compatible with this predatory mite. In another study, [22] found that exposure to bifenazate did not reduce fecundity, longevity, or prey consumption of adult female *P. persimilis* or *N. californicus*. Moreover, exposure to bifenazate, etoxazole, acequinocyl, and chlorfenapyr under laboratory conditions did not indirectly affect fecundity or reproduction of *N. womersleyi* females. In addition, females that fed upon treated prey were in no way indirectly affected [151]. Overall, the miticide bifenazate appears to be compatible with a variety of predatory mite species.

8. Fungicides

Although, in general, fungicides may be considered less harmful to natural enemies than insecticides and miticides [18] it is still critical to determine any indirect effects and thus compatibility with natural enemies since fungicides are extensively used in agricultural and horticultural production systems and as such it is justifiable to evaluate their indirect effects on natural enemies. It may be that the fungicide type will determine compatibility with natural enemies as 'older' fungicides could be more indirectly harmful to natural enemies than 'newer' fungicides, which may be associated with the mode of action or any

metabolites. Although similar to other pesticides, this may depend on the natural enemy type and species, timing of application (spatially and temporally), and life stage exposed. For example, mancozeb was shown to negatively affect fecundity and reproduction of the predatory mites, *Amblyseius andersoni*, *G. occidentalis* [42,152] and *Euseius victoriensis* [45] under laboratory and field conditions and benomyl indirectly inhibited reproduction of female *Amblyseius fallacis* [153] and *G. occidentalis* [148]. However, mancozeb did not indirectly affect longevity or reproduction of two leafminer parasitoids, *Hemiptarsenus varicornis* and *Diglyphus isaea* [57].

It was determined that the 'newer' fungicides, azoxystrobin and fosetyl-aluminum did not inhibit prey consumption (fungus gnat larvae) of rove beetle, *A. coriaria* adults under laboratory conditions [122]. Bostanian et al. (2009) [154] reported that none of the fungicides evaluated including myclobutanil, propiconazole, fenhexamid, and pyraclostrobin, had any indirect effects on the fecundity of the predatory mite, *G. occidentalis*, and the fungicides captan, mancozeb, and myclobutanil did not indirectly affect longevity and fecundity of *A. fallacis* females [155]. Exposure to the fungicides boscalid and kresoxim-methyl, which are relatively 'newer' fungicides did not indirectly affect fecundity of both *E. victoriensis* and *G. occidentalis* [45]; and [148] found that exposure to the fungicides myclobutanil and trifloxystrobin resulted in no indirect effects on fecundity of *G. occidentalis*.

9. Additional factors associated with indirect effects of pesticides on natural enemies

It is important to exercise caution when attempting to translate laboratory evaluations associated with indirect effects into predictions related to field performance of natural enemies [156-159]. Laboratory assays, for example, may fail to take into account the indirect effects of pesticides, which could underestimate their overall impact [18]. In addition, long-term evaluations conducted under field conditions provide more applicable information regarding pesticide-pest-natural enemy interactions [159] including how pesticides indirectly interfere with the synchrony between natural enemies and their prey [99]. Furthermore, field exposure is assumed to be less severe and more variable than laboratory exposure because of factors such as plant architecture (arrangement of leaves and branches), spray application coverage, pesticide degradation, and potential for recolonization [45]. In addition, the methodology used to evaluate indirect effects of pesticides on natural enemies may influence the results obtained [87].

Another potential issue to be considered is that any indirect effects of pesticides on natural enemies may not necessarily be affiliated with the active ingredient but due to inert ingredients in the commercial formulation [2,160-164]. It is possible that formulations such as emulsifiable concentrates (EC) and soluble powders (SP) may contain additives such as adjuvants, surfactants, solvents and/or carriers that are indirectly harmful to natural enemies [45,165]. Studies associated with how inert ingredients affect natural enemies are necessary in order to better understand the actual indirect impact of pesticides on natural enemies.

10. Summary

This book chapter has demonstrated the feasibility of combining or integrating natural enemies with certain pesticides including systemic insecticides, insect growth regulators, selective feeding blockers, microbials, miticides, and fungicides. The information presented clearly indicates that combining pesticides with natural enemies is not straight-forward [2,18] and that compatibility of natural enemies with pesticides depends on a range of factors including class of pesticide applied, natural enemy type (parasitoid or predator), natural enemy species, pesticide formulation, concentration in which natural enemies are exposed to, exposure time, timing of application (spatially and temporally), and developmental life stage (early vs. later instars) exposed to pesticide. In addition, more than one physiological or behavioral parameter (longevity, reproduction, fecundity, and/or searching efficiency) of a given natural enemy may be indirectly affected by pesticides. As such, there are three primary means by which natural enemies may be integrated with pesticides including pesticide selection (using non-nerve toxin or “selective” pesticides), spatial separation (applying pesticides to localized areas of infestation) of natural enemies and pesticides, and temporal discontinuity (applying pesticides when natural enemies are absent or when tolerable life stages are present) between natural enemies and pesticides [2,132].

As [27] indicated, any indirect effects must be evaluated to determine if pesticides are compatible with natural enemies so as not to compromise long-term success of biological control programs. However, many pesticide manufacturers and suppliers make unsubstantiated claims that pesticides are safe to natural enemies without any references to testing methodology, which fails to take into consideration that results obtained associated with any indirect effects may vary depending on concentration, natural enemy species, pesticide exposure time, developmental life stage(s) evaluated, and the influence of residues and repellency [45]. Therefore, compatibility of natural enemies with pesticides is important if both these management strategies are to be integrated into programs designed to regulate arthropod pest populations and minimize plant damage.

Author details

Raymond A. Cloyd

Kansas State University, Department of Entomology, Manhattan, KS, USA

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Photosynthetic Response of Two Rice Field Cyanobacteria to Pesticides

Binata Nayak, Shantanu Bhattacharyya and Jayanta K. Sahu

Additional information is available at the end of the chapter

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1. Introduction

It was 2.4 billion (Ga) years ago that oxygen accumulated atmosphere began in our planet and cyanobacteria (earlier known as Bluegreen algae) are inhabitant of almost 3.5 billions years ago. This oxygenic atmosphere lead to the evolution of life on the earth. The exponential growth rate and long life span of human beings now a days creating a population bomb which is going to affect the environmental stability. In present day scenario of population explosion, it is essential to increase food production to meet the food demands and to maintain the socioeconomic status of the people in all the developing countries including India. In the year 2050, India will reach to the highest population (1.22 billion in 2012), within this globe total population of world will be approx 9.1 billion in 2050 (Carvalho, 2006). The immediate response to increase food production in limited agricultural land areas is possible by intensive use of agrochemicals. Agrochemicals include two large groups of compounds: chemical fertilizers and pesticides. The use of chemical fertilizers tremendously increased worldwide since 1960s and was largely responsible for the “green revolution”, i.e. the massive increase in production obtained from the same surface of land with the help of mineral fertilizers and intensive irrigation. The revolution was assisted also with the introduction of more productive varieties of crops.

The use of pesticides, including insecticides, fungicides, herbicides, rodenticides, etc., to protect crops from pests, allowed to significantly reduce the losses and to improve the yield of crops. The application of different agrochemicals is region specific. In the tropical regions, where insect pests and plant diseases are more frequent, pesticides are generally applied in massive amounts, both in small farms as well as in cash crop. It has been reported that especially the organochlorine and organophosphorus pesticide residues, are found in soils, atmosphere and in the aquatic environment in relatively high concentrations (Carvalho et al., 1997). Pesticides are poisons, intentionally dispersed in the environment to control pests but

they also act upon other species causing serious side effects on non-target species and destabilise the ecosystem. Cyanobacteria the natural nitrogen engineer of the soil are also adversely affected by indiscriminate use of pesticides.

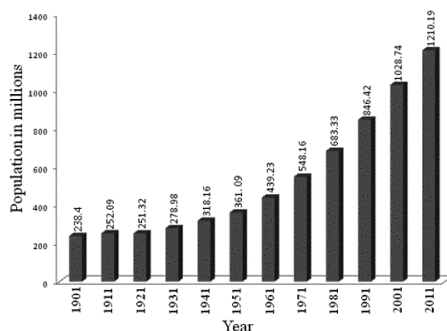


Figure 1. Increasing rate of population

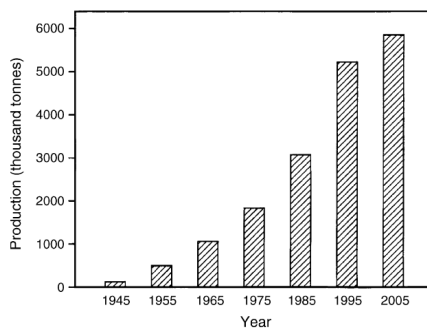


Figure 2. World production of formulated pesticides. Data for year 2005 is estimated (source Agro-chemical service,2000)

Name	Chemical name	Molecular Weight	CAS registry Number	Formula	Structure	Activity	Solubility at 25°C in water (mg/L)
Endosulfan	6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3-oxide	406.96	115-29-7	$C_8H_4O_3S_2Cl_6$		Insecticidal	36%
Monocrotophos	dimethyl (E)-1-methyl-2-(methylcarbamoyl)vinyl phosphate	223.2	2157-98-4	$C_7H_{14}NO_3P$		Insecticidal	35%

Table 1. Physicochemical characteristics of Monocrotophos and Endosulfan

Cyanobacteria are the most diversified ecologically, most successful and evolutionarily most important group of photosynthetic prokaryotes (Peschek et al., 1994) and maintain the homeostasis of nitrogen budget of the rice agroecosystem by photobiological nitrogen fixation in a specialized cell called heterocyst (Fay et al., 1968) at almost zero cost (Mishra & Pabbi 2004). Diversity and evolutionary information of cyanobacteria are available in the internet and for images one can search for “cyanobacteria, images” using Google. Most paddy soils have a natural population of cyanobacteria as they grow and multiply at the simple expense of water, light and air (Fay 1983). Soil nitrogen is the main source of nitrogen for crop growth and rice plant consume 50% of soil nitrogen (Fernandez-valiante et al., 2000). Several reports are available on the adverse effect of agrochemicals on cyanobacteria (Marsac & Houmard, 1993; Das & Adhikary, 1996; Kapoor & Arora, 2000a, 2000b; Shikha & Singh, 2004; Xia, 2005; Kim & Lee, 2006). Although a lot of work has been done on the effect of pesticide in general, no attempt has been made on the effect of pesticide in locally growing cyanobacteria of Western Odisha, India. Farmers of this region use Monocrotophos and Endosulfan on a large scale in rice fields as both the pesticide have broad spectrum activity and they control the attack of insects; the physicochemical properties of both pesticides are given in Table 1.

BGA biofertilizer are added to rice fields to increase the fertility of soil and to minimize dependence on chemical fertilizer. The aim of the present study was to investigate whether *Anabaena sp.* and *Nostoc sp.*, the locally isolated rice field cyanobacteria can be recommended to use as biofertilizer by tolerating the deleterious effect of Monocrotophos and Endosulfan. The chapter presents experimental results to illustrate the effects of Monocrotophos and Endosulfan in time and concentration dependent manner on growth, pigments and photosynthesis of these two alga.

2. Material and methods

Two species of heterocystous cyanobacteria belonging to genera *Anabaena* and *Nostoc* isolated from rice field. Selection of these two genus was based on their relatively better growth rate and wider occurrence. Two commercial grade pesticide i.e. Monocrotophos (organophosphate 36%SL) and Endosulfan (organochlorine 35%EC) were used in the investigation. Fresh stock solutions of these pesticides were prepared in double distilled water and added to the culture medium to obtain the desired concentration. pH of all the medium was adjusted to 7.4 prior to sterilisation. Experiments were conducted in 15×150mm Borosil test tube containing 10 ml of nitrogen free BG11 medium (Rippka et al., 1979) and by inoculating equal amount of homogenized culture suspension (absorbance of the inoculum of each organism from their exponential growth phase at 760nm was 0.4 always). The medium contained various concentration of Monocrotophos (20,50,100 &150ppm) and Endosulfan (1,3,5,10 and 15 ppm),

Growth was measured by light scattering technique by taking the absorbance at 760nm, *Chl a* pigment of the cyanobacterial cells were extracted with 80% chilled acetone. Absorbance of the acetone extract was recorded at 660 nm and the amount of *Chl a* was determined using extinction coefficient of Mackinney, 1941.

Algal suspension was homogenized in a glass hand-homogenizer for 5 minutes and then centrifuged at 3500 rpm for 10 minutes. After centrifugation, the pellet containing algal cells was resuspended in 50 mM tris-HCl buffer, pH 7.8 containing 175 mM NaCl. Room temperature *Chl a* fluorescence emission of algal suspension was measured as per Panda (1999) in a spectrofluorimeter (Hitachi, model, 650-40, Japan). For all scanning, a slit width of 10 nm was used. The whole cell algal suspension equivalent to 10 µg of Chl in a total volume of 3 ml containing 50 mM Tris-HCl buffer and 175 mM NaCl (pH 7.8) was excited at 450 nm and emission was recorded at 685 nm for PS II and 735 nm for PS I emission.

The same algal suspension was also used to measure the excitation emission. During scanning, the emission was monitored at 685 nm and a slit width of 10 nm was maintained. Excitation emission was recorded at 439 nm, 471 nm and 485 nm. The excitation energy transfer from Car to Chl was measured by exciting the algal suspension at 475 nm and 600 nm. The emission was recorded at 685 nm for PS II and 735 nm for PS I. Efficiency of the energy transfer was assessed by calculating the ratio of excitation at 475 nm to 600 nm as described by Gruszeski et al., 1991.

Fluorescence polarization was measured by exciting the algal suspension at 620 nm and polarization was recorded at 685 nm. Polarization (P) was calculated as per the following formula of Swain et al., 1990.

$$P = \frac{I_{vv} - \frac{I_{vh} \times I_{hv}}{I_{hh}}}{I_{vv} + \frac{I_{vh} \times I_{hv}}{I_{hh}}}$$

where, I = intensity of fluorescence
 v = vertical geometry of the polarizer
 h = horizontal geometry of the analyzer

The 2,6-dichlorophenol indophenol (DCPIP) photoreduction was measured spectrophotometrically as described by Swain et al., 1990 with modification. 3 ml of reaction mixture contained whole cell algal suspension (equivalent to 10 µg Chl), 50 mM Tris-HCl buffer (pH 7.8) and 175 mM NaCl. This reaction mixture was illuminated for 30 seconds with saturating white light (7×10^4 ergs $\text{cm}^{-2} \text{sec}^{-1}$) coming from a projector lamp. The incident radiation beam was passed through a water filter to minimize infrared radiation. The photoreduction of the dye was measured at 600 nm. The reduction of the dye is expressed as µ moles DCPIP reduced/mg Chl/hr.

Photosynthetic efficiency of algal suspension in terms of chlorophyll fluorescence was measured at room temperature using a Plant Efficiency Analyzer (Handy PEA, Hansatech Instruments, Norfolk, UK). The Fv/Fm of algal suspension was measured by the Handy PEA after 20 minutes dark-adaption.

3. Results and discussion

The nitrogen-fixing cyanobacteria represent as one of the prominent component of microbial population in wetland soils, especially in rice fields. They significantly contribute to soil fertility as a natural biofertilizer (Kumar & Kumar, 1998). Some cyanobacterial strains that thrive and grow in rice fields release small quantities of the major fertilizing product ammonia and small polypeptides during active growth whereas most of the other fixed products become available mainly through autolysis and decomposition (Hammouda, 1999). Therefore, cyanobacteria are considered as a vital component of the rice agroecosystem. However, excessive use of pesticides has a detrimental effect on the growth of these beneficial microorganisms, soil fertility and ultimately on the crop productivity. The effect of pesticides on the population of nitrogen fixing organisms varies with characteristics of the species and chemical nature of the pesticide.

3.1. Changes in the growth pattern

Growth response of two different species of heterocystous cyanobacteria namely *Anabaena* sp. and *Nostoc* sp. to different concentrations of insecticide Monocrotophos is shown in Fig.3. Experiments showed that *Anabaena* sp. tolerated up to 100 ppm whereas *Nostoc* sp. tolerated upto 150 ppm of the insecticide where as for Endosulfan its limit was 5ppm & 15 ppm respectively. Growth curves indicate that both the algae showed lag phase up to 3rd day of incubation followed by rapid growth up to 12th day in case of control and treated (20 ppm for *Anabaena* sp. and 50 ppm for *Nostoc* sp.) samples. The present study of growth pattern of *Anabaena* sp. and *Nostoc* sp suggest that the tolerance capacity of *Nostoc* is more compared to *Anabaena* for both the pesticides. Both the alga also tolerate higher doses of pesticides Monocrotophos compared to Endosulfan. Endosulfan has more inhibitory effect on growth of both the BGA. These findings support the observation of Das and Adhikary, 1996 that organophosphate insecticide is less toxic than organochlorine. Several authors (Rath & Adhikary, 1994; Goyal et al., 1994; Das & Adhikary, 1996; Anand & Subramanian 1997; Kaur & Ahluwalia, 1997; Kapoor & Arora, 1998; 2000 a, 2000b; Xia, 2005; Chen et al.,2007; Kumar et al.,2008; Bhattacharyya et al., 2011) have reported inhibitory effect of various pesticides on the growth of cyanobacteria. The inhibition of growth in different concentrations of the pesticides is due to alteration in synthesis of nucleic acids, amino acids and proteins (Kumar et al., 2011) as well as due to impairment in photosynthetic activity (Lal & Saxena, 1980) of the BGA.

3.2. Changes in chlorophyll *a* contents

Almost all oxygenic photosynthesizer, with the exception of *Acaryochloris* a cyanobacterium, use chlorophyll *a* (Björn et al., 2009). The amount of Chl content in the photosynthetic unit of cyanobacterial cell indicates its growth and physiological status. Fig.4 depicts the kinetics of *Chl a* accumulation and loss in *Anabaena* sp. and *Nostoc* sp. treated with different concentrations of Monocrotophos (Fig.4B) and Endosulfan (Fig.4A) in the BG11 medium over 15 days of incubation along with the control. The kinetics pattern was closely similar to

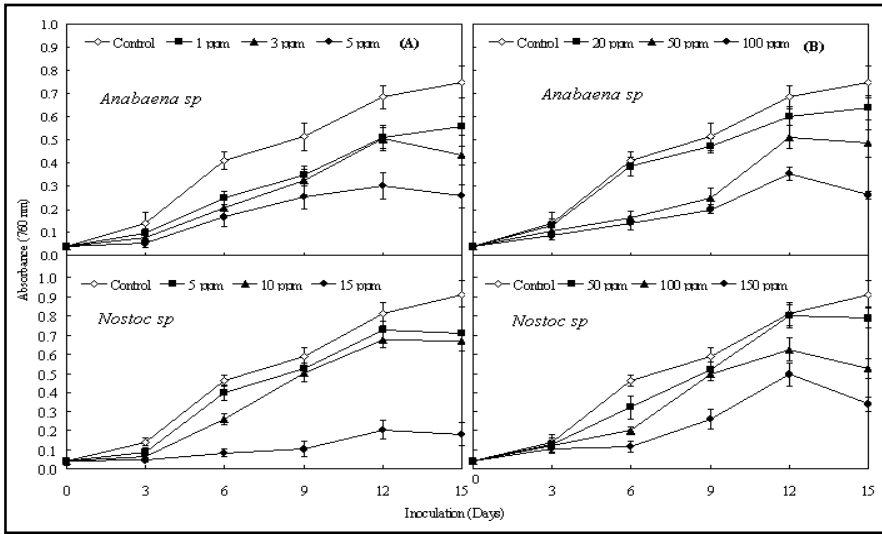


Figure 3. Effect of different concentration of Endosulfan (A) & Monocrotophos (B) on growth of *Anabaena* and *Nostoc* cultured under laboratory condition.

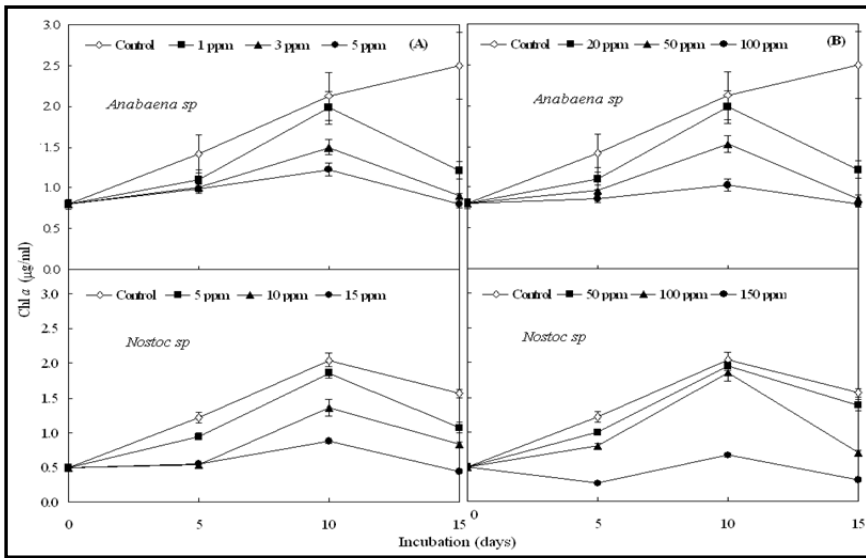


Figure 4. Effect of different concentration of Endosulfan(A) & Monocrotophos (B) on *Chl a* of *Anabaena* and *Nostoc* cultured under laboratory condition.

that of growth kinetics with minor variations for both the treated and control samples. Except in *Anabaena* (control sample), the *Chl a* content reached its maximum level on the 10th day of incubation followed by decline both in control and treated samples. The rate of pigment loss in treated sample was more than that of control. As indicated from the levels, the

pigment synthesis was very less in *Anabaena* with 100 ppm Monocrotophos, 5ppm Endosulfan and *Nostoc* with 150 ppm Monocrotophos, 15 ppm Endosulfan treatment. In these two concentration of pesticides the pigment levels were almost same with the initial level of the pigment in both the samples through out the experimental period of 15 days. The *Chl a* content was maximum on 10th day though growth rate was maximum at 12th day of incubation. The pigment content declined after 10th day of incubation in all treated samples and control of *Nostoc* sp. On the other hand, except in the control samples of *Anabaena* sp. pigment level was highest on 10th day then followed by a sharp decline.

The data indicates that *Chl a* accumulation and loss in the present study is also time and concentration dependent manner (Fig 4). The pesticides are known to interfere with the synthesis of *Chl a* pigment by inhibiting the formation of porphyrin rings (Moreland, 1980; Lal and Saxena, 1980). In the present work, the low level of Chl in pesticide treated samples supports the observations of Das & Adhikary, 1996, Megharaj et al., 2011, Kumar et al., 2008, Battah et al., 2001; Sikha & Singh, 2004 and Xia, 2005.

3.3. Changes in electron transport activity

The DCPIP photoreduction reflects the photochemical potential of PS II. The activities also reflect the coupling between light absorption and photochemical reaction of the thylakoid membrane. In the present study the rate of dye reduction in control and pesticides treated samples of both the alga resemble with the kinetics of *Chl a* accumulation and loss. The rate of dye reduction in treated samples is low compared to the control (Table 2 and 3). This could be due to loss of pigments and protein content of the organism under the pesticide treated conditions due to stress-induced formation of ROS (Behera & Choudhury, 2001; Hideg & Vass, 1996) and possible changes in the thylakoid microenvironment. The degradation of D1 protein under stress condition may be another reason (Long & Humphries, 1994). These observations are similar to the findings of Shikha and Singh, 2004, Bhattacharyya et al., 2011.

µ mol of DCPIP Reduced (<i>Anabaena sp</i>)											
Treatment	Dose (ppm)	0 day	5 day	10 day	15 day	Treatment	Dose (ppm)	0 day	5 day	10 day	15 day
Control		20±1.2	101±3.6	175±3.5	241±5.5	Control		20±1.2	101±1.8	175±5.9	241±8.5
Monocrotophos	20	20±1.2	94±2.7	180±6.2	152±2.9	Endosulfan	1	20±1.2	91±22	165±8	135±6
	50	20±1.2	83±1.1	140±2.8	132±3.6		2	20±1.2	80±0.8	120±3	110±7
	100	20±1.2	65±5.3	95±3.3	85±2.6		3	20±1.2	65±1.6	95±5	75±3

Table 2. Electron transport efficiency of PS II in terms of DCPIP photoreduction of *Anabaena sp* grown under different concentrations of Monocrotophos and Endosulfan in laboratory condition. (±SD)

µ mol of DCPIP Reduced (<i>Nostoc Sp</i>)											
Treatment	Dose (ppm)	0 day	5 day	10 day	15 day	Treatment	Dose (ppm)	0 day	5 day	10 day	15 day
Control		29±2.6	145.8±4.2	190±8	142±6.5	Control		29±2.6	145.8±7	190±11	142±8.2
Monocrotophos	50	29±2.6	94±6	126±6	84±3.2	Endosulfan	5	29±2.6	100.8±3.8	116±7	54.6±5
	100	29±2.6	55.5±4	96±7	49.6±3.6		10	29±2.6	84±3.3	102±6	50±6
	150	29±2.6	25.2±2.6	45.8±5.4	30±6		15	29±2.6	82±3.4	94±8	32.4±4

Table 3. Electron transport efficiency of PS II in terms of DCPIP photoreduction of *Nostoc Sp* grown under different concentrations of Monocrotophos and Endosulfan in laboratory condition. (±SD)

3.4. Measurement of fluorescence characteristics

Analyses of fluorescence emission, excitation emission, fluorescence polarization and excitation energy transfer of thylakoids provide information about the structural organization and the microenvironment of thylakoid membrane. The analyses also give information about the degree of coupling of different pigment complexes. Information about the coupling of light absorption and photochemical reactions could also be obtained by monitoring fluorescence characteristics of whole cells of the cyanobacteria. Therefore, to determine the structural and functional status of the thylakoid, fluorescence excitation, emission and polarization measurements are very much important. Campbell et al., 1998 have opined that fluorescence analysis is an integral part of the studies of photosynthesis in BGA. Shikha & Singh, 2004 have extensively used fluorescence studies to monitor photosynthetic status of *A. doliolum* treated with herbicide glyphosphate.

Cyanobacteria fluorescence characteristics are distinct from those of plants due to their specific structural and functional properties (Campbell et al., 1998). These include significant fluorescence emission from the light harvesting phycobiliproteins, large and rapid changes in fluorescence yield (state transitions) which depend on metabolic and environmental conditions as well as flexible and overlapping respiratory and photosynthetic electron transport chains. In cyanobacteria, the photosynthetic system is tightly linked to other principal metabolic pathways and is itself a major metabolic sink for iron, nitrogen and carbon skeletons. Therefore, Chl fluorescence signals can provide rapid, real-time information on both photosynthesis and overall acclimation status of cyanobacteria.

3.4.1. Fluorescence emission

The fluorescence characteristics of test organisms both in control and insecticides treated samples are shown in Table 4 and 5. There is gradual increase in fluorescence intensity at F685 and F735 in all conditions over 15 days of incubation under laboratory condition except on 5th day of control. The ratios of F685 to F735 increased from 5th day till the end of experiment both in control and treated samples. The ratios were also more in treated samples than in the control. The ratios gradually increased as the concentration of the insecticide and treatment period increased in both the organisms.

		Fluorescence Intensity (Arbitrary unit)											
Treatment	Dose (ppm)	F685				F735				F685/F735			
		0 day	5 day	10 day	15 day	0 day	5 day	10 day	15 day	0 day	5 day	10 day	15 day
Control	0	91.0	88.0	137.0	195.0	26.5	25.0	30.0	36.0	3.4	3.52	4.50	5.40
	20	91.0	100.0	167.0	202.0	26.5	28.0	34.0	40.0	3.4	3.57	4.90	5.05
Monocrotophos	50	91.0	122.0	178.0	240.0	26.5	32.0	36.0	42.0	3.4	3.81	4.94	5.71
	100	91.0	139.0	195.0	270.0	26.5	36.0	39.0	46.0	3.4	3.86	5.00	5.84
Endosulfan	1	91.0	110.0	183.0	299.0	26.5	27.0	37.0	54.0	3.4	4.07	4.90	5.53
	3	91.0	122.0	193.0	314.0	26.5	29.0	38.0	55.0	3.4	4.20	5.07	5.70
	5	91.0	152.0	218.0	338.0	26.5	35.0	41.0	56.0	3.4	4.34	5.30	6.03

Table 4. Chlorophyll a fluorescence emission of *Anabaena* grown in control and different concentrations of Monocrotophos and Endodahan in laboratory conditions.

Fluorescence Intensity (Arbitrary unit)													
Treatment	Dose (ppm)	F685				F735				F685/F735			
		0 day	5 day	10 day	15 day	0 day	5 day	10 day	15 day	0 day	5 day	10 day	15 day
Control		97.5	92.0	146.0	204.0	21.6	20.0	24.6	34.0	4.5	4.6	5.9	6.0
Monocrotophos	50	97.5	108.0	177.0	220.0	21.6	22.1	29.0	36.0	4.5	4.9	6.1	6.1
	100	97.5	134.0	188.0	257.0	21.6	25.2	30.3	39.1	4.5	5.3	6.2	6.6
	150	97.5	147.0	203.0	284.0	21.6	26.3	31.7	42.3	4.5	5.6	6.4	6.7
Endosulfan	5	97.5	102.0	151.0	280.0	21.6	21.8	25.0	44.0	4.5	4.7	6.0	6.4
	10	97.5	114.0	164.0	294.0	21.6	21.6	24.8	42.5	4.5	5.3	6.6	6.9
	15	97.5	148.0	198.0	328.0	21.6	26.6	28.4	45.8	4.5	5.6	6.6	7.1

Table 5. Chlorophyll *a* fluorescence emission of *Nostoc* grown in control and different concentrations of Monocrotophos and Endodahan in laboratory conditions.

Chlorophyll *a* fluorescence emission spectra of whole algal cells measured at room temperature exhibit usually two emission maxima, the first at 685 (F685) nm and the second at 735 (F735) nm. F685 is considered as the emission from PS II and F735 from PS I (Papageorgiou, 1975). A gradual increase in fluorescence intensity (Table 4 and 5) at 685 nm (F685) and 735 nm (F735) is observed over 15 days of incubation of both the alga in control as well as insecticides treated samples. Increase in fluorescence intensity particularly during developmental stage has been ascribed due to improved organization of light harvesting (antenna) complexes of the thylakoid which results in trapping of more solar energy. However, if proportional increase in the photochemical activity will not take place, then the absorbed energy is emitted as fluorescence (Krause & Weis, 1991; Krieger et al., 1992). On the other hand, uncoupling of the photosynthetic pigments and RC during natural ageing or under stress conditions may also lead to increase in the fluorescence intensity. Continuous increase in the fluorescence intensity in the control sample could be due to higher trapping of solar energy as the algal cell improves their thylakoid organization during the culture. However, without proportional increase in photochemical activities, the excitation energy is emitted as fluorescence. On the other hand increase in the fluorescence intensity in the insecticides treated samples is much higher than the control. This suggests that the pesticides have induced uncoupling of light harvesting system and electron transport resulting emission of excitation energy as fluorescence. Higher susceptibility of PS II compared to PS I to different stress such as water stress (Deo and Biswal, 1998), light stress (Behera et al., 2002), oxidation stress (Behera and Choudhury, 2001) etc. have been reported in different plant systems. The gradual increase in the ratio of F685 and F735, when the concentration of pesticides increase suggests that PS II is more affected by the treatments.

3.4.2. Fluorescence excitation

Table-6 depicts the effect of different concentrations of pesticides on the ratio of peak heights of fluorescence excitation emission of *Anabaena* sp. The ratios of 471 nm to 439 nm and 485 nm to 439 nm increased throughout the 15 days of incubation in all concentrations of Monocrotophos and Endosulfan used along with the control sample. However, compared

to the control, the ratio declined with insecticide treatment as well as with the increase in concentration of both pesticides.

Treatment	Dose (ppm)	Ratio of Peak Heights(a.u)							
		E471/E439				E485/E439			
		0 day	5 day	10 day	15 day	0 day	5 day	10 day	15 day
Control		0.489	0.988	1.213	1.500	0.625	1.195	1.325	1.632
Monocrotophos	20	0.489	0.940	1.208	1.478	0.625	1.010	1.217	1.417
	50	0.489	0.891	1.112	1.155	0.625	0.921	1.200	1.253
	100	0.489	0.695	0.789	0.918	0.625	0.900	1.182	1.208
Endosulfan	1	0.489	0.825	1.094	1.132	0.625	1.093	1.131	1.348
	3	0.489	0.821	1.087	1.021	0.625	0.956	1.121	1.187
	5	0.489	0.624	0.721	0.802	0.625	0.795	0.860	0.934

Table 6. Effect of different concentrations of Monocrotophos and Endosulfan on ratio of peak heights of fluorescence excitation of *Anabaena* grown in laboratory conditions.

The ratio of peak heights of fluorescence excitation emission of *Nostoc* sp. is represented in Table-7 both in control and pesticides treated (50, 100 and 150 ppm of Monocrotophos and 5, 10 and 15 ppm of Endosulfan) samples over 15 days of incubation under laboratory condition. Similar trend of increase in the ratio of 471 nm to 439 nm was also noted in Monocrotophos and Endosulfan treated samples as well as in control over 15 days of incubation. However, the increase was less in insecticide treated samples and more so when the concentration of the insecticide was more. On the other hand, except in control, no definite increasing or decreasing trend in the ratio of 485 nm to 439 nm was noted in the treated samples.

Treatment	Dose (ppm)	Ratio of Peak Heights(a.u)							
		E471/E439				E485/E439			
		0 day	5 day	10 day	15 day	0 day	5 day	10 day	15 day
Control	0	0.627	1.055	1.368	1.600	1.304	1.505	1.602	1.678
Monocrotophos	50	0.627	1.040	1.150	1.320	1.304	1.202	1.310	1.408
	100	0.627	0.932	0.983	1.152	1.304	1.084	1.093	1.101
	150	0.627	0.729	0.765	0.997	1.304	0.935	1.012	1.087
Endosulfan	5	0.627	0.757	0.925	1.162	1.304	1.454	0.835	1.303
	10	0.627	0.747	0.854	1.051	1.304	1.359	0.519	1.131
	15	0.627	0.629	0.765	0.908	1.304	0.909	0.429	0.933

Table 7. Effect of different concentrations of Monocrotophos and Endosulfan on ratio of peak heights of fluorescence excitation of *Nostoc* grown in laboratory conditions.

The study of fluorescence excitation characteristics of chloroplast is used to explain the spatial arrangement and coupling of different pigment molecules in the thylakoid membrane (Behera & Choudhury, 1997). The changes in the relative peak values of fluorescence excitation at 471 (E471) nm and 485 (E485) nm with reference to peak at 439 (E439) nm reflects the alterations in pigment protein complexes in the thylakoid domain during development of the organism. Table 6 and 7 indicate the changes in the ratio of peak heights in control and with different concentration of Monocrotophos and Endosulfan. The decrease in the ratio of 471 nm to 439

nm is attributed to gradual decrease in coupling between Chl and Car with the increase of the duration of incubation period with the insecticides.

3.4.3. Efficiency of energy transfer

The simple and direct proof of excitation energy transfer from Car and phycobilisomes (PBS) to Chl comes from the contribution of the light absorbed by the Car and PBS in Chl *a* fluorescence. At shorter wavelength, only Chl, Car and PBS and at longer wavelength only Chl is responsible for the absorption. There is a significant decline in the capacity of energy transfer in PS II for all concentrations of Monocrotophos and Endosulfan in both the alga compared to the control (Table 8 and 9). The temporal kinetics of energy transfer follows similar pattern like the kinetics of DCPIP photoreduction (Table 2 and 3) and photosynthetic efficiency of PS II during the 15 days of incubation. The decrease in the *Chl a* contents in the insecticide treated samples (Fig. 4) may be correlated to certain conformational changes in the pigment protein complex in the photosystem in turns affecting the excitation energy transfer (Gruszieski et al., 1991). The energy transfer from Car to Chl is increasingly hampered (Table 8 and 9) as the concentration of the pesticides increased.

Though the values are different, the kinetics of energy transfer in PS I is similar to that of PS II for both *Anabaena* sp. and *Nostoc* sp. in control and treated samples. However, compared to PS II, PS I is less susceptible to the insecticide treatment in both the alga. Smaller changes in excitation energy transfer in PS I suggest that PS I is less effected even under stress condition. Relatively less susceptibility of PS I compared to PS II to various stress conditions has been shown earlier by various authors (Choudhury & Choe, 1996; Deo & Biswal, 1998; Campbell et al., 1998; Behera et al., 2002)

Treatment	Dose (ppm)	Efficiency of excitation energy transfer (a.u)							
		PS II				PS I			
		0 day	5 day	10 day	15 day	0 day	5 day	10 day	15 day
Control	0	1.48 (100)	1.95 (131)	2.15 (145)	2.10 (142)	1.22 (100)	1.64 (134)	1.83 (150)	1.80 (147)
Monocrotophos	20	1.48 (100)	1.87 (126)	2.00 (135)	1.92 (130)	1.22 (100)	1.59 (130)	1.74 (143)	1.69 (138)
	50	1.48 (100)	1.75 (118)	1.93 (130)	1.80 (122)	1.22 (100)	1.52 (125)	1.67 (137)	1.58 (130)
	100	1.48 (100)	1.67 (112)	1.70 (114)	1.62 (109)	1.22 (100)	1.41 (116)	1.52 (125)	1.46 (120)
Endosulfan	1	1.48 (100)	1.82 (123)	1.98 (134)	1.88 (127)	1.22 (100)	1.58 (129)	1.70 (149)	1.67 (137)
	3	1.48 (100)	1.70 (115)	1.87 (126)	1.82 (123)	1.22 (100)	1.54 (126)	1.64 (134)	1.56 (128)
	5	1.48 (100)	1.55 (105)	1.64 (111)	1.60 (108)	1.22 (100)	1.46 (120)	1.54 (126)	1.50 (122)

Table 8. Efficiency of excitation energy transfer from carotenoids to chlorophyll of PS II and PS I of *Anabaena* grown under control and different concentrations of Monocrotophos and Endosulfan in laboratory conditions.

Treatment	Dose (ppm)	Efficiency of excitation energy transfer (a.u)							
		PS II				PS I			
		0 day	5 day	10 day	15 day	0 day	5 day	10 day	15 day
Control	0	0.558 (100)	0.714 (128)	0.822 (147)	0.812 (145)	0.469 (100)	0.615 (131)	0.724 (154)	0.703 (150)
Monocrotophos	20	0.558 (100)	0.708 (127)	0.794 (142)	0.760 (136)	0.469 (100)	0.600 (128)	0.613 (146)	0.656 (140)
	50	0.558 (100)	0.675 (121)	0.730 (131)	0.712 (127)	0.469 (100)	0.572 (122)	0.637 (136)	0.609 (130)
	100	0.558 (100)	0.655 (113)	0.647 (116)	0.608 (109)	0.469 (100)	0.539 (115)	0.562 (120)	0.529 (113)
Endosulfan	5	0.558 (100)	0.704 (126)	0.783 (140)	0.753 (135)	0.469 (100)	0.605 (129)	0.680 (145)	0.656 (140)
	10	0.558 (100)	0.671 (120)	0.725 (130)	0.704 (126)	0.469 (100)	0.586 (125)	0.635 (136)	0.609 (130)
	15	0.558 (100)	0.619 (111)	0.636 (114)	0.638 (110)	0.469 (100)	0.558 (119)	0.572 (122)	0.548 (117)

Table 9. Efficiency of excitation energy transfer from carotenoids to chlorophyll of PS II and PS I of *Nostoc* grown under control and different concentrations of Monocrotophos and Endosulfan in laboratory conditions.

3.4.4. Fluorescence polarization

Changes in fluorescence polarization of the two algal species under control and insecticides treated conditions give further information on the status of pigment-protein complexes with reference to their microenvironment in thylakoid membrane. The increase in polarization during the initial stage of incubation in all treated samples compared to the control (Fig.5) could be due to the poor coupling between the pigment protein complex and RC (Behera & Choudhury, 1997). On the other hand, increase in polarization in the later phase of growth (10-15 days of incubation) could be due to disorganization of pigment protein complexes and RC, leading to a decrease in quantum migration. Alternatively, peroxidation of lipid during later stage may induce gel phase of the thylakoid membranes restricting the mobility of Chl dipole (Panda & Biswal, 1989 and 1990). This may cause an increase in the polarization value at 100 and 150 ppm of Monocrotophos and 5 and 15 ppm of Endosulfan treatment to the *Anabaena* sp and *Nostoc* sp. respectively. Significant high levels of polarization suggests a greater disorganization of thylakoid membrane due to high lipid peroxidation (Kumar et al., 2008).

3.4.5. Photosynthetic efficiency

Photosynthetic efficiency of PS II can be measured by monitoring the ratio Fv/Fm. It is known that photoinhibition occurs when the rate of excitation energy captured exceeds the rate of consumption in photosynthetic reactions (Osmond, 1981; Powles, 1984). Photoinhibition in terms of Fv/Fm has been found both in higher plants (Panda et al., 2006; Rodrigues et al., 2007) as well as in algae (Ying and Hader, 2002; Xia, 2005; Chen et al., 2007).

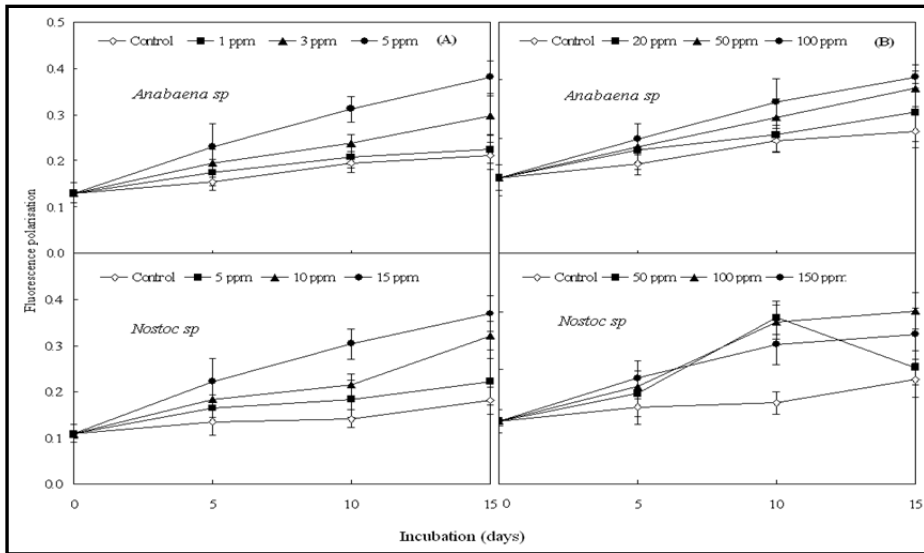


Figure 5. Effect of different concentration of Endosulfan(A) & Monocrotophos (B)on fluorescence polarization of *Nostoc* and *Anabaena* cultured under laboratory condition.

The primary site of photoinhibition is the reaction centre (D1 protein) of PS II (Demming and Bjorkman, 1987; Jordan, 1996). Photoinhibition is manifested as a decrease in oxygen evolution (Krause, 1988) and photochemical efficiency (Falk and Samuelsson, 1992). The data on the measurement of F_v/F_m during the laboratory incubation of the different samples (Table 10 and 11) show similar kinetics like that of the photosynthetic pigment and protein accumulation and loss and DCPIP photoreduction of both the BGA in control and treated (pesticide) samples. As the concentration of pesticides increased, photosynthetic efficiency decreased. This shows that both the species of cyanobacteria are sensitive to higher concentration of pesticides. Pesticides, particularly at higher concentration may (directly or indirectly) cause damage to D1 protein of PS II leading to photoinhibition. The decrease in F_v/F_m ratio in pesticide treated sample could also be due to decrease in Phycocyanin, Phycoerythrin and Allophycocyanin content, which results in a decrease of light energy absorption by phycobilisomes and reduction of photochemical efficiency (F_v/F_m) of PS II. Similar observations have been reported by Xia, 2005 in *N. sphaeroids*. The present finding is in confirmatory to the observation of Xia, 2005.

PS II efficiency											
<i>Nostoc</i>											
Treatment	Dose (ppm)	0 day	5 day	10 day	15 day	Treatment	Dose (ppm)	0 day	5 day	10 day	15 day
Control		0.370 (100)	0.524 (142)	0.562 (152)	0.536 (145)	Control		0.370 (100)	0.524 (142)	0.562 (152)	0.536 (145)
Monocrotophos	50	0.370 (100)	0.500 (135)	0.515 (139)	0.473 (128)	Endosulfan	5	0.370 (100)	0.458 (124)	0.520 (141)	0.467 (126)
	100	0.370 (100)	0.431 (116)	0.500 (135)	0.429 (116)		10	0.370 (100)	0.387 (105)	0.455 (123)	0.375 (101)
	150	0.370 (100)	0.375 (101)	0.404 (109)	0.387 (105)		15	0.370 (100)	0.313 (85)	0.333 (90)	0.316 (85)

Table 10. Photosynthetic efficiency (in term of F_v/F_m) of PS II of *Nostoc* grown under different concentrations of Monocrotophos and Endosulfan in laboratory condition.

<i>Anabaena</i>											
Treatment	Dose (ppm)	0 day	5 day	10 day	15 day	Treatment	Dose (ppm)	0 day	5 day	10 day	15 day
Control		0.350 (100)	0.520 (149)	0.556 (159)	0.515 (147)	Control		0.350 (100)	0.520 (149)	0.556 (159)	0.515 (147)
Monocrotophos	20	0.350 (100)	0.482 (138)	0.500 (143)	0.468 (142)	Endosulfan	1	0.350 (100)	0.404 (115)	0.511 (146)	0.455 (130)
	50	0.350 (100)	0.442 (126)	0.482 (138)	0.429 (123)		3	0.350 (100)	0.316 (90.3)	0.419 (119.7)	0.327 (93.42)
	100	0.350 (100)	0.375 (107)	0.419 (120)	0.351 (100.3)		5	0.350 (100)	0.313 (89.42)	0.375 (107)	0.308 (88)

Table 11. Photosynthetic efficiency (in term of F_v/F_m) of PS II of *Anabaena* grown under different concentrations of Monocrotophos and Endosulfan in laboratory condition.

4. Conclusion

The insecticide Monocrotophos and Endosulfan affected photosynthetic function which may have inhibited the growth of both the cyanobacteria by affecting the production of photosynthetic pigments in the antenna complex, electron transfer, and photosynthetic efficiency of PS-II. Both the cyanobacteria responded differently to both the pesticides with time and concentration dependent manner. Endosulfan has more inhibitory effect than Monocrotophos. Tolerance capacity of *Nostoc sp.* is more than *Anabaena sp.*. Our data indicate that use of Endosulfan may pose a risk to diazotrophic cyanobacterium, and consequently to the nitrogen economy of the soil. It is currently understood from the extensive studies conducted so far on impacts of many pollutants on cyanobacteria and microalgae that evaluation with

a wide taxonomic range in different ecosystem is necessary to arrive at a generalization on the nontarget effects of pollutant (Ramakrishnan et al., 2010) However, the effect of pesticides on the population of nitrogen-fixing cyanobacteria in rice fields also depends on pesticide concentrations, moreover, toxicity is affected not only by the types of pesticide, but also by the taxonomic groups and species. Since Endosulfan has more deleterious effect on natural engineer of the rice field (BGA) it's use be limited to maintain the stability of paddy ecosystem.

Author details

Binata Nayak and Shantanu Bhattacharyya

School of Life Sciences, Sambalpur University Jyoti Vihar, Burla, Odisha, India

Jayanta K. Sahu

Trust Fund College, Bargarh, Odisha India

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Botanical Pesticides and Pest Management

Plant Based Pesticides: Green Environment with Special Reference to Silk Worms

Dipsikha Bora, Bulbuli Khanikor and Hiren Gogoi

Additional information is available at the end of the chapter

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1. Introduction

Pesticides once having entry to an environment either get into the complex web of life through food chain or different components of the environment through physical passages like drifting by air and aquatic runways. Such facts were meticulously described by Rachel Carson [1] in her book 'Silent spring' where she advocated for choosing either the chemical control or biological control to avoid creation of endless problems to mankind owing to pesticide use. Looking back at the history of tremendous potentiality of synthetic chemicals to manage insect pests followed by subsequent cases of failure of chemical control due to development of insect resistance and pest resurgence, here we intend to cite examples of selective toxicity of insecticides and reiterate its importance in management of insect pests. Pesticidal pollution is a global problem. Use of synthetic insecticides to control pest around the world has resulted in disturbances of the environment, secondary pest resurgence, pest resistance to pesticides, lethal effects to non-target organisms as well as direct toxicity to users. It has been reported that about 2.5 million tons of pesticides are used on crops each year and the worldwide damage caused by pesticides reaches \$100 billion annually. The reason behind this amount of cost is the high toxicity and non biodegradable properties of pesticides and the residues in soil, water resources and crops that affect public health [2]. Hence search for the environment friendly, highly selective, newer biodegradable pesticides for pest management programme has been advocated to be essential for last several decades.

South East Asian countries are the hubs for production of raw silks produced by sericigenous insects namely *Bombyx mori* (mulberry silk worm), *Antheraea assama* (Muga silk worm), *Antheraea mylitta* (Tasar silk worm) and *Philosamia ricini* (Eri silk worm). India holds second position in world's raw silk production and contributes to 13.45 % of the total production [3]. Yet the production is not sufficient even to meet the domestic demand of the

raw silk. One of the major constraint in silk production is the susceptibility of the silkworms to attack of different pests, parasitoids, predators and pathogens. Even mulberry silkworms which can be cultured in indoor condition are not free from such constraints. In addition, their host plants are also susceptible to the attack by herbivorous pests (insects, mites) and various pathogens (Nematodes, bacteria, fungus and virus) [4,5]. Growth and development of silkworm to a great extent depend on quality and quantity of food consumed and utilized [6]. In such situations, usually farmers take shelter of spurious chemicals for controlling the pest population unless they are well versed with efficacy of pesticides and their hazardous effects. Application of insecticide in sericulture field is not at all advisable as the leaves of host plants are directly consumed by the silk worms and silk worms become affected either through consumption of contaminated food or contact toxicity of the insecticides. *Bombyx mori* are highly susceptible to insecticides and in China its production is reported to be decreasing by almost 30% per annum because of insecticidal poisoning [7]. Fenvalerate-20EC (Sumicidine-20EC), one of the commonly used pyrethroid, reduced the rate of feeding, assimilation and efficiencies of conversion of ingested and digested food into body substance in late instar larvae of *Bombyx mori* [8]. Hexachlorocyclohexane, an organochloride insecticide was reported to cause decrease in fibroin content, pupal and shell weight, adult emergence percentage, fecundity as well as deterioration in quality and quantity of silk thread in *B. mori* [9]. About 50% of normal water intake of eri silkworm (*Philosamia ricini*) was reported to decrease after feeding with leaves of *Ricinus communis* treated with permethrin, a pyrethroid insecticide which might have resulted due to repellency or disruption of feeding physiology [10]. The chemicals used if are phytotoxic reduce the nutritive quality of the leaves. It is globally accepted that complete elimination of pesticide drift is impossible. More often sericulture fields are contaminated by insecticides sprayed into other crop systems in the neighbouring areas. For instance many of the nonmulberry- sericulture fields in Brahmaputra Valley of Assam are situated mostly in close vicinity of stretches of crop fields like paddy and tea. Thus those sericulture fields cannot be expected to be spared of indirect contamination caused by widespread use of pesticides in paddy fields and tea gardens that warns precautionary measures (Plates 1).



Plate 1. Components of Seri-ecosystem in Assam

2. Pesticides in environment

Introduction of pesticides into a crop system subjects it into a variety of physico-chemical and biological processes which determine the persistence, fate and the ultimate degradation product. Many workers have shown that only a portion of pesticides sprayed onto crops reach their targets, the rest enter the atmosphere by spray drift, volatilization from soil or water, surface runoff, biotransformation by microorganisms, plants, animals, biomagnification through food chain and photodecomposition [11]. One of the major environmental aspect is the effect of sunlight that may lead to various photoprocesses and to photoproducts which are mostly different from parent pesticides in the environmental properties and toxicological significance. The quantum of light energy emitted from the radiation is absorbed by pesticides in environment and this raises the energy state of the molecule, causes excitation of electrons leading to formation or disruption of chemical bonds. Photolysis of pesticides have been studied in water, soil and plant surface. Various sensitizers present in environment such as riboflavin, humic substance etc. absorb light energy and serve as donor of energy to pesticide acceptor and bring about photodecomposition of pesticides. Most organophosphates whose photochemistry have been studied are phosphorothioate and phosphorodithioate compounds. Although not highly susceptible to photodegradation by UV light, malathion degrades to different photoproducts such as malaaxon, malathion diacid, o,o-dimethyl phosphorodithioic acid, o,o-dimethyl phosphorothioic acid and phosphoric acid [12, 13]. Some of the compounds viz. malaaxon are more toxic than the parent compound. Insecticides aimed against pest population may enter non-target arena through spillage at sublethal level but even these sublethal dosages may exert considerable damage on behaviour and activity of non-target population [14]. Pesticidal effect of insecticides at sublethal dosages may have long term effect and they may be expressed at a later part of the insect's life [15,6]. Continuous exposure to sublethal dosages on the environment may on the otherhand help a pest to develop resistance mechanism against the toxic compound. Troitskaya and Chichigina [16] showed that combined use of bacterial and chemical insecticides in silk-producing areas possess a real danger to *Bombyx mori*.

2.1. A study was carried out to evaluate the susceptibility of the silkworm, *Antheraea assama* to sublethal dosages of organophosphorous chemicals, malathion and phosphamidon in terms of certain developmental and biochemical parameters. The insecticides were sprayed to leaves at sublethal concentrations based on LD50 values determined earlier and allowed the fifth instar larvae to feed on them in 12 D: 12L photoperiodic condition. The parameters considered were mean larval growth rate, food consumption and utilization computed by the method of Waldbauer (17). In case of malathion treatment although at the lowest dose applied the mean daily consumption, utilization and mean larval weight did not vary significantly they decreased at higher dosages. The correlation coefficient between the mean daily consumption and utilization of food and concentrations of insecticide was -0.826 and -0.812 respectively. This represented a feeding deterrent effect of malathion and its ability to

interfere with digestive physiology. Accordingly the growth rate and in later part of developmental period, the percentage of pupation and adult emergence decreased even at lower concentration. In case of treatment with phosphamidon, the daily food consumption and utilization decreased but they did not vary at the highest dose. The correlation coefficient between the mean daily consumption and concentrations of phosphamidon were +0.539 which might indicate probable absence of feeding deterrence ability in phosphamidon. The growth rate decreased, but the mean larval weight increased with increase of concentration and the correlation coefficient between larval weight and concentrations of phosphamidon was +0.930. Although variations were observed in effect of different concentration during larval period, in later stage of development, similarly with the effects of malathion, the percentage of pupation and adult emergence decreased significantly (Figure.1). The mechanism in which the two insecticides interfered with the insect's body physiology was probably different. In order to study the effect of LD40 dosages of malathion and phosphamidon on tissue weight and different bioconstituents, the early instar larvae were allowed to feed on treated leaves and grow till adult. Analysis in late fifth instar larvae before silk spinning revealed that the tissue weight decreased along with total lipid, protein, Glycogen (female) and cholesterol (male) (Figure 2).

3. Insecticide mechanism of action

The major classes of synthetic pesticides are organochlorines, organophosphates, carbamates and pyrethroides. Preliminary survey revealed that organophosphates and pyrethroides are two of the most common pesticide classes used by common farmers against pests of paddy and other crops and vegetables in Assam. They also belong to the most commonly used pesticide groups in tea gardens. Organophosphates like malathion, phosphamidon and dimethoate even at sublethal dose have been reported to be highly toxic against the larvae of *A. assama* [6]. Organophosphates (OPs) are known to cause inhibition of esterases in silk worms [18]. Carboxylesterases constitute a class of the metabolic enzymes involved in insecticide resistance to OPs, carbamates, and pyrethroids through gene amplification, upregulation and coding sequence mutations [19]. The major function of acetylcholine esterase (AChE) is hydrolysis of the neurotransmitter acetylcholine bounded at cholinergic synapses in the central nervous system of insects [20] and the latter confers target site for susceptibility to organophosphorous insecticides which in *Bombyx mori* is reported to act through inhibition of *BmAChE1* responsible for expression of acetylcholinesterase [19,21,22]. Like DDT, Pyrethroides are axonic poisons. But in contrast to the residual persistence and biomagnification effect of DDT for which its use in agricultural crop has been banned over the globe, pyrethroides are the fastest developing group of modern insecticides primarily because of their effectiveness and safety application [23]. The synthetic pyrethroid deltamethrin (Decis) although is effective against a notorious parasitoid of silk worm, *Exorista sorbillans* with LC50 value at 0.106%, the insecticide has been found to be more highly toxic (LC50= 9×10^{-5}) to the larvae of muga silk worm, *Antheraea assama* [24]. Aerial application of organophosphates to agricultural field and their

drifting to nearby mulberry plantation was reported to influence food consumption and utilization of silk worms [25]. Lipophilic insecticides are mainly carried in blood [26] in protein and protein associated forms [27]. From blood, insecticides are redistributed to gastrointestinal system, adipose tissue and brain [28-30]. Toxic effect of insecticides and oil pollutants are reported to cause changes in levels of different bioconstituents and metabolic processes in insects[30-34] finally leading to growth inhibition (Figure.1& 2) [6,15,35].

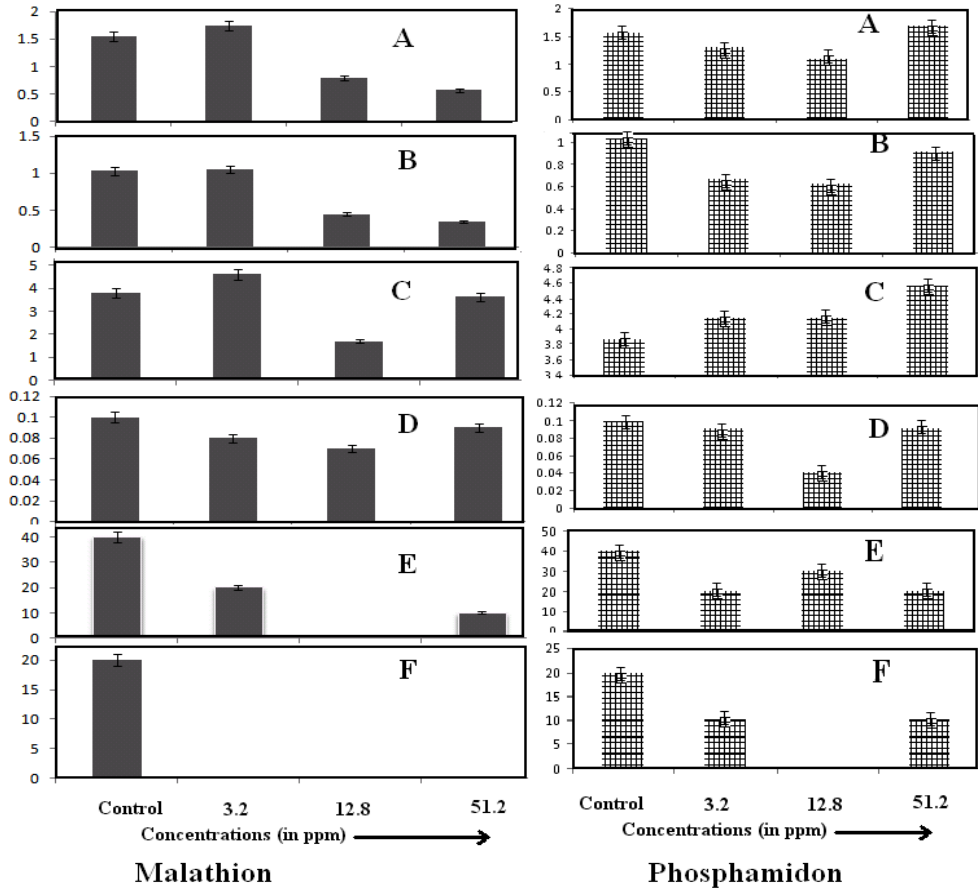


Figure 1. Effect of malathion and phosphamidon on developmental parameters of *A. assama*. A. Daily consumption, B. Daily utilization, C. Mean larval weight, D. Growth rate, E. Percentage of pupation, F. Percentage of emergence

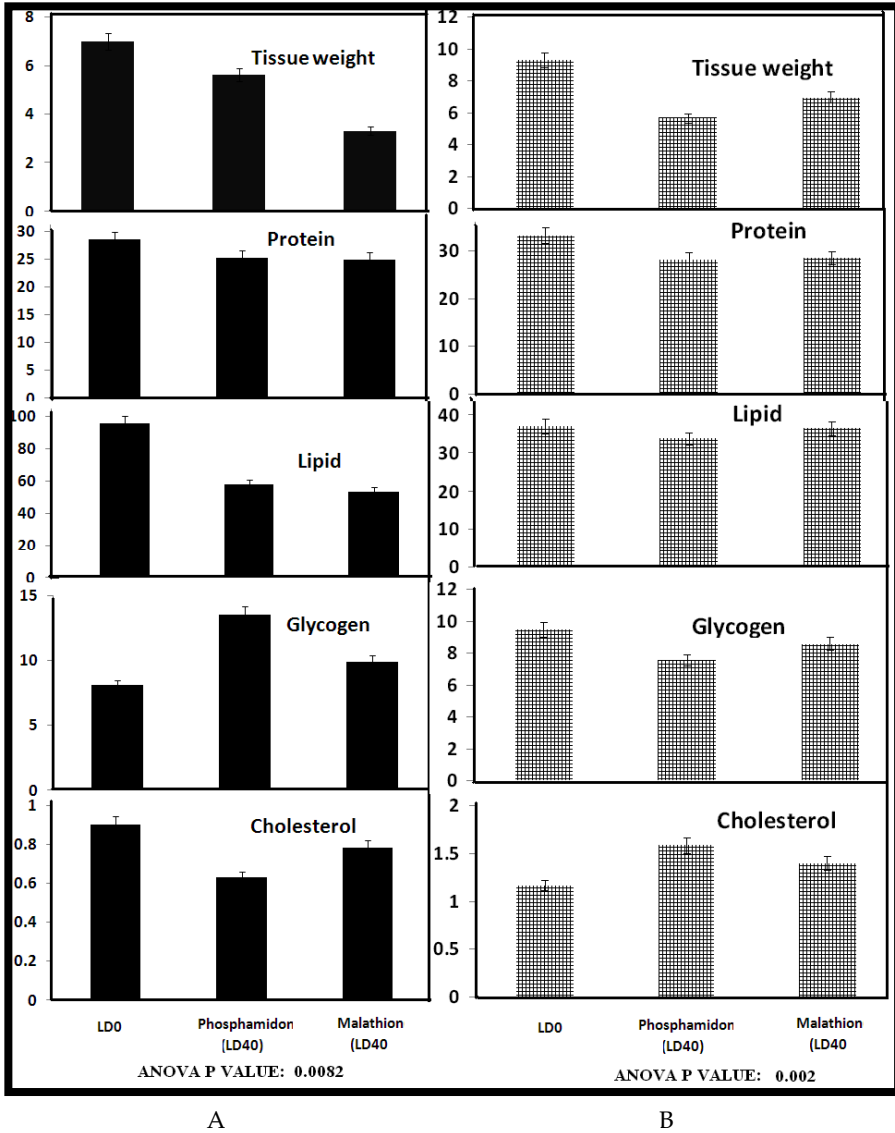


Figure 2. Effect of phosphamidon and malathion on tissue weight and level of biochemical constituents, Tissue weight (mg), Total Protein (mg/weight), Total lipid (mg/ml), Glycogen (mg/ml) and cholesterol (mg/ml). A. Male, B. Female.

4. Natural products

Natural products are excellent alternative to synthetic chemical pesticides. Plants exhibit enormous versatility in synthesizing complex materials which have no immediate obvious

growth or metabolic functions. These complex materials referred to as secondary metabolites are produced as constitutive and induced defense as a result of co evolution arising out of millions of years of plant-herbivore interaction [36-41]. They may be exploited for the management of insect pests owing to their ability to act as toxicant, repellent, antifeedant and insect growth regulators. They are non phytotoxic, biodegradable and have little or no mammalian toxicity [42,43]. Plant extracts and essential oils come under the category of “ Green pesticides” as they are safe, eco friendly and more compatible with environmental components compared to synthetic pesticides. 20th century witnessed an increasing trend in use of botanicals with more than 2000 bioactive plant species identified for their insecticidal and anti - pathogenic properties [39,40,44,45]. Natural insecticides such as pyrethrum, rotenone and nicotine have been used extensively for insect control [39]. Limnoids such as azadirachtin and gedunin, present in species from Meliaceae and Rutaceae are recognized for their toxic effects on insects and are used in several insecticide formulations in many parts of the world [46,47]. Neem formulations have been found effective against the mulberry hairy caterpillar, *Eupteromollifera* Walker [48]. Pink mealy bug, *Maconellicoccus hirsutus* (Green) is one of the major pest which infests the mulberry plants and cause Tukra diseases leading to qualitative loss of leaves. Leaf extracts of *Andrographis* (99.0%), *Leucas* leaf extract, NSKE (99.0%), *Vitex* leaf and *Ocimum* (90.1%) have been reported to act as repellent against the bug [49].

Grasserie (viral), Flacherie (bacterial), Muscardine (fungal) and Pebrine (protozoan) are four common diseases of silkworm and they have been causing heavy loss to silkworm crops in silk producing countries like India and China. Herbal extracts have been tried for control of these diseases. Isaiarasu *et al.* [50] reported efficacy of aqueous and alcoholic crude extracts of *Acalypta indica*, *Ocimum sanctum* and *Tridax procumbens* against flacherie and muscardine diseases in silkworm. The alcoholic extract of the plant *Tridax procumbens* were reported most effective followed by alcoholic extract of *Ocimum sanctum* and *Acalypta indica*. The reported zone of inhibition (area mm²) of *Acalypta indica*, *Ocimum sanctum* and *Tridax procumbens* at a dose of 50 µl against flacherie causing bacteria were 29.9± 9.7, 42.4±6.8, 74.6±6.6 respectively and against muscardine causing fungus were 13.1±6.3, 23.6±3.4, 23.1±9.1 respectively. Aqueous extracts of thirteen plants were tested against cytoplasmic polyhedrosis virus (AmCPV) in tasar silkworm, *Antheraea mylitta* and out of them 2% concentrations of *Aloe barbedensis* (AKP 3), *Psoralea corylifolia* (AKP13) and *Bougainvillea spectabilis* (AKP 9) were reported effective in suppressing virosis. They reduced mortality of larvae due to virus infection of 66.17%, 64.47% and 57.19% respectively. Total haemocyte count and haemolymph protein were also reported to increase in treated larvae which is considered as expression of immune response against the attack of pathogen [51]. Extracts of *Terminalia chebua* has been recommended against a potent bacterial strain, *Pseudomonas aeruginosa* (strain AC3) causing flacherie disease in *Antheraea assama* [52].

4.1. Plants of ethnic importance

Indigenous knowledge (IK) is unique to a particular culture and society. IK is embedded in community practices, institutions, relationships and rituals [53]. It forms the basis for local decision-making in agriculture, health, natural resource management and other activities

and constitutes an important component in the global knowledge system. In most cases, IK is an underutilized resource in the development processes [53]. Learning from indigenous knowledge of specific communities used for generations after generations can improve the understanding of their local conditions and saves time, effort and money besides constituting the foundation for activities designed to address regional and global problems. Thus, the natural products based on the indigenous use of botanicals could be one way of mitigating the problems associated with inappropriate use of synthetic chemicals [54].

4.2. Botanicals in sericulture field

In this chapter we restrict our discussions to candidate plants for being used against a parasitoids of silk worm. In sericulture field, farmers of Assam traditionally sprinkle extracts of tulsi (*Ocimum sanctum*) leaf over egg bunches (Kharika) of *A. assama*. No other plants were recorded to be used against pests and pathogens of this silkworm in a survey carried out in Upper Brahmaputra valley of Assam during 2007-2011. Uzi fly, *Exorista sorbillans* (Diptera: Tachinidae) is a parasitoid of silkworm and a serious threat to sericulture industry. The mated adult female fly lays eggs on the integument of third to fifth instar larva of *Antheraea assama*. The maggots after hatching pierce through the integument and grow inside the body of the silk worm by feeding on the fat body. The matured maggot pierce through the shell of the silk cocoon and crawl away from the site of the cocoonage in search of suitable place for pupation (Plate 2). The piercing of the cocoon shell renders the silk cocoon unreliable. In this way, the fly is reported to cause 20-80% loss of seed crop of *A. assama*. The fly infestation is reported in all the commercial silkworm varieties and from almost all silk producing countries of the world [55,56]. A total of ten plants possessing insecticidal and medicinal values including *O. sanctum* were bioassayed against *E. sorbillans* (Table 1). Leaves of the plants were shade dried, ground to fine powder and extracts were prepared by using ethanol, water and hydroalcohol (50:50). Ethanolic extract of three plants namely *Catheranthus roseus*, *Ocimum sanctum* and *Ageratum conyzoides* proved effective by causing 53.33 %, 22.17%, 57.41% mortality after 24h at 10% concentration. The order of toxicity was *Ageratum conyzoides* > *Catheranthus roseus* > *Ocimum sanctum* > *Melia azedarach* > *Paederia foetida* > *Eupatorium odoratum* > *Polygonum hydropiper* > *Vitex negundo* = *Leucas aspera*.

Further fractionation of ethanolic extract of the effective plants using an elutropic series of solvents viz. petroleum ether, chloroform, butanol and water followed by subsequent bioassay showed that the petroleum ether extract of *Catheranthus roseus*, *Ocimum sanctum* and *Ageratum conyzoides* caused 46 %, 6.67%, 86.21% respectively after 24h and 100%, 33.79%, 100% mortality respectively after 48h at 5% concentration. Results of other solvent fractions were found negligible. The LC50 value of the most effective petroleum ether extract of *Ageratum conyzoides* was recorded as 0.74 percent [57]. Qualitative phytochemical studies of petroleum ether extract of the plants showed positive results for the presence of flavonoids, alkaloids, phenols and terpenoids. *Ocimum sanctum* has been reported to be effective against other Dipterans and many other pests and microorganisms [58-61]. In addition to *Ocimum* the other two plants found effective were *Ageratum conyzoides* (Asteraceae) and *Catharanthus roseus* (Apocynaceae). Extracts of different species belonging to Asteraceae were earlier found effective against mosquitoes. *Ageratum conyzoides* with a

long history in medicinal use is a common weed species and naturally grows in abundance in Assam. It is widely distributed in tropics and subtropics and has been used in various parts of Africa, Asia and in South America for curing various diseases like purulent ophthalmia, ulcers, wound caused by burns, asthma, dyspnea, pneumonia and also as purgative, febrifuge, antiinflammayory, analgesic, anti-diarrheic etc. Both essential oil and the major component precocene have antijuvenile hormone activity on a variety of insects [62]. In addition to their medicinal importance *Catharanthus roseus* has been reported effective against many insect pests including Dipterans. Volatile components obtained by hydrodistillation was found to contain 76 compounds [63].

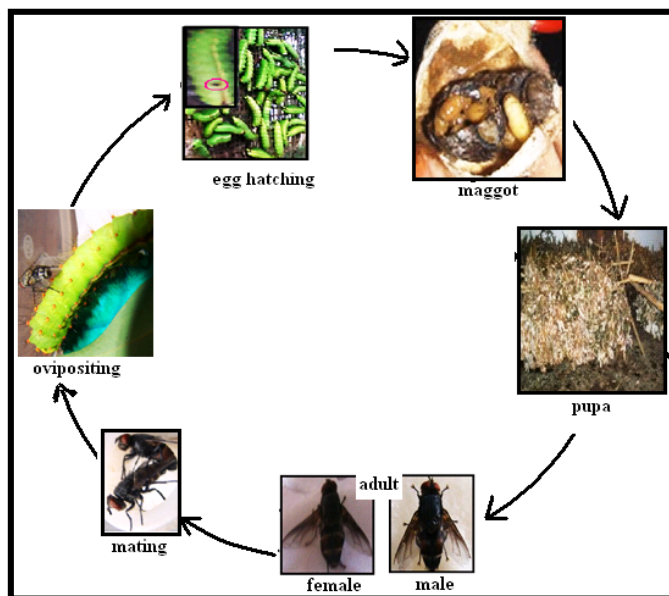


Plate 2. Life Cycle of *Exorista sorbillans* (Uzi fly)

Sl. No.	Plant	Cold water (Mean±SE)	Hot water (Mean±SE)	Hydroalcohol (Mean±SE)	Ethanol (Mean±SE)
1.	<i>O.sanctum</i>	0±0	3.33±3.33	3.33±3.33	22.17±4.54
2.	<i>O.gratissimum</i>	0±0	13.33±3.33	6.67±3.33	15±5.01
3.	<i>A.conyzoides</i>	0±0	10±5.78	13.70±3.17	57.41±4.31
4.	<i>C.roseus</i>	0±0	3.33±3.34	3.33±3.34	53.33±21.88
5.	<i>E.odorum</i>	0±0	3.33±3.34	0±0	3.33±3.34
6.	<i>L.aspera</i>	0±0	0±0	0±0	0±0
7.	<i>M.azedarach</i>	0±0	0±0	0±0	6.67±3.3
8.	<i>P.foetida</i>	0±0	10±0	0±0	3.33±3.34
9.	<i>P.hydropiper</i>	0±0	3.33±3.34	0±0	0±0
10.	<i>V.negundo</i>	0±0	0±0	0±0	0±0

Table 1. Comparative toxicity of crude extracts of plants (10%) to *E.sorbillans* (contact residual film technique)

4.3. Botanicals in paddy cultivation

Farmers in some locations of Brahmaputra valley of Assam use plant materials in rice fields for prevention of pest infestation. For instance, robub tenga (*Citrus maxima*), chuka tenga (*Citrus medica*), jora tenga (*Citrus spp.*), leaf of baghdhaka (*Chromolaena odorata*), patharua bihlongoni (*Polygonum hydropiper*), chirata tita (*Andrographis paniculata*) etc. But, no systematic study has been carried out so far to ascertain if farmers are deriving any benefit or there is any scientific basis behind the beliefs of the farmers. A survey done by interactions with 200 farmers in 44 villages revealed that 23% farmers used synthetic chemical pesticides, 37% used traditionally used plant parts, 27% used both synthetic chemicals and traditionally used plant parts, while 13% adopted neither synthetic chemicals nor traditionally used plant materials (Table 2) [64].

Similarly with the discussions in context with pests and their control in sericulture field, here we limit our discussions regarding traditional use of plants and their scientific validation in context with a representative pest of paddy. *Nymphula depunctalis* (Guenée) [= *Parapoynx stagnalis* (Zeller)] (Lepidoptera; Pyralidae) is a serious pest of paddy that attacks young rice plants in waterlogged paddy fields of the Oriental region [65-70]. It also thrives on various other grasses [71]. A number of related species occur in Asia but *N. depunctalis* is most widely distributed occurring in South and South East Asia, China, Japan, Australia, South America and Africa [72-74]. In many parts of Asia, *N. depunctalis* has been reported as a major pest [75-79]. In India, it is one of the serious pests in the states of Andhra Pradesh, Assam, Bihar, Karnataka, Orissa, Tamil Nadu, Manipur and Kerala. Damage to the plant occurs mainly due to defoliation by scraping of the green tissues by the larvae leaving only the white papery epidermis behind. Upon hatching, the first instar larvae climb onto a leaf and begin feeding on the green tissues by scraping the leaf surface. They then move to the leaf tip and cut a slit on the margin at a location 2–3 cm below the leaf tip. Then they make another cut about 1 cm below the first cut. Due to a lack of turgor pressure, the cut leaf segment rolls around a feeding larva to form a tubular case that is secured by silk spun by the larva. The inner surface of the leaf case is lined with silk to hold a thin film of water that is essential for respiration and to prevent desiccation of the larva [80]. Pupal period is spent within a closed case. (Plate 3).

Twenty two plants were found to be used traditionally in paddy fields against *N. depunctalis* and out of them extracts of 13 plants were selected for bioassay against *N. depunctalis*. The methods used for bioassay were residual film technique [81] and case dip technique [82]. The selected plant parts were shade dried, ground to powder and used to prepare extracts using water, hydro-alcohol (50:50) and ethanol. While hot water extract was the least toxic, both hydro-alcohol and ethanolic extracts were highly toxic. Out of thirteen plants tested, ethanol extract of seven plants were found highly effective (90–100% mortality), five plant extracts were found moderately effective (50–89% mortality) and one was found the least effective (mortality less than 50%). The highly effective ones were leaves of *Calotropis procera*, root-bark of *Zanthoxylum nitidum*, leaves of *Zanthoxylum rhesta*, stem bark of *Crataeva nurvala* and leaves of *Croton tiglium*, *Vitex negundo* and *Chromolaena odorata*.

The moderately effective ones were leaves of *Melia azedarach*, *Dryopteris filix-mass*, *Polygonum hydropiper* and *Tephrosia candida*, roots of *P. hydropiper*. Leaf of *Premna latifolia* was found the least effective. The degree of toxicity of the effective plants were *Calotropis procera* > *Zanthoxylum nitidum* > *Zanthoxylum rhesta* using residual film technique. The degree of toxicity in bioassay using case dip technique was *Calotropis procera* > *Zanthoxylum nitidum* > *Zanthoxylum rhesta* > *Crataeva nurvala* > *Croton tiglium* > *Vitex negundo* > *Chromolaena odorata*. Phytochemical analysis for alkaloid, flavonoid, tannin, saponin, sterol and glycosides using crude ethanol extract showed the presence of flavonoid, tannin, saponin and glycoside in leaf extract of *Calotropis procera*; flavonoid, saponin, sterol and glycoside in the leaf extract of *Chromolaena odorata*; alkaloid, flavonoid, tannin and sterol in the stem-bark extract of *Crataeva nurvala*; flavonoid, tannin and sterol in the leaf extract of *Croton tiglium*; tannin and sterol in the leaf extract of *Vitex negundo*; alkaloid and sterol in the root-bark extract of *Zanthoxylum nitidum* and flavonoid, tannin, saponin and glycoside in *Zanthoxylum rhesta*. (Figure 1 and 2)[59].

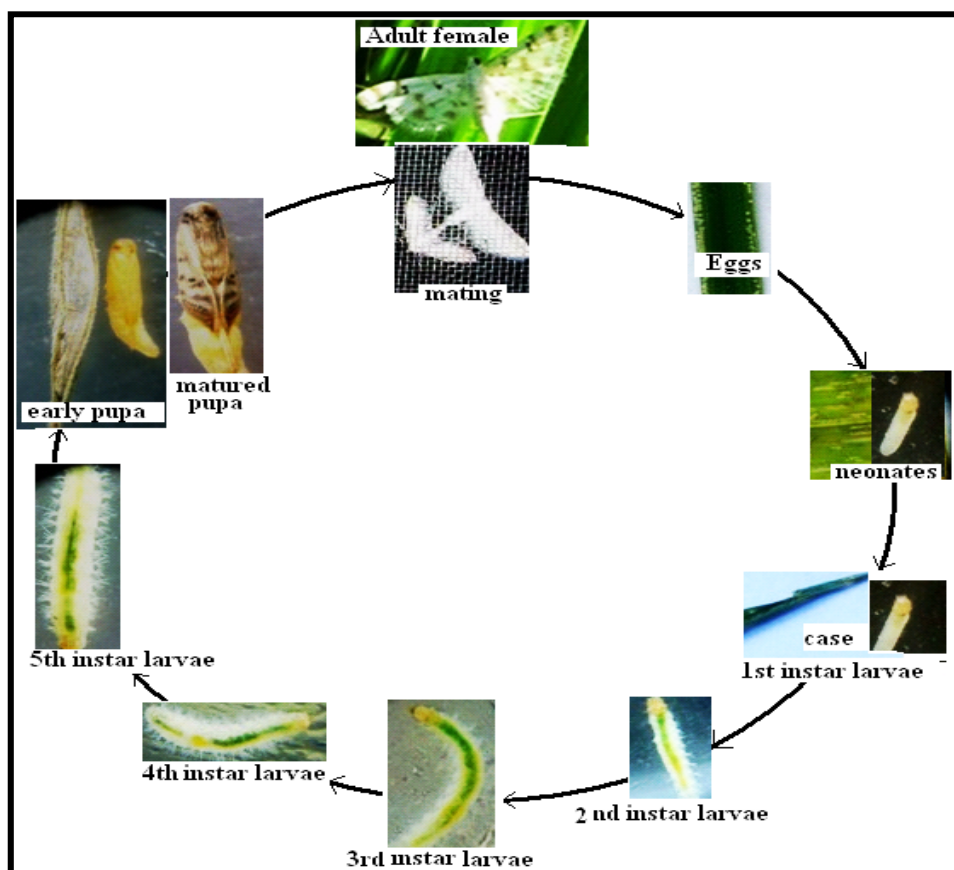


Plate 3. Life cycle of *Nymphula depunctalis*

Scientific Name	Common Name	Family	Part Used	Farmer s in %
<i>Ageratum conyzoides</i>	Gundhua ban	Asteraceae	Leaf (LF)*	01.0
<i>Andrographis paniculata</i>	Chirata	Acanthaceae	Leaf	03.0
<i>Azadirachta indica</i>	Mahaneem	Meliaceae	Leaf and Seed	05.5
<i>Calotropis procera</i>	Akan	Asclepiadaceae	Leaf and stem	04.0
<i>Chromolaena odorata</i>	Germany ban	Asteraceae	Leaf	42.0
<i>Citrus grandis, C. medica</i>	Rebab and Bor Tenga	Rutaceae	Fruits and Fruit peel	11.5
<i>Colocasia spp.</i>	Kachu	Araceae	Stem	01.5
<i>Crataeva nurvala</i>	Varun	Capparidaceae	Stem-Bark (SB)*	03.5
<i>Croton tiglium</i>	Koni bih	Euphorbiaceae	Leaf and Seed	03.0
<i>Cymbopogon nardus</i>	Citronella	Gramineae	Leaf	01.5
<i>Dryopteris filix-mas</i>	Dhekia bilohngoni	Aspidiaceae	Leaf	03.0
<i>Euphorbia neriifolia</i>	Siju	Cactaceae	Stem	00.5
<i>Jatropha gossypifolia</i>	Bhoot ara	Euphorbiaceae	Leaf and Stem-Bark	03.0
<i>Melia azedarach</i>	Ghora neem	Meliaceae	Leaf	05.0
<i>Murraya koenigii</i>	Narasinga	Rutaceae	Leaf	00.5
<i>Polygonum hydropiper</i>	Patharuabihlongoni	Polygonaceae	Leaf, Stem, Root (RT)*	07.5
<i>Premna latifolia</i>	Pitha	Lamiaceae	Leaf	21.00
<i>Tephrosia candida</i>	Kuku mah	Fabaceae	Stem-Bark and Leaf	06.0
<i>Thevetia neriifolia</i>	Rakta karabi	Apocynaceae	Stem-Bark, Seed	02.00
<i>Vitex negundo</i>	Pasatia	Verbenaceae	Leaf	13.00
<i>Zanthoxylum nitidum</i>	Ricom, Tezmui	Rutaceae	Root-Bark (RB)*	03.00
<i>Zanthoxylum rhesta</i>	Ongare	Rutaceae	Leaf and Stem Bark	02.00

Table 2. List of plants used by the farmers against insect pests of paddy in North East India

4.4. Botanicals in tea gardens

Tea, *Camellia sinensis* (L.) O. Kuntze is grown in a monoculture system and about 1031 arthropod species are reported to be associated with the crop [83]. Cultivation and production of Assam tea was started by Assam company under the British regime around 1840 and the company monopolized the industry till 1860. With the advent of synthetic pesticides and their tremendous potentiality for controlling insect pests, during 50's tea industries started applying insecticides against tea pests which boosted production [83]. With the growing concern over pesticidal residue in tea leaves and environmental impact of pesticides, various international regulatory bodies have fixed maximum permissible limit of residues in tea leaves. Synthetic pesticides are still being used in tea plantations although in limited quantity. These pesticides however do not remain confined to the tea plantations only and contaminate the host plants of silkworms grown in sericulture fields lying adjacent to the tea gardens [6]. Literature reveals that several plants have been found to be effective against pests of tea [83-86].

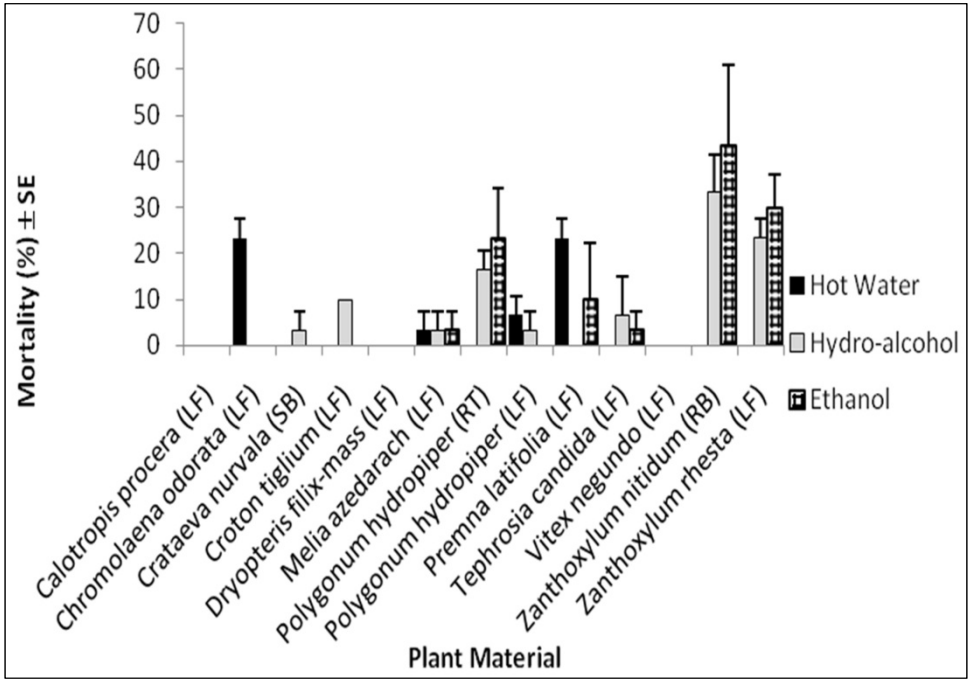


Figure 3. Effects of plant extracts on percent mortality of larvae of *N. depunctalis* in residual film technique

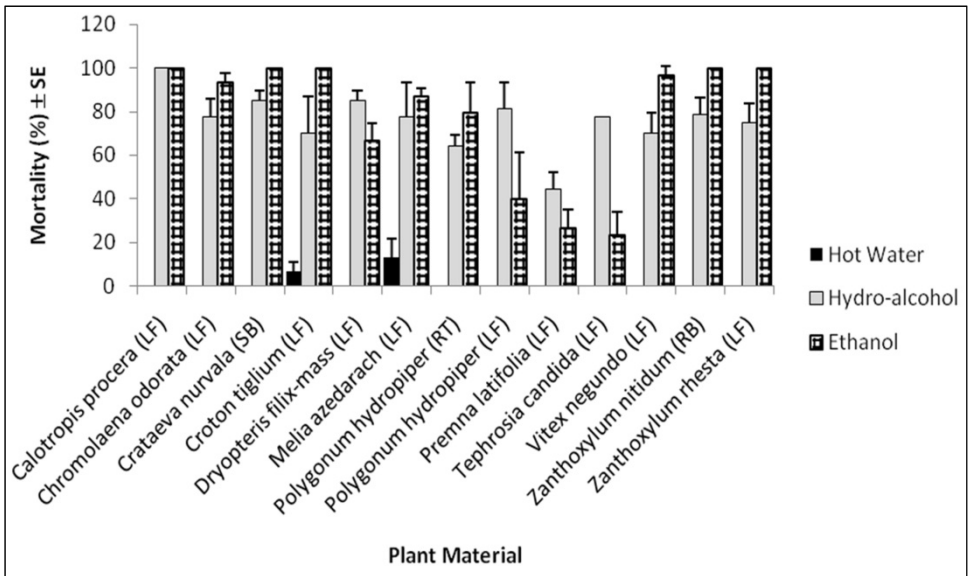


Figure 4. Effects of plant extracts on percent mortality of larvae of *N. depunctalis* in case dip technique.

5. Selective toxicity of plant products

Integrated pest management emphasizes on use of pesticides having selective toxicity as against the use of broad spectrum pesticides. This reminds us of classical examples of using different insecticides for controlling aphids and thrips in potted chrysanthemum in glass houses of Southern Britain in 70's at the advent of development of the concept of integrated pest management [87]. Apart from the aspects why plant products are considered better options for pest control as discussed above, if they can be categorized for having differential efficacy against different insects, the latter can give an added value to plant products for being used in pest control. However the efficacy also depends not only on the compound(s) present in the plant preparation but also on the ability of the insect to defend against the compounds they are subjected to. Less tissue susceptibility, presence of detoxifying enzyme, high immune response, development of alternative physiological pathways, reduced penetration of pesticides through the cuticle and intestine, lower transport of pesticides to the target sites, storage of pesticides in fat body or other inert organs, genetically determined modified behavior in response to pesticide etc. may be factors responsible for conferring resistance to certain insects against high susceptibility of other insect species [23,88,89].

Silk worms have a bitrophic relationship with the Dipteran, *Exorista sorbillans* (Widemann) (Uzi fly) as the later is an endoparasitoid of the silk worm. As silkworms are beneficial insects, before recommending use of any botanical it is desirable to investigate whether the recommended plant or its product found effective against pest and pathogens in sericoculture possess selective toxicity or not. An investigation carried out to evaluate selective efficacy of petroleum ether extract of certain plants against the component insects of the bitrophic system, *Exorista sorbillans* and larvae of *A. assama* revealed no mortality of late instar larvae of *A. assama* till 48h at 10% concentration while they were highly toxic to the parasitoid *Exorista sorbillans*.

Essential oils are volatile mixtures of hydrocarbons with diverse functional groups. Essential oils are defined as any volatile oil(s) that have strong aromatic components and that give distinctive odour, flavor or scent to a plant. These are the byproducts of plant metabolism and are commonly referred to as volatile plant secondary metabolites. Essential oils are found in glandular hairs or secretory cavities of plant cell wall and are present as droplets of fluid in the leaves, stems, bark, flowers, roots or fruits in different plants. The aromatic characteristic of essential oils attract or repel insect, protect plant from heat or cold and the chemical constituents of the oil act as defense material. Their probable diverse mode of action and use in food and pharmaceutical industry justify their status as possible environmentally benign candidate for pest management. *O.sanctum* and *A.conyzoides* are plants with rich source of essential oils. The toxicity of these oils were evaluated by both contact residual and topical application method against the parasitoid of silkworm, *E.sorbillans*. These oils were found more effective than petroleum ether extract of the respective plants and the calculated LC50 value of essential oil of *O. sanctum* and *A. conyzoides* in contact residual film method were recorded as 0.15% and 0.05% respectively.

But, topical application of the oil showed a different order of toxicity and reflected the fact that degree of toxicity depended on method of application. The Lethal time for *O. sanctum* oil was 4 ± 0.58 minutes and that of *A. conyzoides* oil was 118 ± 12.45 minutes after topical application of $1 \mu\text{l}$ of oil on thorax of the fly and hence degree of toxicity of oil on topical application was $O. sanctum > A. conyzoides$. When toxicity of these oils were compared against the larvae of *A. assama*, it was found that late instar larvae could survive till 48h after application of 0.5% concentrations of oil of the plants. The same concentration of oil of *A. conyzoides* while were applied against early instar larvae 100% mortality was caused with LC50 value at 0.19% concentration (Table 3) [81]. But toxicity of oil of *O. sanctum* against *A. assama* larvae was lower and at 0.5 percent concentration, it could only cause a maximum of 5.78% mortality of early instar larvae at 48h of treatment and could cause no mortality in late instar larvae till 48h of observation period (Figure 5). This may suggest less tissue sensitivity of *Antheraea assama* against the action of extract and oil of *O. sanctum* applied [90]. The plant *Ocimum spp.* is known to comprise more than thirty species, but only a few have been subjected to phytochemical studies (Grayer et al. 1996). The whole plant of *Ocimum* is rich in essential oil and based on composition of volatile principles of essential oil, intraspecific chemotypes of several species of *Ocimum* have been described [91-93]. The plant is a part of Indian tradition as a holy substance and its essential oil is larvicidal against both *Aedes* and *Culex* mosquitoes [94].

Plant material	<i>Exorista sorbillans</i>			<i>Antheraea assama</i>		
	Regression equation	LC ₅₀ (%)	95% Fiducial limit	Regression equation	LC ₅₀ (%)	95% Fiducial Limit
<i>A. conyzoides</i> oil	$Y=8.72+2.87X$	0.05	2.603-3.075	$Y=7.10663+2.92014X$	0.19	2.394-2.837
<i>O. sanctum</i> oil	$Y=9.15452+4.97895X$	0.146	8.929-11.172	-	-	-
<i>A. conyzoides</i> petroleum ether	$Y=5.17654+1.27665X$	0.72	1.220-1.352	$Y=3.62422+0.625415X$	154.88	0.311-0.937
Deltamethrin	$Y=5.90148+9.044456x$	0.103	1.688-1.969	$Y=7.46583+.612381x$.00009	0.572-0.656

Table 3. Efficacy of extract & oil of *A. conyzoides* and *O. sanctum* as compared with Deltamethrin [() in cells indicate zero mortality].

5.1. Identification of haemocytes of *A. assama* by light microscopic and transmission electron microscopic studies (TEM) studies

In order to understand the possible effects of the extracts of *O. sanctum* and *A. conyzoides* at cellular level electron microscopic studies of haemocytes were carried out after topical application of the extracts on thoracic surface of fifth instar larvae of *A. assama*. Five types of haemocytes of *A. assama* were identified under light microscopic and transmission electron

microscopic studies. The identified cells were-1. Plasmatocyte 2. Granulocyte 3. Spherulocyte 4. Prohaemocyte 5. Oenocyte (Plate-4-9)

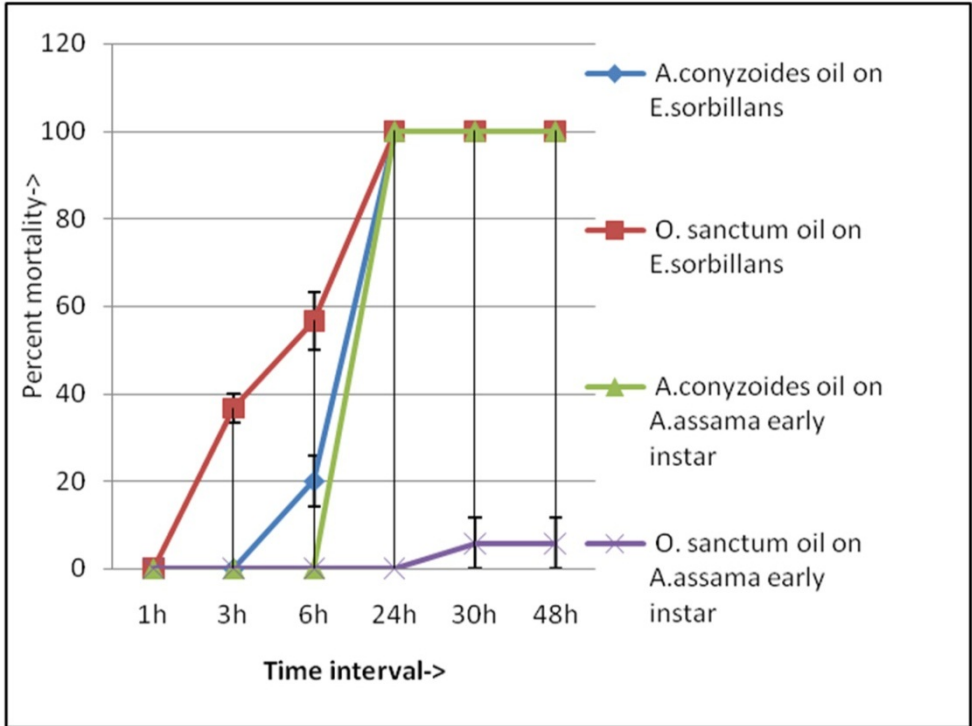


Figure 5. Comparative percent mortality of *A. assama* and *E. sorbillans* at 0.5% concentration of oil of *O. sanctum* and *A. conyzoides*

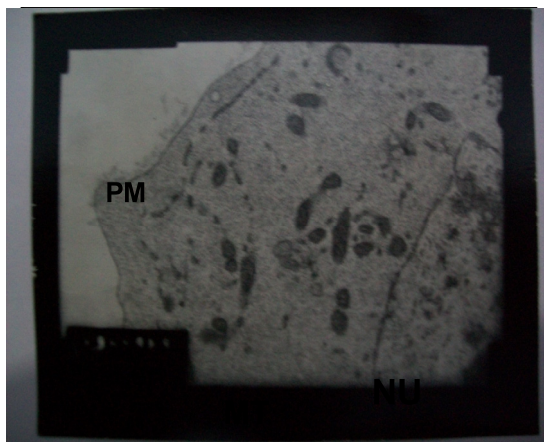


Plate 4. Plasmatocyte in *A. assama*

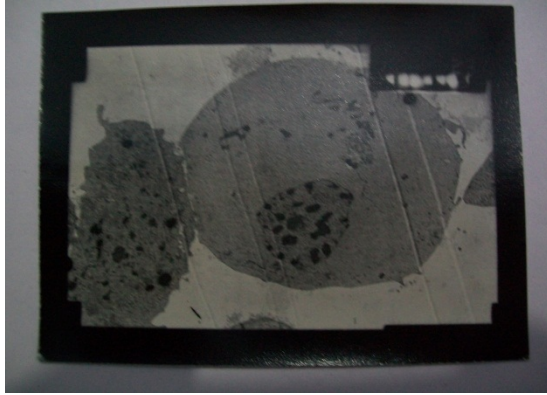


Plate 5. Plasmotocyte and Oenocyte

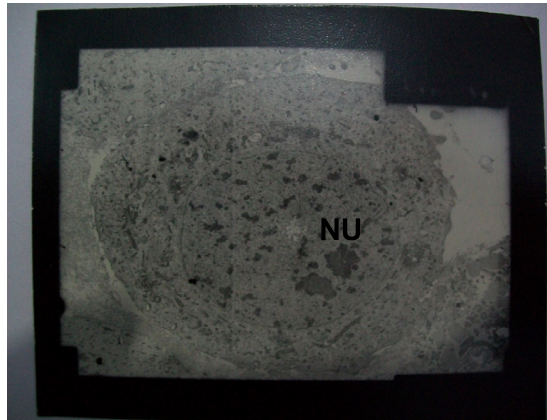


Plate 6. Prohaemocyte of *A. assama*

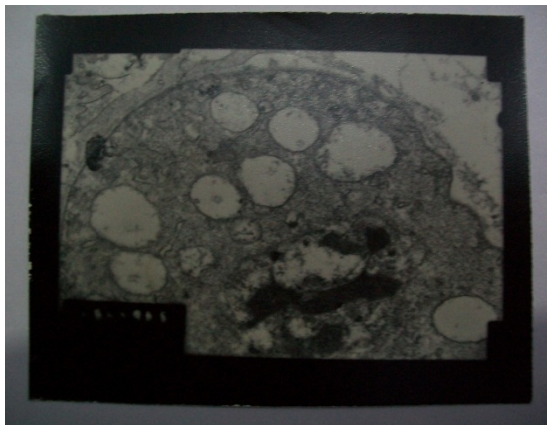


Plate 7. Spherulocyte of *A. assama*

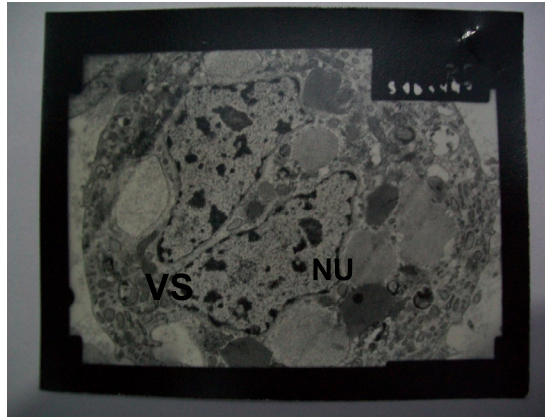


Plate 8. Granulocyte of *A.assama* under TEM

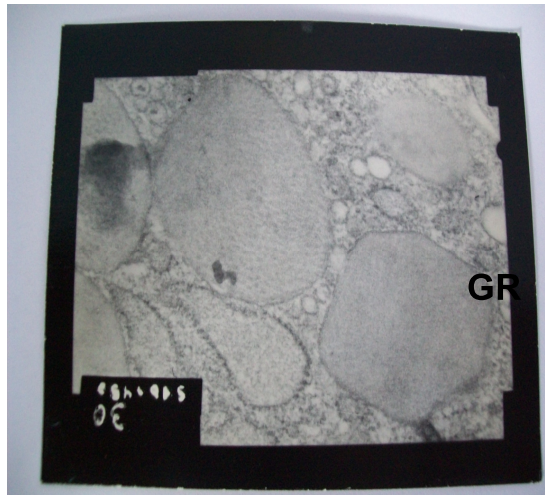


Plate 9. Structured granule

5.1.1. *Plasmatocyte*

Plasmatocytes are polymorphic cells with round, ovoid, elongate and spindle shaped structures with filopodia. The cells are small to moderate size. The long axis is 25 μm and the short axis is 14.74 μm . Surface of PLs is not smooth and have ridges much flatter than those of granulocytes. Nucleus is dense, round, ovoid or elongated with distinct membrane. The cytoplasm is generally abundant, granular and agranular. Cytoplasm contains a good number of mitochondria and extensive rough endoplasmic reticulum. Golgi body recorded was in less number. Vacuole is rare in normal cell. The nucleus is mostly centrally placed, prominent, usually round but changes shape with the change in shape of the cell.

5.1.2. *Granulocyte*

They are rounded or oval in shape with long and short filopodia. The long axis is 8.382 μm . Two types of membrane bound granules were observed, electron dense and structured. Cytoplasm contains large number of mitochondria, endoplasmic reticulum, free ribosomes, lysosomes and small number of vacuoles. Binucleate granulocyte was also recorded. Nucleus generally small takes various shapes, round, ovoid or irregular. The cell surface contains many elevations and depressions giving a conspicuous pattern.

5.1.3. *Spherulocyte*

These are spherical or ovoid or elongated cells with long axis 7.21 μm . The cell surface contains pits. Wavy edges show presence of spherules. Nucleus is mostly centrally placed, sometimes obscured by spherules. Mitochondria, rough endoplasmic reticulum and golgi complex were observed in cytoplasm when spherule number was less.

5.1.4. *Prohaemocyte*

They are round, oval in shape and long axis is 7.94 μm . The nucleus occupies major area of the cell. Plasma membrane is almost smooth. Cytoplasm is thin, almost homogeneous, contains few number of mitochondria, Golgi bodies and endoplasmic reticulum. SEM shows a spherical surface.

5.1.5. *Oenocytoid*

These are large rounded or ovoid cells with eccentric nucleus. Nucleus is small, nucleoli were prominent. The plasma membrane is smooth and regular. Cytoplasm uniformly distributed and contains extensive rough endoplasmic reticulum. Filopods are generally absent. Other cellular organelles like Golgi bodies and mitochondria are less in number.

5.2. TEM studies on effect of petroleum ether extract on haemocytes of late instar larvae

Transmission electron microscopic studies of haemocytes of fifth instar silk worm larvae was done after application of petroleum ether extracts of *O. sanctum* and *A. conyzoides* at different hours of treatment (Plates 10-21).

Application of petroleum ether extract of *O. sanctum* at 0.25 h caused less damage of PL and GR. Mitochondria and nucleus was almost intact in both types of cells. Nuclear membrane and cell membrane damage was negligible. Cell attachment was less in number. Release of material from GR was observed in some points. Golgi body was in formative phase in GR. PL cells in dividing stage and dividing nucleus in GR was recorded.

Cell membrane breakdown of PL was observed at 1h of treatment while mitochondria was intact. Filopods are less. But cell attachment was marked. GR cell was less affected and

filopod like extension was many toward cell attachment site. Granules were released from the area of damage of the cell membrane. Dissolution of nuclear membrane was observed at certain points. OE, SP, PRO was less affected. At 6h of treatment PL was less affected. Golgi body was observed at formative phase. Filopods are less in number. Export of material from the cell was observed. GR cell was also less affected. Cell membrane, mitochondria were well preserved. Nucleus in dividing stage was recorded. In some GR, granules release from the cell. Some GR attach to one another. Other cells are less affected. But at 12 h of treatment period, cell membrane breakage, mitochondrial damage vacuolization of PL cell was observed while GR cell was less affected. But again at 48h no effect on cell membrane, nuclear membrane, mitochondria was observed. Golgi was found to be in formative stage. Release of material was not recorded. GR cell was looking intact where cell membrane, mitochondria and nuclear membrane were not affected. Aggregation of cell was less. OE cell membrane was ruptured and released some cytoplasmic materials but mitochondria, nuclear membrane were well preserved. Attachment of OE with other cells was observed. SP and PRO were less affected.

At 0.25h of *A. conyzoides* petroleum ether extract treatment, heavy loss of the cell membrane of PL cell was noticed but mitochondrial structure was found almost in good condition. ER long while many Golgi bodies were found in formative stage casting off numerous vesicles. Cell attachment was very pronounced with release of flocculent material. Many vesicles were observed in cytoplasm. Extensive damage of the cell membrane of GR cell was observed. Some of the mitochondria was found in a state of completely dissolved mitochondrial membrane. But nuclear membrane was in good condition. Cell attachment was observed while ER and GB was looking normal. OE was less affected.

At 1h of treatment of *A. conyzoides* petroleum ether extract, damage of cell membrane of PL in some parts were noticed but mitochondria was unaffected. Nuclear membrane was damaged and Golgi body was in formative stage. Filopods present and large vesicles in the cytoplasm was observed. GR cell membrane was found totally damaged state and some of the mitochondria was also affected. But nuclear membrane was less affected. ER short and GB was not observed. Attachment of the cells were observed and numerous vesicles were seen. Other cells were less affected. At 12h of treatment also, damaged PL cell with ruptured cell membrane, affected mitochondria, large vesicles and filopod were recorded. GR cell was also affected with mitochondrial damage, cell membrane rupture, heavy vacuolization. Release of cytoplasmic material to outside of the cell and import of material was noticed.

At 1h of acetone treatment as control almost intact cell membrane, mitochondria, ER, nuclear membrane was observed. Cell attachment was less with few filopods in PL cell. Granules and vesicles number were less and release of cytoplasmic material were not observed in PL cell. But GR cell was affected and plasma membrane rupture and damage in mitochondria was observed. Plenty number of ER but few vesicles were observed. Both types of granules structured and electron dense were observed. Cell aggregation with release of flocculent material was noticed. OE was less affected than PL and GR. But at 6h of acetone treatment PL and GR were found intact with intact plasma membrane, nuclear membrane, normal mitochondria, few vesicles and very less cell attachment. A medium

number of filopods were observed but release of cytoplasmic material was not observed. OE cell was also found intact with unaffected mitochondria. After 48h treatment of acetone also, the nuclear membrane and mitochondria of both PL and GR were found intact with less cell attachment. Cell membrane damage of PL was less. Golgi body was found in formative phase and few number of filopods and vesicles were observed but release of flocculent material was not recorded. Nuclear division of PL cell was observed. Cell membrane rupture of some of the GR cell was recorded with discharge of granule while other cells were intact with almost absence of filopods. Plenty number of ER and large sized vesicles were seen. Both types of granules structured and electron dense were noticed.

A comparative assessment of the effects made showed that the rupture of plasma membrane (PM) in plasmatocyte (PLs) was maximum in treatment with *A. conyzoides* while it was comparatively less in treatment with *Ocimum sanctum*. Symptoms of immune response i.e. cell attachment between PLs and GRs and among the plasmatocytes along with release of cytoplasmic and granular material from granulocytes were observed in case of treatment with all the plant extracts. Toxic symptoms like highly affected mitochondria with break down of nuclear membrane, dissolution of cristae became evident in case of treatment with *A. conyzoides*. In case of treatment with *Ocimum sanctum* the affects were comparatively less and at 48h the PLs were exactly similar with those of control insects. Similar effects on ultrastructure PLs and GRs were reported to be caused in lepidopteran insect by neem gold [95,96]. Plant based pesticides were earlier reported to decrease prohaemocyte, plasmatocyte and spherulocyte and increase granunocyte and oenocyte population [95,97]. Gupta(1998) reported on involvement of plasmatocytes and granulocyte in cell mediated immune response. In our investigation also cell lysis, release of granular material, vacuolization and clumping of PLs and GRs were observed after treatment with the plant extracts.

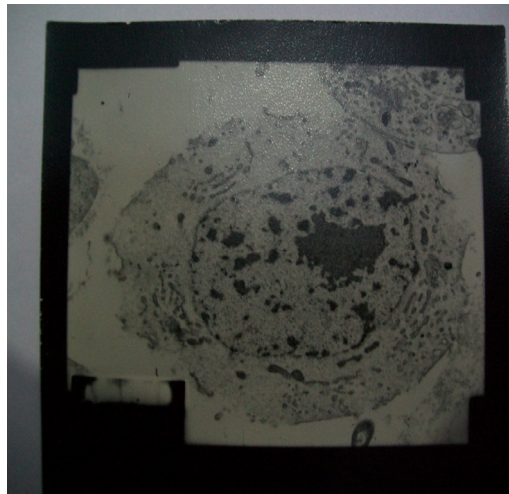


Plate 10. Plasmamembrane rupture in *A.conyzoides* PL under TEM



Plate 11. Nuclear membrane rupture *A. conyzoides* treated PL under TEM

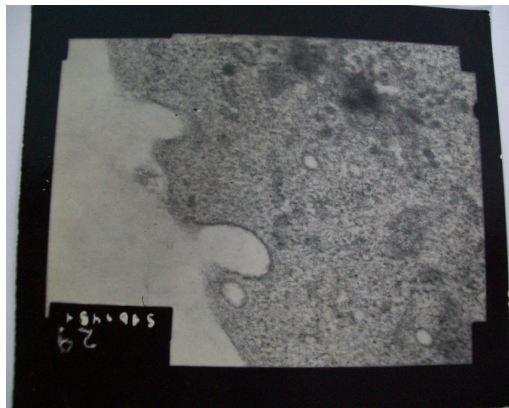


Plate 12. Pinocytotic vesicle formation of PL at 12 h of treatment with *A. conyzoides*

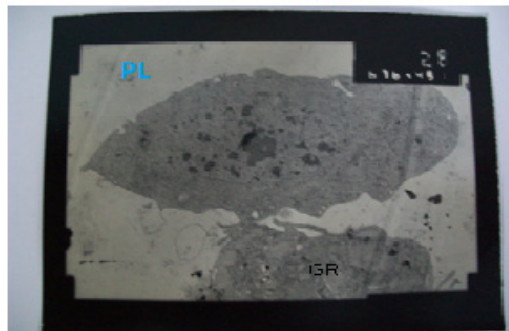


Plate 13. Cell clumping between PLs and GRs at 12 h of treatment with *A. conyzoides*

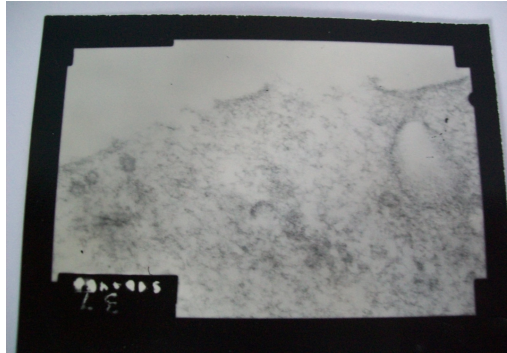


Plate 14. PM rupture at 12 hr after treatment with *O. sanctum*

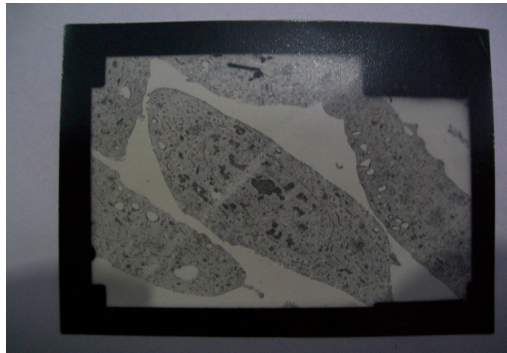


Plate 15. Normal PLs at 48hr of treatment with *O. sanctum* under TEM

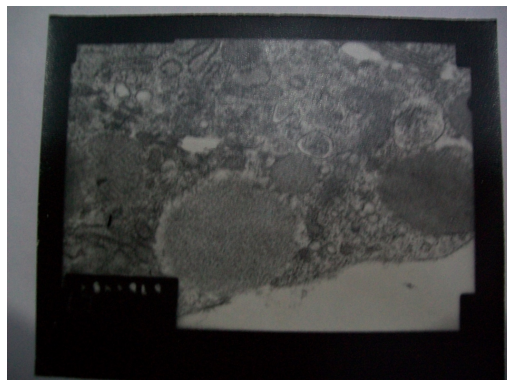


Plate 16. GR at 1hr of *O. sanctum* treatment under TEM

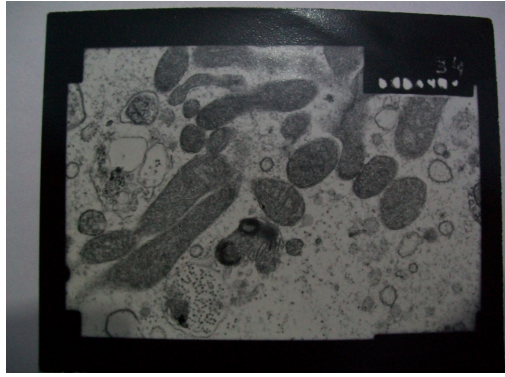


Plate 17. Mitochondria at 15min of *O. sanctum* treatment

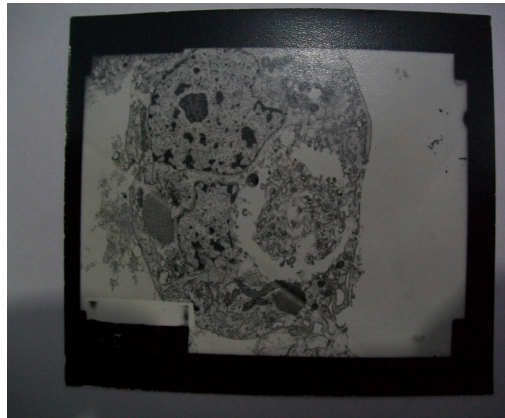


Plate 18. Granulocyte damage at 15min of *O. sanctum*

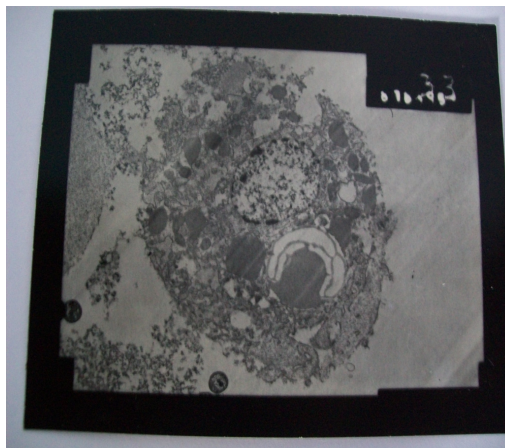


Plate 19. Granular material release at 12hr of treatment in *O. sanctum*

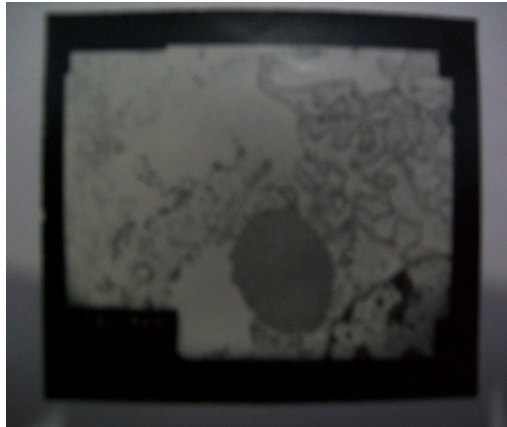


Plate 20. GR breakdown at 1hr of treatment in *A. conyzoides* *A. conyzoides* under TEM

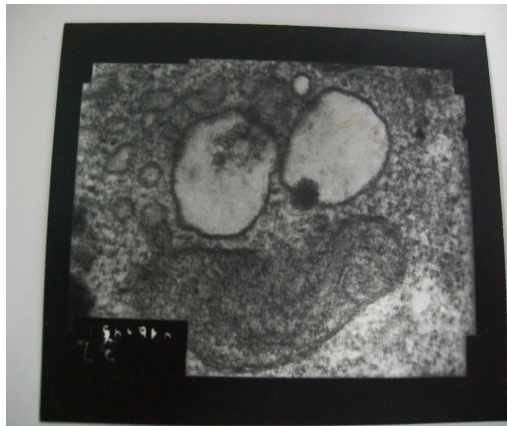


Plate 21. GR mitochondria damage at 12hr of treatment in *A. conyzoides*

5.3. Effect of essential oil on economic characters of silk worm

Study on impact of essential oils of *O. sanctum* and *A. conyzoides* after topical application at the dosages of 0.2, 0.1, 0.02 and 0.01 μlg^{-1} on 2nd day old *A. assama* fifth instar larvae showed that there was no significant difference on developmental period of fifth instar larvae from that of control in case of treatment of essential oil of *O. sanctum*. A significant decrease in developmental period was caused by essential oil of *A. conyzoides* at the highest dose. Early spinning of larvae after application of essential oil of *A. conyzoides* might be due to its effect on endocrine system. Precocene found in essential oil of *Ageratum* species is known to cause precocious metamorphosis through its action on *Corpora allata* [23,99].

Economic characters of silkworm like cocoon weight and pupal weight of treated larvae did not vary significantly from that of control larvae. But significant decrease of shell weight was recorded after application of essential oil of *A. conyzoides* at all dosages [24]. Reduction in shell

weight might be due to reduction of spinning period causing precocious metamorphosis which might be caused by precocene II found in *A. conyzoides* [99]. Thus in comparison to *A. conyzoides*, *O. sanctum* is a preferable candidates for being used against *E. sorbillan*. However, nutritional indices like efficiency of conversion of ingested food into body matter (ECI), efficiency of conversion of digested food into body matter (ECD), approximate digestibility (AD), consumption index (CI), relative growth rate (RGR) were not found to differ significantly from that of control at lower dosages (0.02, 0.01 $\mu\text{l/g}$) after treatment of both essential oils of *O. sanctum* and *A. conyzoides* [24]. There are also reports stating beneficial effects of plant products on silk worms which is supportive of use of plant products in sericulture field. Botanicals like *Curcuma longa*, *Phyllanthus ambilica*, *Asparagus racemosus*, *Aegle marmelos*, *Boerhavia diffusa*, *Allium sativum* and *Ocimum basilicum* applied against flacherie infested *Bombyx mori* larvae increased larval weight, cocoon weight, shell weight, silk ratio [100]. Similar studies on beneficial effects of plant products on growth, development and economic characters of silk worms have been carried out by several workers [101-103]. Synergistic actions of plant chemicals might be responsible for growth enhancement of certain insects [104].

Table: Variation of cocoon weight of <i>A. assama</i> late instar larvae after treatment with essential oil of plants (P<0.05).				
Dose→	0.2 $\mu\text{l/g}$	0.1 $\mu\text{l/g}$	0.02 $\mu\text{l/g}$	0.01 $\mu\text{l/g}$
Plant(oil)↓	Cocoon weight (Mean±SE)	Cocoon weight (Mean±SE)	Cocoon weight (Mean±SE)	Cocoon weight (Mean±SE)
<i>O. sanctum</i>	5.15±.45	4.85±.44	5.13±.44	4.85±.44
<i>A. conyzoides</i>	4.81±.31	5.30±.29	4.81±.31	5.3±.29
Control	5.15±.66	5.15±.66	5.15±.66	5.15±.66
Table: Variation of pupa weight of <i>A. assama</i> late instar larvae after treatment with essential oil of plants (P<0.05).				
Dose→	0.2 $\mu\text{l/g}$	0.1 $\mu\text{l/g}$	0.02 $\mu\text{l/g}$	0.01 $\mu\text{l/g}$
Plant(oil)↓	Pupal weight (Mean±SE)	Pupal weight (Mean±SE)	Pupal weight (Mean±SE)	Pupal weight (Mean±SE)
<i>O. sanctum</i>	4.69±0.42	4.47±0.46	4.73±0.41	4.47±0.46
<i>A. conyzoides</i>	4.50±0.31	4.96±0.26	4.50±0.31	4.96±0.26
Control	4.75±0.64	4.75±0.64	4.75±0.64	4.75±0.64
Table: Variation of shell weight of <i>A. assama</i> after treatment with essential oil of plants (P<0.05).				
Dose→	0.2 $\mu\text{l/g}$	0.1 $\mu\text{l/g}$	0.02 $\mu\text{l/g}$	0.01 $\mu\text{l/g}$
Plant(oil)↓	Shell weight (Mean±SE)	Shell weight (Mean±SE)	Shell weight (Mean±SE)	Shell weight (Mean±SE)
<i>O. sanctum</i>	0.34±0.01	0.34±0.04	0.33±0.01	0.34±0.04
<i>A. conyzoides</i>	0.26±0.04	0.29±0.02	0.28±0.01	0.29±0.02
Control	0.32±0.02	0.32±0.02	0.32±0.02	0.32±0.02
Table: Variation of developmental period after treatment with essential oil of plants (P<0.05).				
Dose→	0.2 $\mu\text{l/g}$	0.1 $\mu\text{l/g}$	0.02 $\mu\text{l/g}$	0.01 $\mu\text{l/g}$
Plant(oil)↓	Dev period (Mean±SE)	Dev period (Mean±SE)	Dev period (Mean±SE)	Dev period (Mean±SE)
<i>O. sanctum</i>	11.67±.33	11.67±.33	11.33±.33	11.67±.33
<i>A. conyzoides</i>	10±0*	10.67±.33*	11.00±.58*	11.67±.33
Control	11.67±.33	11.67±.33	11.67±.33	11.67±.33

SE→ Standard error; *→ significant at 95% Confidence Interval

Table 4. Variation of cocoon weight of *A. assama* late instar larvae after treatment with essential oil of selected plants (P<0.05)

6. Mechanism of action of botanicals

The efficacy of plant products depend on presence of specific organic compound (s) which may interfere with the body physiology of the target organism. The compounds may belong to different chemical groups of secondary metabolites of plants viz. Alkaloids, phenolics, flavonoids and terpenoids. The ethanolic extract of *O. sanctum* is reported to have greater and broader spectrum of activity against tested organisms [106]. Literature reveals that in comparison to essential oil less works have been carried out to isolate and characterize bioactive compound of *Ocimum sp.* having insecticidal potency through solvent extraction and hence knowledge regarding the use of solvent extracts of *Ocimum* is comparatively less. Petroleum ether and acetone extract of *A. conyzoides* earlier was reported to have juvenile hormone activity against *Culex quinquefasciatus*, *Aedes aegypti* and *Aedes stephensi*. Hexane extract was found effective against *Musca domestica* and methanolic extract caused abnormal development and suppression of population of *Anopheles stephensi* [99,106,107]. Thiophene derivative, a class of compound found in many Asteraceae species has been attributed for the toxic effect of the plant. The hexane fraction of acetone extract of *C. roseus* containing alpha-amyrin is postulated to be active IGR against many pests [63]. Moreira et al. [107] showed the hexane extract of *Ageratum conyzoides* to have insecticidal activity and purified the compounds using IR, ¹H NMR, ¹³C NMR, HMBC. The compounds were 5,6,7,8,3,4,5/ heptamethoxy flavone, 5,6,7,8,3/ pentamethoxy-4,5/-methylene dioxyflavone and coumarin out of which only coumarin showed insecticidal activity against dictyopteran, lepidopteran and dipteran.

The mechanism of toxic effect of essential oil and oil compounds on insect at present is not well known. Insects vary enormously in their response to different essential oils and oil compounds. Essential oils are liquid in room temperature and get easily transformed from a liquid to a gaseous state at room or slightly higher temperature without undergoing decomposition. The quantity of essential oil found in most plants is 1 to 2% but can contain amounts ranging from 0.01 to 10%. Most essential oils comprise of monoterpenes with 10 carbon atoms, sesquiterpenes with 15 carbon atoms and rarely diterpenes or higher terpenes. The most predominant groups are cyclic compounds with saturated and unsaturated hexacyclic or an aromatic system. Bicyclic and acyclic components are also present [2]. Monoterpenes are common essential oil constituents and several hundred naturally occurring monoerpenes are reported. They are biosynthesized from geranyl pyrophosphate of the isoprenoid pathway. These can be classified into two major groups- monoterpene hydrocarbons and oxygenated monoterpenes. Monoterpene hydrocarbons include acyclic aliphatic, monocyclic aliphatic and dicyclic aliphatic while oxygenated monoterpenes include acyclic monoterpene, monocyclic monoterpene and dicyclic monoterpene [41]. Some of the major oil constituents of *O. sanctum* are methyl eugenol, caryophyllene, germacrene D, β -elemene, eugenol, caryophyllene epoxide and α -cadinol [24,108,109]. Oil of *A. conyzoides* plant collected from South-East Asian countries mostly contains sesquiterpene Beta-caryophyllene, demethoxyageratochromene and many monoterpenes [24,110]. The monoterpenes in Indian oil of *A. conyzoides* have been reported to be mainly Ocimene (5.3%), alpha-pinene (6.6%), eugenol (4.4%), methyl eugenol (1.8%) and the sesquiterpenes are Beta-caryophyllene(1.9%), Delta- cadinene(4.3%), sesquiphellandrene (1.2%) and caryophyllene

epoxide (0.5%) [62]. Eugenol was earlier reported to be effective against mosquitoes in Michigan [60]. Compounds like coumarin and mainly furanocoumarins can alter the detoxication capability of an organism by reversibly or irreversibly inhibiting cytochrome P450 detoxication enzymes [111-112]. They are also reported to have neurotoxic mode of action probably through binding with different types of octapamine receptors and interference with octapamine activity [113-115] or interference with GABA-gated chloride channels [2,115] or inhibition of acetylcholine esterase [116-118]. Such activity of the oil may be attributed to action of a single major compound or synergistic actions of group of compounds [119].

The root-bark extract of *Zanthoxylum nitidum* having efficacy against rice pest contains several alkaloids [120,121] out of which benzo[c]phenanthridine alkaloids (sanguinarine and chelerythrine) are reported to have the molluscicidal activity [122]. Alkaloid is a large group of plant secondary metabolites and its occurrence has been reported in approximately 20% of all plant species. Most of the alkaloids exert their effect on different region of the nervous system. Different neuroreceptors like alpha, serotonin, muscarinic, nicotinic acetylcholine receptors, adrenergic receptor and enzymes involved in synaptic transmission are some of the target sites of alkaloids. Basic molecular targets are DNA intercalation, protein biosynthesis and membrane stability [123].

The structures of phenolic allelochemicals and their mode of action are diverse [124]. In lepidopteran larvae, phenolic toxicity might occur in the form of oxidative stress [125]. However, *Heliothis virescens* larvae after feeding with high phenolic foliage exhibited improved total Trolox equivalent antioxidant capacity (TEAC) in haemolymph [126]. Therefore it has been proposed that the elevated foliar phenolics in some plants might have beneficial antioxidant properties for herbivorous insects. Flavonoids are known to inhibit cholinesterases [127] and might be responsible for insecticidal action [128]. Various forms of saponins and sterols derived from different plants have been reported to act as insect growth regulator [129].

7. The future of prospects of botanicals in seri-ecosystem

An important essence of integrated pest management is to consider the whole ecosystem as the management unit. A seri-ecosystem should necessarily include not only the sericulture field or the silkworm culture units, but also the agricultural field in the neighbouring areas. A consensus approach is to keep the natural ecosystem largely intact. While attempts are made to control pests and pathogens in the neighbouring agricultural fields, issues regarding their impact on sericulture activities must be taken into consideration or vice-versa. Emphasis is to be given on studies associated with efficacy of plant and plant products against pests and pathogens in the whole management unit, their mode and mechanism of action, identification of target sites and use of resistant strains. Life scientist and chemist need to act coherently to make effective use of botanicals. With the knowledge in hand we hypothesize that holistic approach involving in depth research for using botanical in both sericulture field and the other crop systems in the vicinity might be able to provide a green environment to the silk worms.

Author details

Dipsikha Bora and Hiren Gogoi

Department of Life Sciences, Dibrugarh University, Dibrugarh, Assam, India

Bulbuli Khanikor

Department of Zoology, Gauhati University, Guwahati, Assam, India

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Plants as Potential Sources of Pesticidal Agents: A Review

Simon Koma Okwute

Additional information is available at the end of the chapter

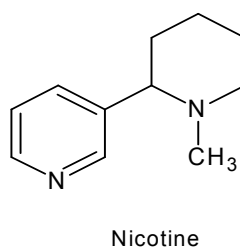
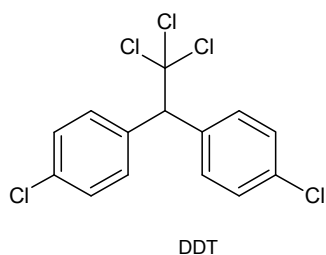
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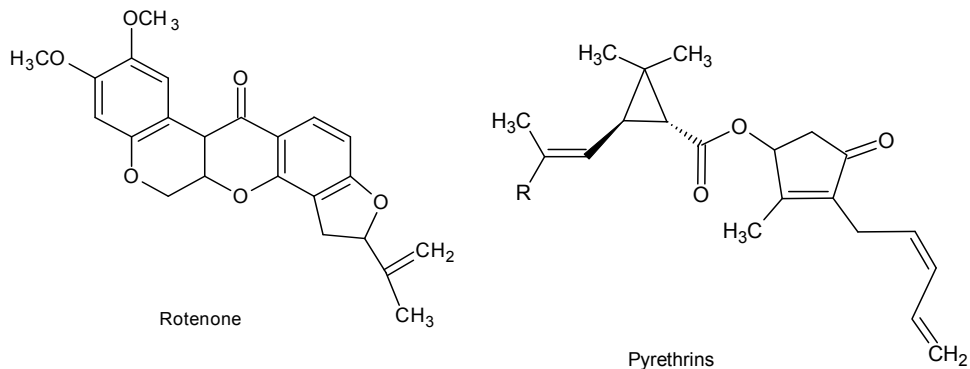
1. Introduction

For global food security, the agricultural sector of the world economy must achieve a production level that ensures adequate food supply to feed the increasing population as well as provides raw materials for the industries. This is particularly so as the energy sector is vigorously pursuing research into the use of grains and root crops as sources of starch for conversion into bio-fuels. Coincidentally, these crops (maize, rice, millet, *sorgum*, soybeans, cowpeas, sugarcane, groundnuts, e.t.c.) are the staple foods in most parts of the developing countries of the world such as Africa, South America and Asia. In addition to the above new development in the industrial utilization of these crops, they are frequently and vigorously attacked by *herbivorous insects* and other pests such as *phytopathogens* and mollusks. In fact the loss due to pests and diseases is about 35% on the field and 14% in storage, giving a total loss of about 50% of agricultural crops annually. Thus the world food production is adversely affected by insects and pests during crop growth, harvest and storage [1]. Apart from the farm environment insects and pests constitute serious menace in the home, gardens and bodies of water, and transmit a number of diseases by acting as hosts to some disease-causing parasites. Thus elimination of these insects and pests or mitigation of their activities will go a long way in reducing world food crisis as well as improve human and animal health.

Insects and other pests have been in existence since the creation of the universe, and of course man. The threat of insects and other pests such as mosquitoes, cockroaches, rodents, parasitic worms, pathogens and snails, has been well known and challenged by man. The ancient man had deployed different methods of control, including prayers, magic spells, cultivation systems, mechanical practices as well as application of organic and inorganic substances to protect his crops from the attack of weeds, diseases and insect pests [1].

Between 500 BC and the 19th century a number of substances classified as pesticides and defined as “any substances or mixture of substances intended for preventing, destroying, repelling or mitigating any pest” were used to control pests. They included sulphur, arsenic, lead and mercury [2]. In 1874 DDT (dichlorodiphenyltrichloroethane) was synthesized and during the second half of World War II its insecticidal activity was discovered and was effectively used to control malaria and typhus diseases among the troops. It became the first synthetic organic pesticide and was used after the war for agricultural purposes [3]. There is no doubt that the use of insecticides has contributed immensely to the increase in agricultural productivity and to the improvement in human health, particularly the eradication of malaria in the developed countries of the world in the 20th century and beyond [4]. However, it has been established that use of synthetic organic pesticides, particularly the chlorinated hydrocarbons such as DDT and derivatives has led to serious environmental pollution (water, air and soil), affecting human health and causing death of non-target organisms (animals, plants, and fish). This situation led to the Stockholm Convention in 2001 and the eventual ban of DDT in 2004 [5]. Before the ban efforts were already made by researchers for alternative sources of pesticides due to other reasons including (a) non-selectivity/specificity, (b) ineffectiveness, (c) not many of the synthetic compounds have been successfully marketed due to lack of interest by potential users, (d) high cost of synthetic chemicals and (e) development of resistance [6-7]. Natural products from plants have attracted researchers in recent years as potential sources of new pesticides. The folkloric use of higher terrestrial plants by the natives of various parts of the world as *pesticidal* and antimicrobial materials has been well known [8-9]. Perhaps, one of the early plants so recorded as *pesticidal* material was tobacco (*Nicotiana tabacum*). The use of tobacco leaf infusion to kill aphids led to the isolation of the alkaloid, *nicotine*, while the chemical investigation of the Japanese plant, *Roh-ten* (*Rhododendron hortense*) in 1902 showed *rotenone*, as the active constituent [10]. In this class of age-old *pesticidal* plants are species belonging to the genus *Chrysanthemum* found in Kenya and other highlands in Africa, which are the sources of the all purpose and very successful insecticidal extract, *pyrethrum*, and the active constituents, the *pyrethrins* [11].





Tens of thousands of natural products have been identified from plants and hundreds of thousands are yet to be isolated and screened for their bioactivities. This large reservoir of organic chemicals is largely untapped or under-tapped for use as pesticides. In this chapter the traditional applications of native plants as pesticidal agents and the results of biological and chemical studies on these plants in the past few decades are examined with a view to assessing their potential use in agriculture and related fields. The factors influencing efficacy, the advantages of and problems associated with the use of plant-based *pesticidal* products are also discussed.

The pesticidal agents that will be dealt with will include insecticides (insect killers including adults, ova, and larvae) insect *repellents*, *antifeedants*, *molluscicides*, *fungicides* and *phytotoxins* (herbicides). It must however be stated at this stage that although much work has been done in the past decades to show that indeed plants have the potentials to provide alternative and safe pesticides to replace the synthetic ones not enough work has been done in the area of identifying the active components. Whether or not it is very necessary to utilize pure constituents will be discussed later from the point of view of safety, cost and effectiveness (synergism). It is equally important to note that this review will be restricted to those plant-based pesticides that have the potential to be used as extracts (solutions), smoke or dust that have the potential of killing pests or their hosts or mitigating their effects. Consequently, although plant materials that act against worms that destroy crops of economic importance may be discussed, *anthelmintics* for intestinal worms in humans and other animals will not be included in the discussion.

2. Pesticidal plants

There is no doubt that a number of plants possess pesticidal activity and investigations by various research groups in different parts of the world have confirmed this. One of the most recent studies was the survey by Mwine *et al.* which established that thirty-four species belonging to eighteen families are used in traditional agricultural practices in Southern Uganda [12]. Also, Rajapake and Ratnasekera studied the toxicity of the ethanol extracts of the leaves of twenty plant species from different families to

Callosobruchus maculatus and *Callosobruchus chinensis*. It was observed that mortality reached a maximum level in 72 hours of exposure to the leaves oils which indicated a high level of lethality [13]. Similarly, Lajide *et al.* and Fatope *et al.* have investigated the protectant effectiveness of some plants native to Nigeria against the maize weevil, *Sitophilus zeamais* Motsch, and the cowpea weevil, *Callosobruchus maculatus* F, respectively[14-15]. On the basis of the results of pesticidal screenings it has been established that a number of plants have broad pesticidal activity and those commonly used in traditional agricultural applications in many parts of the developing countries, particularly in the tropical areas, are shown in Table 1 which are only representative but not exhaustive of the thousands of plants so far screened [13-16]. From various investigations it has been established that activity is usually distributed in most cases among the various parts of the same plant though the lethality and quantities of the active components may vary [13].

Having provided a background to the potential use of plant materials as pesticides we shall now look at efforts made in the last few decades by researchers to give us hope that if we return to the ways of our ancestors in combating pests by applying science and technology the terrestrial environment which is our home will be protected against the harmful effects of synthetic pesticides.

2.1. Insecticidal plants

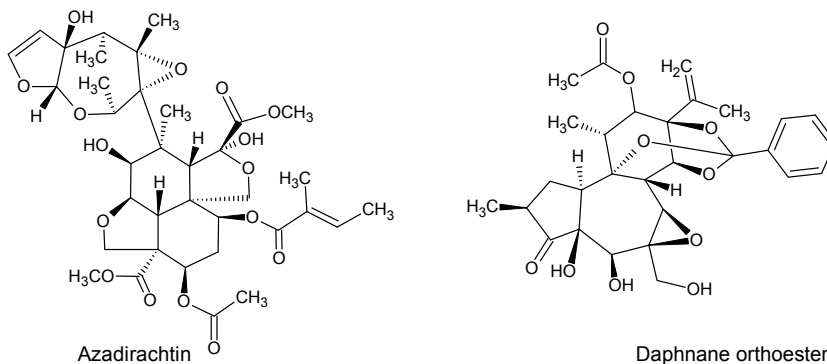
In the past decades, apart from the *pyrethrum* which has attained international and commercial acclaim due to its high effectiveness and broad spectrum insecticidal activity (repels and kills insects depending on concentration) very few natural insecticides have been developed. Of particular economic significance among the plants in common use today is the tropical plant *Azadirachta indica*, popularly known as the neem tree. In India as well as in Nigeria the plant is effectively used to control over 25 different species of insect pests. The activity has been associated with the presence of *azadirachtin*, which is said to be highest in the kernel than in the leaves and other tissues of the plant [1,13]. The effectiveness of nine insecticidal species of Chinese origin has been compared with synthetic insecticides against 40 species of insects. Three of the plants *Milletia pachycarpa* Benth, *Trpterygium Forrestii* Loes and *Rhododendron molle* G. Don were studied in detail. The finely ground powder when applied as spray in suspension or as dust were highly active against *aphids*, *pentatomids* and leaf-beetles as well as against *caterpillars*, body lice and plant lice. Among the plants *R. molle* displayed specific toxicity against certain species of *lepidopterous* larvae, *pentatomids* and leaf-beetles. The three plants were shown to contain *rotenone*, [17].

Investigation of the Sri Lankan plants showed that extracts of three plants, *Plearostylia opposita* (Wall) Alston (Celastraceae), *Aegle marmelos* Correa (Rutaceae) and *Excoecaria agallocha* (Euphorbiaceae) were insecticidal. For the first time three compounds possessing the *daphnane orthoester* skeleton, which are constituents of the ethyl acetate extract of *E. agallocha*, were found to be insecticidal[18-19].

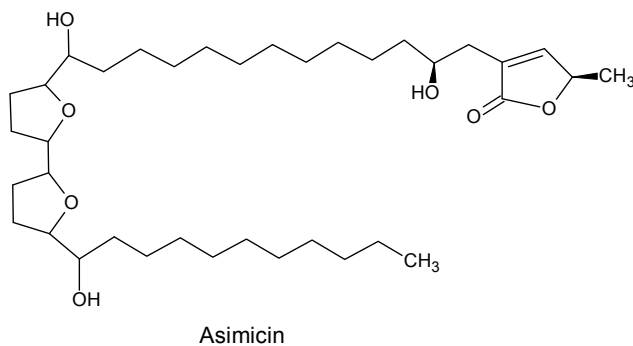
Species	Families	Parts
<i>Abrus precatorius</i> L	Fabaceae	L, S
<i>Allium sativum</i> L	Alliaceae	L
<i>Anacardium occidentale</i> L	Anacardiaceae	L
<i>Annona senegalensis</i> Pers.	Asteraceae	S, B
<i>Artemisia annua</i> L	Asteraceae	L, B
<i>Azadirachta indica</i> A. Juss	Meliaceae	L, B, R, F
<i>Balanites aegyptiaca</i> Linn Bel.	Zypophyllaceae	R
<i>Bidens pilosa</i> L	Asteraceae	L
<i>Cannabis sativa</i> L	Cannabaceae	L, S, F
<i>Capsicum frutescens</i> L	Solanaceae	F
<i>Carica papaya</i> L	Caricaceae	R, B
<i>Chrysanthemum coccineum</i> Wild	Asteraceae	L, F
<i>Clausena anisata</i>	Rutaceae	L, R
<i>Dalbergia saxatilis</i>	Fabaceae	L, B
<i>Dannettia tripetala</i>	Annonaceae	L
<i>Eucalyptus globules</i> Labill	Myrtaceae	L, B
<i>Gmelina arborea</i> Juss.	Verbenaceae	L
<i>Hyptis suaveolens</i> Poit.	Labiata	shoot
<i>Jatropha curcas</i> L	Euphorbiaceae	sap, F, S, B
<i>Khaya senegalensis</i> A. Juss	Meliaceae	S, B
<i>Lannea acida</i>	Anacardiaceae	B
<i>Lawsonia inermis</i>	Lythraceae	L
<i>Melia azadarach</i> L	Meliaceae	L, R, B
<i>Mitracarpus scaber</i> Zucc	Rubiaceae	shoot
<i>Nicotiana tabacum</i> L	Solanaceae	L
<i>Ocimum gratissimum</i> L	Limnaceae	L
<i>Parkia clappertoniana</i> Keay.	Mimosaceae	S, B
<i>Phytolacca dodecandra</i> L'Herit	Phytolaceae	L, F
<i>Piper guineense</i> Schum & Thonn	Piperaceae	F
<i>Piliostigma thonningii</i>	Caesalpinaceae	R, B
<i>Prosopis africana</i> Linn.	Mimosaceae	S, B
<i>Sphenoclea zeylanica</i> Gearth	Sphenocleaceae	shoot
<i>Tagetes minuta</i> L	Asteraceae	L
<i>Tephrosia vogelii</i> Hook	Fabaceae	L
<i>Vernonia amygdalina</i> L	Asteraceae	L

Key: L=Leaf, B=Bark, S=Seed, R=Root, F=Fruit

Table 1. Species, families, parts used and evaluated

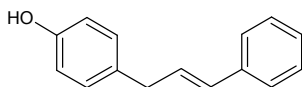


The pawpaw tree, *Asimina tribola* (*Annonaceae*), a plant found in various traditional communities, particularly in Africa and the Americas, has been investigated and found to possess *antitumor*, *pesticidal*, and *anti-feedant* properties. The pesticidal activity is known to reside in the seeds and bark and the focus has been on *asimicin*, which is the major bioactive component. It is active against blowfly larvae, *Calliphora vicina* Meig, the spotted spider mite, the *melon aphid*, the mosquito larvae (*A. aegypti*), the Mexican *bean beetle*, *nematodes*, and many pests of agricultural concerns [20].



Some of the investigations have revealed the mode of action of some of the plant products. N. M. Ba and co-workers have studied *Cassia nigricans* V., *Cymbopogon schoenanthus* S. and *Cleome viscosa* L from Burkina Faso for their insecticidal potentials and established that they were reasonably active and that they were most effective by inhalation. Consequently, such plants are not suitable for field applications. The plants however showed potent stomach and contact toxicity on 1st instars larvae irrespective of the crude extract and therefore good for cowpea protection in storage [21]. Similarly, Okwute *et al.* have demonstrated the *protectant* property of the powdered dry leaves of *Dalbergia saxatilis* against the cowpea *bruchid*, *Callosobruchus maculatus* and established that *oviposition* and damage to seeds was less and mortality higher with *D. saxatilis* as a contact poison than as a respiratory poison (Table2)[22]. It was also shown that the treated seeds were quite viable after the treatment with over 70% germination rate after 5 days exposure to planting (moist) conditions [22]. For

Dalbergia spp. the insecticidal activity against adult mosquitoes and houseflies has been demonstrated (Figure 1) and the activity has been attributed to the presence of *cinnamylphenols*, [23].



Cinnamylphenol

The genus *Piper* (family Piperaceae) is probably one of the most studied. With over 1000 species, about 112 genera have been screened for *pesticidal activity* and over 611 active compounds have been isolated and identified from various parts of the species [24]. Perhaps, of great significance are extractives from *Piper guineense*, *Piper longum*, and *Piper retrofractum* which are known to be active against *Callosobruchus maculatus*, the garden insect, *Zonocerus variegatus* L, and the mosquito larvae causing 96-100% mortality rate in 48 hours mostly as solution sprays [25-26]. From the chloroform and petroleum extracts of *P. guineense* fruits were isolated two *Piper amides*, *guineensine*, and *piperine*, having terminal isobutyl and piperidyl basic moieties, respectively. In these experiments *piperine* was shown to be a synergist rather than an insecticide in the crude extracts. The significance of this co-occurrence in the efficacy and efficiency of crude drugs and bio-pesticides will be discussed later. In an effort to enhance the insecticidal activity of the *piperine amides* some workers have embarked on structure- activity relationships (SAR) studies and have come to the conclusion that the piperonyl group does not influence activity and that the isobutyl group does not confer any special advantage as previously reported [27]. However, using *piperine* (95% mortality) as a template pesticide and replacing the piperidyl group gave a higher insecticidal activity (97.5% mortality) with N-diethyl moiety than the isopropyl analogue (95% mortality) against *Aedes monuste erseis* [28].

Treatment (gm)	No. of eggs laid on seeds	No. of damaged seeds	Insect mortality	% Germination
2.00	3.2 ±0.84	0±0.00	10.0±0.00	83.3
1s.75	3.4±0.55	0±0.00	10.0±0.00	91.7
1.50	3.6±0.95	0±0.00	10.0±0.00	83.3
1.25	3.8±1.00	0±0.00	10.0±0.00	75.0
1.00	4.8±1.64	0±0.00	10.6±0.89	100
0.75	5.8±1.48	0±0.00	12.0±0.71	100
0.50	10.6±2.70	9.8±1.30	14.0±1.92	100
0.25	16.4±3.11	10.8±1.95	0.0±0.00	91.7
0.0g(Control)	17.0±3.16	32.0±3.49	0.0±0.00	91.7

Values are means of 5 replicates

Table 2. Evaluation of *protectant* potentials of *Dalbergia saxatilis*

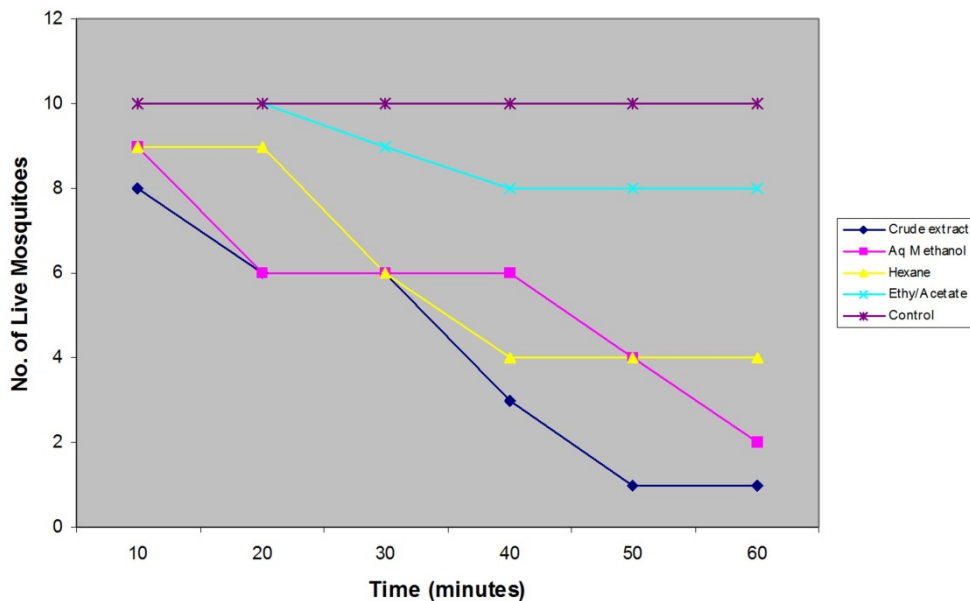
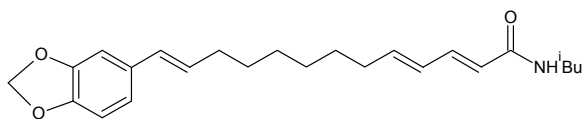
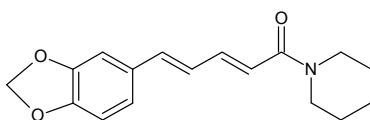


Figure 1. Mortality rate of mosquitoes exposed to 0.2% solutions of the crude extract and fractions of *Dalbergia saxatilis*



Guineensine



Piperine

A new class of insecticides was recently discovered by Beltsville researchers led by Puterka in the U.S.A. that offers a safe and effective alternative to commercial insecticides. They are polyesters of sugars and include *sucrose* and *sorbitol octanoates*. They were isolated from the poisonous hairs on the tobacco leaves which hitherto were assumed to contain nicotine, a popular insecticide. When insects were contaminated by rubbing they caused death of the insects by a dehydration process, and rapidly degraded to harmless sugars and fatty acids. These polyesters are known to be effective against a variety of farm and domestic insect pests and the deadly parasitic *Varroa* mite which usually settles on the back of honey bees [29].

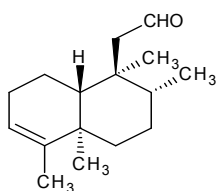
2.2. Repellent and anti-feedant plants

Closely related to the insecticidal agents and sometimes used in combination with insecticides in pest management strategies are some classes of pesticidal agents with interesting and peculiar biological activities. They include insect *repellents*, *anti-feedants* or *deterrents*, and *attractants*. These classes are far less common in plant sources than the insecticides but will be given some attention. Sometimes, a given insecticide may act as an insecticide or as a *repellent* depending on the concentration. The major difference between the two is that a repellent does not kill insects but keeps them away by exuding pungent vapours or exhibits slightly toxic effects [13]. By these activities a *repellent* prevents insects from perching or landing on the surfaces of targets. Thus *repellents* can be used to prevent and control the outbreak of insect borne diseases such as malaria. The insects of interests in this regard include mosquito, flea, fly, and the *arachnid tick*. [30]. The use of plant materials as *insect repellents* is increasingly receiving attention, particularly in the developing countries. For example Seyoun *et al.* reported that in Western Kenya the natives employ direct burning of the species *Ocimum americana* L, *Lantana camara* L, *Tagetes minuta*, and *Azadirachta indica* A. Juss against the malaria vector, *Anopheles gambiae* S.S.Giles [31]. Some recent studies on *repellent* plants have led to the isolation and characterization of some active components. Prominent among these compounds are *callicarpenal*, and *intermedeol*, from the species *Cymbopogon nardus* which showed promising alternative in the control of infestations by *Amblyomma cajennense* [32]; *nepetalactone*, a *catnip* compound for the control of the Asian adult male and female Lady beetle as well as cockroaches, flies, termites and mosquitoes [33-34]; and *geraniol*, and *p-menthane-3,8-diol* (PMD), monoterpene alcohols from the *citronella* and *lemon oils*, respectively [35]. Some researchers have found that products containing 40% *lemon eucalyptus oil* are as effective as products containing high concentrations of *DEET* and that *neem oil* can give up to 12 hours protection against mosquitoes in cage experiments [36-37].

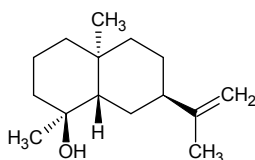
Literature on the direct production of chemicals with specific activity to act as insect *anti-feedants* is very scanty probably as *anti-feedancy* and *repellency* are closely related bioactivities. However, a number of plants produce *polyphenols* called tannins which confer astringency or bitter taste on such plants and consequently herbivores stay away from eating such plants [38]. Among the few plants studied for feeding *deterrence* or *anti-feedancy* the species *Xylopiya aethiopica* is very significant. The hexane and methanol extracts of the fruits and seeds have been shown to possess strong termite *anti-feedant* activity and *ent-kauranes* and some *phenolic amides* have been implicated. Among the *ent-kauranes* the activity was significantly dependent on the structures and that (-)-*kau-16-en-19-oic acid*, had the strongest anti-feedant activity [39]. Another species with promise is *Jatropha podagrica* cultivated in West Africa. The organic extracts showed reasonable *anti-feedant* activity against *Chilo partellus*, the maize stem borer, at concentrations of 100 %/leaf disc, the chloroform extract being the strongest. The most active compound isolated was *15-epi-4E-jatrogrossidentadione*, [40].

Attractants are *semio-chemicals* produced usually by some insects with effect on other insects as a communication tool and can be used to determine or control insect populations,

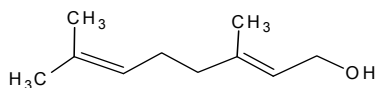
particularly by disrupting their mating patterns. Rarely do plants produce chemicals that attract insects that are natural enemies of other insects that feed on the plants except the tea tree [41]. Thus field application of this phenomenon is not common and therefore will not be discussed further.



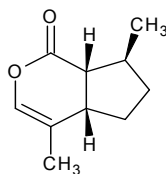
Callicarpenal



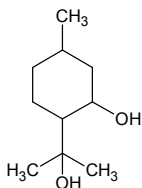
Intermedeol



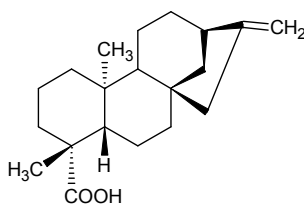
Geraniol



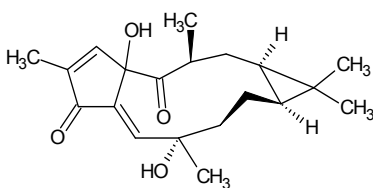
Nepetalactone



p-Menthane-3,8-diol(PMD)



(-)Kau-16-en-19-oic acid



15-epi-4E-jatrogrossiden-tadione

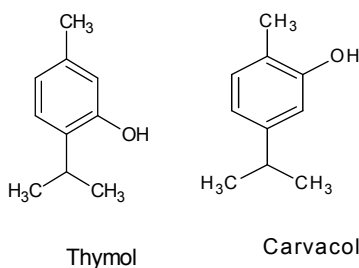
2.3. Fungitoxic plants

Plant diseases, particularly fungal infections, contribute significantly to agricultural crop losses globally. Research has been on to utilize botanicals in plant disease control worldwide and extracts from many plant species have been found to be active against many *phytopathogenic fungi* without imposing ill side effects[42]. In some cases the active components have been identified and tested directly. The results so far are quite encouraging and some are discussed in this chapter.

Many plants produce *essential oils* as secondary metabolites but their exact role in the life processes of the plants has been unknown. There is however no doubt as revealed in this survey that the results of various investigations have overwhelmingly implicated essential oils of many species as possessing *fungitoxic activity*. They are therefore agents of protection in plants against diseases. Consequently, since the leaves, resins, and latices of plants contain essential oils more commonly than other parts of plants they have been more commonly investigated for *fungitoxicity* [43-44].

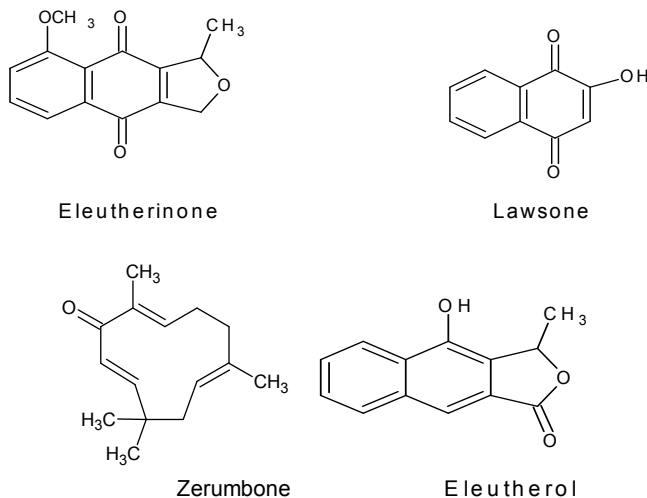
Typical studies included the investigation of the essential oil of the leaves of *Phenopodium ambrosioides* which has been shown to exhibit strong fungitoxic activity against mycelia growth of *Phizoctonia solani*, the causative organism of damping off disease of seedlings, at 1000 ppm without any phytotoxicity on the germination and seedling growth of *Phaseolus aureus*[45]; the activity of the steam-distillate and hot-water extracts of fresh leaves of *Cymbopogon*, *Ocimum gratissimum*, *Chromoleana odorata* and fruits of *Xylophia aethiopica* against *Ustilago maydis*, *Ustilaginoidea virens*, *Curvularia lunata*, and *Phizopus spp*, reducing growth by 10-60%[46]; the screening of the leaves of 30 angiospermic taxa against *Pythium aphanideratum*, *P. debaryanum* with *Hyptis suaveolens*(Labiatae), *Murraya knoenigii*(Rutaceae), and *Ocimum canum*(Labiatae) which displayed strong toxicity at 43-86% inhibition in soils infected with *P. debaryanum* [47]; the use of *Ocimum gratissimum* and *Eucalyptus globules* water extracts to control cowpea seedling wilting induced by *Sclerotium rolfsil* from 39.6% for untreated to 4-12% for treated[48]; and the tomato fruit rot, which is commonly observed in local markets in many parts of Africa, can be significantly reduced with the extracts of a number of local plants such as *Cassia alata*, *Alchornea cordifolia* and *Moringa oleifera* as post-harvest agents[49]. Of particular interest and importance is the availability of some species such as the popular neem tree (*Azadirachta indica*) and the pawpaw leaves extracts which are known to act against the yam rot. Yam is an important tuberous food crop of the tropical South America and Africa where both plants are found commonly around villages and within family compounds. The pawpaw leaves extract at the various concentrations of 20, 40, 60, and 80% were found to be more active than the neem against *Alternaria solani* [50].

Efforts have been made by some researchers to investigate the active constituents of some of the fungitoxic plants. The constituents of the essential oils of 9 Turkish species including *Thymbra spicata* were investigated using GC-FID technique. At least 20 components were identified and the activity was attributable to the presence of phenolic agents such as thymol, and carvacol, [51].



In other studies the fungitoxic chloroform extract of the underground parts (bulbs) of *Eleutherine bulbosa*(Miller) Urban(Iridaceae) gave 4 compounds of which three naphthaquinones, including *eleutherinone*, were active at 100µg/ spot(bioautography). The fourth compound, *eleutherol*, which lacked the quinone moiety was not active, showing the strategic role of this group in the bioactivity of the series[52]. The relationship between fungitoxicity and the quinone skeleton is also exhibited in the broad spectrum fungitoxicity of *lawsone*,

isolated from the leaves of *Lawsonia inermis*, against eight different *phytopathogenic fungi*(Table 3) [53]. Working on the rhizomes of *Zingiber cassumunar* N. Kishore and R.S. Dwivedi isolated the *fungitoxic* and *non-phytotoxic monocyclic sesquiterpene*, *zerumbone*, which was active at 1000 ppm against *Rhizoctonia solani*, a *damping-off pathogen*[54][55].



<i>Fungi</i>	PPM of <i>lawsone</i>		
	1000	2000	4000
	Inhibition %		
<i>Alternaria solani</i>	60	100	100
<i>Alternaria tenuis</i>	100	100	100
<i>Aspergillus niger</i>	65	100	100
<i>Aspergillus wanti</i>	100	100	100
<i>Absidia ramosa</i>	100	100	100
<i>Absidia orymbifera</i>	100	100	100
<i>Absidia</i>			
<i>crophalophora</i>	100	100	100
<i>fusispora</i>			
<i>Circinella umbellate</i>	84	100	100

Table 3. *Fungitoxicity* measured as % inhibition of *lawsone* against eight different fungi (% inhibition)

2.4. Molluscicidal plants

Biharzia affects millions of people, particularly children who play or swim in infected freshwaters in the developing countries of Africa, Asia and Latin America. The disease was discovered in 1851 by Theodor Bilharz as the cause of urinary *schistosomiasis*. It is associated with certain species of aquatic snails of the genera *Biomphalaria*, *Bulinus* and

Oncomelania. Therefore, one way of attacking the disease is to eliminate the host snails [56-57]. Chemicals that kill snails are called *molluscicidal agents*. Most of the *molluscicidal agents* in use today are synthetic and like most synthetic pesticides are harmful to man and the environment. *Molluscicidal agents* of natural origin are important in the widespread control of *Schistosomiasis*. Mirazid, an Egyptian drug from *myrrh* was being developed as an oral drug until 2005 when it was found to be only 8 times as effective as *praziquantel*, a synthetic chemical, and has therefore not been recommended by WHO. However, other plants have been studied and some have demonstrated potential activity which may provide leads for future drugs but more importantly are the searches for molluscicidal agents from plants to eliminate the host snails. This is the focus of the presentation in this chapter.

Adesina and Adewunmi, and Kloos and McCullough, have separately investigated the species *Clausena anisata* and found it to possess *molluscicidal activity* which is distributed among the root, leaves, bark and stem in a decreasing order of potency [58-59]. Adedotun and Alexander evaluated the *molluscicidal activity* of the aqueous and ethanolic extracts of fruits and roots of *Dalbergia sissoo* against the egg mass and adults of *Biomphalaria pfeifferi* and found that only the ethanolic extracts showed significant activities. Thus ethanol extracts the active constituents more than water [60]. Similar observations have been recorded for *Clausena anisata* parts and *Tetrapleura tetraptera* fruits, particularly when the active components are *glycosides* (Table 4) [58,61].

<u>extracts</u>			
<u>Plant parts</u>	<u>Concentration</u>	<u>% Mortality</u>	<u>Solvent</u>
<i>Clausena anisata</i>			
Root	6-10 ppm	100	Methanol
Leaves	1000 ppm	53.3	Water
Stem	1000 ppm	7	Water
Bark	1000 ppm	40	Water
<i>Tetrapleura tetraptera</i>			
Fruit	100%	100	Water
Fruit	10%	100	Methanol

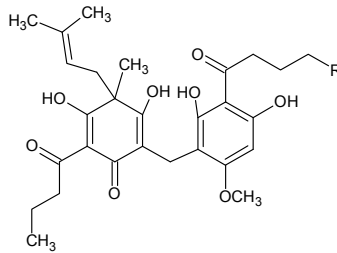
Table 4. Molluscicidal activity of *Clausena anisata* and *Tetrapleura tetraptera*

Investigation of the extracts of the Argentine collection of the fern *Elaphoglossum piloselloides* led to the isolation of two new *bicyclic phloroglucinols* which showed acute *molluscicidal activity* against the *Schistosomiasis* vector, *Biomphalaria peregrina* [62]. Other phytochemical and biological investigations have implicated *jatrophone*, as one of the *molluscicidal agents* in the active crude ethanol extract of *Jatropha elliptica*, while a *monodesmosidic saponin*, and *thujone*, have also been identified as the active constituents of the bark powder of *Saraca asoca* and the leaf powder of *Thuja orientalis*, respectively, against the freshwater snail *Lymnaea acuminata* [63-64]. Finally, the *molluscicidal properties* of the leaves of *Alternanthera sessilis*, a plant found in West Africa, have been investigated and confirmed. The effect of heat on the stability of the product has been determined by comparing the activities of the unevaporated and evaporated

aqueous extracts which showed that the unevaporated has higher activity than the evaporated and the fresh leaves higher than the dry leaves (Table 5)[65].

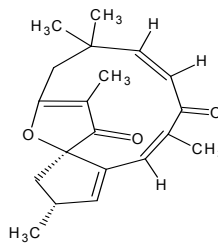
<i>Molluscicide</i>	<i>LC50 and limits(mg/ml)</i>
Crude unevaporated fresh leaves extract	32.57(25.15-39.08)
Crude unevaporated dry leaves extract	40.42(35.15-46-47)
Crude evaporated fresh leaves extract	43.57(38.38-49.46)
Crude evaporated dry leaves extract	48.07(42.81-54.28)

Table 5. Expected effective lethal concentrations of *A. sesselis* extracts of dry and fresh leaves on adult *B. globosus*.

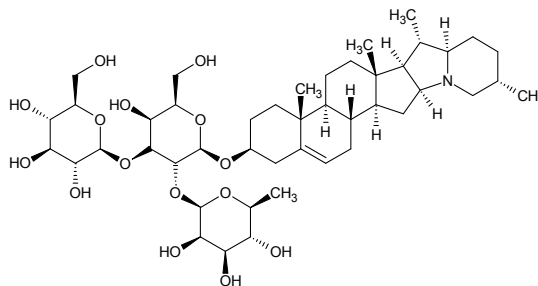


Phloroglucinol I, R=H

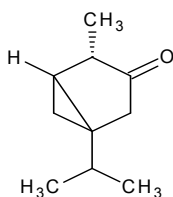
Phloroglucinol II, R=Et



Jatrophone



Monodesmosidic saponin

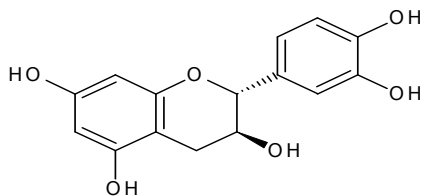


Thujone

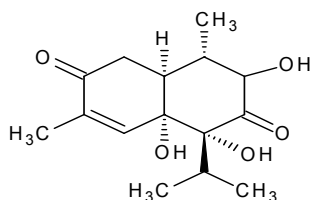
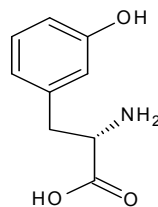
2.5. Herbicidal plants

Apart from insects and diseases disturbing crop plants and animals on the farm and in the environment weeds need also to be controlled because they retard plant growth and therefore reduce crop yields. *Herbicides*, also commonly known as *weed-killers*, are pesticides used to kill unwanted plants. *Selective herbicides* kill specific targets while leaving the desired crop relatively unharmed. Some of these act by interfering with the growth of the weed. Some plants produce *natural herbicides*, and such action of *natural herbicides* (interfering) is called *allelopathy*. Herbicides are widely used in agriculture and in landscape turf management.

The plant *Centaurea maculisa* provides a good example of *allelopathy*. The root secretes (+) and (-) *catechins*, but it is (-) *catechin* which is phytotoxic and accounts for the invasive behavior in the rhizosphere [66-67]. The phenolic root exudate of Buckwheat (*Fagopyrum esculentum*) has been studied using HPLC and GC-MS and *palmitic acid methyl ester* and a *gallic acid derivative* have been implicated as the active constituents [68]. *Allelopathic properties* have also been found among some *terpenoids*. Investigation of the aerial part of *Eupatorium adenophorum* led to the isolation of eleven components of which *5,6-dihydroxycadinan-3-ene-2,7-dione*, was the only active herbicidal compound [69]. It has been observed that certain varieties of common fescue lawn grass come equipped with their *natural broad-spectrum herbicide* that inhibits the growth of weeds and other plants around them. A group of Cornell researchers led by Frank Schroeder has identified the *natural herbicide* to be the *amino acid, m-tyrosine*, and that the grass exudes the compound from the roots. The compound is toxic to plants but not to fungi, mammals or bacteria. The major drawback is its high solubility in water, making it ineffective if applied directly as a herbicide in the field [70]. On the other hand the *spotted knapweed plant* spreads over large areas because it releases *catechin* through its roots into the soil that kills the surrounding plants. Unlike *m-tyrosine*, *catechin* is quite stable and does not kill certain species of grass and grass-like plants like wheat. Therefore, it can be sprayed or added to soil to maintain lawns and wheat fields and is environmentally friendly [71]. Thus it has great potentials as experiments have shown that it is as effective as 2,4-D against weeds, kills weeds within one week of application and ordinary tapping of the leaves activates the plants' chemical response [71].



(-) Catechin

5,6-dihydroxycadinan-
3-ene-2,7-dione

m-Tyrosine

3. Commercial botanic pesticides

Plant-based pesticides (botanic pesticides or botanicals) have been in use as pesticides for over 150 years. It was only very recently that the synthetic insecticides effectively became the prominent agrochemicals for controlling all forms of agricultural pests and have assumed a very important position in the marketplace. However, in the past three decades so much has been reported in literature in respect of natural products that were identified with potent pesticidal activity such as feeding detergency and toxicity to insects in laboratory assays. In spite of the above success not many of the isolated compounds or the crude material (extract or dust) have really found the market due to regulatory procedures associated with product development, particularly in the United States of America. For example, in the past twenty years probably only very few new sources of botanicals have been developed to the commercial status. Thus the four major commercial products today include the *pyrethrum/pyrethrins*, *rotenone*, *neem* and the *essential oils*. Three others in limited use or importance include *ryania*, *nicotine* and *sabadilla*, while *garlic oil* and *Capsicum oleoresin* are relatively new extracts [72]. *Botanical pesticides* are processed in various ways, principally (a) as crude plant material in the form of powder or dust; (b) as extracts from plant resins, formulated into liquid concentrations; and (c) as pure isolated constituents by extraction/chromatographic techniques or hydro-distillation of the plant tissue, particularly the leaves. For a pesticide to be considered safe for use and be registered as a commercial product, the LD_{50} , the term used to describe the lethal dose required to kill 50% of the test animals expressed in milligrams (mg) per kilogram (kg) of body weight, must be determined. Technically, the lower the value the more toxic the sample is to

mammals. Although botanical pesticides are generally considered safer than their synthetic counterparts, some have much lower LD₅₀ than standard synthetic insecticides like *carbaryl* and *malathion*. The *pesticidal* characteristics of some of the current *commercial botanicals* are outlined below and summarized in Table 5. [73-74].

The *pyrethrins*, account for about 80% of global use of botanicals. Kenya is the major supplier, followed by Tanzania and the Botanical Resources Australia. The material is highly degradable under sunlight, oxygen and moisture. It therefore requires frequent applications. Its activity is usually enhanced by incorporating *piperonyl butoxide*(PBO) as a synergist. It acts against a wide range of pest. *Rotenone*, on the other hand, is available mainly from *Lonchocarpus* spp and the *Derris* spp. found in East Indies, Malaya and South America (Venezuela and Peru). It is obtained by solvent extraction to yield resins containing about 45% *rotenoids* of which the major component, *rotenone*, is 44%. Its activity and persistence are comparable to DDT and it is used to protect lettuce and tomato crops. It is slower acting than any of the *botanicals* currently in use and yet readily degradable, taking several days to kill insects. The *neem* product has become popular commercially in recent time because of its broad spectrum activity and low mammalian toxicity. There are two neem-based products, the first being the neem oil from the cold pressing of seeds for the management of *phytopathogens* while the other product is medium- polarity extract containing the potent compound azadirachtin,⁵ (0.2-0.6% of seed/weight). The actual commercial product is a 10-50% concentration using solvents. Although it has a half-life of about 20 hours its systemic action on foliage ensures reasonable persistence in field applications.

The *essential oils* are products of steam distillation of aromatic plants, mostly of the family *Lamiaceae*, giving *monoterpenoid phenols* and *sesquiterpenes*. Examples are *thymol* and *carvacol*. They possess high volatility and therefore are not suitable for field applications but appropriate for stored grains. The essential oils are components of many commercial foods and beverages and are therefore more readily approved for use without going through the rigorous regulatory procedures even in the USA.

The other botanicals, though not very important commercially have some advantages in applications [74]. They include the following:

- a. *Ryania* has a low mammalian toxicity but has the longest residual activity, providing up to two weeks of control after an initial application. It works best on caterpillars and worms but also kills a number of other pests with the exception of spider mite.
- b. *Nicotine*, a constituent of *Nicotiana tabaccum*, is the most toxic of all botanicals and extremely harmful to humans. It is a very fast-acting nerve toxin and is most effective on soft-bodied insects and mites.
- c. *Sabadilla* is available from the seeds of the plant *Schoenocaulon officinale* which is cultivated in Venezuela. It is one of the least toxic of the botanicals. It is toxic to honeybees, caterpillars and leafhoppers as well as beetles.

From the above it is clear that a serious drawback to the commercialization of botanicals is the high cost of processing plant materials to meet World Health Organisation(WHO) and Food and Agriculture Organisation(FAO) safety standards.

Plant Name	Product/Trade Name	Group/Mode of Action	Targets
1. <i>Lonchocarpus spp</i> <i>Derris elliptica</i>	Rotenone	Insecticidal	Aphids, bean leaf beetle, cucumber beetles, leafhopper, red spider mite
2. <i>Chrysanthemum cinerariaefolium</i>	Pyrethrum/Pyrethrins	Insecticidal	Crawling and flying insects such as cockroaches, ants, mosquitoes, termites
3. <i>Nicotiana tabaccum</i>	Nicotine	Insecticidal antifungal	Aphids, thrips, mites, bugs, fungus gnat, leafhoppers
4. <i>Azadirachta indica</i> [<i>Dogonyaro</i> (Nigeria)]	Azadirachtin/Neem oil Neem cake Neem powder Bionimbecidine(GreenGold)	Repellent Antifeedant Nematocide sterilant Anti-fungal	Dandruffs(shampoos) eczema, nematodes, sucking and chewing insects(caterpillars, aphids, thrips, maize weevils)
5. Citrus trees	d-Limonene, Linalool	Contact poison	Fleas, aphids, mites, paper wasp, house cricket, dips for pets
6. <i>Shoenoecaulon officinale</i>	Sabadilla dust	Insecticidal	Bugs, blister beetles flies, caterpillars, potato leafhopper
7. <i>Ryania speciosa</i>	Ryania	Insecticidal	Caterpillars, thrips, beetles, bugs, aphids
8. <i>Adenium obesum</i> (<i>Heliotis sp</i>)	Chacals Baobab(Senegal)	Insecticidal	Cotton pests, particularly the larvae of ballworm

Table 6. List of some commercial botanical pesticides

Given the large number of plants traditionally used as *pesticidal agents* by various local communities globally, particularly in the developing countries, the number of plants so far investigated and the products developed from them, the impact on agricultural production from this source is very insignificant. Therefore, there is need for more plants to be harnessed for use in agriculture and related fields. However, there is need to examine the modalities for their utilization, particularly with respect to consistency of constituents as well as efficacy and quality of the products, vis-à-vis the production of bioactive plant-based products using western models or utilize the plants according to traditional procedures that eliminate purification. For example, the *anti-sickle cell anaemia* drug, *NICOSAN* has been found to be less potent and more toxic on separation into individual components [75]. Thus, there are some advantages in the traditional procedures of preparing herbal products in a manner that preserves the constituents of the plants and hence enhances synergism and potency. However, while appreciating the low cost of production of *botanic products* by

eliminating sophisticated purification and formulation procedures, a middle of the road approach that ensures consistency of active constituents and enhances efficacy and safe delivery is necessary. This may be achieved by using bioassay-guided fractionation which has been shown by some workers to ensure that bioactive compounds of the same chemical class in a crude plant extract are consistently pooled together. The procedure has been shown to improve activity dramatically and has been used to obtain active compounds from plants that were previously considered to be inactive [76].

Thus, cheap plant-based bioactive products may be prepared with improved efficacy if processed using bioassay-guided fractionation of the crude extract and classified as *orphan pesticide* as is sometimes done in drug development. The content of the identified components can be used to standardize the crude pesticide as *gedunin* has been proposed for crude *neem-based antimalarial drugs* [77]. It is only in this way will the abundant plant-based natural resources of the developing countries be readily and cheaply made available for agricultural production without polluting the environment.

4. Conclusion

The results of *pesticidal* and *phytochemical screenings* of a number of higher plants based on traditional knowledge strongly indicate that plants are endowed with *pesticidal* properties that can be harnessed cheaply for use in agriculture and related fields. The need to use plant-based products arises from the fact that the synthetic pesticides are harmful to humans, and the entire ecosystem due to high toxicity and persistence. Also, they are too expensive for the poor farmers in the developing countries of the world. On the other hand, plant-based products are cheap and bio-degradable and are therefore environmentally friendly. However, an agricultural programme that depends essentially on plant-based materials must be backed-up by a vigorous research programme into new plant sources. As revealed in this review traditional knowledge has so far guided studies on possible active plants and the results have overwhelmingly confirmed the activity of a reasonable percentage of the plants. The results have equally established that plants belonging to certain families of plants are more likely to possess *pesticidal* activity. Thus, these results will serve as useful guides in the collection of plants for laboratory and field research studies.

One area of difficulty in laboratory research studies is the *bioassay* of plant extracts. It has been established that certain crude extracts contain active components but may appear inactive in primary screens due to *antagonistic actions* of the constituents. Such problems may be overcome, particularly in screening against plant pathogens, by the application of *bio-autographic techniques*. Associated with the detection and the determination of level of activity in crude extracts is the appropriateness of the solvent for the extraction of plant materials. Use of a less desirable solvent can lead to low extract activity due to low concentration of the active principle. For example, aqueous alcoholic extracts have been found to be more active than aqueous extracts as most of the active compounds are *lipophilic* in character and are therefore more readily extracted into an organic medium.

For the poor countries it would be more expensive to use the plant extracts or the pure constituents than the plant powder or dust in large-scale field applications. Crude extracts can however be cheaply used if a readily available solvent such as water is the solvent of choice. Use of extracts also allows for easy dosage calculation and spraying applications which need to be done repeatedly due to high volatility of plant-based pesticidal products. The efficacy of such products can be enhanced by *bioassay-guided fractionation* which is known to concentrate activity and promote synergism between structurally related constituents.

Obviously, in large-scale field utilization of botanic agricultural chemicals there must be adequate and constant supply of candidate plants to the areas in need. This means that since plants grow well usually in areas of natural habitat effort should be made to invest in large-scale cultivation of such plants in their various localities as is the practice in China, Japan and Kenya. This will be of great economic advantage in the developing countries as such programmes can lead to economic empowerment of the poor farmers and ultimately improve the economies of these countries.

Author details

Simon Koma Okwute

Department of Chemistry, University of Abuja, Gwagwalada, Federal Capital Territory, Abuja, Nigeria

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Neem Crude Extract as Potential Biopesticide for Controlling Golden Apple Snail, *Pomacea canaliculata*

Rosdiyani Massaguni and Siti Noor Hajjar Md Latip

Additional information is available at the end of the chapter

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1. Introduction

Paddy is the staple food of more than 60% of the world population, and mainly produced and consumed in the Asian region which over 90% of the crop grown in Asia. In Malaysia, the cultivation of paddy rice have covers 204,246 ha area of land and principally planted in the eight granary areas; Muda Agricultural Development Authority (96,558 ha), Kemubu Agriculture Development Authority (32,167 ha), Kerian Sungai Manik Project (27,829 ha), Northeast Selangor Project (18,482 ha), Penang Integrated Agricultural Development Project (10,305 ha), Seberang Perak (8,529 ha), Kemasin Semerak Integrated Agricultural Development Project (5,220 ha) and North Terengganu Integrated Agricultural Development Project (5,156 ha) [1]. Most of the paddy in Malaysia is planted as wet paddy, while dry land paddy is very small acreage, and mostly in Sarawak and Sabah. The continuously increasing population caused the increase of the volume in production of rice is an immediate requirement in Malaysia, in order to attain self-sufficiency in food. Since the possibility of extending area under cultivation has practically been exhausted, the only alternative is to enhance crop productivity per hectare. However, achieving this task seems impossible due to various obstacles. One of the main problems is the paddy field has been destroyed by many weeds and pests, such as insects, birds, rodents, and snails especially golden apple snail.

The golden apple snail is a freshwater mollusc that native from Northern Argentina and Southern Brazil [2]. The intention of its introduction in Asia in early 1980s, it has been considered for use as an aquaculture species that provide dietary high-protein supplement for local consumption and as an income earner for the rural poor [3]. Unfortunately, the low market value due to the unexpectedly poor consumer reception resulted in the elimination of its existence in Taiwan [4]. Nevertheless, many snail-farming projects were abandoned

and the golden apple snail escapes into irrigation ditches and the natural waterways, and subsequently it invaded the rice fields. The initial introduction is thought to have been from Argentina to Taiwan, but by 1982, the golden apple snail had been introduced from Taiwan to the Philippines and continued to China (1985), Korea (1986), Sarawak and Peninsular Malaysia, Malaysia (1987), Java and Sumatra, Indonesia (1989), Thailand (1989), Vietnam (1989) Hong Kong (1991), Laos (1992), and Cambodia (1995) [5]. The golden apple snail has been reported as an important pest of paddy rice in all of these countries and the damage is clearly sufficiently serious to warrant major concern.

In Malaysia, reference [6] was reported that this golden apple snail was firstly presented to be the caused of rice field's damage in 1992 when it was first seen in Keningau, Sabah. After a year from the first invasion, it devastated to Selangor state with an estimated damage about 48 ha of irrigated rice. The golden apple snail was then spread rapidly to other rice growing areas which able to establish easily and rapidly in wet lowland areas where as the water is stagnant and shallow. Once this pest infested in new areas, it was difficult to manage due to its biological and morphological characteristics [7]. According to reference [2], the characteristic possess by golden apple snail such as high fecundity, voracious appetite, fast growth and reproduction, and able to aestivate in soil during dry season have led to its rapid multiplication and widespread distribution. A female snail start to lay eggs at two months old and can laid 50–500 eggs per cluster at one time, with 80% hatchability rate and the incubation ranges from 10 to 15 days characteristics [7]. An adult of golden apple snail can live up to 3 years with the size up to 3 cm in height. In addition, it also has a gill and a lung-like organ which make it being able to survive in and out of water. It also can withstand drought for several months by closing its operculum and aestivate in the soil.

Golden apple snail was reported as a major and serious pest in paddy field as it can caused severe damages by completely eliminate the young leaf and stem from plant bases which will result in the death of damaged plants [5,8]. It cuts the base of young seedlings with its layered tooth (radula) and munches on the succulent tender sheath of rice. The damage intensity of the infestation are depends on snail density and size and the growth stage of the rice plant [9]. Reference [10] reported that crop stand was reduce by over 90% when a density of golden apple snail is 8 per m² with the size of snail is from 10 to 40 mm. The golden apple snail are most damaging to young rice seedlings which is up to 15 days after transplanting because the young, tender leaves and stems favour the snail's feeding habits [11]. This snail is a nocturnal herbivore which unlike as other species of slugs of water and land, where it has a highly voracious appetite. Reference [12] has stated that it can consume a blade of rice in just 3 to 5 minutes. They can even consume the young seedling in a whole field overnight and the obvious signs of severe damage are characterized by missing hills and floating fragments of rice plants. It can destroy newly transplanted or direct-seeded rice as long as there is water in the field.

Therefore, the successful establishment and invasion of the golden apple snail in irrigated rice systems have led to significant economic damage. Farmers in the infested areas are faced with the options of paying additional costs to control the spread of golden apple snail, replanting damaged areas of paddy, or ignoring the problem all together at the risk of

potentially large yield losses. The economic analysis on yield losses and severe damage cause by golden apple snail was reported by [10] and [13]. Reference [10] reported the yield loss of rice by golden apple snail in Philippines for year 1990 was at 70,000 to 100,000 tons valued at US\$12.5-17.8 million. The total cost due to the golden apple snail including yield loss, replanting cost and the cost of control such as molluscicides and handpicking was estimated at US\$28-45 million. According to another estimate on cost of control the golden apple snail in two countries; Japan and Philippine which farmers spent US\$64,385 and US\$10 million respectively only for pesticides [13].

There are a diversity of management approach has been conducted, including chemical, biological, physical, and cultural methods in order to prevent losses due to these pests. These pests can be effectively controlled by application of pesticides, however, this has long-term toxicity effects, particularly for livestock grazing on pastures following rice production, fish population and also will effect on human health. In another study, cultural control practices were investigated for control of pest population which includes mixed cropping, planting methods (transplanting or direct seeding), age of seedlings at time of transplanting, water management, fertilizer management, crop rotation, number of rice crops per year, planting time, synchronous or asynchronous planting over a given area, trap crop, tillage, weeding and growth duration of the crop. The selection of a particular control method or a combination of methods will depend largely on the management strategy to be adopted which in turn depends on the nature of the paddy rice industry being affected and the costs versus benefits of the whole operation.

Cultural and mechanical control methods will make the environment less favourable for the golden apple snails to establish their colonies. Wire mesh grills were highly recommended to construct at water inlets which it can minimize the entry of golden apple snail into the rice fields and prevent invasion of golden apple snails, but small snails can still enter unnoticed. Hand picking of eggs and destroying of golden apple snails to reduce population levels are highly labour intensive practices and unfeasible in large paddy fields. Among the recommended cultural control measures; crop establishment, planting methods, seedling rate, good leveling the field to remove snail refuges and facilitate drainage, planting at higher densities, burning straw, are the most used methods whereas farmers have started to use older seedlings (more than 30 days old) as a way of minimizing golden apple snail damage [14]. Reference [15] also reported that the use of roto-tiller during plant preparation is beneficial as it resulted to about 27% golden apple snail mortality as compared to the unploughed fields.

The golden apple snail can be utilized as an animal feed and considered as a replacement for duck and fish meal. Herding duck in paddy fields during the fallow period is advised because ducks was consumed snail's shell and meat. Therefore, duck herding together with feed supplementation during their confinement can increase the side income for paddy farmers. However, [16] was stated that this method was not practical in some areas such as Japan whereas there is little market for duck in Japan. Another biological agent is fish which a carp with pharyngeal teeth has a high potential for preying on golden apple snails. However, utilization of fish may not be practical, since fish culture requires keeping deep

water in fields [13], but this is often not compatible with modern farming methods. Natural enemy fauna against golden apple snail are very poor in paddy fields and, thus, population explosions of golden apple snail always occur there.

These methods are only partially effective and very labour intensive. Chemicals are still used extensively and inappropriately. Rice farmers mostly rely on commercial available synthetic molluscicides for the immediate control of the golden apple snail in lowland rice fields, without considering the toxic hazards to themselves and non-target organisms. Reference [12] was stated the most common synthetic insecticides used are Brestan® (Triphenyl tin acetate), Aquatin (triphenyltin chloride) and Namekill® (metaldehyde). Reference [17] and [18] were added another molluscicides which is niclosamide (2',5'-dichloro-4'-nitrosalicylanilide), recommended for control of golden apple snail in transplanted and direct-seeded rice while metaldehyde has been found to be effective in controlling golden apple snail in transplanted. However, this chemicals were used abusively that causing excessive environmental pollution and extremely toxic to non-target organisms. For example, niclosamide which is the only compound recommended for the control of aquatic snails by the World Health Organization (WHO) is effective against golden apple snail at 0.5–1.0 mg a.i./L, but the LC₅₀ for carps is only 0.14 mg a.i./L [5]. This means that no fish must be present in the rice fields while the product is applied. Furthermore, the cost of niclosamide in Malaysia is about RM85-95/ha, which is unaffordable to many farmers.

Therefore, the new approach emphasized on environmentally friendly control measures was adopted to replace the chemical oriented control program such as biopesticide or botanical pest control [16]. Botanical pesticide is a biopesticide which extracted directly from the plants that contain toxic compound which use for pest control. It was slow-acting crop protectants which provide an alternative to the synthetic pesticides [19]. Regarding botanical pesticides, a recent review shows that although some plants are used locally against golden apple snail, very little research has been published [5]. In the Philippines, the use of botanicals has been focused recently not only for insect pests but also for golden apple snail control. Reference [20] had found that use of eco-friendly pesticides of plant origin is safer not only to users but also to non-target organisms and the environment in general. As many plant products have been reported to possess pest control properties in various crop plants. Hence, in recent years there is an increased awareness on the use of plant products in pest management strategies such as *Derris elliptica* [21], *Curcuma longa*, *Blumea balsamifera* [22], *Phytolacca dodecandra* [23], *Melia azedarach* [24], *Nicotiana tabacum* [25], *Chenopodium quinoa* [5], *Azadirachta indica* [26], *Barringtonia racemosa* [27], *Blumea mollis* and *Hygrophila auriculata* [28].

The neem tree known botanically as *Azadirachta indica* A. Juss. belong to family Meliaceae, tribe Melieae and the genus *Azadirachta* is a tropical evergreen related to mahogany. The tribe Melieae are consists of two genera *Azadirachta* and *Melia*. Reference [29] were reported the species belonging to *Melia* genus are distributed in Indo-Myanmar, Indonesia, Philippines, China, Fiji, Malaysia, Mexico and Africa. *Melia azedarach* Linn. also called as 'gora neem' or 'bakayan' (Persian Lilac) is often confused with neem (*Azadirachta indica* A. Juss). However, these two species are quite different, the former being a native of Middle

East. Reference [30] reported two varieties of neem *Azadirachta indica* A. Juss which one of it is *Azadirachta indica* Juss var. *Siamensis* Valetton (Siamese neem tree). This variety was found throughout South-east Asia (Cambodia, Laos, Myanmar and Thailand). The *siamensis* variety is phenotypically different from the Indian variety and is characterized by less branching, longer and thicker leaflets, a larger and denser inflorescence and larger fruit.

This plant is native to the coastal fringe forests of the drier tropical region of east India, Sri Lanka and Burma. It is currently widespread in Pakistan, Myanmar, Thailand, Malaysia and Indonesia [31]. The neem tree is undemanding and grows well on moist, dry, stony, clayey or shallow soils. Therefore, it is able to grow almost anywhere in the lowland tropics. However, it generally performs best in areas with annual rainfall of 400-1,200 mm [32]. Extracts or crude parts of neem often used for protecting stored grains against insects by mixing them together with seeds. Reference [33] was found that the leaf powder, the seed oil and all kinds of extracts do indeed have a negative effect on the seed-eating insects. However, if this plant parts are used to treat stored seeds against insects, the mammalian consumer of these seeds especially human ought not to be affected by residues of this treatment.

Neem appears to be safe for humans and the environment as it has not been found to possess toxic compound. Reference [34] was stated that neem has oral LD₅₀ in rats of >5000 mg/kg which making it essentially nontoxic to mammals. In fact, neem leaves and other plant parts are valued for their therapeutic properties and extensively used for medicinal especially in India. Reference [35] reported that many disorders like inflammation, infections, fever, skin diseases, dental disorders and others have been treated with different parts of neem tree. In addition, neem also exhibits a wide range of pharmacological activities such as blood sugar lowering properties, anti-inflammatory, antihyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic anticarcinogenic and immunomodulatory [33].

Nowadays, the dependency on synthetic chemicals has prompted the large scale synthesis of newer chemicals. Eventhough these synthetic pesticides valued for effectiveness and convenience but it also pose certain problems including phytotoxicity and toxicity to non-target organisms, environmental degradation and health hazards to farmers. In addition, they also may accelerate development of resistant pests to specific pesticidal chemicals.

Therefore, the cost-effective, nontoxic, biodegradable, eco-friendly and botanical 'soft pesticides' are the need of present day agriculture as an alternative to hazardous and recalcitrant synthetic pesticides [35]. Neem was stated on the tops of the list of 2,400 plant species that are reported to have pesticidal properties and is regarded as the most reliable source of eco-friendly biopesticidal property. Neem based pesticides are systemic in nature which have no ill effects on humans and animals, and have no residual effect on agricultural produce. Besides, it is also easy to prepare, cheap and highly effective and thus constitute as an important source of pesticide for economically poor farmers.

There are a lot of researches have been done to discover and determine the potential of neem as a biopesticide in against a variety of rice insect pests. Reference [36] reported the

effects of two different neem products (Parker Oil™ and Neema®) on mortality, food consumption and survival of the brown planthopper (*Nilaparvata lugens*) were studied in the field. The experiment with nymph and adult reared in cages that set out in the paddy field showed immediate mortality after treatment application. The results clearly indicate the neem-based pesticide (Parker Oil™ and Neema®) containing low lethal concentration can be used effectively to inhibit the growth and survival of *Nilaparvata lugens*. Besides, reference [15] and [37] also have reviewed the effectiveness of neem insecticidal properties that successfully against white-backed planthopper (*Sogatella furcifera*), green leafhopper (*Nephotettix virescens*) and the rice water weevil (*Lissorhoptrus oryzophilus*).

Reference [12] tested aqueous neem leaf and seed extracts, neem oil and Bioblitz (EC formulation) against golden apple snail in the laboratory. Leaf and seed extracts were the most toxic causing 100% snail mortality at 100 ppm after 48 hours. Aqueous neem seed and leaves extract was tested against golden apple snails in the field (Rejesus and Punzalan, 1997). Treatment with concentration 20, 90 and 100 kg/ha of neem seeds were the most effective aqueous extract which inhibited feeding of the golden apple snails. However, effects of neem treatments on the ecology of the golden apple snails are still to be investigated.

In Thailand, reference [38] revealed the toxicity of leaf crude extracts from neem tree and garlic (*Allium sativum* L.) on mortality rate of golden apple snails at concentrations of 50, 250, 500, 750 and 1000 mg/l. High concentration of neem tree leaf extract (1000 mg/l) killed 95.83% of golden apple snail in 95 hours and high concentration of garlic (1000 mg/l) killed 91.66% of golden apple snail in 96 hours.

Besides against rice insect pests, neem also has shown activity on a wide range of insect pests of many crops worldwide. Reference [35] stated that neem products are effective against more than 350 species of arthropods, 12 species of nematodes, 15 species of fungi, three viruses, two species of snails and one crustacean species. Some naturally occurring compounds have been isolated from neem plants and shown to be active against different species of insect pests. It is well recognised that azadirachtin is the active ingredient of neem [34].

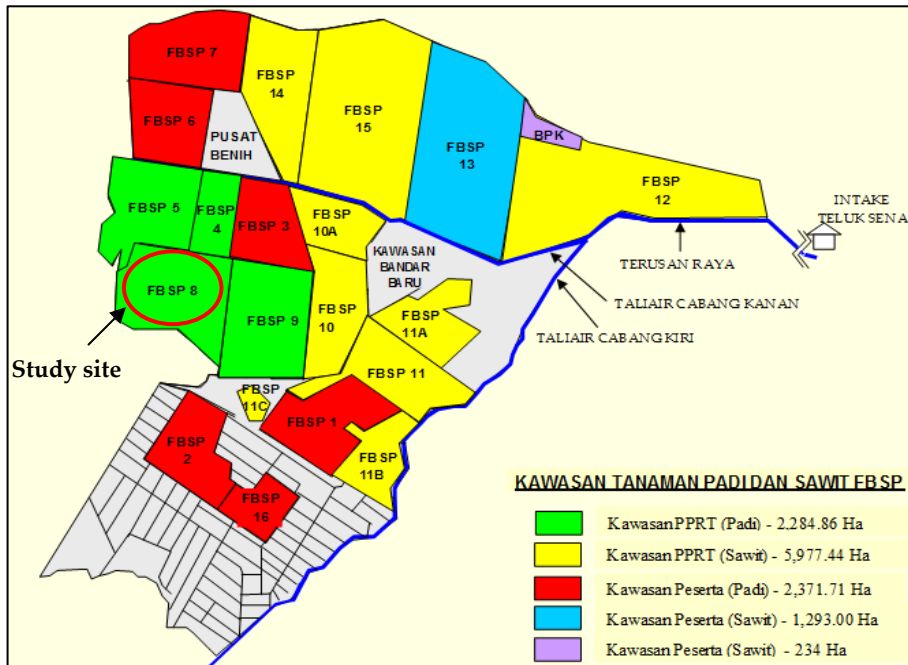
Azadirachtin (C₃₅H₄₄O₁₆) is a tetranortriterpenoids (limonoids) which extractable from *Azadirachta* plant species. This compound in neem have insecticidal properties as an antifeedant, repellence, oviposition deterrent, molting inhibition and a growth retardant for a variety of insects and arthropods [26,39,40,41]. Although every plant part of the neem tree contains azadirachtin substance, but most of previous research stated that the substance was much more concentrated in the seed kernels [32,33]. Its presence in the neem seed kernel is to the extent of 0.1% to 0.5% by weight. Besides the azadirachtin, neem also contains more than 20 compounds that responsible for the characteristics smell of crushed seeds and neem oil.

The increasing amount of research on insect-plant chemical interactions has unveiled the potential of utilizing botanicals insecticides in the form of secondary plant metabolites or allelochemicals [36]. These naturally occurring biocidal agents have been shown to be selective, readily biodegradable and safe to human. The neem tree has been proposed in this study as the azadirachtins of this tree have been recognised for their insecticidal properties.

2. Material and methods

2.1. Sampling location

The samples were collected from irrigated lowland rice field of Federal Land Consolidation and Rehabilitation Authority (FELCRA) Seberang Perak which located within Kg. Gajah Sub-district, Perak. FELCRA was cover the area about 17,698 ha by planting two types of major crops; paddy and oil palm (Figure 1). Total area under paddy cultivation is about 4,656.57 ha of which 3,413.78 ha are under an estates central management and the rest under semi-estate management.



Source: Administration of FELCRA Seberang Perak (2009)

Figure 1. Map of FELCRA Seberang Perak that showing lot area for its two major crops

The sample collections of golden apple snail have been conducted from the lot FBSP 8 (Figure 1) with total area 712.59 ha (669 lots). The sampling collections were done at block L1B7 (191.94 ha). The leaves and seeds of neem were also collected from the surrounding area of FELCRA Seberang Perak. They were brought to the laboratory and placed in room temperature (30-35°C) to dry and stored in an airtight container until used.

2.2. Tested mollusc

A population of golden apple snail were collected within the study area: L1B7- FELCRA Seberang Perak. Then, they were sorted for standard sizes (small and large) using a digital

vernier caliper (range of $\pm 1\text{mm}$) (Figure 2). The golden apple snails were assorted into size class with the size range within 20-40 mm. The size of golden apple snail was selected by the height of shell which provided convenience in selecting snails of uniform size. These golden apple snails were used for the toxicity test in laboratory conditions.



Small (20-30 mm)

Large (30-40 mm)

Figure 2. Measurement of golden apple snail shell height for size determination

2.3. Extraction procedure

The concentrations used in the bioassays were prepared from raw plant parts extraction, in successive dilutions with distilled water (aqueous extract). Before the extraction process, the neem leaves and seed samples were air-dried at room temperature, and grounded into fine powder. Then, the fine powder was sieved for extraction. Crude plant extracts were prepared by filling up distilled water on weighed plant material to get desired concentration rate. The solution was left overnight and filtered through plastic net. Liquid detergent was added to the final extract to act as a surfactant. The solutions were independently sprayed on the plants and pure distilled water was used for control with the same volume of the solution.

2.4. Toxicity test

Two plant parts which is neem seeds and leaves in combinations with application of required concentration rates of neem extract solution including the control (untreated) were carried out on two different sizes of golden apple snail (Table 1). The results from range-finder test were used to establish a more narrow concentration range for the definitive toxicity test. Once this range was determined, four narrow concentrations were developed to test the toxicity of leaves and seed crude extract and the experiment was replicated three times. A total of 135 golden apple snail with five snails per treatment were tested, which golden apple snail were exposed to the test substance in one plastic cage. Then, they were fed with 14 day-old of paddy seedlings that had been transplanted from nursery with 30 seedlings per plastic cage. A various concentrations of crude aqueous extract solutions from each of the raw materials were sprayed evenly on these paddy seedlings. After that, distilled water was flooded into the plastic cages to mimic the natural conditions for golden apple

snail's growth. Mortality and survival of golden apple snail was assessed at 24, 48, 72 and 96 hours after treatment application. The mortality of the golden apple snail during the tests were confirmed by the opening of operculum and the head did not respond when pushed.

Treatment	Neem plant parts	Concentration (%)
P ₀ C ₀	-nil-	0 (water only)
P ₁ C ₁	Leaves	0.75
P ₁ C ₂		1.5
P ₁ C ₃		2.25
P ₁ C ₄		3.0
P ₂ C ₁		Seeds
P ₂ C ₂	1.5	
P ₂ C ₃	2.25	
P ₂ C ₄	3.0	

Table 1. Experimental treatment on two different sizes of golden apple snail, small and large.

2.5. Statistical analysis

The observation on golden apple snail mortality was carried out for four consecutive days after treatment application and data was recorded based on the number of golden apple snail's mortality in every 24 hours up to 96 hours exposure period. For the toxicity test, the concentration-mortality regression analysis were developed using the mortality data of golden apple snail after 96 hours treatment. This regression analysis was conducted to determine the values of concentration of neem crude extract that caused 50% and 90% (LC₅₀ and LC₉₀) mortality of the golden apple snail. Probit Analysis was used to analyse statistically the data and calculated together with their 95% fiducial limits. Then, the variances within the treatments in terms of concentration of neem crude extract, type of plant parts and size of golden apple snail were evaluated in analysis of variance (ANOVA) by General Linear Model and when the significant differences were observed, further multi-comparison test was applied through Pairwise comparison analysis. This analysis could determine which means were significantly different and classified them in a group. The t-test at 95% confidence interval was used to compare LC₅₀ valued between neem leaves crude extract and neem seeds crude extract, and to compare LC₅₀ valued in different size of golden apple snail. Probit Analysis, ANOVA and t-test analysis were undertaken using the Minitab® 14.1 version (Minitab, Inc.).

3. Result and discussion

3.1. Mortality of golden apple snail

The mortality gathered from this experiment was used to identify the potential of each neem leaf and seed extracts in controlling golden apple snail. The percentages of mortality for

both sizes (small and large) on the control treatment (P₀C₀) were significantly lower than on all the neem-treated plants ($p \leq 0.005$) (Table 2). Approximately 6.7% of the golden apple snail mortality percentage on the control treatment for both sizes respectively, and those treated with aqueous neem extract is in the range of 89.5% to 92.9%. The results showed that golden apple snail was susceptible to neem extraction as the golden apple snail mortality relatively high on the treated plants compared with the control treatment.

$$\% \text{ mortality} = \frac{\text{mean mortality in treatment} - \text{mean mortality in control}}{\text{Mean mortality in treatment}} \times 100$$

Treatment	Percentage of mortality (%)	
	Small golden apple snail	Large golden apple snail
P ₀ C ₀	6.7	6.7
P ₁ C ₁	92.6	92.6
P ₁ C ₂	92.9	91.3
P ₁ C ₃	93.1	92.3
P ₁ C ₄	92.6	89.5
P ₂ C ₁	89.5	90.5
P ₂ C ₂	90.5	89.5
P ₂ C ₃	92.6	91.7
P ₂ C ₄	89.5	90.9

Table 2. Mortality of golden apple snail treated with different treatments.

Results obtained from these experiments noted that the aqueous neem extract has the potential to reduce golden apple snail infestation. According to reference [12], the tested of small-scale plot showed the neem treated plots had lower missing hill damage compared to control that plots, even the golden apple snail mortality is very low. Besides golden apple snail, neem tree extract also could help controlling other paddy insect pests as it is typically control a broad-spectrum of pests. This can result in the need for use of additional application of pesticides.

3.2. Mortality pattern of golden apple snail

The mortality pattern as a result of the exposure of different size ranges of golden apple snail to different concentrations with different neem plant parts is demonstrated. Data mortality of the snail was taken every 24-hours interval in four days and the results were shown in Figure 3 and 4. The analysis of data demonstrated in Figure 3 and Figure 4 as distribution of golden apple snail mortality in relation with the time and different treatment concentrations for both neem plant parts.

Based on the observations, it showed that the effectiveness of neem extract on the snail tested was both relatively slow and not highly varies and took 72 to 96 hours to reach end-point mortality. The findings were contradicted to that finding by [12], where the 48 hours

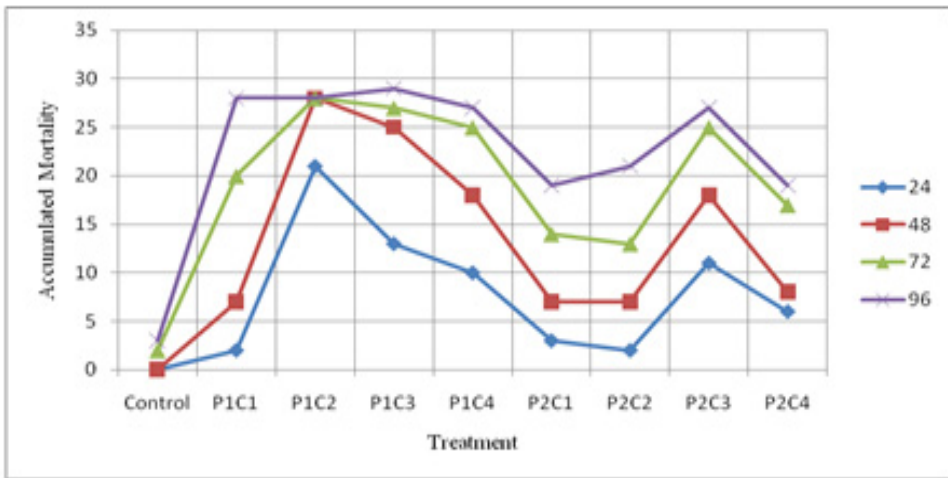


Figure 3. Accumulated mortality of small golden apple snail on different treatments

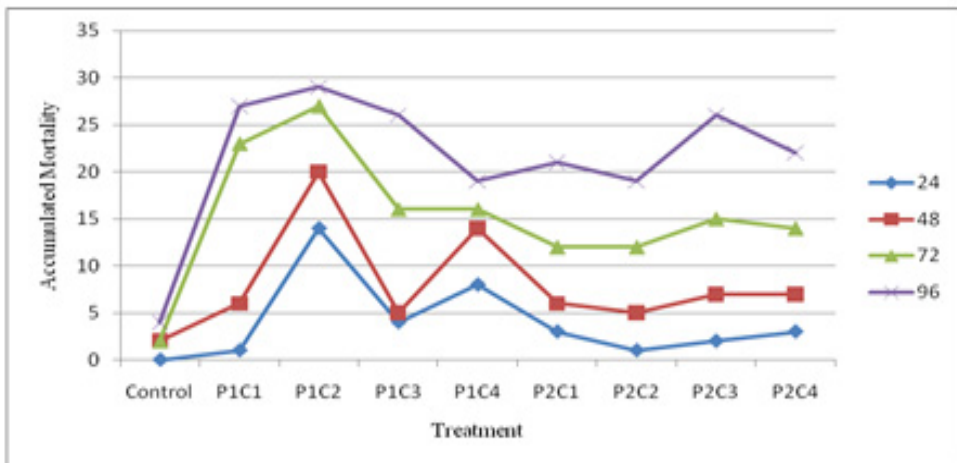


Figure 4. Accumulated mortality of mixed sizes of golden apple snail on different treatments

as end-point mortality for golden apple snail. However, a similar situation was also observed in another study [38] which neem tree leaf extract was killed 95.83% of golden apple snail in 95 hours. Reference [42] was stated that the effectiveness varies according to the insect pest species, its life stage and environmental factors.

In this study, the effectiveness of neem extracts varies for between the plant parts tested and the small size and large size of golden apple snail for the same plant parts. Results from the studies demonstrated small sizes of golden apple snail were highly mortality (25.19%) at 24 hours and 24.07% at 72 hours for large sizes of golden apple snail. These results were due to

different food intake rates between the small and large golden apple snail. The mortality of small golden apple snail was higher and faster than that the large golden apple snail as small golden apple snail has high appetite for development process. This result agreed to that reference [43], who found that smaller golden apple snail had a greater relative foraging capacity on macrophytes than adult. They also added that the juvenile of golden apple snail could consume an approximately 12 times more resources by mass than adults.

Different parts of neem plant also express different potencies of molluscicides. Thus, the golden apple snail mortality was also comparable to the different plant parts which 93.33% of small golden apple snail was dead with leaves crude extract and only 71.67% in seeds crude extract. For the large sizes of golden apple snail, 84.17% was dead in leaves crude extract and 73.33% when treated with neem seeds crude extract. This can be stated that leaves crude extract are more effective compared to the seeds crude extract in controlling small golden apple snail. A similar situation happened in controlling large sizes of golden apple snail, but the mortality rate for small golden apple snail was much larger than large golden apple snail.

The evaluation on the molluscicidal test data of aqueous neem extract was revealed that the plant parts vary considerably in their degree of activities. Result from the studies shows that the aqueous neem leaves crude extract was more effective than neem seeds crude extract for both sizes of golden apple snail. However, reference [22] classified the seeds had the highest molluscicidal toxicity than the leaves on the death of 80-100% at 48 hours for different concentrations. Furthermore, reference [12] also added that, among the aqueous extracts, neem leaf extract was the least toxic even at 0.1%.

3.3. Analysis of variance (ANOVA) in experimental treatment

The analysis of variance within treatments in term of concentrations of neem crude extract, different types of neem plant parts and sizes of golden apple snail were analysed statistically by determination of ANOVA using General Linear Model (GLM). Then, when the significant difference was observed, further multi-comparison test was applied through Pairwise comparison analysis to determine means were significantly different and classified into groups.

From the analysis of variance in Table 3 was obtained the F-statistic of 7.08 with p-value is 0.000 and Table 4 with the F-statistic of 3.93, p-value is 0.008. This value indicates that there are very strong evidences to suggest that the means of variable for small and large sizes of golden apple snails are not similar to each other and it required discovering which treatment has significantly difference in means.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	8	192.963	192.963	24.120	7.08	0.000
Error	18	61.333	61.333	3.407		
Total	26	254.296				

Table 3. Analysis of variance for treatments application on small sizes of golden apple snail.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	8	146.667	146.667	18.333	3.93	0.008
Error	18	84.000	84.000	4.667		
Total	26	230.667				

Table 4. Analysis of variance for treatments application on large sizes of golden apple snail.

Figures 5 and 6 summarize the results of Tukey’s Simultaneous Test (Pairwise comparison) for all the tested neem concentrations for both plant parts against the golden apple snail. The results indicate that leaves and seeds of neem plants extracts were significantly effective than the control treatment. A comparison of treated plants with control revealed significant difference in the number of mortality for golden apple snail. In Figure 5, shows that the mean level for control treatment (P₀C₀) is significantly the lowest among other treatments, while Figure 6 shows the mean level for P₀C₀ is lower than other six treatments except for P₁C₄ and P₂C₂. However, another treatment was not significantly different from each other.

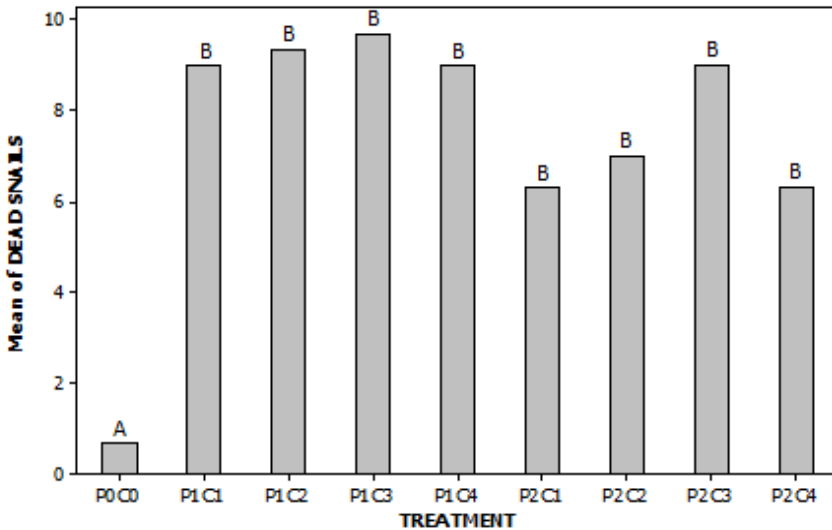


Figure 5. Pairwise comparisons among levels of treatment on small sizes of golden apple snail.

The comparison of the different treatments revealed that the neem has a positive effect against golden apple snail. Findings from the study suggested that the neem tested had a feeding deterrent effect on the golden apple snail. Reference [12], while studying the effect of *Phytolacca dodecandra* and *Azadirachta indica* on the reproduction of the golden apple snail reported that their active molluscicidal compounds such as triterpenoid and azadirachtin [34] caused a significant reduction in the survival of young and matured golden apple snail. The compounds have many properties including insecticidal activity, antifeedant, acting as a phago- and oviposition deterrent [36], growth retardant, moulting inhibitor, and sterilant as well as having anti-fungal, anti-viral and anti-bacterial properties against pathogens [33].

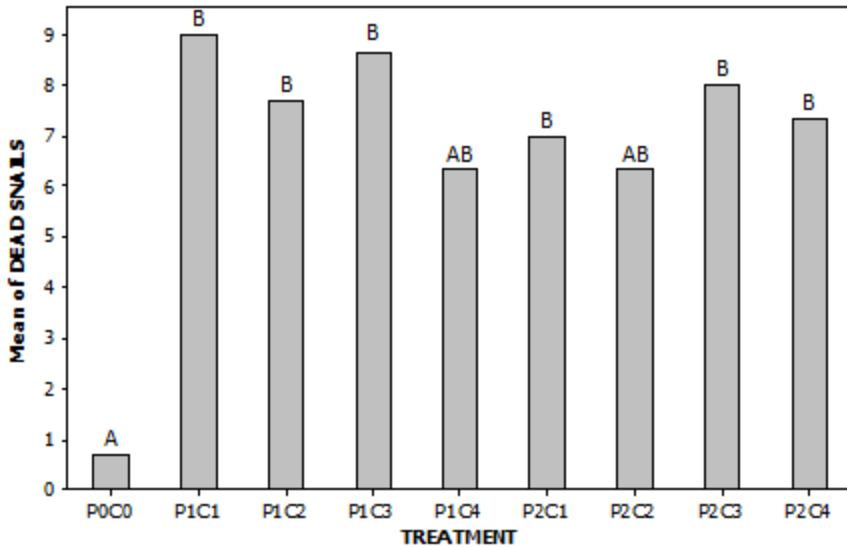


Figure 6. Pairwise comparisons among levels of treatment on large sizes of golden apple snail.

3.4. LC₅₀ value of neem crude extract

Table 5 and 6 summarize the results on the effect of neem leaves and seeds aqueous crude extract to two different golden apple snail’s mortality. Results from Table 5 indicate that the estimate of the LC₅₀ value for neem leaves crude extract against small golden apple snail is 0.442% with a 95% confidence interval (CI) of (0.012 – 0.743%) while for neem seeds crude extract is 1.036% with a 95% CI of (0.444 – 1.456). The LC₅₀ value in large golden apple snail for neem leaves crude extract is 0.498% with a 95% CI of (-0.714 – 1.065%) and 1.045 with a 95% CI of (0.489 – 1.449%) for neem seeds crude extract (Table 6).

Neem plant parts	Conc. (%)	LC ₅₀ (%) 95% CI	LC ₉₅ (%) 95% CI
Leaves	0.75	0.442 (0.012 – 0.743)	2.266 (1.878 – 2.912)
	1.5		
	2.25		
	3.0		
Seeds	0.75	1.036 (0.444 – 1.456)	4.279 (3.391 – 6.282)
	1.5		
	2.25		
	3.0		

Note: CI=Confidence Interval; LC=Lethal Concentration

Table 5. Toxicity of neem crude extract against small sizes of golden apple snail

Neem plant parts	Conc. (%)	LC ₅₀ (%) 95% CI	LC ₉₅ (%) 95% CI
Leaves	0.75	0.498 (-0.714 – 1.065)	4.632 (3.469 – 8.007)
	1.5		
	2.25		
	3.0		
Seeds	0.75	1.045 (0.489 – 1.449)	4.149 (3.307 – 6.001)
	1.5		
	2.25		
	3.0		

Note: CI=Confidence Interval; LC=Lethal Concentration

Table 6. Toxicity of neem crude extract against large sizes of golden apple snail

Based on the LC₅₀ values after 96 hours exposure period, the aqueous extract of neem leaves demonstrated more potent molluscicidal activity than neem seeds aqueous extract, which can be attributed to the different extracting plant parts. Comparison of the LC₅₀ and LC₉₅ of aqueous neem crude extract on leaves and seeds at 96 hours exposure showed that, golden apple snails were more sensitive against the aqueous leaves crude extract than that aqueous seeds crude extract. The aqueous extract of the neem leaves was 2.34 and 2.10 times more toxic than those from the seeds against small and mixed sizes of golden apple snail, respectively.

Finding from this study was contradicted to that study by reference [12], aqueous neem seed extract was more toxic than neem leaves extract. The seed extract was also the most toxic and causing 100% mortality after 24 hours of exposure at 20,000 ppm for both golden apple snail size and the neem leaf extract the least toxic that caused 100% mortality at 30,000 ppm and 40,000 ppm for small and mixed golden apple snail sizes, respectively. Reference [22] found that, the seed extract has high toxicity with toxic more than 200-1,000 ppm and leaves extract was in inactive status when the toxicity more than 10,000 ppm.

The factor causing differences in the findings probably due to the azadirachtin content in each of the different neem plant parts. All parts of the neem tree contain azadirachtin, and more concentrated in the seed kernel [32,33]. Finding of the study was expected considering the fact that the content of azadirachtin in neem tree varies greatly between locations and other factors may also contribute to variability [42]. Difference in trees maturity, application technique and perhaps environmental factors would probably contribute to these inconsistencies. This study did not analyze on the active ingredient from prepared the extract; however, it is possible that the azadirachtin content in seeds and leaves could raise the efficiency of extraction.

Results from the research also demonstrated that, snail mortality using the neem extracts at 96 hours was dependent on the sizes of golden apple snail's sizes. The higher lethal

concentration was observed in the large golden apple snail's size (30-40 mm) than the small golden apple snail (20-30 mm), which suggesting that lethal concentration increased as the size of the golden apple snail is increased. A similar situation was also observed in another study [12] where the leaf extract caused 100% mortality at 30,000 ppm for small golden apple snail and 40,000 ppm for mixed sizes of golden apple snail. Reference [44] was stated that, the size of golden apple snail at 10 mm, started eating paddy plants. Therefore, control measures should be implemented when the size of the golden apple snails was smaller or younger stage to reduce crop losses by using lower concentration of neem extract.

3.5. Correlation between treatment and size of golden apple snail

The interaction between treatments can be visualized by plotting the number of snail mortality as dependent variable against two types of plant part with one line for each level of the concentration as illustrated in the Figure 7 and 8. As the difference between any leaves and seeds means changes with the concentration level; which the leaves were the high ranking in the observation for both sizes of golden apple snail.

The interaction plot in Figure 7 indicates that the ordinal interaction as the lines is not parallel. The difference between neem leaves and neem seeds extracts was essentially close for concentration 2.25%, whereas the difference on concentrations 0.75% and 3.0% ppm was much larger for the neem leaves crude extract than that neem seeds crude extract.

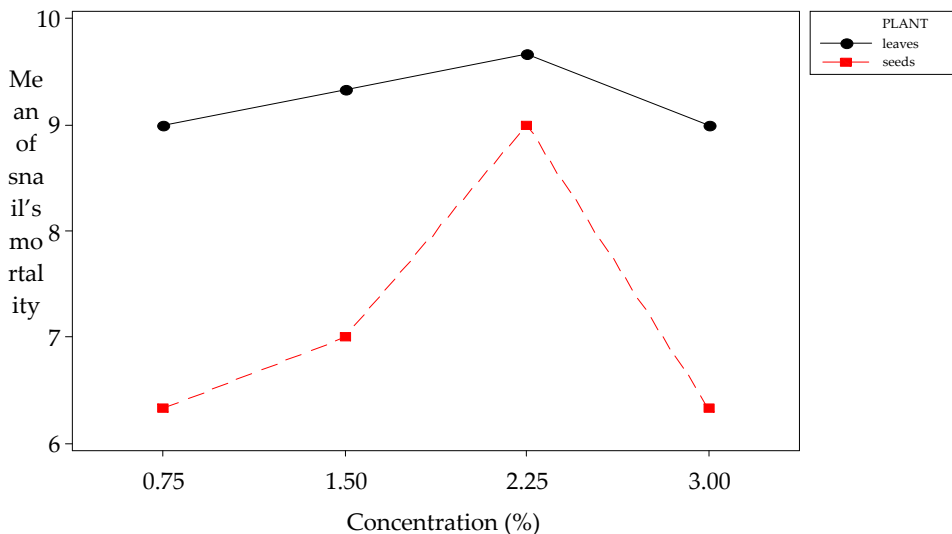


Figure 7. Interaction plot for treatment and mortality rate of small golden apple snail

Figure 8 shows the lines are not parallel but crossed each other; mean that there is an interaction. From examining this interaction plot, it appears that neem leaves crude extract has the highest mortality rate compared to the neem seeds crude extract for three different concentrations of 0.75%, 1.5% and 2.25% ppm. However, it was not for concentration 3.0% where as neem seed extract higher than neem leaves extract.

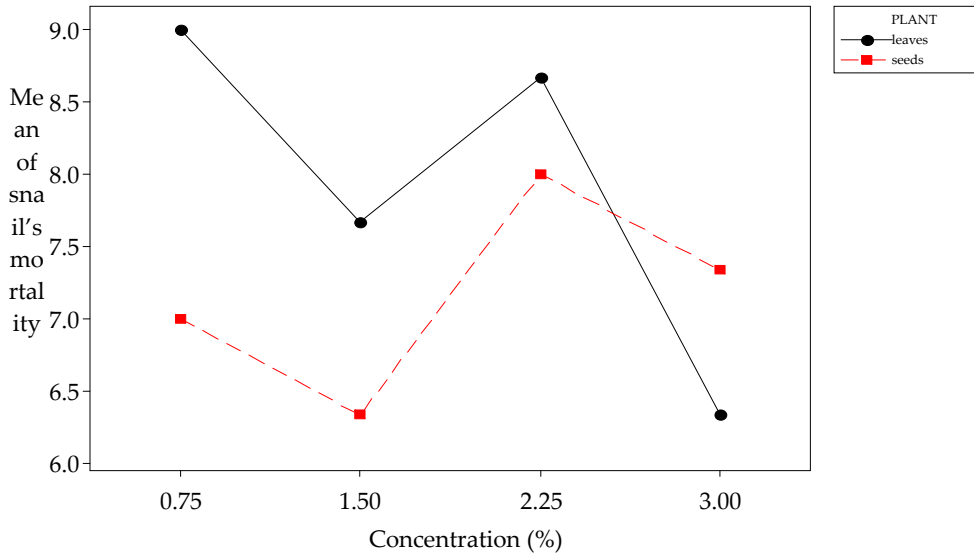


Figure 8. Interaction plot for treatment and mortality rate of mixed sizes of golden apple snail

4. Conclusion

The comparisons of LC_{50} values between neem leaves and seeds extract were not substantially different from each other and it showed no significant difference (T-test: 0.56; $p=0.677$ and T-test: 0.86; $p=0.549$). However, aqueous neem leaves extract have the potency in controlling both sizes of golden apple snail in using low concentration compared to aqueous neem seeds extract. The LC_{50} value of aqueous neem leaves extract after 96 hours exposure period was 2.34 times and 2.10 times more toxic than those from the seeds for small and large sizes of golden apple snail, respectively. On the other hand, aqueous neem leaves extract was caused high snail mortality with 93.33% and 84.17% of small and large size of golden apple snail was dead compared to aqueous seeds crude extract, 71.67% and 73.33% respectively. In the context of effectiveness, neem leaves extract also showed the ability to cause high mortality in a shorter time than seeds crude extract by killed 38.3% of the small golden apple snail within 24 hours and 30.83% at 72 hours for large sizes of golden apple snail, while neem seed extract was only 24.17% at 72 hours and 29.17% at 96 hours for small and large size of golden apple snail, respectively.

From the result, both plant parts have ability in controlling golden apple snail but aqueous neem leaves extract was expressed the effectiveness as a molluscicide than neem seeds extract for both size of golden apple snail.

In this study, all the tested neem concentrations for both plant parts affected the golden apple snail mortality (small and large) and were significantly different from the control treatment (ANOVA: $F=7.08$, $p=0.000$ for small snail and $F=3.93$, $p=0.008$ for large size of golden apple snail). The result was revealed that control treatment caused the lowest mortality number of golden apple snail among other treatments. However, the four concentrations for the extraction of leaves and seeds showed no significant difference in the mortality rate of golden apple snail.

Toxicity study of neem leaves and seeds crude extract in small and large size of golden apple snail exhibited the statistically not significant difference of the LC_{50} values between two different sizes of golden apple snail (T-test: - 1.02; $p=0.494$ and T-test: - 1.02; $p=0.494$). Half of the small golden apple snail populations were appeared to be affected by the neem leaves extract at 0.442% and 1.036% for neem seed extract. While for large size of golden apple snail, the concentration of neem leaves extract at 0.498% and 1.045% for neem seed extract was needed to cause 50% mortality of golden apple snail. The higher lethal concentration was observed in the large size of golden apple snail than the small golden apple snail, which suggesting that lethal concentration increased as the size of the golden apple snail is increased. Therefore, the neem crude extract application to small golden apple snail is the appropriate application because the farmer can use the lower concentration for crop damage protecting. In the observation of the speed of action of neem extracts, the study was showed that 25.19% of the small sizes of golden apple snail were highly dead at 24 hours and 24.07% at 72 hours for large sizes of golden apple snail.

In conclusion, the molluscicidal potential of neem has been proven, beyond doubt by the present investigations. This both neem plant parts can be used as alternative molluscicides to harmful synthetic chemical molluscicides that are widely used today to eradicate unwanted golden apple snail in the paddy field. Utilization of neem-based biopesticide early in the rice growing season when young golden apple snail at the predominant life stage would provide effective control and, due to their minimal effects on other aquatic life will reduce the resurgence problem that always occurred when chemical molluscicides are used early in the rice growing season. Therefore, the use of neem as molluscicides are highly recommended because they are toxicologically safe, environment friendly, easy to use and have a wide range of insecticidal activity.

The research has attempted to provide some information on the potential and toxicity of neem trees against the golden apple snail. In addition, this research also provides an idea for the development of locally produced, cheaper and safer biopesticide formulation which its application can provides an alternative way for synthetic pesticide application in pest management control at paddy field. Therefore, the results gathered from this research need to be confirmed on paddy field trial site, as well as investigate the environment impact from the extract application by determining the acute toxicity effect and chronic toxicity effect on

non-target organism such as fish. It is also recommended to extend neem extraction research to other extraction solvent and method, as solvent and extraction procedure may largely influence azadirachtin production. Moreover, the evaluations in this study were done using determination of golden apple snail mortality and different amount of organic compound in neem might influence the pesticidal activities of neem on golden apple snail. The study on the mechanism or mode of action of neem crude extract on golden apple snail should be tested to ensure the main factor of golden apple snail mortality.

Author details

Siti Noor Hajjar Md Latip and Rosdiyani Massaguni

Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia

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Evaluation of *Combretum micranthum* G. Don (Combretaceae) as a Biopesticide Against Pest Termite

Annick Tahiri

Additional information is available at the end of the chapter

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1. Introduction

In tropical agroforestry, although expensive and complex, chemical inputs are most widely used by farmers to control pest termites (1). Despite the many chemical treatments used, the damage and losses caused by termites remain high (2). With the intensification of agriculture and restrictions in the use of pesticides for their toxic effects on the environment and humans (3), we wanted to develop the local knowledge of plants with pesticidal properties. We have focused our work to search for biopesticides. Important prerequisites required before the use of a natural substance termiticide are: dose, lethal concentration, action on pest population, ways of action, transmission, assignment to the task of termites foraging and persistence of efficiency.

We chose the kinkeliba, *Combretum micranthum* G. Don (Combretaceae).

In West African Sahel, this shrub has shown potential in many medicines, construction, handicrafts, energy and agroforestry.

The virtues in traditional medicine and modern pharmacopoeia of Kinkeliba are well known. It possesses tonic, diuretic, antidiarrhea, antibiotic, antigonorrheal, antirheumatic and vermifuge (4, 5, 6). The leaves in decoction and chewed fresh leaves are used to treat coughs, bronchitis, hepatobiliary diseases, hepatitis and malaria (7). In combination with other plants, the leaves of Kinkeliba treat gonorrhoea. A leaf infusion is prescribed to prevent the contamination of leprosy (8). The root is prescribed in the diet of infertile women as energy medicine. The decoction of the root is anthelmintic and bactericidal for wounds.

Wood obtained from *Combretum* was in demand as it is mostly used as building materials for habitats and granaries in Sahelian country (9). Young stems, hard and soft, are used in basketry and carpentry (9). In the energy sector, the stems are used as firewood and charcoal

(10). In agroforestry, this shrub is highly resistant to fires and drought, is often found on abandoned agricultural land and is considered as a sterile or nearly sterile soil indicator (5). In biotoxicity, in-vitro research has shown that decoction and alcohol extract of leaf exhibits strong antimalarial properties against strains of *Plasmodium falciparum* (11). The methanol extract of the leaf showed potent acaricidal activity against the spider mite *Tetranychus urticae* (12). However, its pesticides properties and its use in protection against the attacks of insects and especially termites are less accurate. The wood of *C. micranthum* is sought as building material for its strength and its resistance to borers and termites in particular (9). However, other studies indicate that the roots of this shrub are very susceptible to termites' attack (10).

Also, we checked the basis of traditional use of this plant in the country for anti-termite and its mode of action on termites. The choice of this study is based on the following observation: Traditional methods from herbal products against insect pests are more and more abandoned to the profit of chemical inputs. The overall objective of this study is to propose an efficient, more accessible and cheaper alternative to farmers, without threat to the environment and human-being in order to reduce the use of chemical inputs.

This study aims to examine the question of the effectiveness of Kinkeliba as a termiticide, by a series of bioassays in the laboratory.

The working hypothesis is that the Kinkeliba *C. micranthum* can provide effective protection against attack by termite pests and be an alternative to the use of chemical inputs.

More specifically, it is to study:

- The direct toxicity by contact of various total aqueous, alcoholic and hexane leaf extracts of *C. micranthum* on adult workers of termite species, *Macrotermes bellicosus* Rambur;
- Whether the termite mortality may be the result or not of the consumption of the extract (consumption test);
- Whether the extract is toxic without being in contact with the termite (inhalation test);
- Whether the workers when are able to detect the product and avoid it (test of choice);
- Assess the lethal dose 50 (LD50) in 24 hours;
- Determine the active extracts of this plant's persistence of efficiency;
- Identify the main classes of secondary metabolites contained in the most active extracts of this plant.

2. Materials and methods

2.1. Study site

The work took place in Côte d'Ivoire, West Africa. The biological tests, extractions and chemical analysis of extracts were performed at the University of Cocody in Abidjan.

2.2. Plant material

Combretum comprises about 370 species, with approximately 300 species in Africa alone. We chose, in particular, the Kinkeliba *Combretum micranthum* G. Don (Combretaceae) (Figure 1).

It is a bushy shrub or creeper, top 2 to 5 m, but up to 10 m if hugs trees (6). Widely spread throughout Africa, the local plant is particularly dense on the cuirasses (6). This species is socio-economically important and frequently used in the Sahelian countries. It produces good firewood and charcoal (10). A study shows the operation impact of this wood in building homes and granaries in the Sahel region (9).



Figure 1. Stems with leaves of Kinkeliba *Combretum micranthum* G. Don

C. micranthum is highly ranked among the sahelians preferred plants for several basis criteria. Its wood is strong, durable, it does not rot, is resistant to termites and borers, the port is straight and easy to work. It is also appreciated in wicker basketry. Yet, production following natural seeding is most of the time compromised by climatic factors. This species breeds in very little by seeding, but so abundant by stump and natural layering, especially west of the Niger and southern Burkina Faso (13).

This medical plant has tonic, diuretic, antimalarial, antibacterial, antidiarrhea, antibiotic, antigonorrhoeal, antirheumatic and vermifuge properties (5, 7). It is more used in West Africa as an antimalarial drug and this antimalarial activity has been demonstrated by several authors. In-vitro research has shown that aqueous extract of leaf showing high antiplasmodial properties with an IC₅₀ inferior to 5 g/ml (7, 13) and against strains of *Plasmodium falciparum* resistant to chloroquine, at IC₅₀ values lower than that of *Azadirachta indica* (12). Alcoholic extract of leaf exhibits also strong antimalarial properties against strains of *Plasmodium falciparum* (11).

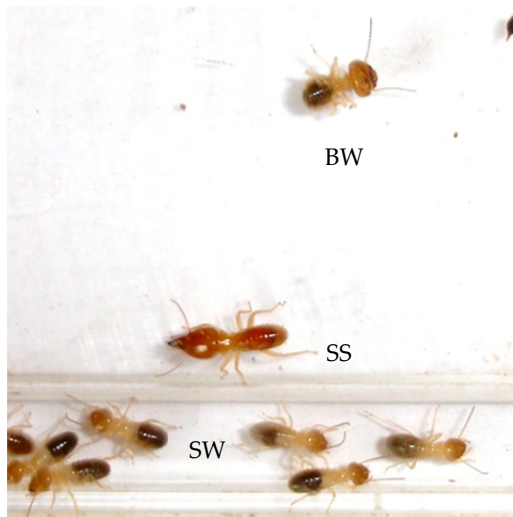
By cons, kinkeliba has not been systematically studied to determine its pesticides properties. The methanol extract of the leaf showed potent acaricidal activity against the spider mite *Tetranychus urticae* (12). Apart from its known particular resistance to termite attacks, we have no data about its anti-termite activities.

The leaves of the plant tested in its work come from Ouagadougou, in Burkina Faso.

2.3. Animal material

The termite is one of the greatest scourges of tropical agroforestry. Ten percent of termite species described to date are pests (13). Termites can cause damages to crops, buildings, pastures, forests and even non-cellulose materials such as electric cables. In many African countries, with the intensification of agriculture and restrictions in the use of pesticides, termite attacks are considerable. The most important damages are caused by species belonging to the subfamily of fungus-growing termites, the Macrotermitinae. Over 60% of destructive species belong to this subfamily (1). *Macrotermes bellicosus* Rambur 1842, formerly *Bellicositermes natalensis*, belongs to this Macrotermitinae. It was chosen for biotesting because of its impact on many crops in various countries of Africa and Côte d'Ivoire in particular. The attacks by this species involve very different cultures including groundnuts, maize, millet, sorghum, sugar cane, tobacco, tea, coffee, coconut and eucalyptus (1, 14). This termite is responsible of external attacks on the plants it covers by veneer of soil. It also penetrates the roots and stems of plants (14). The fungus of the genus *Termitomyces* with whom it develops a symbiotic relationship allows it to assimilate easily complex compounds such as wood cellulose or lignin, by predigested food on wheels.

This specie is also abundant in the study area: 1.85 nests per ha has been counted in the south and 1.7 in Lamto in the center of Côte d'Ivoire (15); 2.7 live nests per ha in Booro Borotou in the north west of Côte d'Ivoire (16). Density can reach 37.5 nests per hectare in the north-eastern Côte d'Ivoire (17). It epigeous slender and shaped cathedral nest, described by several authors whose (17), allows a large number of workers capture.



Big worker (BW); Small worker (SW) and Small soldier (SS)

Figure 2. Population sample of *Macrotermes bellicosus* Rambur

The workers studied in this work (Figure 2) come from the same colonies from the same nest on the campus of Cocody (Figure 3). Dimorphism between the size of the head of small and large workers in this specie has allowed separating in experiences.



Figure 3. Nest of *Macrotermes bellicosus*

2.4. Preparation of total extracts

The leaves of the plant are harvested in September 2009. They are spread out on sheets of paper, dried in the shade and then sprayed with an electric grinder. The total aqueous, alcoholic and hexane leaves extracts are produced by the method of extraction with successive conventional solvents of different polarity. This protocol allows a selective separation of the components of the organ. The collected solutions were evaporated by a rotary evaporator to obtain total extracts hexane (oil), methanol and aqueous dried under vacuum (18).

2.5. Calculate the rate (in %) performance in the extraction

The performance of each extraction fraction (R) is defined as the ratio of the amount of the extract (E) on the dry matter of the product (DM):

$$R = E / DM \times 100$$

2.6. Preparation of formulations

A stock solution of 10% was prepared from each dried extract with the corresponding solvent before being tested on 1 gram fresh weight of young workers (PO) of the adult termite *M. bellicosus* (or on 136 workers) at doses of 10, 20, 50 and 100 μl per box, so at four levels: 1, 2, 5 and 10 mg of extract / l.

2.7. The biological tests

Four bioassays were performed according to protocols described in (19):

1. The direct toxicity test measures the response of termites in soil treated with the insecticide;
2. The consumption toxicity test is to determine whether mortality of small workers is the result or not of insecticide consumption and specifies the importance of consumption of the product in this mortality;
3. The inhalation toxicity test is to investigate whether the fumes insecticide are toxic;
4. The avoidance test of the extract is to determine whether the workers, when a choice is given, are able to detect the product and to avoid it.

The tests are performed at the laboratory room temperature between 27 and 28 °C. Direct Toxicity and consumption test are made in a small rectangular plexiglass box, with 95 x 65 x 20 mm high, containing 7 grams of soil moistened with 2 ml of distilled water. Tests of inhalation and avoidance are performed in a large box of plexiglass 180 x 120 x 70 mm high, containing 17 grams of sieved soil and moistened with 5 ml of distilled water.

Using a micropipette, the doses are deposited either on land (for direct toxicity), or on pieces of Whatman paper No. 1 of 4 cm² (for tests of consumption, inhalation and avoidance). After deposition, the boxes are dried in the open air for 1 hour. Small workers of *M. bellicosus* are then introduced into these devices that are closed and do not allow air circulation. Each extract solution is tested with four doses previously mentioned. Each dose is repeated ten times for all tests. Each control box is treated with the corresponding solvent. The mortality of young workers was determined 24 hours after treatment. The LD50 is calculated. The surface of each Whatman paper covered by veneer and consume (mm²/worker) were measured daily with an ocular micrometer adapted to a magnifying glass. The amount of extract ingested per worker per day is calculated in ppm.

2.8. Persistence of efficiency (in days) of the extract the most active

In a small box of plexiglass 95 x 65 x 20 mm high containing 7 grams of soil moistened with 2 ml of distilled water, the soil is treated in the most effective dose of the extract. Small workers are removed and replaced by new every 24 hours for 7 days. Control box were treated with the corresponding solvent. Small dead workers are counted until the amount in the boxes in the testing and control is not significantly different. The mortality rate is calculated.

2.9. Calculation of mortality percentage

The mortality rate (Pc) is calculated using the ratio of individual dead observed on the total number of termites.

$$Pc = \text{observed mortality} / \text{total termites} \times 100$$

2.10. Chemical characterization tests for the secondary metabolites extracted from the plant

A phytochemical screening was performed on the leaves of *C. micranthum* to highlight the major chemical groups. The tests were performed using the technique described in (20). These chemical characterization tests, for the alkaloids from disclosure by the Dragendorff reagent, flavonoids with the perchloride test, saponins by the index of foam; tannins by reaction with ferric chloride at 1%, terpenoids by reaction with acetic anhydride - sulfuric acid.

2.11. Statistical analysis

Data collected during the biological tests are processed using the software Statistica (2001). The box plot, the estimated bootstrap, the nonparametric tests of Newman-Keuls and Kruskal-Wallis (at 5%) and correlation tests were implemented here. The LD50 is calculated by Probit analysis based on the mortalities obtained after 24 hours in different doses.

3. Results

3.1. Yield of successive extractions of the leaves of *Combretum micranthum*

The successive quantities extracted by each of the three solvents, expressed as a percentage of the total amount extracted, are higher in aqueous and alcohol solvents (respectively 9 and 8%) compared to the hexane solvent (3%).

3.2. Direct toxicity of extracts of *Combretum micranthum*

Three classes of extracts were prepared from the most efficient to the less active according to their toxicity (LD50) of the termite *M. bellicosus* stand. Topping the list is to find the alcoholic extract of leaves. This extract has greater toxicity than all other extracted plants. Secondly, we have to find the aqueous extract of leaves. However, the extracted hexane is less toxic (Table 1). The mortality of small workers total is obtained between 48 and 96 hours after treatment with the different extracts. When the toxicity of kinkeliba is compared to other plants having termiticides properties such as neem and papaya, tested under the same experimental conditions and the same species of *M. bellicosus*, the LD50 of alcoholic extract of kinkeliba leaves is 2.5 to 2.7 times lower than that of the alcoholic extract of neem and of papaya leaves (Table 2).

Extract	LD50 (mg/l)
Alcoholic leaf	3.69 ± 0.03 a
Aqueous leaf	6.49 ± 0.00 b
Hexane leaf	10.22 ± 0.03 c

Within a column values followed by same letters are not significantly different at the 5% according to the Kruskal-Wallis ($p < 0.05$, ANOVA - analysis of variance). LD50: 50 lethal dose, quantitative indicator of the toxicity of a substance.

Table 1. LD50 values of *Combretum micranthum* extracts tested on small workers of *M. bellicosus*.

Alcoholic leaf extract	LD50 (mg/l)
<i>C. micranthum</i>	3.69 ± 0.03 a
<i>A. indica</i>	9.52 ± 0.68 b
<i>C. papaya</i>	10.32 ± 0.03 c

Within a column values followed by same letters are not significantly different at the 5% according to the Kruskal-Wallis ($p < 0.05$, ANOVA - analysis of variance). LD50: 50 lethal dose, quantitative indicator of the toxicity of a substance.

Table 2. LD50 values compared of different plants tested on small workers of *M. bellicosus*.

3.3. Persistence of efficacy (in days) of different extracts of *Combretum micranthum*

The persistence of efficacy is the duration of the residual activity which generates a sample rate of higher mortality in the control at 5%. Leaf extracts of kinkeliba (aqueous and alcoholic) have persistent efficacy among the lowest (between 3.4 and 3.6 days). The hexane extracted from the leaves is the best (4 days).

3.4. Action of the most active alcoholic extract of leaves of *Combretum micranthum*

a. Toxicity consumption

Papers witnesses and papers treated with the extract are visited by the termite, as shown by the veneer of soil. By cons, the termite does not consume treated papers. In contrast, the average area of paper consumed in the control is 1.6 mm² or 0.012 mm² / worker (Table 3).

The amount of extracts ingested by the workers at the end experience is zero. However, the percentage of mortality obtained from the workers treated are significantly higher than that of the control ($F = 18.455$, $p = 0.000$, $N = 50$). So there is no correlation between the mortality of workers and the consumption of the extract for 48 hours at doses tested ($R = 0.483$, $N = 50$, $p = 0.262$). The toxic effect of the alcoholic extracts of leaves of *C. micranthum* is not related to its ingestion by the termite. Other ways of action of the product will be specified by the following tests.

Dose of alcoholic extract of leaf of <i>C.micranthum</i> (mg/l)	cumulative surface veneer (mm ² /w)	cumulative surface of paper consumed (mm ² /w)	cumulative quantity of extract ingested (ppm/w)
0	0.73 ± 0.07 b	0.01 ± 0.01 b	0 a
1	0.97 ± 0.31 b	0 a	0 a
2	0.44 ± 0.20 a	0 a	0 a
5	0.36 ± 0.21 a	0 a	0 a
10	0.47 ± 0.10 a	0 a	0 a

Measurements made at the time corresponding to 50% mortality of the workers. Average of 10 repetitions ± SD (N = 50). Within a column, values followed by same letters are not significantly different at the 5% according to the Kruskal-Wallis (p < 0.05, ANOVA, analysis of variance). Surface Veneer (F = 13.171, p = 0.000, N = 50); surface consumed (F = 9.486, p = 0.000, N = 50); quantity ingested (F = 0, p = 0.000, N = 50). w = worker.

Table 3. Effect of alcoholic extract of leaves of *Combretum micranthum* on the harvesting of *Macrotermes bellicosus* workers (consumption test)

b. Inhalation toxicity

The extract does not act by ingestion; it is interesting to see if it can be toxic without being in contact with the termite. At doses of 2, 5 and 10 mg / l, the inhalation of the extract results in a mortality rate significantly higher than that obtained in the control at 24, 48 and 72 hours after treatment (F = 22.832, p = 0.000, N = 50).

c. Avoidance of the extract

When a choice is presented, control papers and treated papers with the extract are visited by the termite, as shown by the veneer of soil. But no treated paper is consumed by the workers of *M. bellicosus*. By cons, the control paper placed nearby is (Table 4).

Dose of alcoholic extract of leaf of <i>C.micranthum</i> (mg/l)	Cumulative surface veneer (mm ² /w)	Cumulative surface of paper consumed (mm ² /w)
Control	1.12 ± 0,15 c	0.03 ± 0 c
1	0.09 ± 0.02 b	0 a
Control	1.10 ± 0,88 c	0.01 ± 0b
2	0.02 ± 0.02 a	0 a
Control	0.75 ± 0,09 c	0.01 ± 0 b
5	0.02 ± 0.03 a	0 a
Control	0.74 ± 0.58 c	0.01 ± 0 b
10	0.03 ± 0.03 a	0 a

Measurements made at the time corresponding to 50% mortality of the workers. Average of 10 repetitions ± SD (N = 80). Within a column, values followed by same letters are not significantly different at the 5% according to the Kruskal-Wallis (p < 0.05, ANOVA - analysis of variance). Surface Veneer (F = 14.259, p = 0.016, N = 80) - surface ingested (F = 22.563, p = 0.000, N = 80).

Table 4. Effect of alcoholic extract of leaves of *Combretum micranthum* on the harvesting of *Macrotermes bellicosus* workers (avoidance test)

3.5. Phytochemical screening of the plant

The leaves of the Combretaceae *C. micranthum* have all the desired compounds (alkaloids, flavonoids, tannins and terpenoids) except saponins. Analyses indicate that the most active alcoholic extract contains phenolic compounds (flavonoids and tannins) and alkaloids. It has no terpenoids (Table 5 and 6).

Extract	Alkaloid Dragendorff	Flavonoid Perchloride	Tannin Chloride Ferric	Terpenoid Acetic anhydride - Sulfuric acid	Saponin Index of foam
Leaf					
Aqueous solvent	+	+	+	+	-
Methanolic solvent	+	+	+	-	-

Table 5. Main classes of secondary metabolites in *Combretum micranthum*

Compound
Proteins
Fat
Carbohydrates
Salts and other minerals
Aliphatic alcohols (C16 au C30)
Phenolic compounds
Alkaloids
Organic acids
Gallic acid
Acid amyène
Triterpenoid
Beta-sistostérol
Potassium nitrate

(Source: 6, 25, 26)

Table 6. Some chemical data on the leaves of *Combretum micranthum*

4. Discussion

The leaves of the kinkeliba have interest termiticides properties. This result sets a different scope of kinkeliba still underexploited in the context of an overall recovery of natural vegetation. Alcoholic extract of leaves is the most toxic on the termite. The hexane extract (oils) of leaves is less toxic (LD50 2.8 times lower).

The performance of the alcoholic extract of this plant has been mentioned by other authors as potent acaricidal activity against the spider mite *Tetranychus urticae* (12). On insects, we have few other references. In other fields, the alcoholic extract from this plant was successful such as antimalarial drugs (21). According to these authors, this polar solvent is able to extract a wide range of chemicals substances as in traditional mixtures and foster its effectiveness (21). These results confirm also the effectiveness of methanolic leaf extract of medical plants with anti-termite properties and termite-resistant formulations (24).

The extract of leaves, especially the alcoholic extract, is more active in kinkeliba than in neem and papaya. At high doses, this extract has a linear dose-effect. However, these two last plants have other total extracts more efficient on termite than the kinkeliba which are the alcoholic extract of papaya seeds (22) and the aqueous extract of neem seeds (23).

The alcoholic extract constituents of kinkeliba leaves help to explain its potential insecticide action. The extract contains phenolic compounds (flavonoids and tannins) and alkaloids. This result is consistent with the compounds isolated from this plant by other authors (6, 25, 26). These authors state that flavonoids are myricetin glycosides and vitexin; the alkaloids are the stachydrine, 4-hydroxystachydrine and choline (12, 26). A new series of type of piperidine flavan alkaloids, kinkeloids A, B, C, and D have been recently identified (27).

The insecticidal effects of these components were mentioned by several authors. Phenolic compounds have both pesticides and fungicides properties (28). The tannins possess insecticidal, larvicidal and repellent properties (29). Alkaloids induce toxic effects on insects (30, 31). The extracts are total, so several other substances can also induce a toxic effect on the termite.

This kinkeliba alcoholic extract of leaves affect the mortality of the termite, has an extraction yield among the best and persistence of a lower efficiency than the other extracts. This extract is more easily degradable. Its use in field can reduce the potential risk of residual pollution and can be much more environmental friendly as synthetic drugs. The results also show that the contact and inhalation are the two essential ways to the effectiveness of this extract on the termite. Toxic substances from the extract can therefore penetrate through the cuticle and the spiracles of the insect. The alcoholic extract does not act by ingestion and is an inhibitor of food intake by the termite following the experimental doses used. But, inhibition of food intake by the extract is exerted only on the consumption of treated paper and not that of the untreated paper placed nearby. These results indicate that the extract can be efficiently used for soil treatments to protect a food substrate against *M. bellicosus* infestation. However the use of bait in this extract against this insect can cause problems.

C. micranthum leaves could be effective in providing a barrier to prevention and effective control of termite infestation in agronomy cropping systems. With the used doses, the extracts have no effect about 4 days after treatment. The persistence of low efficiency of the extract can be explained. The termite may also be able to detoxify toxic compounds like many other herbivores by producing enzymes that are involved in the mechanisms of metabolic detoxification of organic pollutants (32).

5. Conclusion

Simple techniques for managing termite attacks in the field by leaf extracts like kinkeliba can be more practiced by farmers. The application rates are achievable (in the laboratory, the experimental dose of 1.6 kg of extract per hectare gave satisfactory results). The identification of active compounds on the termite, capabilities of termiticide formulations, the factors that may compromise the effectiveness of kinkeliba on the ground and its impact on soil, insects other than termites are to be assessed in a natural environment.

Author details

Annick Tahiri

University of Cocody - Faculty of Biosciences -

Laboratory of Endocrinology and Reproductive Biology, Abidjan, Côte d'Ivoire

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Biotechnological Approaches for the Control of Insect Pests in Crop Plants

Jackie Stevens, Kerry Dunse, Jennifer Fox, Shelley Evans and Marilyn Anderson

Additional information is available at the end of the chapter

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1. Introduction

Each year billions of dollars are spent worldwide on insect control in agriculture [1]. Despite this expenditure, up to 40% of a crop can be lost to insect damage, particularly in developing countries [2]. Some of the most damaging insect species belong to the Lepidoptera, the second largest insect order comprised of moths and butterflies. The larval stage of moths cause major damage to an array of economically valuable crops including cotton, tobacco, tomato, corn, sorghum, lucerne, sunflower, pulses, and wheat [3]. Until recently, broad spectrum chemical insecticides have been the primary control agent for agricultural pests, with about 40% targeted to the control of lepidopteran insects [4]. Over the years the widespread use of pesticides has led to pesticide resistant insects, a reduction in beneficial insect populations and harmful effects to humans and the environment [5-8]. These problems have led researchers to develop different insect control strategies using both synthetic and natural molecules that are more environmentally friendly.

One such approach has been the use of transgenic plants expressing plant defence molecules. Genetic modification can potentially provide a much larger array of novel insecticidal genes that are otherwise beyond the scope of conventional breeding. The first transgenic plant that expressed an insecticidal gene was produced in 1987. The transgenic tobacco plant produced cowpea trypsin inhibitor at levels of up to 1% of the soluble protein and had enhanced protection against the lepidopteran pest *Heliothis virescens* [9,10]. The gene encoding the cowpea trypsin inhibitor was subsequently transferred into rice [11] and potato [12,13], but did not provide sustainable insect protection and was thus not commercially viable. Commercial development of insecticidal genes has focused on the *Bacillus thuringiensis* (Bt) toxins [14,15]. In 1987, genes encoding the Bt endotoxins were also transformed into tobacco and tomato plants [16-18]. Since the commercialisation of biotech crops in 1996, farmers have adopted the technology at such a dramatic rate, that in 2011, 16.7

million farmers in 29 counties planted 160 million hectares of the biotech crops. This has led to a reduction in chemical pesticide use of 443 million Kg and an additional financial gain for farmers of US \$78 billion in the last 15 years [19]. In India alone, Bt-cotton has increased cotton yields by up to 60%, and has reduced insecticide sprays by around half. This in turn has led to an income increase of up to US \$11.9 billion per annum [19]. The reliance of a worldwide industry on one insect resistance trait has led to real concerns about the development of Bt-resistant insects [20], especially since at least four cases of field based resistance have already been documented [21-23]. This in turn has led to a search for new insecticidal proteins and their encoding genes that have commercial potential for plant protection [8,24]. They include α -amylase inhibitors [25,26], vegetative insecticidal protein [27,28], chitinases [29] and protease inhibitors [30,31], as well as several other proteins directed to targets in the insect gut (Table 1).

Transgene	Source and Mode of Action	Example of use
<i>Bacillus thuringiensis</i> (Bt) endotoxin	See section "The <i>Bacillus thuringiensis</i> endotoxin"	See section "The <i>Bacillus thuringiensis</i> endotoxin"
Vegetative insecticidal protein (VIP)	VIPs are produced by <i>Bacillus cereus</i> and <i>Bacillus thuringiensis</i> . They have similar activity to endotoxins from Bt. Vip1/Vip2 are toxic to coleopteran insects and Vip3 is toxic to lepidopteran insects [32].	VIP was highly toxic to <i>Agrotis</i> and <i>Spodoptera</i> species. VIP induced gut paralysis, complete lysis of the gut epithelial cells and resulted in larval mortality [33].
		<i>Agrotis ipsilon</i> and <i>Spodoptera frugiperda</i> larvae suffered gut paralysis, disruption of midgut epithelial cells and mortality on Vip3A [34].
		Vip3A was toxic to <i>A. ipsilon</i> and <i>S. frugiperda</i> . Larvae of <i>Ostrinia nubilalis</i> and <i>Danaus plexippus</i> were insensitive [35].
		Vip3Aa14 was toxic to <i>Spodoptera litura</i> and <i>Plutella xylostella</i> . Larvae of <i>Helicoverpa armigera</i> and <i>Pieris brassicae</i> were insensitive [27].
		VIP3Ac1 had insecticidal activity against larvae of <i>S. frugiperda</i> , <i>Helicoverpa zea</i> and <i>Trichoplusia ni</i> , but low activity against <i>Bombyx mori</i> and <i>O. nubilalis</i> . The chimeric protein Vip3AcAa was insecticidal to <i>O. nubilalis</i> [28].
		Vip3LB resulted in growth inhibition of <i>Spodoptera littoralis</i> when incorporated into a semi solid artificial diet [36].

Transgene	Source and Mode of Action	Example of use
Biotin binding proteins (avidin and streptavidin)	Biotin is an essential vitamin for insects. It functions as a covalently-bound cofactor in various carboxylases, which have major roles in gluconeogenesis, lipogenesis, amino acid and fatty acid catabolism, and the citric acid cycle.	Avidin and streptavidin increased mortality in four Lepidoptera; <i>Epiphyas postvittana</i> , <i>Planotortrix octo</i> , <i>Ctenopseustis obliquana</i> and <i>Phthorimaea operculella</i> when incorporated into artificial diets [37].
	Avidin is a water-soluble tetrameric glycoprotein from chicken egg, which binds strongly to biotin. Streptavidin is a homologous protein found in the culture supernatant of <i>Streptomyces avidinii</i> .	Transgenic plants with leaves expressing avidin in the vacuole halted growth and caused mortality in <i>H. armigera</i> and <i>S. litura</i> larvae [38].
		Transgenic tobacco plants expressing either avidin or streptavidin increased mortality of the potato tuber moth (<i>P. operculella</i>). Similarly, transgenic apple expressing either avidin or streptavidin increased mortality and decreased growth of the lightbrown apple moth (<i>E. postvittana</i>) [39].
		Transgenic tobacco expressing avidin reduced <i>S. litura</i> larval mass [40].
Transgenic tobacco expressing three variants of biotin binding proteins in the vacuole increased mortality of <i>P. operculella</i> larvae [41].		
Chitinase (enzyme)	Chitinase catalyses the hydrolysis of chitin, which is one of the vital components of the lining of the digestive tract in insects and is not present in plant and higher animals.	Transgenic tobacco plants expressing <i>M. sexta</i> chitinase caused a reduction in survival and growth of <i>H. virescens</i> , but not <i>M. sexta</i> larvae [42].
		<i>Lacanobia oleracea</i> larvae exposed to diet containing recombinant <i>L. oleracea</i> chitinase had a reduction in weight gain and consumption compared to control-fed larvae [43].
		Transgenic rapeseed (<i>Brassica napus</i>) expressing <i>M. sexta</i> chitinase and scorpion insect toxin increased mortality and reduced growth of <i>Plutella maculipennis</i> [44].

Transgene	Source and Mode of Action	Example of use
		Oral injection of <i>B. mori</i> chitinase (<i>Bm</i> -CHI) caused high mortality in Japanese pine beetle, <i>Monochamus alternates</i> (Coleoptera). The peritrophic membrane chitin was degraded by <i>Bm</i> -CHI, but the midgut epithelium was not affected [29].
Cholesterol oxidase (enzyme)	Cholesterol oxidase is a bacterial enzyme that catalyzes the oxidation of cholesterol and other 3-hydroxysterols, resulting in production of the corresponding 3-hydroxysterols and hydrogen peroxide. Functions by damaging midgut membranes.	Cholesterol oxidase from <i>Streptomyces</i> caused stunting of <i>H. virescens</i> , <i>H. zea</i> and <i>Pectinophora gossypiella</i> when incorporated into an artificial diet [45].
		Cholesterol oxidase expressing tobacco leaves that were incorporated in artificial diets caused mortality and severe stunting of neonate <i>Anthonomus grandis</i> larvae [46].
Lipoxygenases (enzyme)	Dioxygenase enzymes are widely distributed in plants and catalyse the hydroperoxidation of cis-cis-pentadiene moieties in unsaturated fatty acids. Functions by damaging midgut membranes.	Lipoxygenase from soybean retards the growth of <i>Manduca sexta</i> when incorporated into artificial diet [47].
Alpha-amylase inhibitors	Alpha-amylase inhibitors block starch digestion. Widespread in microorganisms, plants and animals, [25,26].	Development of pea weevil larvae (<i>Bruchus pisorum</i> ; Coleoptera) was blocked at an early stage after ingestion of transgenic peas expressing an alpha-amylase inhibitor from the common bean (<i>Phaseolus vulgaris</i>) [48].
Alpha-amylase inhibitors	Alpha-amylase inhibitors block starch digestion. Widespread in microorganisms, plants and animals, [25,26].	Alpha-amylase inhibitor protects against predation by certain species of bruchids (Coleoptera: Bruchidae) and the tomato moth, <i>L. oleracea</i> (Lepidoptera) [49].
		Alpha-amylase inhibitor 1, from the common bean (<i>P. vulgaris</i>), provided complete protection against pea weevil (<i>B. pisorum</i> ; Coleoptera) in transgenic peas. Whereas alpha-amylase inhibitor 2 delayed maturation of larvae [50].
		The alpha-amylase activity in <i>Tecia solanivora</i> larvae was inhibited by alpha-amylase inhibitor from amaranth seeds [51]

Transgene	Source and Mode of Action	Example of use
Protease inhibitors	See section Protease inhibitors for the control of insect pests	See Table 2
Lectins	Multivalent carbohydrate-binding proteins. Some bind to midgut epithelial cells, disrupting their function, causing breakdown of nutrient transport, and absorption of potentially harmful substances [25,52].	Lectin from soybean seed inhibited larval growth of <i>M. sexta</i> [47].
		Wheatgerm agglutinin was toxic when fed to <i>O. nubilalis</i> . Formation of the peritrophic membrane was disrupted in the anterior midgut microvilli [53].
		<i>O. nubilalis</i> growth was strongly inhibited by wheat germ agglutinin (WGA), whereas <i>M. sexta</i> was not affected. In <i>O. nubilalis</i> larvae, WGA caused hypersecretion of unorganized peritrophic membrane in the anterior midgut lumen, disintegration of microvilli and cessation of feeding [54]
		The snowdrop lectin (<i>Galanthus nivalis</i> , agglutinin, GNA) reduced <i>L. oleracea</i> larval biomass and slowed larval development when in an artificial diet or expressed in potato plants [55].
		Transgenic potato expressing snowdrop lectin (<i>G. nivalis</i> agglutinin; GNA) reduced development of <i>L. oleracea</i> larvae. Transgenic plants were significantly less damaged [56].
		Transgenic tobacco plants expressing leaf (ASAL) and bulb (ASAI) agglutinins from <i>Allium sativum</i> retarded <i>S. littoralis</i> larval development and growth [57].
Trypsin-modulating ostatic factor (TMOF)	A peptide that blocks trypsin biosynthesis in mosquitoes (<i>Aedes aegypti</i> ; Diptera [Aea-TMOF]) and fleshflies (Sarcophaga; Diptera) [59].	Injection or oral ingestion of Aea-TMOF caused inhibition of trypsin biosynthesis and larval growth in <i>H. virescens</i> . Mortality of <i>H. virescens</i> increased when fed transgenic tobacco plants expressing Aea-TMOF [60].

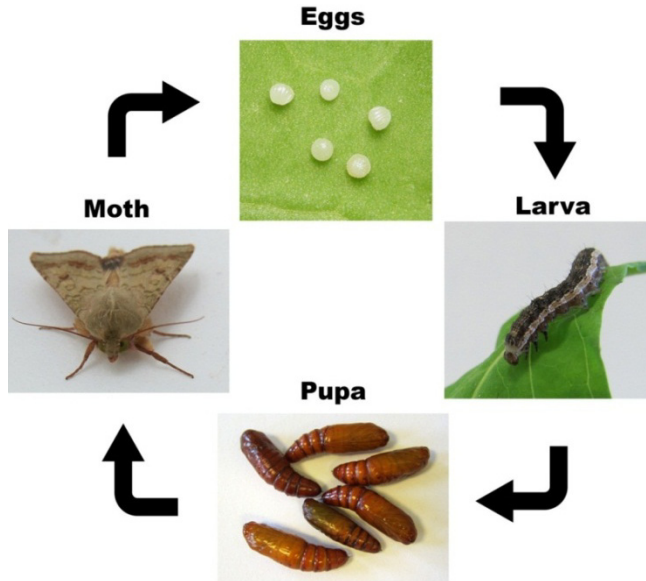
Transgene	Source and Mode of Action	Example of use
Isopentenyl-transferase gene (ipt)	Microorganism-derived gene from <i>Agrobacterium tumefaciens</i> . Codes for a key enzyme in the cytokinin-biosynthetic pathway.	Ipt expressed in tobacco and tomato decreased leaf consumption by <i>M. sexta</i> and reduced survival of the peach potato aphid, <i>Myzus persicae</i> (Hemiptera) [61].
RNAi constructs: 1) Vacuolar ATPase	Nutrient uptake by midgut cells is energized by the electrical difference created by the K ⁺ pump. The K ⁺ pump also regulates midgut lumen pH and determines the potassium concentration in blood, epithelial cells and midgut lumen [62]. The primary motor for transport is a vacuolar-type proton ATPase.	Transgenic corn plants expressing dsRNA of a V-ATPase from <i>Diabrotica virgifera</i> (western corn rootworm [WCR], Coleoptera) showed significant reduction in WCR feeding and plant damage [63].
2) Cytochrome P450 monooxygenase	Cytochrome P450 monooxygenase permits insects to tolerate otherwise inhibitory concentrations of the cotton metabolite, gossypol.	<i>H. armigera</i> fed on plants expressing cytochrome P450 dsRNA had retarded growth. Growth inhibition was more dramatic in the presence of gossypol [64].
3) Hemolin	Recognition of microbial infection is an essential first step in immunity in insects. Induction of this protective effect is associated with up-regulation of microbial pattern recognition protein genes such as hemolin.	Pupae of the giant silkworm (<i>Hyalophora cecropia</i>) were injected with hemolin dsRNA and developed normally into moths. After mating, no larvae emerged from the eggs which had malformed embryos [65]. Prior infection of <i>M. sexta</i> larvae with non-pathogenic <i>E. coli</i> , elicited effective immunity against subsequent infection by the lethal pathogen <i>Photobacterium luminescens</i> . Injection of hemolin dsRNA left the insect more susceptible to <i>P. luminescens</i> infection than insects that had not experienced prior infection with <i>E. coli</i> [66].

Table 1. Biotechnological approaches for the control of lepidopteran insects with transgenes

1.1. *Helicoverpa* species

Helicoverpa species (Figure 1) are polyphagous pests of at least 181 plant species from 49 families including cotton, corn, soybeans, tobacco and chick-pea [67-69]. They are one of the

most serious pests in cotton-producing countries like Australia, India and China, causing enormous economic problems [70,71].



The lepidopteran species, *H. armigera*, progresses through four stages of development; egg, six larval instars, pupal and adult. The time frame of each of these stages varies with environmental conditions. Over the warmer months, the life cycle can be completed in 30-40 days and each female moth can lay from 500-3000 eggs.

Figure 1. *Helicoverpa armigera* life cycle

One of the reasons these pests are so damaging is the larva's feeding preference for plant structures that are high in nitrogen, principally reproductive structures and growing points such as cotton buds and bolls, corn ears, tobacco buds, and sorghum heads. Damage to these structures has a direct influence on yield [67]. *H. armigera* larvae are foliar feeders at the early instar stage and shift to developing seeds or bolls at later stages [72]. *H. armigera* is a major problem in Australia because it has developed resistance to many of the chemical insecticides that have been used for its control [68,73]. Unlike other lepidopteran species, *H. armigera* larvae don't migrate far from their original host plant, consequently their populations in agricultural areas are exposed to consistent selection pressure, leading to greater resistance to insecticides [5].

In the 1995/96 growing season, transgenic cotton known as Ingard that expressed the *Cry1Ac* gene became commercially available in Australia [71]. To preserve the susceptibility of lepidopterans to Bt toxins, a conservative resistance management plan was imposed, where planting of Ingard cotton was restricted to 30% of the cotton production area per farm [71]. The average amount of insecticide used per hectare was 44% lower on Ingard cotton compared to conventional cotton [71]. In the 2004/05 growing season, Ingard cotton was replaced by Bollgard II, which expressed both the *Cry1Ac* and *Cry2Ab* genes [71].

Restrictions were not placed on this new variety and Bollgard II cotton comprised around 80% of the total cotton area planted in Australia during the 2004/05 and 2005/06 seasons [71] and 95% of the total cotton area in the 2010/2011 season [19]. This reduced the average amount of insecticide used per hectare by 85% compared to conventional cotton [71]. So far, there have been no reported field failures of Bollgard II due to resistance. However, while alleles that confer resistance to Cry1Ac in *H. armigera* are rare in the field, alleles that confer resistance to Cry2Ab are more common.

2. The use of genetically modified plants for control of lepidopteran insects

As mentioned previously, insects are responsible for major crop losses worldwide. In addition to direct impacts on yield, insects also reduce yields by making crops more susceptible to disease causing pathogens [8]. Last decade, most control measures focused on the use of chemical pesticides, a curative pest control strategy that was useful for rapid control of certain pest outbreaks. However, excessive and indiscriminate large-scale use of pesticides has led to development of pesticide-resistant insects [74]. Additionally, the long-term and extensive use of synthetic chemicals has led to concerns regarding their impact on food safety, associated human health and the environment [8]. As the use of pesticides for prevention of insect-associated losses cannot be overlooked in agriculture, there is a greater need to develop alternative or additional technologies which would allow a more selective use of pesticides and provide sustainable crop protection [52]. To achieve this objective, it is necessary to enhance the resistance of plants to pests and pathogens through integrated pest management (IPM) programs. They will need to consist of a combination of control strategies including (A) the use of natural biocontrol factors such as pathogens, predators or parasites [75]; (B) various preventive pest control strategies including crop rotation, intercropping, and cultivation of pest-resistant varieties of plants [8] and (C) genetic control via the release of sterile insects and also the use of natural insecticides. The latter includes secondary metabolites [52,76], viruses [77,78] and transgenes.

As the products of most transgenes are ingested by the insect pest and therefore act through the gut, most of the focus has been on transgene encoded proteins that target the insect midgut and/or the peritrophic membrane to disrupt digestion or nutrition [53,54,79-81]. Generally, the detrimental effects on larval and insect growth result from limited assimilation of nutrients [82-85]. Furthermore, any severe delay in growth and development, in a natural setting, lengthens the period in which the larvae are vulnerable to natural predators such as mice, spiders and predaceous insects [30,86,87]. The use of transgenic plants that express insecticidal agents thus reduces the population of insect pests and reduces the usage of chemical insecticides. This extends the useful life of the insecticides and also reduces the ecological damage they may cause [61]. As with any new method of insect control, the impact of transgenic plants on non-target and beneficial insects, particularly pollinators such as honey bees, needs to be assessed [88-90]. Table 1 lists a number of biotechnology approaches tested on lepidopteran insects. Since the discovery that dsRNA

can silence genes, RNA interference (RNAi) has been developed as an effective tool for regulating gene expression in plants and animals. RNA interference or gene silencing has been used to inhibit virus replication and spread in transgenic plants and has potential to be developed commercially for disease control [91]. The use of RNAi for insect control is less well developed. Insect genes can be down-regulated by injection of dsRNA or by oral administration of high concentrations of exogenously supplied dsRNA as part of an artificial diet, but a much more efficient method of delivering dsRNA is needed before RNAi technology can be used to control pests in the field [64,65]. To date, the most successful transgenes for insect control have been the genes encoding insecticidal toxins from the soil bacterium *Bacillus thuringiensis*.

2.1. The *Bacillus thuringiensis* endotoxins

The use of genes encoding endotoxins from *Bacillus thuringiensis* is now a well-established technology for producing transgenic plants with enhanced resistance to the larvae of lepidopteran insect pests [92]. Bt cotton was first released for commercial production in the USA in 1996 and subsequently grown in several countries including Argentina, Australia, China, Colombia, Indonesia, Mexico, South Africa, and India [93]. Since then other transgenic crop species producing Bt toxins have been commercialized including maize, tomato and potato (<http://cera-gmc.org>). The adoption of Bt crop varieties by farmers has been rapid reflecting the benefits of these crops such as reduced insecticide use, lower production costs and higher yields [94]. Only two Bt crops are grown in Australia (Table 2). In the most recent season (2011/2012) approximately 80% of the cotton grown in Australia was Bollgard II® [95].

B. thuringiensis, a Gram-positive soil bacterium, produces a proteinaceous parasporal crystalline inclusion during sporulation [96]. There are two main categories of Bt toxins: Cry and Cyt. These two groups are classified further by a detailed nomenclature system that describes groups Cry1 to Cry55 and Cyt1 to Cyt2 [97-99]. The Cry toxins are divided into three larger families that are not related phylogenetically. The largest Cry family is the three domain family, and genes from this family are present in the majority of commercialised Bt crops [100].

The larvae of insect orders primarily affected by Bt toxins are Lepidoptera (butterflies and moths), Diptera (mosquitoes) and Coleoptera (larval and adult beetles) [101]. However, Bt toxins are not toxic to people, wildlife, or most beneficial insects [102,103] and therefore the opportunities for biological control are great. The effect of Bt toxins on a range of lepidopteran insects has been studied including: *Bombyx mori* [104], *Helicoverpa armigera* [105], *Heliothis virescens* [106,107], *Manduca sexta* [108,109], *Ostrinia nubilalis* [110-113], *Plutella xylostella* [114,115], *Sesamia nonagrioides* [115], *Spodoptera exigua* [116], *Spodoptera frugiperda* [117] and *Spodoptera littoralis* [118]. The Cry toxins produced in Bt crops generally target lepidopteran pests, although some also target coleopteran pests [100]. The first commercialised Bt crops contained only one Cry toxin, but second generation Bt crops have between two to six different toxins [100].

Trade name	Crop	Bt protein	Company	Year released
Ingard®	cotton	Cry1Ac	Monsanto	1996
Bollgard II®	cotton	Cry1Ac, Cry2Ab	Monsanto	2003

This table lists the transgenic crops in Australia producing Bt proteins.

Table 2. Bt crops grown in Australia

2.2. Mechanism of action

The Bt toxin mechanism of action is described by two models: The pore formation model and the signal transduction model. The initial steps of both models are the same. Upon ingestion by insects the crystalline inclusion is solubilised in the midgut [119]. Most target insects have a high gut pH [120] that is crucial for the efficacy of Bt toxins since most Bt-prototoxins are only soluble above pH 9.5 [121]. The 130 kDa prototoxins are activated by insect gut proteases, which typically cleave from both the C- and N-termini resulting in a 43-65 kDa protease-resistant active core [122-125].

The pore formation model has been the accepted mode of action for 20 years and is supported by numerous publications [96,126-128]. In this model the activated toxins bind to the primary receptors in the brush border membrane of the midgut epithelium columnar cells [14]. The major receptors for Cry toxins in lepidopterans are cadherin-like proteins [129-133]. The binding site of Cry toxins varies depending on the structure of the Cry toxin [105,110]. Binding to cadherin facilitates further proteolytic cleavage of the toxin and promotes the formation of oligomers [128,134]. The toxins then interact with secondary receptors in the midgut larval membrane. These secondary receptors are GPI-anchored proteins; either aminopeptidases or alkaline phosphatases [119,128,131,135]. Following secondary receptor binding, the toxin inserts into the membrane and creates pores [128]. These pores lead to the disruption of membrane integrity and cause an electrolyte imbalance that ultimately leads to death by starvation or septicaemia [136,137]. It is likely that there are more receptors involved in Bt toxicity since insects lacking the cadherin receptor are still killed by modified Bt toxins [138,139].

An alternative model for the Bt toxin mechanism of action proposes that Cry toxins trigger a signalling cascade pathway [140,141]. This model differs from the pore formation model in that it does not involve toxin oligomerisation, secondary receptors or the formation of pores in the membrane. Instead, in this model, binding to the cadherin receptor initiates a Mg²⁺ dependent signal cascade pathway that includes a guanine nucleotide-binding protein, adenylyl cyclase, and protein kinase A which ultimately results in cell death.

2.3. Resistance of lepidopteran insects to Bt toxins

More recently there have been reports of field resistance to Bt crops in pink bollworm (*Pectinophore gossypiella* [142,143]), cotton bollworm (*Helicoverpa* spp [144-147]), armyworm (*Spodoptera frugiperda*[22]) and western corn rootworm (*Diabrotica virgifera virgifera* [148]. Some insects collected from the field have Bt resistance that has been characterized in the

laboratory. However, there is debate about the relevance of this laboratory resistance in the field [149]. A decrease in field performance of Bt corn against *S. frugiperda* was observed in Puerto Rico [150] and against *Busseola fusca* in South Africa [23,151]. In southeastern US problems with control of *H. zea* on Bt cotton have also been reported [144-146].

The most common mechanism of resistance is the disruption of binding of Bt toxin to receptors in the midgut membrane. This disruption may be caused either by mutations in the receptor that blocks binding (reviewed in [20]) or changes in expression of the receptors [152,153]. Mutations in cadherin genes are responsible for Bt resistance in *Heliothis virescens* [154], *Helicoverpa armigera* [155] and *Pectinophora gossypiella* [156]. Another resistance mechanism associated with an ABC transporter locus has been reported in three lepidopteran spp (*H. virescens*, *P. xylostella* and *T. ni* [157]). Resistance to Bt in *Ostrinia nubilalis* is due to reduced midgut protease activity resulting in less activation of the protoxins [111,158,159].

2.4. Management of resistance to Bt crops

There are two main strategies for management of insect resistance to Bt crops: Refuge and pyramiding. The main approach for delaying evolution of resistance to Bt crops is the refuge strategy [21]. Farmers are mandated to maintain an abundance of host non-Bt crops as a refuge surrounding their Bt crops. The theory behind this strategy is that any Bt resistant larvae that arise on the Bt crops will mate with susceptible individuals from neighbouring non-Bt crops. As long as inheritance of resistance remains recessive the offspring will be susceptible to Bt crops [160-162]. This strategy is then combined with several other mandatory farming practices that include control of volunteer and ratoon plants that arise post-harvest, planting within a defined period of time to restrict the exposure of the Bt crop to the insect pests, restricted use of foliar Bt and the cultivation of crop residues [95]. The other major strategy to combat the evolution of Bt resistance is gene pyramiding. For example, the development of second generation Bt cotton that has at least two Bt toxins such as the Monsanto Bollgard II cotton variety, but up to six Bt toxins [100]. Another resistance management strategy which is still in the research phase of development is the use of insecticidal genes with completely different modes of action such as proteinase inhibitors. The success of combining multiple Bt genes for resistance management is contingent on the individual toxins having different targets to prevent cross resistance developing [163-165]. Binding studies with various Cry toxins have been used to identify toxins with different binding sites in the lepidopteran midguts [105,166,167]. This information can be used to design combinations of Cry toxins that complement each other to delay the development of resistance to Bt crops.

In addition to the resistance management plan for Bollgard cotton outlined above, farmers also use integrated pest management (IPM) systems as a sustainable approach to control all pests. IPM systems deploy a tactical combination of biotechnological, chemical, biological and cultural control methods to avoid pest problems [168]. Some of the major IPM strategies and tools include maintenance of beneficial insect populations, ensuring healthy plant growth, managing weed hosts and monitoring pest populations and plant damage regularly. All these

additional practices lead to better control of insect populations in general and therefore helps prevent the development of resistance in insect populations to Bt.

3. Protease inhibitors for the control of insect pests

Protease inhibitors are one component of a plant's natural defence mechanism against herbivores and pathogens [169]. Plants protect themselves directly by constitutively expressing protease inhibitors [170] and by inducing protease inhibitors in response to mechanical wounding or insect attack [169,171]. They may also release volatile compounds after insect damage that function as potent attractants for predators of insect herbivores [172]. The release of volatile compounds after wounding, such as methyl jasmonate also triggers the production of proteinase inhibitors in neighbouring unwounded plants essentially prearming the local population against insect attack [173].

3.1. Mechanism of action of protease inhibitors on lepidopteran insects

Protease inhibitors when incorporated into artificial diets or expressed in transgenic plants increase mortality [174] and reduce the growth and development of larvae from many insect pest species including Coleoptera [175,176], Orthoptera [177] and Lepidoptera [178,179](Table 2). The mechanisms by which ingested PIs mediate their effects on insect physiology differs between insect species [180]. Proteinase inhibitors bind to insect digestive proteases, preventing proteolysis which blocks digestion of protein [181]. This effectively starves the larvae of protein and essential amino acids required for insect growth, development and reproduction [182-185]. To compensate for this inhibition, several insect species increase production of proteases to swamp the ingested PIs [186,187]. This in turn can lead to a limitation in bioavailability of essential amino acids for protein synthesis, impairment of growth and development, and potentially death [182,186]. The loss of the sulphur-containing amino acids (cysteine and methionine) is critical because the sulfhydryl content in trypsin and chymotrypsin is high and reprourement of the sulphur-containing amino acids is difficult since cysteine and methionine are in relatively low concentrations in the diet, especially if the food source is plant material [186]. Broadway and colleagues confirmed this hypothesis in bioassays with *Spodoptera exigua* where the weight-reducing effects obtained with soybean trypsin inhibitor were eliminated when the diets were supplemented with methionine [186].

3.2. PIs in transgenic plants for plant protection: success and failure

Several groups have reported enhanced protection of plants against lepidopteran pests after transformation with genes encoding PIs (Table 3). Despite this substantial body of work, defense strategies based on PI expression in plants have not resulted in any commercial application so far [61,214,215]. This relates to two distinct problems: (1) the levels of PI-expression in transgenic plants and (2) the pest's capacity to react to PI consumption. Most problems arise from the use of a single transgene producing a PI that targets only one protease or one class of protease in the insect midgut.

Protease inhibitor	Protease family	Proteases inhibited	Transformed plant	Insect species used in bioassay	Effect of PI on larval growth
<i>Arabidopsis thaliana</i> serpin 1 [AtSerpin1]	alpha-1-peptidase inhibitor	Chymotrypsin	<i>Arabidopsis</i>	<i>Spodoptera littoralis</i>	38% biomass reduction after feeding for 4 days [188]
Barley trypsin inhibitor [BTI]	Cereal trypsin inhibitor	Trypsin	Tobacco	<i>Spodoptera exigua</i>	29% reduction in survival [189]
			Wheat	<i>Sitotroga cerealella</i>	No effect on growth or mortality [190]
Bovine pancreatic trypsin inhibitor [BPTI]	Kunitz (animal)	Trypsin, chymotrypsin, plasmin, kallikreins	Tobacco	<i>Spodoptera exigua</i>	Reduced trypsin activity; induced leucine aminopeptidase and carboxypeptidase A activities; chymotrypsin, elastase, and carboxypeptidase B proteases not affected [190]
			Sugarcane	<i>Scirpophaga excerptalis</i>	Significant reduction in weight [191]
Bovine spleen trypsin inhibitor [SI]	Kunitz (animal)	Trypsin, chymotrypsin	Tobacco	<i>Helicoverpa armigera</i>	Reduced survival and growth [192]
Cowpea trypsin inhibitor [CpTI]	Bowman-Birk	Trypsin	Tobacco	<i>Heliothis virescens</i>	Increased mortality [9]
			Tobacco	<i>Helicoverpa zea</i>	Increased mortality [193]
			Rice	<i>Chilo suppressalis-Sesamia inferens</i>	Growth not monitored [11]
			Potato	<i>Lacanobia oleracea</i>	45% biomass reduction [13]
			Tobacco	<i>Spodoptera litura</i>	50% biomass reduction [194]
			Potato	<i>Lacanobia oleracea</i>	Decreased weight and delayed development [12]
Giant taro proteinase inhibitor [GTPI]	Kunitz (plant)	Trypsin, chymotrypsin	Tobacco	<i>Helicoverpa armigera</i>	Decreased growth, no increase in mortality [195]

Protease inhibitor	Protease family	Proteases inhibited	Transformed plant	Insect species used in bioassay	Effect of PI on larval growth
Mustard trypsin inhibitor 2 [MTI-2]	Brassicaceae proteinase inhibitor	Trypsin, chymotrypsin	Tobacco, <i>Arabidopsis</i> and oilseed rape	<i>Spodoptera littoralis</i>	Increased mortality; surviving larvae up to 39% smaller after 10 days [187]
				<i>Mamestra brassicae</i> , <i>Plutella xylostella</i> , <i>Spodoptera littoralis</i>	<i>P. xylostella</i> : 100% mortality on <i>Arabidopsis</i> ; high mortality & delayed development on oilseed rape. <i>M. brassicae</i> : increased mortality & weight of survivors on <i>Arabidopsis</i> and tobacco, no effect on oilseed rape. <i>S. littoralis</i> : delay in development on oilseed rape [178].
			Tobacco	<i>Spodoptera littoralis</i>	No effect on growth; reduction in fertility [196]
			Oilseed rape	<i>Plutella xylostella</i>	Reduction in survival and weight [30]
<i>Nicotiana glauca</i> protease inhibitor [NaPI]	Proteinase inhibitor II	Trypsin, chymotrypsin	Tobacco	<i>Helicoverpa punctigera</i>	Decreased weight; increased mortality [197]
			Tobacco and peas	<i>Helicoverpa armigera</i>	Increased mortality; delayed growth [198]
			'Royal Gala' apple	<i>Epiphyas postvittana</i>	Larval and pupal weights reduced; developmental abnormalities [31]
			Cotton	<i>Helicoverpa armigera</i>	A higher number of cotton bolls were recorded in plants expressing NaPI and a PotI inhibitor from potato, StPin1A [199].

Protease inhibitor	Protease family	Proteases inhibited	Transformed plant	Insect species used in bioassay	Effect of PI on larval growth
Potato inhibitor II [Pin II, PPI- II, Pot II, PI-II]	Proteinase inhibitor II	Trypsin, chymotrypsin, oryzin, subtilisin, elastase	Tobacco	<i>Manduca sexta</i>	Growth retarded [200]
			Tobacco	<i>Chrysodeixis eriosoma</i> , <i>Spodoptera litura</i> , <i>Thysanoplusia orichalcea</i>	<i>C. eriosoma</i> larvae grew slower; <i>S. litura</i> and <i>T. orichalcea</i> growth either unaffected or enhanced [201]
			Tobacco	<i>Spodoptera exigua</i>	Growth not affected [202]
			Rice	<i>Sesamia inferens</i>	Decreased weight [74]
			<i>Brassica napus</i>	<i>Plutella xylostella</i>	Lowered growth rates however more plant tissue consumed [203]
			Tomato	<i>Heliothis obsoleta</i>	Increased mortality and decreased weight on homozygous plants expressing PI-II and potato carboxypeptidase inhibitor (PCI), opposite effect on hemizygous plants [204]
<i>Solanum americanum</i> proteinase inhibitor [SaPIN2a]	Proteinase inhibitor II	Trypsin, chymotrypsin	Tobacco	<i>Helicoverpa armigera</i> , <i>Spodoptera litura</i>	Reduction in larval weight and pupation rate [205]
Soybean Kunitz trypsin inhibitor [SBTI, SKTI]	Kunitz (plant)	Trypsin, chymotrypsin, kallikrein, plasmin	Poplar	<i>Clostera anastomosis</i> , <i>Lymantria dispar</i>	Mortality and growth not significantly affected [206]
			Potato	<i>Lacanobia oleracea</i>	Survival and growth decreased by 33% and 40% respectively after 21 days [174]
			Tobacco	<i>Spodoptera litura</i>	Increased mortality and delayed development [207]
			Tobacco	<i>Helicoverpa armigera</i>	Development unaffected [208]

Protease inhibitor	Protease family	Proteases inhibited	Transformed plant	Insect species used in bioassay	Effect of PI on larval growth
			Tobacco and potato	<i>Spodoptera littoralis</i>	High mortality on tobacco and up to 50% weight reduction on potato [209]
			Sugarcane	<i>Diatraea saccharalis</i>	Increased mortality; retarded growth [210]
Soybean Bowman-Birk trypsin inhibitor [SBB1]	Bowman-Birk	Trypsin, chymotrypsin	Sugarcane	<i>Diatraea saccharalis</i>	Growth severely retarded [210]
Sweet potato trypsin inhibitor [SWTI, Sporamin]	Kunitz (plant)	Trypsin	Cauliflower	<i>Plutella xylostella</i> , <i>Spodoptera litura</i>	Increased mortality [42]
			Tobacco	<i>Spodoptera litura</i>	Growth and survival severely retarded [211]
			Tobacco	<i>Helicoverpa armigera</i>	Increased mortality and delayed growth and development in larvae on plants expressing sporamin and a phytocystatin from taro, CeCPI [212]
			Brassica	<i>Plutella xylostella</i>	Survival rate and body mass was significantly lower in larvae fed plants expressing sporamin and chitinase [213]
Tomato inhibitor I [Tom1]	Proteinase inhibitor I	Chymotrypsin subtilisin, trypsin	Tobacco	<i>Manduca sexta</i>	Little effect on growth [200]
Tomato inhibitor II [TPI-II]	Proteinase inhibitor II	Chymotrypsin trypsin, subtilisin	Tobacco	<i>Manduca sexta</i>	Growth retarded [200]

This table lists plant and non-plant serine protease inhibitors expressed in transgenic plants that have been tested in bioassays with lepidopteran larvae. The major enzymes targeted by each PI are given, however other enzymes may be weakly inhibited or have not been tested.

Table 3. Serine protease inhibitors that have been tested for their effect on growth and development of lepidopteran larvae

The first problem of inadequate levels of PI expression is best exemplified by studies with *P. xylostella*, the diamondback moth. When larvae of the diamondback moth consumed transgenic plants expressing the chymotrypsin and trypsin specific potato type II proteinase inhibitor, Pot II, they suffered lower growth rates. However, this did not confer an advantage to the plants because the larvae consumed more tissue to compensate for their decrease in metabolism [13,203]. As a result, the insects maintained population growth rates similar to those of larvae on non-transgenic plants. Growth enhancement has been reported after PI ingestion in insects from a number of orders [201,216]. Larvae that consumed tobacco leaves expressing low levels of mustard trypsin inhibitor 2 (MTI-2) developed faster, had an increased mean weight and caused more damage to leaves compared to control larvae on non-transgenic tobacco [187]. The increase in leaf surface consumption observed with plants expressing low levels of MTI-2 may have resulted from a decrease in available protein due to the presence of MTI-2 and/or to an increase in gut proteolytic capacity induced by PI consumption [187].

The second problem, the pest's capacity to react to PI consumption, is exemplified by the observation that several PIs that are potent inhibitors of insect proteases *in vitro* fail to produce any deleterious effect when fed to larvae [187]. Several mechanisms have been reported for this lack of effect (Figure 2). For example, the complement of proteolytic enzymes in the insect midgut can be altered after PI ingestion [183,214,217]. This could involve a switch to enzymes of different substrate specificity, but the same mechanistic class. For example, production of a chymotrypsin-like enzyme rather than a trypsin-like protease [195,218]. Another mechanism used to detoxify the PIs is degradation via endogenous proteases within the insect midgut [214,219]. Insects that feed regularly on a particular host plant are generally not affected by the PIs produced by the host. For example the PIs from chickpea, a host plant for *H. armigera*, are rapidly degraded by the *H. armigera* gut proteases [219,220]. Similarly, single domain cystatins from potato multicystatin are degraded when fed to larvae of *Diabrotica* spp (Coleoptera). Sometimes non-host PIs are also rapidly degraded. Human stefin A, a potent inhibitor of human cysteine proteases, was degraded by cystatin-insensitive proteases in the gut of Colorado potato beetle (*Leptinotarsa decemlineata*) and black vine weevil (*Otiorynchus sulcatus*) [221]. Another anti-PI mechanism is the production of midgut inhibitor-resistant serine proteases [182,222-224]. Some insect larvae adapt to the presence of PIs by replacing the inhibited enzymes with other PI-resistant proteases and can exhibit increased ingestion rates and faster development than larvae fed on control diets lacking PIs [202,204,225,226]. Some classic examples of this phenomenon are as follows. Soybean Kunitz trypsin inhibitor (SKTI) is normally an effective inhibitor of protease activity in gut extracts from *H. armigera* larvae, this insect is not seriously affected by ingestion of this PI because it responds to chronic ingestion of SKTI by increasing activity of an SKTI-resistant trypsin [227]. Similarly, growth and development of *S. exigua* larvae was not impacted when fed leaves from tobacco plants transformed with the chymotrypsin/trypsin specific potato proteinase inhibitor II (Pot II) [202]. Analysis of the trypsin activity in the gut of these insects demonstrated that only 18% of the trypsin activity of insects reared on these

transgenic plants was inhibited by Pot II, whereas 78% of the trypsin activity in the gut of insects reared on control plants was Pot II-inhibitable [202]. The larvae had compensated for the loss of the PI-inhibitable trypsin by a 2.5-fold induction of new activity that was resistant to inhibition by Pot II [202]. Another observation of induction of PI-resistant enzymes was made by Markwick and coworkers who reported that the trypsin in three species of leaf rollers (Tortricidae) that had fed on diets containing SKTI was less inhibited by bovine pancreatic trypsin inhibitor (BPTI) compared to the trypsin in control larvae [228]. These responses have been reported for lepidopteran species that have ingested PIs in native plants, transgenic plants, and artificial diets [195,229]. In summary, potent inhibition of an insect digestive enzyme *in vitro* by a particular PI is not a good prediction that the PI will be useful when expressed as a transgene for crop protection. That is, expression and regulation of midgut serine proteases in herbivorous insects is tightly regulated and is heavily influenced by the levels and the nature of ingested PIs [230,231]. The mechanism by which changes in protease levels and protease isoforms is regulated in response to PI ingestion is still unknown for phytophagous insects. An overview of the effects of PIs on insects is presented in Figure 2.

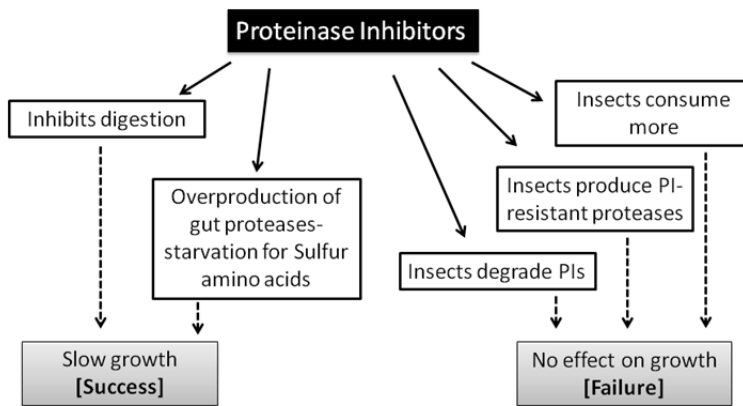
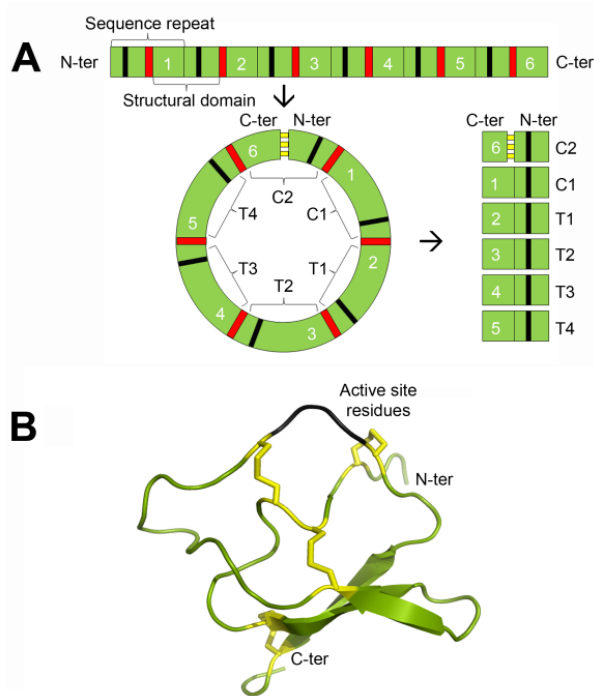


Figure 2. Outline of the various effects of ingested PIs on insect pests leading to success or failure in plant protection

3.3. Proteinase inhibitors from *Nicotiana glauca* as defence molecules against insect pests

Female reproductive tissues and wounded leaves of the ornamental tobacco, *Nicotiana glauca* amass high levels of serine proteinase inhibitors for protection against insect pests and pathogens [232]. These serine proteinase inhibitors (NaPI) belong to the Potato type II family (Merops family I20) which have only been described in the Solanaceae. The NaPI precursor protein (NaProPI; 43 kDa), is composed of an ER signal peptide (29 amino acids), six repeated domains each with a potential PI-reactive site, and a 25 residue C-terminal domain that is essential for vacuolar targeting (VTS) [232-234] (Figure 3).



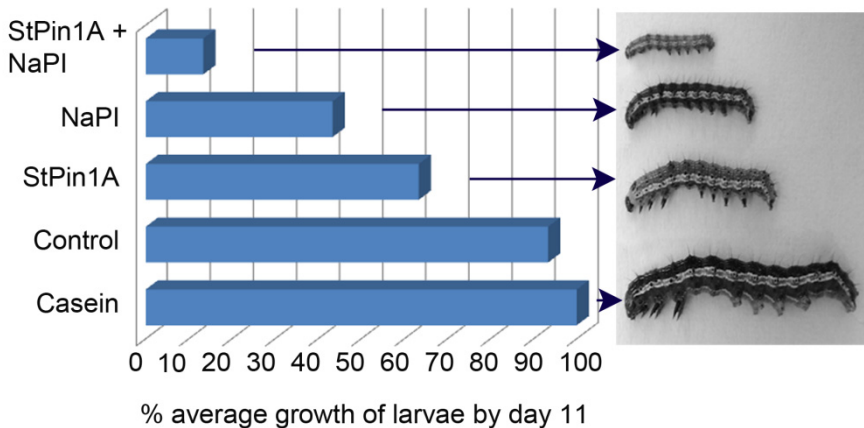
(A) The NaPI precursor protein, shown as a linear gene product, forms a circular 'bracelet' structure that is 'clasped' by three disulphide bonds (yellow) between the N- and C-terminal repeats. Each repeat (labeled 1-6) contains a protease-reactive site (black), which is specific for either chymotrypsin (C1 and C2) or trypsin (T1-4). The six linker regions (red), with sequence EEKKN, are cleaved to release the six active inhibitor domains. The N-terminal signal sequence and the C-terminal vacuolar targeting signal have been omitted for clarity. Figure adapted from Scanlon *et al.* [235]. (B) Ribbon view of T1 showing the major secondary structural element, a triple stranded β -sheet (green) and the cysteines involved in disulfide bonds (yellow). The reactive site residues (black) are positioned between two cysteines that anchor the reactive loop to the central coil [236]. The other five inhibitors have the same structure [236-238].

Figure 3. Diagrammatic representation of the domain organisation of NaProPI and the structure of the T1 inhibitor domain

Processing of NaProPI in the secretory pathway removes the ER signal peptide and VTS, and releases six PIs [232,239]. Processing of the six repeat NaProPI occurs at sites located within, rather than between, these repeated regions [232,239]. Complete removal of the linker sequence (Glu-Glu-Lys-Lys-Asn) contained within each repeated region [239], generates five contiguous inhibitors, a chymotrypsin inhibitor (C1) and four trypsin inhibitors (T1-T4), and two flanking peptides from the N- and C-termini. The flanking peptides form a novel two-chain chymotrypsin inhibitor (C2) that can only be formed if NaPI adopts a circular structure (Figure 3; [240]). The peptides have very similar amino acid sequences [239]. The three-dimensional structures of C1, C2, T1, T2, T3 and T4 have been determined by NMR spectroscopy [234,236,240]. A triple stranded β -sheet is the dominant secondary structural feature; several β -turns and a short region α -helix are also present (Figure 3B; [238]). The reactive site is located on an exposed loop which has a higher degree

of mobility than other regions of the protein (Figure 3B). This is a common feature of PIs and is thought to allow the inhibitor to adapt to slightly different enzymes [239].

Atkinson and colleagues suggested NaPIs may be involved in deterring insects from feeding on stigmas or in protecting the stigma from pathogen invasion since the related type-II PIs from potato and tomato are effective against proteases of fungal, bacterial and insect origin [232,241]. The PIs from *N. alata* inhibit the digestive gut proteases from five insect orders *in vitro* and display significant inhibitory activity against the midgut proteases of *H. punctigera* and *T. commodus* [197,198]. Significant mortality was recorded when *H. punctigera* larvae were fed transgenic tobacco [197] or transgenic peas [198] expressing the NaPI precursor. More recently, the response of *Helicoverpa* larvae to ingestion of NaPI has been more thoroughly characterized. Following ingestion of NaPI, all surviving *Helicoverpa punctigera* larvae produced high levels of a chymotrypsin that was resistant to inhibition by NaPI [199]. However this NaPI-resistant chymotrypsin was strongly inhibited by a potato type 1 inhibitor which is also produced by solanaceous plants, but belongs to a different class of serine proteinase inhibitors. When presented to *H. armigera* larvae in an artificial diet the combination of NaPI and the potato type I inhibitor had a much more dramatic effect on growth and development of the larvae compared to either of the inhibitors alone (Figure 4).



Neonates were transferred to cotton-leaf based artificial diets containing 0.3% of PIs (NaPI, StPin1A) and growth (mg) measured every 2nd day until day 11. Day 11, the % average weights compared to casein control are shown with representative larvae from each treatment (adapted from [199])

Figure 4. Percentage of *Helicoverpa* larval growth on day 11.

This laboratory result was then translated to transgenic plants in the field. Transgenic cotton plants expressing both PI classes, NaPI and StPin1A performed better than transgenic cotton plants expressing either PI alone. The improved performance of the transgenic cotton plants with both PIs was measured by an increase in cotton boll number per plant and increased yield of lint at the end of the cotton growing season (Figure 5)[199].



Figure 5. A higher number of cotton bolls were produced on field grown transgenic cotton producing NaPI and StPin1A (A) compared to Coker (B) the control non-transgenic parent

3.4. Commercialisation of PIs and strategies to avoid resistance

Since the first transgenic plants appeared almost two decades ago, this technology has contributed to the development of new approaches for crop protection [25]. There are numerous reports showing that expression of PIs in transgenic plants confers resistance to the intended target insects (see Table II; reviewed in [61,215,242,243]). However, many of the candidate genes that have been used in genetic transformation of crops have limited application because they do not have broad spectrum activity against the major insect pests or are only mildly effective against the target pests [52]. To overcome the development of insect resistance to transgenic plants expressing PIs, it is necessary to develop PIs that have broad activity against most or all of the proteases that the insects use for digestion. Several strategies have been proposed.

3.4.1. *Selecting second generation protease inhibitors from novel sources*

PI-resistant proteases probably result from the selection pressure imposed on insects when they encounter high endogenous PI levels in certain host plants [170]. Such selection for PI-resistant proteases does not occur for PIs from non-host plants. Therefore, one approach to obtain better inhibitors for a particular insect pest is to search for PIs in plant species that are unrelated to the plant that is the normal host for that pest [10,74,170]. Another approach is to select PIs from synthetic libraries of mutant inhibitors for insect control [170].

3.4.2. *Use of multiple inhibitors*

Another strategy for controlling resistance development is to use at least two inhibitors that have different targets. This can be achieved by producing chimeric proteins, gene stacking (pyramiding) or the use a single inhibitors that have dual targets. Some examples of

bifunctional inhibitors are alpha-amylase/trypsin inhibitors [8] and trypsin/ carboxypeptidase A inhibitors [244]. Similarly, expression of a fusion protein composed of a cystatin and a serine PI has been used to control certain nematode pathogens in transgenic plants [245]. Oppert and colleagues [246] demonstrated synergism between soybean Kunitz trypsin inhibitor and the cysteine protease inhibitor L-trans-epoxysuccinylleucylamide [4-guanidino] butane (E64) in artificial diet bioassays with *Tribolium castaneum* (red flour beetle, Coleoptera).

Transgenic tobacco plants expressing both a Bt-toxin and a cowpea trypsin inhibitor (CpTI) were more protected from *H. armigera* damage compared to transgenic tobacco expressing the Bt-toxin alone [247]. The enhanced insecticidal activity was attributed to enhanced stability of the Bt-toxin when the gut protease activity had been lowered [248,249]. In a separate set of experiments, *H. armigera* and *S. litura* larvae that consumed leaves from transgenic tobacco expressing avidin (from chicken egg white) that had been painted with Cry1Ba protein died significantly faster than larvae given either of the two treatments alone [38]. When used together in bioassays with artificial diet, the different and complementary action of Pot I (a chymotrypsin inhibitor) and CPI (a carboxypeptidase inhibitor) also resulted in a synergistic effect at reducing the growth rate of *Cydia pomonella* (codling moth) larvae [250]. However, the protective effects observed with PI gene constructs have not been sufficient to lead to a serious attempt at commercialising these transgenic crops.

4. Summary

The usefulness of insect-resistant transgenic plants has been widely demonstrated with the highly successfully implementation of crops that produce the Bt toxin. The current fear is that although Bt toxin has defended crops in the field for nearly 10 years now, the discovery of Bt resistance in *H. zea* populations in crop fields in the USA [251] and Bt resistance in populations of *D. virgifera* found in corn fields [148] might lead to widespread development of resistance to the Bt toxin. We have reported that two structurally different PIs that target different enzymes greatly improved the protection of transgenic cotton plants in the field. This supports the general consensus in the literature that no single insect trait will provide sustainable crop protection and that stacking of multiple insect traits that target different mechanisms should be employed.

Author details

Jackie Stevens, Kerry Dunse, Jennifer Fox, Shelley Evans and Marilyn Anderson*
La Trobe Institute for Molecular Sciences, La Trobe University, Melbourne, Australia

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Limited Receptive Area Neural Classifier for Larvae Recognition

Tatiana Baidyk, Oleksandr Makeyev,
Ernst Kussul and Marco Antonio Rodríguez Flores

Additional information is available at the end of the chapter

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1. Introduction

During the last years many agrochemicals (pesticides, herbicides, insecticides, so on) have been used for pest control in the world, and in Mexico too. This causes great damage to the health of people who has contact with pesticides and to the environment.

In recent years, large amounts of pesticides are used to achieve record harvests (for example, DDT has been used in Mexico for more than 50 years) [1], [2]). DDT is forbidden in the U.S., Canada and Europe because causes cancer. To reduce the pesticide amount it is necessary to locate precisely the distribution of insects and caterpillars. This task is very important not only for crops but also for monitoring forests. The forest health demands the efforts to fight threats of different kinds of insects and caterpillars [3], for example with Emerald ash borer, *Agrilus planipennis* Fairmaire [4].

For reducing the amounts of pesticides it is needed more precisely locate the distribution of pests in crops, in order to exterminate them effectively and to achieve the least damage. For this reason we need to develop an image recognition system intended to achieve the least possible exposure and contact of the persons who works in agriculture with any type of pesticide, as well as decreased crop areas where apply.

The task is not easy because many insects use the technique of mimesis (imitation) to hide them among the foliage. In this work the characteristics of the textures are used to find the insects. For this reason we are dedicated to development of computer vision system based on neural networks to locate the insects. This will permit us to avoid using large amounts of pesticides that are harmful to both farmers and crops.

Man's efforts in creation of computers have allowed the construction of machines able to solve automatically and quickly certain operations that are tedious. With the construction of

the first computers in XX century, advances in science and technology have had a boom, allowing to easily implementing algorithms to solve many problems. Current developments are directed to study human capabilities as a source of new ideas for the design of new machines. Thus, the artificial intelligence is an attempt to discover and describe aspects of human intelligence that can be simulated by machines. This discipline was developed in recent years and has applications in some fields such as computer vision, theorem proving, information processing, neural networks as part of artificial intelligence among others methodologies.

After thorough investigation of related literature dedicated to neural networks and their applications, making a focus on pattern recognition, we selected the structure of LIRA neural classifier as the basis for our computer vision system.

It is interesting for us to investigate the recognition of the larvae. There is wide variety of larvae and caterpillars, and some of them are very dangerous. The most common of the insects are larvae in foliage of forests, gardens and trees.

The aim of this chapter is to propose an alternative for pest control in crops (in this case caterpillars and/or larvae) and avoid using large amounts of pesticides. It is also necessary to monitor the areas of localization of these pests to know how they are distributed. With this knowledge it is possible to dose the amounts of pesticides applied to the field.

The monitoring of forests and fields with different types of agricultural plants is very important. Mobile robot (1), for example, in Fig.1 may inspect the plants with a camera (2) on the board. It is possible to do the monitoring with airplanes and video observations of some regions of interest.

Artificial neural networks (ANN) are currently an active multidisciplinary field, involving researchers from different areas such as electronics, physics, mathematics, engineering, biology or psychology because they present a way to emulate certain characteristics of humans such as the ability to memorize and associate events to carry out certain tasks.

2. Artificial neural networks

Neural networks are a simplified model of human brain, which is an example of a system that is able to acquire knowledge through experience. An ANN is a system for the treatment of information whose basic unit is inspired by the fundamental cell of the human nervous system: the neuron.

Historically two groups of researchers have worked with artificial neural networks. One group was motivated by the aim of using ANNs to study and to model the different biological learning processes. A second group was motivated by the aim of obtaining highly effective machine learning algorithms, regardless of whether they reflect the biological processes.

The importance of ANNs is because they are an alternative solution to complex problems related to the recognition of shapes or patterns, prediction, coding, control and optimization.

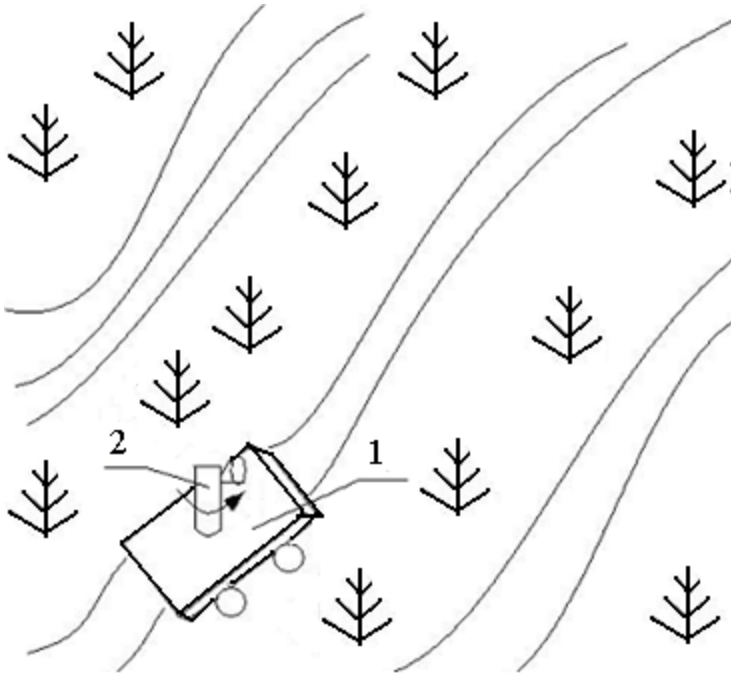


Figure 1. Environmental monitoring (1- mobile robot; 2- TV camera)

The evolution of computer systems inspired by the human brain, and therefore endowed with certain "intelligence", is the combination of simple elements of process (neurons) interconnected and operated in parallel. These new methods are applied to resolve the different problems.

The first studies on ANNs started in the earliest 40s of XX century and from there have been strongly increasing till today, thanks to the works of many scientists and advances in hardware. Many scientists have developed the neural networks, due to this fact the neural networks have taken an important place in science.

Warren McCulloch and Walter Pitts made the first mathematical model of neuron [5]. Pitts and McCulloch model is based on the idea that neurons operate using binary pulses. This model introduces the idea of a threshold function later used by many models such as, for example, the Hopfield neural networks (discrete bidirectional associative memory) [6], [7]. This model generated great interest to provide sophisticated behavioral measures through simple calculations. The model is a key factor in learning ability.

Donald Hebb developed a mathematical model of learning procedure [8]. Hebb studied on neurons and the classic conditions for learning. He developed a learning paradigm that now has his name: Hebbian learning.

Inspired by the work of McCulloch and Pitts, Minsky and Edmonds designed a machine with 40 neurons whose connections were adjusted according to a series of events that

occurred when performing certain tasks. The machine was built with tubes, motors and relays, and was able to successfully model the behavior of a rat looking for food in the maze. Marvin Minsky and Papert proposed the results of artificial neural networks analysis [9], they argued that there were a number of fundamental problems with the network research program.

Albert Uttley began to develop new paradigms of artificial neural networks, creating a theoretical machine that consists of processing elements. The processing element was a linear separator that fit input parameters using the Shannon entropy measure. These machines have been used to simulate atmospheric phenomena, as well as adaptive recognition of patterns.

In 1957, Frank Rosenblatt generalized the McCulloch-Pitts model of cells by adding learning, calling this model the PERCEPTRON [10]. A two-level model was developed, which adjusted the weights of the connections between input and output levels in proportion to the error between the desired output and the output obtained. Rosenblatt attempted to extend their learning process to a three-level PERCEPTRON, but he did not find a solid mathematical method to train the hidden layer connections.

Bernard Widrow proposed an artificial neural network design very similar to PERCEPTRON, called Adaptive Linear Element or ADALINE [11]. The ADALINE had two levels, much like the PERCEPTRON, adjusted the weights between the input and output levels according to the error between the expected value and the obtained output. The difference between these two models is very small, but the applications that are addressed to are very different. In 1960, Widrow and his colleagues mathematically proved that the error between the desired output and that obtained in certain circumstances can be minimized to the extent we want. Both the PERCEPTRON as the ADALINE maintained the problem of linear separability. The ADALINE has been used for adaptive signal processing, control systems and adaptive antenna systems.

Steinbuch was among the first researchers who developed the methods of information encoding in neural networks. Steinbuch networks were applied to handwriting recognition, to machinery fault diagnosis and control of multiple production processes.

Stephen Grossberg is the most influential and formal of all researchers in artificial neural networks [12]. Grossberg made important studies on psychological processes and phenomena (mind) and biological (brain) of human information processing and tried to bring the two (mind and brain) in a unified theory. Grossberg's work included strict mathematical analysis that allowed the realization of new paradigms of neural networks. These made it possible to have direct access to information while operating in real time.

Shun-Ichi Amari combined the activity of biological neural networks with rigorous mathematical models of neural networks. One of these is a solution to the famous problem of credit allocation. His studies include the treatment of dynamic neural networks and randomly connected, competitive learning studies and mathematical analysis of associative memories.

James Anderson worked with a memory model based on the association of activation of the neuron synapse, performed a linear associative memory model, following the Hebb approach. He used a new method of error correction, and linear threshold function substituted by another ramp function, creating a new model called Brain-state-in-a-box.

Kunihiko Fukushima began work on artificial neural networks in the latest 60's, studying spatial and space-time vision systems, and brain [13]. His most notable work was the creation of an artificial neural network paradigm for multi-view, which has been improved over time. Fukushima called his first project COGNITRON and made an improved version called NEOCOGNITRON [14].

A. Harry Klopff studied the relationship between the psychology of the mind and brain biology since 1969. He theorized that the neuron is a component that moves the brain searches for targets. It is an adaptive system that increases the effectiveness of excitatory synapses when depolarized, and increases the efficiency of inhibitory synapses when hyperpolarized.

Teuvo Kohonen began his research on artificial neural networks with random connections paradigms in 1971 [15]. Kohonen's work focused on associative memories and correlation matrices, similar to the work of Anderson, Steinbuch and Piske. Kohonen and Ruohonen later extended the model of linear associative memory, which linearly independent vectors required for good performance. He later did research on teaching methods and developed the LVQ (Learning Vector Quantization), a competitive learning system.

Robert Hecht-Nielsen was the principal designer of one of the first neural computer dedicated to processing the neural network paradigms [16]. The neuro-computer, the TRW MARK III, is supported by a DIGITAL VAX computer, and was commercially available from 1986.

John Hopfield described the method of state analysis of auto-associative networks [6], [7]. He introduced an energy function in his studies of systems. Hopfield shows that you can build an energy equation that describes the activity of a single layer neural network in discrete time, and that this energy can be dissipated and the system converge to a local minimum. This analysis raised the interest to apply artificial neural network paradigms for difficult problems that conventional computers cannot solve. Hopfield extended his model to consider continuous time.

The artificial neural networks (ANNs) are computer systems, which mimics the computational abilities of biological systems by using a number of interconnected artificial neurons. Artificial neurons are simple emulations of biological neurons; they take the information from sensors or other artificial neurons, perform simple operations on the data and pass the result to other artificial neurons. ANNs operate by artificial neurons and have processing their data in this way. They use both the parallel logic (all neurons are functioning in the same layer) combined with the serial operations (information from one layer is transferred to neurons in another layer). The three main characteristics, which describe a neural network, and thereby contribute to their functional abilities, are: structure, dynamics and learning.

ANNs offer specific advantages of information processing, which makes it the technology of decision making in many application areas. These advantages include:

- Adaptive Learning,
- Self-organization,
- Fault tolerance via redundant information coding,
- Real-time operation,
- Easy integration into existing technology.

During the last few years we have investigated the neural classifiers that have demonstrated good results in recognition of different objects, for example, handwritten digits (on MNIST database), texture recognition in micromechanics, recognition of pin-hole relative position, etc. [17] - [20].

LIRA neural classifier permits us to create a new system to recognize larvae. This work is very important to reduce pesticide application in fields, forests, and so on [1] - [4]. If we can localize the distribution of larvae we can reduce the pesticide quantity. The pesticides are very dangerous for people's health. In the literature, there are different methods of larvae recognition [21], [22]. This work is done to keep the trees healthy and to obtain good harvests. We propose using the LIRA neural classifier for larvae recognition during field inspection.

3. Image database

We used the image database from [23] (www.forestryimages.org) as color images (in this article we will present several photos from this database). We selected 79 images for the first image database and 55 images for the second image database to test our LIRA neural classifier. The first image database contains images with different number of larvae on the image (Fig.2). The number of larvae may vary from one to dozens in one image.

The second image database contains only one larva. The task of larvae recognition is very difficult because the caterpillars, larvae have different forms. The larvae have very different textures (Fig.3). Some of them contain bristles, others are smooth.

The caterpillars vary in color (Fig.4) and size (Fig.5). All these factors make the recognition process difficult.

To work with this database of images we have to form two sets of images: one for system training and another set to test the system.

We used 79 images and divided them in two parts: for the training process and for recognition (for example, 10 images for training and 69 for recognition, or 20 for training and 59 for recognition). All images were presented in BMP format with resolution of $H_1 \times W_1 = 768 \times 512$ pixels.

To investigate our neural classifier with these images we have marked the images. In Fig.6 we present an original image and the marked larvae. In this case we can train our system with "teacher" (supervised training), all larvae are marked with white (Fig. 6).

We will describe the results of recognition after LIRA neural classifier description and will analyze the possibility of improving of the obtained results.



a) Image Number: 2089019
Polyphemus moth
Image Citation:
Lacy L. Hyche, Auburn University,
www.forestryimages.org



b) Image Number:3057061
Pinkstriped oakworm
Image Citation:
James Solomon, USDA Forest Service,
www.forestryimages.org

Figure 2. Images with different number of larvae



a) Image Number: 1368001
American dagger moth
Image Citation:
Joseph Berger,
www.forestryimages.org



b) Image Number: 1791015
Copper underwing
Image Citation:
Lance S. Risley, William Paterson University
www.forestryimages.org

Figure 3. Images of larvae with different textures.



a) Image Number: 2721072
Peigler's oakworm moth
Image Citation:
Paul M. Choate, University of Florida
www.forestryimages.org



b) Image Number: 1160018
Luna moth
Image Citation:
David J. Moorhead, The University of Georgia,
www.forestryimages.org

Figure 4. Images of larvae with different color



a) Image Number: 1791018
Eight-spotted forester
Image Citation:
Lance S. Risley, William Paterson University,
www.forestryimages.org



b) Image Number: 1748032
Polyphemus moth
Image Citation:
Robert L. Anderson, USDA Forest Service,
www.forestryimages.org

Figure 5. Images of larvae of different size



a) original larvae;

b) larvae marked with white color

Figure 6. Original and marked larvae:

4. LIRA neural classifier

There are two types of image recognition systems. The first type contains a feature extractor and a classifier (Fig.7). The feature extractor transforms the image into a parameter vector. Each component of this vector corresponds to a specific feature used to solve different problems of recognition.

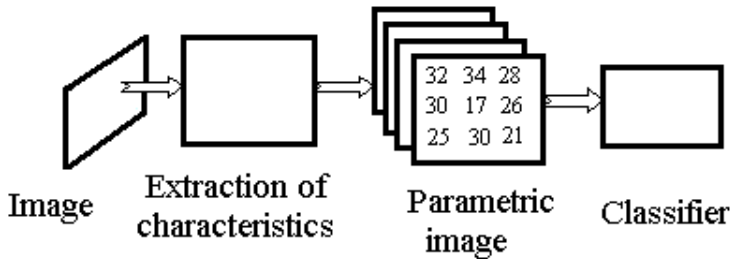


Figure 7. Structure of recognition system

The second type of image recognition system contains only the classifier. Systems for image recognition such as Rosenblatt's Perceptron [10], Fukushima's Neocognitron [14] among others, belong to this type of system. These systems are characterized by the feature extractor that is incorporated in their internal structure.

The Limited Receptive Area (LIRA) neural classifier belongs to the second type of system, it was developed and proposed in [17] - [19], it is based on Rosenblatt's Perceptron [10].

Two major differences of this classifier are based on how an image is presented at the input of the classifier and the encoder. If the image is presented in binary form, i.e. the input values can be only black and white (binary), we call it the Binary LIRA. If the input image is presented with gray scale values, it is called the Grayscale LIRA. The LIRA neural classifier has been tested in handwritten digit recognition tasks [17], microscrews image classification [18], micro devices assembly tasks [19] and has demonstrated good results.

It is necessary to say that science and engineering of image and pattern recognition have developed rapidly in recent years. The digital image processing is a set of techniques applied to the digital representation of an image that makes up a scene, in order to recognize or classify some elements of interest to facilitate further analysis by a computer vision system [24]. The image processing techniques are applied when it is necessary:

- Improve or modify an image,
- Highlight some aspect of the information contained therein,
- Measure, compare or classify an item in the image,
- Recognize image content.

The LIRA neural classifier is described in detail in [25] - [27]. Here we give only the main characteristics of the LIRA neural classifier. It consists of 3 layers as perceptron of Rosenblatt. We included our modifications to the structure and algorithms of the perceptron [26]. So, we can speak about 4 layers (Fig.8): sensor layer *S*, intermediate layer *I*, associative layer *A*, and response layer *R*. The sensor layer *S* in our modifications is scanned with window of size ($H \times W$) that we define for a task. Inside of the window ($H \times W$) we generate many small windows ($h \times w$). The position of the window $h \times w$ we select randomly (position is the position of the left upper corner of the window). The range was [0, *W*] and [0, *H*]. Between *S* and *I* layer we randomly generate connections once and do not change them during the experiments with the neural classifier. The neurons in layer *I* can be ON and OFF neurons. In Fig.8, layer *I*, we present two ON-neurons and three OFF-neurons. ON and OFF-neurons work in a following manner:

$$\begin{aligned} \Psi_{ON}(x_i) &= \begin{cases} 1, & x_i \geq T_i \\ 0, & x_i < T_i \end{cases} \\ \Psi_{OFF}(x_j) &= \begin{cases} 1, & x_j \leq T_j \\ 0, & x_j > T_j \end{cases} \end{aligned} \quad (1)$$

where Ψ is the output value of the neuron; x_i is input value of the neuron *i*; T_i is the threshold of the neuron *i*. These neurons permit us to extract the features of the image. The neurons of associative layer *A* present the binary code of the image. Every neuron a_i has the output only when all ONN and OFF neurons respond. If any neuron of ONN or OFF neurons has no answer ($\Psi = 0$), the neuron a_i has output equal to "0". So in associative layer *A* we have a small number of active neurons (with output equal to "1").

In many experiments which we made to resolve different recognition tasks it was demonstrated that the number of associative neurons may vary from various tens thousands to various hundreds thousands. Large number of neurons in associative layer permits us to improve the recognition rate. The acceptable time of calculations we obtain due to rare coding principle when the number of active neurons in associative layer *A* is much less than the total number of neurons. The calculations are made only for active neurons.

Connections between neurons of *A* layer and *R* layer (the rule of connecting is "all neurons of *A* layer are connected with all neurons of *R* layer") have weights (w_{ij}) that are changed

during the training process. The rule of weight changing is the following. If the system recognizes the class correctly we do not change the weights. If the system recognizes the class incorrectly we change the weights to the incorrect class (decreasing them) and change the weights to the correct class (increasing them). The recognized class is defined with the neuron of layer R that has maximal excitation. In our task the layer R contains only two neurons that correspond to two classes: class of larvae and class of background.

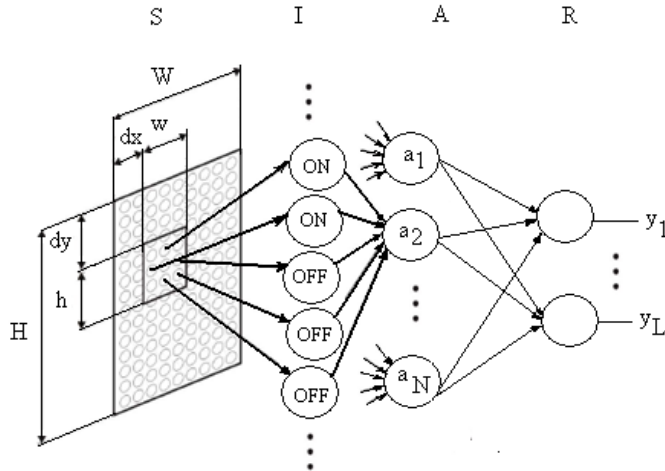


Figure 8. LIRA neural classifier structure.

Then with these rules we developed and programmed the computer vision system based on neural classifier LIRA for image recognition.

The work of the system is divided into two phases: the first phase is the training process of the artificial neural network using a larvae image base that contains larvae of different shapes and colors, with different amounts and with different positions. The second phase is a verification of the system with the other part of the image base of caterpillars (larvae).

The main objective of this investigation is design and programming of the computer vision system based on the LIRA neural classifier. This classifier is useful for the task of caterpillar recognition on crops, considering the different characteristics of larvae and diversity in size and shape that they may have.

The program of the neural model was written in C++ Borland 6 and the experiments were realized with a computer that contains a processor Intel Pentium Dual Core @2.20GHz with 1Gb RAM memory, Windows XP.

We can summarize the working algorithm of the system as follows. An image is presented as input to the LIRA neural classifier. Then the algorithm calculates the image code and it is processed with the LIRA neural classifier to obtain the output of the neural classifier, i.e., the class to be recognized by the classifier. It means the presence or absence of larvae/caterpillar(s) in the image. The image code is stored in memory for using in

successive cycles of training process instead of the input image. This gives us possibility to save significantly the computing resources and time.

The methodology for the construction of the system is as follows:

- Step 1.** Initialize the weights of connections between layers A and R.
- Step 2.** Input the image to the input layer S of the system.
- Step 3.** Encode the image by means of layer I to the feature vector.
- Step 4.** Calculate the vector of characteristics of the layer A (obtain the properties of the image).
- Step 5.** Get the class type recognized in output layer R.
- Step 6.** Modify the weights W_{ij} according to the obtained output.
- Step 7.** Repeat step # 2 to # 6 until you reach the fixed number of cycles.

The training process is supervised training (for this purpose we used marked images). During this training process the system makes adjustments of the connection weights between A and R layers of internal structure of the LIRA neural network. In the training process a training set of images is presented. Every window ($h \times w$) of these images must have a label associated with the class it represents. With this the classifier can be trained.

The training process consists of several cycles, in each cycle every image from the training set is presented to a classifier together with its label. After calculating one neuron with maximum excitation of the output layer R of the classifier is selected as response of the system. The correct class corresponding to the input image is read as the label associated with this. If the response of the system coincides with label, nothing to be done. If we have different answers, the system need to be trained more. The training process is repeated until a convergence criterion will be satisfied:

- 1 st After completing a certain number of cycles.
- 2 nd The error value is zero.

Once the algorithm satisfies any convergence criterion the training process ends.

To test the system or make the system verification we need to apply another set of images that were not participated in the training process. The objective of this stage is to verify the efficiency of the LIRA neural classifier. The purpose of this stage is obtaining of the system recognition rate. The response of the system is a neuron with maximum excitation values in the R layer (Fig.8). If E_i is an excitation of i neuron in the R layer, than the recognized class y_w has the maximum value (equation (2)).

$$y_w = \max_i(E_i). \quad (2)$$

Using the formula (2), R-layer neuron with the highest output value y_w is detected, and called the winner neuron. This neuron represents the recognized class for the input image. For images from the recognition set we calculate number of errors (incorrect responses). According to the obtained results, the efficiency of the system for the proposed task may be validated.

5. Experimental results

In this article we present the results obtained with LIRA neural classifier in the recognition of larvae. The characteristics of LIRA neural classifier are the following: 32,000 associative neurons with two "ON" neurons and three "OFF" neurons. We did 150 cycles of the training process. The error number we calculated for all images. The number of errors is number of samples that were recognized incorrectly.

The tests were carried out in two phases:

- Varying the number of images and allowing a fixed number of training cycles.
- Varying the number of training cycles and fixing the number of images.

The first phase consisted of varying the number of images for training that is, taking a different number of images from the base 79. In this case it took 10/79 images (Number of Images for Training / Total Number of Images), 20/79 images for each test. In all these tests a number of training cycles was 150.

In the second phase the number of training cycles was varied, i.e., from 50 cycles to 300 cycles, leaving a fixed number of 10 images for training in both cases.

Finally, we calculated the percentage of error for each training process according to the following formula: the number of error divided on the total number of windows per image generated in each process.

The window size we changed from (20 x 20) pixels to (120 x 120) pixels. The results obtained in each test are shown below in Tables 1, 2.

Window	Error	% error
20 x 20	1084	10.53
40 x 40	488	11.1
60 x 60	274	10.59
80 x 80	187	12.27
100 x 100	128	11.78
120 x 120	93	18.74

Table 1. Errors obtained for different window size (10/79 images).

We decided to reduce the image database to preserve images, for example, with one larva on the image. So we prepared a new database with 55 images from the previous database. The results are presented in Table 3. We can see that in this case we have improvement in recognition. But, in the future it is necessary to improve the recognition quality of the LIRA neural classifier for the database of 79 images.

With these results for the first phase, we can do the experiments with changing the number of training cycles. You can see also that the window size for the image coding is very important too. For the window of (20 x 20) pixels the error number is less than for a window of (120 x 120) pixels. This is because for a relatively small window the image characteristics are presented in better way than for a much larger window.

Window	Error	% error
20 x 20	1922	10.53
40 x 40	826	11.13
60 x 60	428	10.73
80 x 80	389	11.82
100 x 100	205	12.63
120 x 120	168	13.21

Table 2. Errors obtained for different window size (20/79 images).

Window	Error	% error
20 x 20	1235	8.29
40 x 40	502	8.71
60 x 60	291	8.36
80 x 80	205	10.18
100 x 100	149	12.7
120 x 120	108	10.31

Table 3. Errors obtained for different window size (10/55 images).

In Tables 4 and 5 the results obtained for different number of cycles are shown.

From the Tables 4 and 5 we can see that 50 cycles of training is sufficient to train the LIRA neural classifier. It is not necessary to increase the number of cycles of training process.

We measured the response time for the system with the following features: windows with size (40 x 40) pixels, 150 cycles of training, 10/79 images for training, and 69/79 images for recognition. The time for the recognition of 69/79 images is only 9.26 s. Coding of images with smaller windows requires more time and uses more computer resources. So the response time achieved by the system for training is 0.292 s and for image recognition is 0.042 s for each sample.

Window	Errors
20 x 20	1112
40 x 40	474
60 x 60	269
80 x 80	187
100 x 100	128
120 x 120	108
140 x 140	77
160 x 160	66
180 x 180	40
200 x 200	34

Table 4. Errors obtained for 50 cycles (10/79 images)

Window	Errors
20 x 20	1196
40 x 40	491
60 x 60	261
80 x 80	179
100 x 100	117
120 x 120	99
140 x 140	78
160 x 160	58
180 x 180	46
200 x 200	34

Table 5. Errors obtained for 300 cycles (10/79 images)

So we can do the following conclusion about the LIRA neural classifier quality.

For the first experiments we selected different window size of $(H \times W)$ pixels (Fig.8) that scanned the image from the database. For this purpose we selected 10 images from the database for training of the LIRA neural classifier and the rest of the images ($79 - 10 = 69$) we used for recognition and calculation of errors. The best result of 10.53 %, i.e. the minimal number of errors, we obtained for a window of $(H \times W) = (20 \times 20)$ pixels. The recognition rate in this case is of 89.47%.

We have to explain that the absolute number of errors is maximal for the minimal window (20×20) pixels but in relation to the number of samples for every image the error number is minimal number. For example, for window (20×20) pixels we have 3750 samples for image. For window (200×200) pixels we have 24 samples. Every window we move with a step equaled to the half of the window size. So if the window has size of $(H \times W) = (20 \times 20)$ pixels, the movement step is 10 pixels. If the window size is $(H \times W) = (200 \times 200)$ pixels, the movement step is 100 pixels. The window size is very important in pattern recognition because from this area we collect the features (ON- and OFF-neurons). So if we have a smaller size of window the more precise description of the image we obtain.

We investigated the influence of the number of images in the training set on the recognition rate. The second step we made with 20 images in the training set and 59 images in the recognition set. We change the window size in the same way as in the previous experiment.

In this case the number of errors is increased for the sizes of windows $(H \times W = 80 \times 80)$ pixels and $H \times W = 120 \times 120$ pixels) and in other cases the errors have almost the same value.

We can explain this by the fact that in the training set we had different images that instead of improving the results made them worse. This is connected with very different images of the database.

The recognition rate was improved to 91.71%. It is the best result that our algorithm demonstrated in this investigation.

6. Conclusion

A system for larva recognition is presented in this article. This system is based on LIRA neural classifier. The best result that we obtained in this investigation is about 10.53 % of errors for the database of 79 images (Table 1 and Table 2). In this case we have 89.47% of recognition rate. The worst result was 18.74 % of errors that means 81.26% of recognition rate. The best result for the database of 55 images is 8.29% (91.71% of recognition rate). For a task of this complexity level the result is not bad. But, the LIRA neural classifier can be improved and can demonstrate better results in the future.

7. Impact of the investigation

The prerequisite in the field before using LIRA technique is the necessity to train the LIRA neural classifier with real images of larvae, insects or/and caterpillars. With this training the users can adapt the LIRA neural classifier to new environment, new field conditions.

To reduce the errors in the proposed task it is needed to continue this investigation. Firstly, it is important to obtain more representative image database. The neural networks, especially the neural classifiers, can be trained better with increasing number of images.

Secondly, the further improvement of the computer vision system is connected with investigation and selection of the system parameters, for example, the number of ONN and OFF neurons in *I* layer of LIRA. The number of neurons in *A* layer can vary and has a great influence on the training process.

New ideas in classification and recognition, new methods of feature extraction can be combined with LIRA neural classifier in future.

Author details

Tatiana Baidyk, Ernst Kussul and Marco Antonio Rodríguez Flores
Center of Applied Research and Technological Development, UNAM, Mexico City, D.F., Mexico

Oleksandr Makeyev
Rhode Island University, USA

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Biomarkers in Pesticide Assay

Evolution and Expectations of Enzymatic Biosensors for Pesticides

Rafael Vargas-Bernal, Esmeralda Rodríguez-Miranda and Gabriel Herrera-Pérez

Additional information is available at the end of the chapter

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1. Introduction

The successful use of pesticides around the world has been due to their excellent control of pests such as insects, algae, bacteria, viruses, rodents, or nematodes in agriculture, medicine, household, and industry. Since 9 of the 12 most dangerous and persistent organic pollutants are pesticides, therefore their qualitative and/or quantitative detection continue being one of the most strategic technological areas, given that these can be found in substances in contact with humans and other animals. The effects associated with their consumption and control, are related to human health and environmental toxicity. One of the most important contributions of the environmental chemistry is the control of pesticide residues and metabolites in food, water and soil; where plants, animal and human contacts are possible. Several methods to detect pesticides have been developed, chromatographic methods such as gas chromatography (GC) and high performance liquid chromatography (HPLC), which are coupled with mass spectrometry (MS). Although, these methods are very sensitive and reliable, they are very complex. In addition, they consume a lot of time to realize the analysis, require highly trained technicians for their use, and do not allow on-site or in-field detection. Biosensors represent an interesting technological alternative to determine the presence qualitative and quantitative of pesticides. Their operation is based through a self-contained integrated device including all subsystems required to realize the measurement and transfer of results in an electronic manner. They are a solution of low-cost, fast, high portability, and them do not require trained technicians to be used. In specific, both electrochemical as optical biosensors for pesticides, that use the enzyme immobilization by means of catalytic activity, will be studied with the aim of visualizing their evolution, advances, and perspectives in the near future. Finally, it is illustrated that research in this area might be directed with the aim of optimizing the performance desired.

This chapter is divided as follows: In Section 2, basic concepts related with pesticides and enzymes are described. Next, the history of the enzymatic pesticide biosensors is analyzed

with the aim of identifying the contributions realized in this area, in Section 3. In Sections 4 and 5, the contributions of the optical and electrochemical biosensors in the detection of pesticides in the last years are discussed, respectively. Future perspectives and advances associated with enzymatic pesticide biosensors are described in Section 6. Finally, in Section 7, conclusions highlighting the importance of the study realized here are given.

2. Basic concepts about pesticides and enzymes

A pesticide is any substance or mixture of substances used to prevent, destroy, repel or mitigate any pest. In United States of America, a pesticide can also be used as a plant regulator, defoliant, or disinfectant. Different substances can be used as pesticides: chemical, biological agent, antimicrobial or disinfectant. A pest is a living organism that appears where it is not wanted or that causes damage to crops, animals, or humans. Among the pests can be found: insects, plant pathogens, weeds, mollusks, birds, mammals (rodents), fishes, nematodes, and microbes. Pesticides are categorized in accordance with their chemical substituents: herbicides, fungicides, insecticides and bactericides, as shown in Figure 1. An herbicide is a pesticide used to kill unwanted plants or to reduce the growth of the weed, which leaves unperceived secondary effects. These pesticides are used in different tasks such as forestry, pasture, control of wildlife habitats, and cleaning of waste grounds, industrial sites, railways and railway embankments. A fungicide is a pesticide used to kill or inhibit fungi and fungal spores, which damages the quantity, quality and profit of yield. Such pesticides are used in agriculture and livestock to fight against fungal infections. An insecticide is a class of pesticide used against insects in all stages of growth: egg, larva, and insect. Almost all insecticides modify the ecosystem where they are used; a lot of them are toxic for humans; and much of them are involved in the food chain. A bactericide is a pesticide that kills bacteria. This class of pesticide is used as disinfectant, antiseptic or antibiotic.

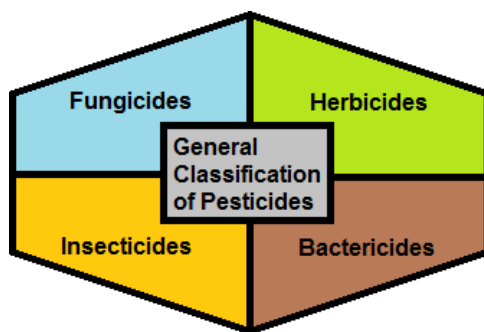


Figure 1. General classification of pesticides by chemical substituents.

The main types of pesticides are illustrated in Figure 2. Among the main pesticides that have been or can be detected are organophosphates (acephate, azinphos-methyl, bensulide, chlorethoxyfos, chlorfenvinphos, chlorpyrifos, chlorpyrifos-methyl, coumaphos, demeton-S-methyl, diazinon, dicrotophos, diisopropyl fluorophosphate, dichlorvos, dimethoate,

dioxathion, disulfoton, dursban, ethion, ethoprop, fenamiphos, fenitrothion, ferthion, fosthiazate, isoxathion, lorsban, malathion, methamidophos, methidathion, metyl parathion, mevinphos, monocrotophos, naled, omethoate, oxon, oxydemeton-methyl, parathion, parathion-methyl, paraoxon, phorate, phosalone, phosmet, phostebupirim, phoxim, pirimiphos-methyl, quinalphos, temephos, terbufos, tetrachlorvinphos, triazophos, tribufos, trichlorfon), carbamates (aldicarb, carbendazim, carbofuran (Furadan), carbaryl (Sevin), ethienocarb, fenobucarb and methomyl), organochlorides (aldrin, beta-HCH, carbon tetrachloride, chlordane, cyclodiene, 1,2-DCB, 1,4-DCB, 1,1-DCE, 1,2-DCE, DDD, DDE, DDT, dicofol, dieldrin, endosulfan, endrin, heptachlor, kepone, lindane, methoxychlor, mirex, pentachlorophenol, tertadifon, and toxaphene), phosphorothioate (coumaphos, pirimiphos-methyl) and pyrethroids (allethrin, bifenthrin, cyfluthrin, cypermethrin, cyphenothrin, deltamethrin, esfenvalerate, etofenprox, fenpropathrin, fenvalerate, flucythrinate, imiprothrin, lambda-cyhalothrin, metofluthrin, permethrin, prallethrin, resmethrin, silafluofen, sumithrin, tau-fluvalinate, tefluthrin, tetramethrin, tralomethrin, and transluthrin).

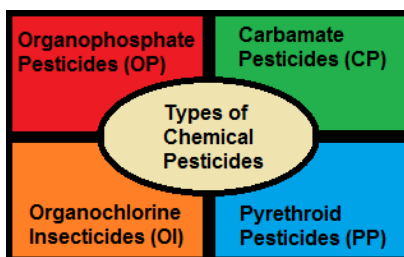


Figure 2. Main types of chemical pesticides applied in agriculture and industry.

An organochloride is an insecticide containing at least one covalently bonded chlorine atom. Their use is not recommended in food animals inasmuch as their persistence in animal tissues conducts to their input in the human food chain. Such pesticides are still industrially applied and although they have a non-animal use, the intoxication of animals can be presented. Among the effects produced by their intoxication are nervous excitement, tremor, convulsions, and death. They can inhibit different enzymes being acetylcholinesterase one of them. An organophosphate is an organic ester of phosphoric or thiophosphoric acid, which is the basis of many insecticides, herbicides and nerve gases. In accordance with the U.S. Environmental Protection Agency (EPA), these pesticides are very highly toxic to bees, wildlife, and humans due that they are organophosphorus compounds very pervasive. Fortunately, in a biosensor they can be detected with facility, since they inhibit the reaction of hydrolysis catalyzed by acetylcholinesterase. A carbamate is any organic ester derived of carbamic acid, which is used as insecticide to kill insects. These have been used in certain medications and insecticides. They are toxic and may cause convulsions and death through ingestion or skin contact. Such pesticides can cause reversible inhibition of acetylcholinesterase and cholinesterase. A pyrethroid is a synthetic substance used as commercial household insecticide. They are generally harmless to human beings in low doses but can harm sensitive individuals. However, such pesticides are toxic to aquatic

organisms. Although few studies have been realized, enzymes such as acetylcholinesterase (AChE) are inhibited by these pesticides.

The rapid detection of organophosphates in the environment and public places is important for homeland security, human and animal water consumption, food safety, and health protection. Regularly, the determination of them is realized by the inhibition of the activity of selected enzymes such as acetylcholinesterase, cholinesterase, acid phosphatase, ascorbate oxidase, acetolactate synthase, urease, and aldehyde dehydrogenase. In addition, these pesticides can also be found in solvents (liquids, solids and gases that dissolve another solid, liquid, or gaseous solute giving place to a solution that is soluble in a certain volume of solvent at a specified temperature, which are used in dry cleanings, spot removers, glues, nail polish removers, detergents, and perfumes), plasticizers (additives used to increase the plasticity or fluidity of the materials to which they are added such as plastics, cement, concrete, wallboard, and clay), and extreme pressure additives (additives for lubricants used to decrease wear of the mechanical parts exposed to very high pressures). Although, organophosphorus and carbamate pesticides are commonly detected by means of inhibition of enzymes such as AChE, the substrates used to immobilize such chemicals have been modified and are being redesigned with the aim of increasing the efficiency of such detection, as well giving portability and reducing the cost.

An enzyme can be defined as a protein that catalyzes or modifies the rate at which chemical reactions proceed; hence it is not consumed during the reaction. The reaction rate can be slow or fast in accordance with the effect desired in the particular reaction. The initial molecules used in the catalysis are called substrates and the molecules produced during this process are called products. A substrate is a molecule upon which an enzyme acts. When a unique substrate is used, it binds with the enzyme active site, giving place to an enzyme-substrate complex. The substrate is converted into one or more products that are released from the active site or part of an enzyme where substrates are bound. Next, the active site is free to accept other substrate molecules during the process of catalysis. The enzyme and the substrate have specific complementary geometric shapes that fit exactly into one another. When more than one substrate is involved, the molecules are bound in a specific order to the active site, before that they react together to produce products more elaborated. In active sites, the substrates undergo the chemical reaction associated with the catalysis, as shown in Figure 3. Almost all chemical reactions in a biological entity need enzymes in order to be developed at adequate rates. Since enzymes are choosy in the type and composition of the substrates, then there exists a small set of possible reactions, and therefore, the possible products are known for each pair involved in the enzyme-substrate complex. Enzymes reduce the activation energy for a chemical reaction, and it is increased due to them reach their equilibrium state more rapidly, and the products are formed faster.

Enzymes are formed by long linear chains of amino acids that can be folded to generate a three-dimensional product. Thanks to that each amino acid sequence is unique, different enzymes can be even used to fulfill the specific properties required for each application. Almost always, the size of enzymes is much larger than the substrates where they will act on, and the active site involved in the catalysis contains only a small portion of the enzyme

composed of 2 to 4 amino acids which are bound to the substrate. In some cases, the enzyme contains cofactors, which are non-protein chemical compounds, being either organic or inorganic, that are required for the protein's biological activity.

Enzymes can be generally considered as globular proteins containing from 62 amino acids to over 2,500 depending of their biological origin. Its activity is determined by biochemical composition and three-dimensional structure. These proteins are more or less soluble in aqueous solutions, inasmuch as they form colloidal solutions. Enzymatic activity is affected in two main types of chemical modifiers: the inhibiting molecules decrease the activity; while activator molecules, increase the activity. Most enzymes can be denatured, that is, they lose the tertiary structure and secondary structure that they have in their native state by means of the application of some external compound or heat. The chemical compounds that can be applied to denature enzymes are acids, salts or organic solvents. Depending on the nature of the enzyme, denaturation may be reversible or irreversible. In addition, such activity can be modified by temperature, chemical environment, and the substrate concentration. The complementary shape, charge and hydrophilic/hydrophobic characteristics of enzymes and substrates are responsible of the specificity found in the catalysis.

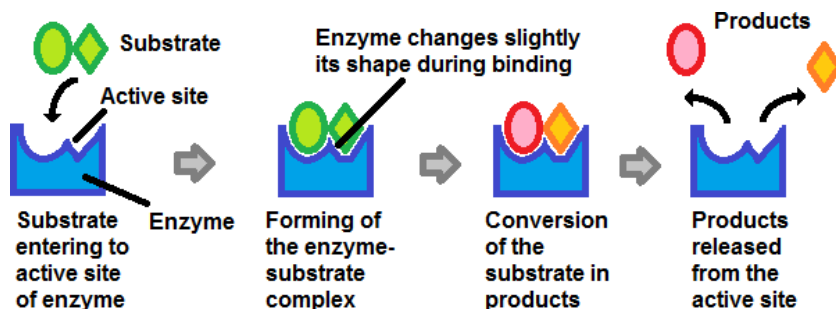


Figure 3. Process realized by an enzyme during the catalysis procedure (James & Tawfik, 2003).

The International Union of Biochemistry and Molecular Biology has classified to enzymes by the mechanism used in the catalysis:

- EC 1 Oxidoreductases: Enzymes that catalyze oxidation/reduction reactions to generate by means of the substrate a new product.
- EC 2 Transferases: Enzymes that transfer a functional group to the substrate to form a new product.
- EC 3 Hydrolases: Enzymes that catalyze the products using the cleavage of the substrate and the addition of water.
- EC 4 Lyases: Enzymes that catalyze the products by means of cleaving several bonds using hydrolysis and oxidation.
- EC 5 Isomerases: Enzymes that catalyze geometrical or structural changes within a substrate molecule with the aim of forming a single product.
- EC 6 Ligases: Enzymes that can catalyze the joining of two substrate molecules by forming a new chemical bond by hydrolysis.

Main enzymes used to enzymatic pesticide biosensors are acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and cholinesterase (ChE), which belong to the type of enzymes considered as hydrolases whose classifications are EC 3.1.1.7, EC 3.1.1.8 and EC 3.1.1.8, respectively (Figure 4). Cholinesterase is a family of enzymes used to catalyze the hydrolysis of the neurotransmitter acetylcholine (ACh) into choline and acetic acid. AChE is found in the blood and neural synapses in multiple molecular forms. Two different three-dimensional forms can be identified: tetrameric G4 form (10) and monomeric G1 (4S). Such enzyme hydrolyses ACh more quickly. BChE is found primarily in the liver. This enzyme hydrolyses butyrylcholine more quickly.

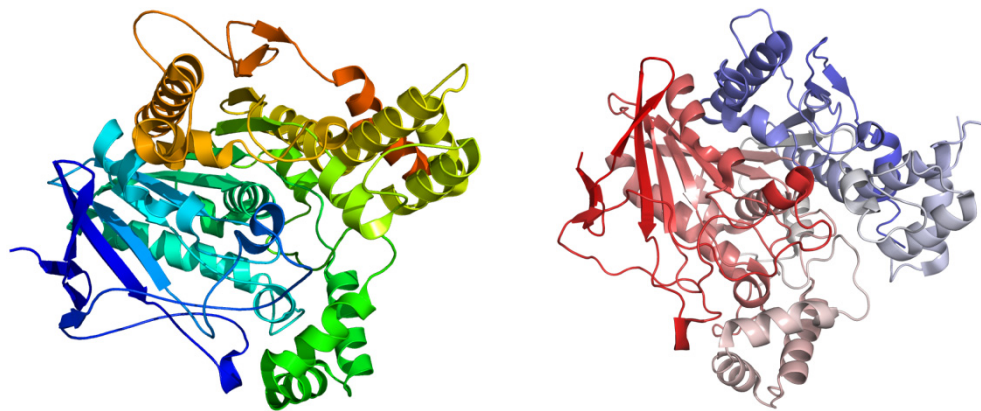


Figure 4. Enzymes used in pesticide biosensors: (left) acetylcholinesterase and (right) butyrylcholinesterase. Source: Protein Data Bank (PDB) (www.wwpdb.org).

Enzymes used in biosensors are highly selective to the substrates and sensitive to pH, temperature, inhibitors, denaturing and chelating agents. Two conditions must be fulfilled to adopt a specific immobilization method in a biosensor: 1) it must provide good mechanical stability, and 2) it must supply excellent conformation and freedom to the enzymes in their catalytic activity.

The cholinesterases (ChE's) have acted as key enzymes in areas such as neurobiology, toxicology, and pharmacology (Miao et al., 2010). AChE or true ChE is found in the central nervous system, bound to the cellular membranes of excitable tissues and associated with nerve transmission processes. Among the pesticides, organophosphates and carbamates form an important class of toxic compounds; their toxicity is based mainly on the inhibition of AChE during synapsis. However, small amounts of these classes of chemicals can also disrupt hormones and reduce their ability to successfully reproduce (Bonde and Storgaard 2002; Claman, 2004); and have been associated with special AChE specific cancers (Alavanja et al., 2004).

ACh is secreted by pre-synaptic membrane into synaptic cleft in neuromuscular junctions and cholinergic neurons. This neurotransmitter binds to ACh receptors (AChR) on the post-synaptic membrane, relaying the signal from the nerve as shown in Figure 5.

Although all AChRs respond to ACh, they also respond to different molecules that in physiological conditions are not present in synaptic cleft. In fact the AChR can be classified according to their affinities and sensitivities in muscarinic (an alkaloid from *Amanita muscaria* mushroom) and nicotine (from Solanaceae plants), or nicotinic receptors (nAChRs) and muscarinic receptors (mAChR). The nAChR (also known as "ionotropic" acetylcholine receptors) are ligand-gated ion channels permeable to Na⁺ and K⁺. In contrast, the mAChRs (also known as "metabotropic" acetylcholine receptors) are not ion channels, but belong instead to the superfamily of G-protein-coupled receptors that activate other ionic channels via a second messenger cascade. Both mAChR and nAChR are two main kinds of "cholinergic" receptors. In order to avoid over-stimulated AChR, ACh must be released from its receptor. This occurs only when the concentration of ACh in the synaptic cleft decreases.

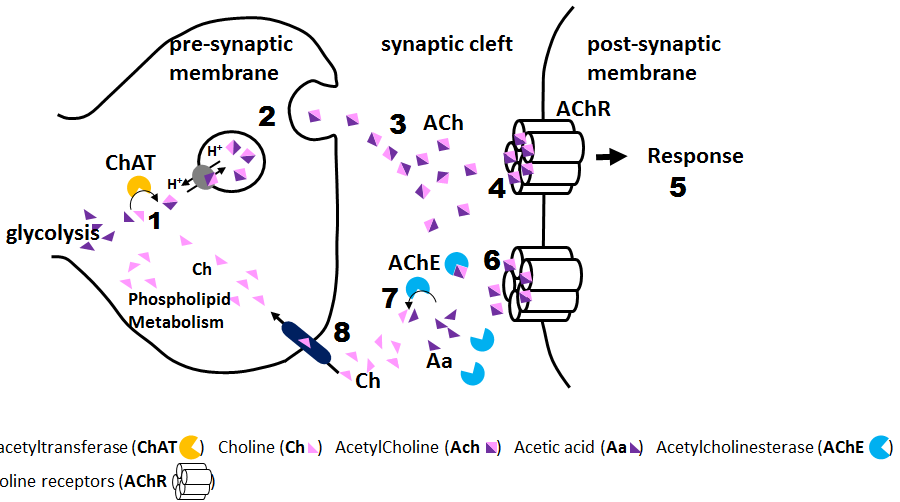


Figure 5. ACh production by Choline acetyltransferase in pre-synaptic terminal (1), ACh secretion to synaptic cleft (2), ACh accumulation in synaptic cleft (3), ACh binds to its receptor in post-synaptic membrane (4), Cellular response (5), ACh release from its receptor (6), AChE hydrolyzes ACh in choline and acetic acid (7), and Choline reuptake into the presynaptic membrane to make more ACh (8).

AChE catalyzes the hydrolysis of ACh into choline and acetic acid, an essential process for removing ACh from the nerve junction (see Figure 6). AChE can catalyze until 25000 molecules of acetylcholine (ACh) per second, whose value is the limit allowed by diffusion of the substrate. The active site of AChE contains 2 subsites: an anionic site and an esteratic subsite.

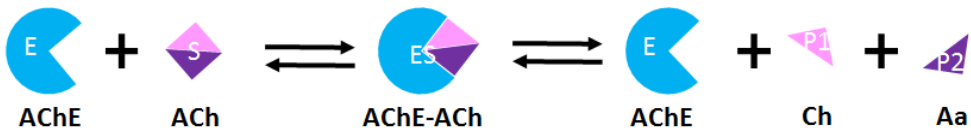


Figure 6. Hydrolysis of ACh by AChE in synaotic cleft.

Inhibition of AChE leads to accumulation of ACh in the synaptic cleft and results in impeded neurotransmission, as shown in Figure 7. During neurotransmission, ACh is released from the nerve into the synaptic cleft and binds to ACh receptors on the post-synaptic membrane, relaying the signal from the nerve. AChE found on the post-synaptic membrane, terminates the signal transmission by hydrolyzing ACh. The produced choline is carried again by the pre-synaptic nerve and ACh is synthesized by combining with acetyl coenzyme A used in metabolism (acetyl-coA) through the action of choline acetyltransferase (enzyme synthesized within the body of a neuron).

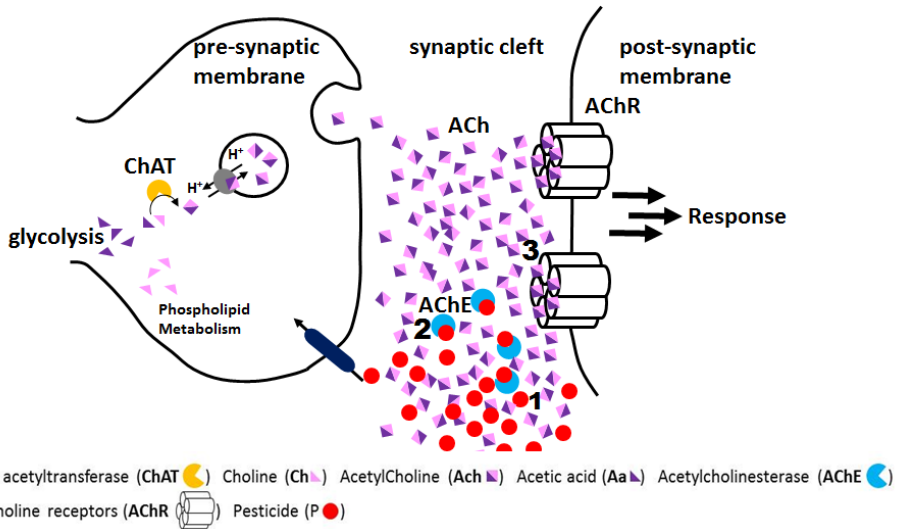


Figure 7. Pesticide accumulation in synaptic cleft (1), AChE inhibition by pesticide (2), and constant activation of AChR (3).

If AChE is unable of removing ACh, the muscle can continue moving uncontrollably.

Some pesticides such as organophosphates and carbamates can bind or inhibit AChE making unable to break down ACh. Organophosphates particularly include some of the more toxic pesticides, which can enter the human body through different routes such as skin absorption, inhalation and ingestion.

Repeated or prolonged exposure to organophosphates may result in the same effects as acute exposure including the delayed symptoms. Other effects include impaired memory and concentration, disorientation, severe depressions, irritability, confusion, headache, speech difficulties, delayed reaction times, nightmares, sleepwalking and drowsiness or insomnia.

For detecting the presence of pesticides in a biological entity, the essential step consists in realizing the immobilization of the enzyme on the transducers of the biosensors, as shown in Figure 8. Such immobilization can be obtained by means of dissolution of the enzyme in a buffer solution that is placed in an electrode surface, physical entrapment of the enzyme

inside a gel, chemical bond between the enzyme and a membrane or an organic or inorganic support or directly to the transducer made of Pt, Au, C, etc. The concentration of the pesticide in any biological entity has a linear or nonlinear correlation with the inhibition of the process of catalysis realized by the enzyme.

Chemicals that modify the action of cholinesterase are efficacious neurotoxins, which produce in humans and animals excessive salivation and eye-watering in low doses, after muscle spasms and finally death. Pesticides such as organochlorines, organophosphates, carbamates and pyrethroids can be used as insecticides, since they operate by combining with a residue of serine in the active site of acetylcholine esterase, so that the set inhibits the enzyme completely. Thus, the enzyme acetylcholine esterase breaks down the neurotransmitter ACh, which is delivered at nerve and muscle junctions in order to carry the muscle or organ to repose. AChE-based biosensors have higher sensitivity due to the more energetic enzyme inhibition by organophosphate pesticides. Other enzymes that have been used in pesticide biosensors are acetolactate synthase, acid phosphatase, alkaline phosphatase, tyrosinase, ascorbate oxidase, etc. Luciferase is used for biosensors based on bioluminescence. Organophosphorus hydrolase (OPH) is used as a recognition receptor in biosensors based on spectroscopic or electrochemical methods. Pairs of enzymes (bienzymatic biosensors) or set of three or more enzymes (multienzymatic biosensors) can be used to detect one or more types of pesticides in a same biosensor. Examples of systems are acetylcholinesterase and choline oxidase, acetylcholinesterase and cytochrome P450 BM-3 (CYP102-A1) mutant, acid phosphatase (AP) and glucose oxidase (GOD), phospholipase D and choline oxidase (ChO), etc.

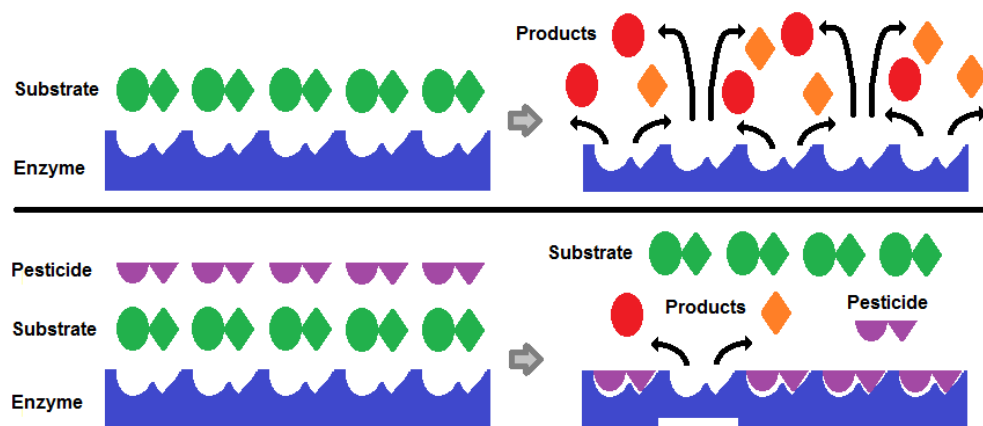


Figure 8. Inhibition of the catalytic reaction by means of pesticides: (up) absence of pesticide in biosensor, and (down) presence of pesticide in biosensor.

3. History of enzymatic pesticides biosensors

AChE has been isolated from several organisms to be used in almost all pesticide biosensors. In the early 1950s, potentiometric biosensors were the first devices used to detect pesticide.

Until middle of the 1980s, it was introduced the first integrated pesticide biosensor, whose operation depended on the inhibition of AChE. Advances in the science and technology have allowed obtaining genetically modified AChE and other enzymes, which have been used in the design of biosensors. Later on, different detection methods were introduced to realize the interface between the analyte and the biosensor: amperometric, conductometric, differential pulse voltammetry (DPV), chemiluminescence, piezoelectric, surface plasmon resonance, thermometer, resonant mirror, phosphorescence, fluorescence, etc. The main drawback of enzymatic pesticides biosensors is non-selectivity, i.e. they have only a unique or reduced signal transduction pathway. With the aim of increasing the selectivity of pesticide biosensors have been proposed alternative solutions: 1) the use of immunosensors, where antibodies operate as recognition receptors, i.e. organic compounds that control the physicochemical superficial properties and grafting process to improve the detection sensitivity and selectivity, and 2) the use of multiple enzymes in the same biosensor to increase the signal transduction pathways. The sensitivity of a biosensor and the stability of the catalyst depend of the immobilization method employed to design the interface between the analyte and the biochemical compounds involved in the detection. Despite of advances achieved until now in enzymatic pesticide biosensors, there are still a lot of technological aspects that require scientific research to carry on biosensors into exhaustive commercialization.

Commonly, most pesticides are measured by analytical techniques of laboratory such as processes based on gas chromatography or liquid chromatography, which are coupled with mass spectrometry. However, more and more requirements linked with the sample preparation, specialized chemical analytical equipment and personal with very high expertise are restricting the application of these techniques for realizing field-based detections. Therefore, it is necessary the use of rapid, reliable and low-cost methods that can be applied in the detection of pesticides in any place where a pesticide could be found. Hence, methodologies based on miniaturization of systems capable of determining precisely the presence or quantity of pesticide found in a sample even in insignificant quantities of matter and especially in very short times is the main objective to fulfill. An excellent possibility to obtain a very small system is a biosensor.

A biosensor can be defined as an analytical device capable of detecting an analyte (substance or chemical constituent interesting from an analytical point of view) that combines a biological material (nucleic acids, natural products, antibodies, enzymes, cell receptors, organelles, microorganisms, tissues, etc.), a biologically derived material (engineering proteins, aptamers, recombinant antibodies, biomass, bone, wood, etc.) or a biomimic (imprinted polymers, combinatorial ligands, biomimetic catalysts, synthetic receptors, etc.), with or integrated within a physicochemical transducer that will produce either discrete or continuous electronic signals proportional to the quantity of analyte present in a sample (see Figure 9).

Transducers in biosensors may be optical (optical fibers, waveguides, interferometers, fiber gratings, ring resonators, and photonic crystals), electrochemical (pH, polarographic,

capacitive, potentiometric or conductometric probes, amperometric, etc.), micromechanical, piezoelectric, magnetic or thermometric. Last two types of biosensors are rare. Biosensors have been applied to detect the presence of pesticides in a large variety of biological samples such as body fluids, food samples, cell cultures and environmental samples.

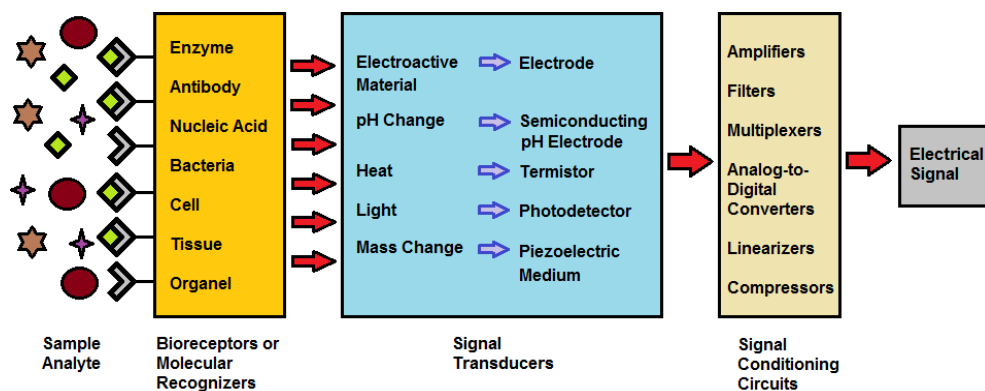


Figure 9. Biosensor operating principle: main subsystems.

A great number of different technologies have been developed to design biosensors, unfortunately, not all them are used in the different application types. In Figure 10, the key biosensor technologies that are being used today are illustrated. Biosensors applying electrochemical, piezoelectric, and optoelectronic principles have experienced the highest technological performance until now. A bioluminescent biosensor exploits the phenomena of visible light emission in biological entities due to the oxidation of organic compounds mediated by a catalytic enzyme. The light generation depends on the chemical reaction kinetics. This type of biosensor requires a photodetector to transduce the light emitted by the biological entity to an electrical signal. An electrochemical biosensor exploits the detection of physicochemical properties of electroactive substances to realize the biorecognition that provides the measurable signal: electrical current, voltage, resistance or superficial charge. An optical biosensor exploits two different strategies: 1) changes in light absorption between the reactants and products of a chemical reaction mediated by enzymes or 2) measurements of the output light by a luminescent process. A piezoelectric biosensor makes use of the change in frequency of a piezoelectric crystal, which is proportional to the mass of absorbed material as product of the chemical reaction catalyzed by enzymes. A special case is associated with a resonant mirror (RM) biosensor that uses the evanescent field emitted in a waveguide to determine changes in the refractive index at the sensing biochemical surface. In a thermistor-based biosensor, the temperature change induced by the enzyme-catalyzed reaction is exploited to determine the concentration of pesticide.

Pesticide biosensors can make use of the approach called flow injection analysis (FIA), which injects a plug of sample into a flowing carrier stream to realize a chemical analysis (Marinov et al., 2011). In this method, the sample is injected into a continuous flow of carrier solution that mixes the analyte with other continuously flowing solutions before reaching

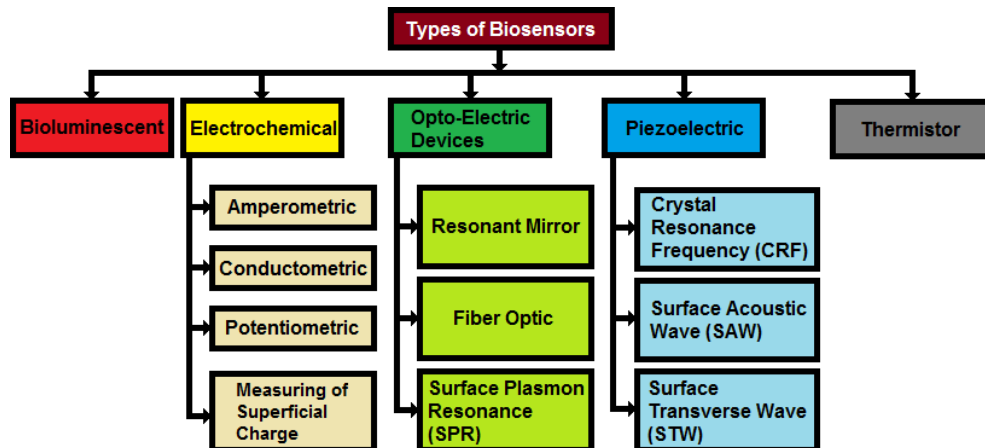


Figure 10. Types of Biosensors applied in several fields of engineering.

any type detector contained in a biosensor. Thus, this method dramatically increases the precision of the measurement and enormously reduces the response time. In addition, this technique allows designing non-disposable or reusable biosensors. In miniaturized systems, exhaustive use of microfluidic devices such as microvalves, micromixers, and micropumps is associated with the design of this system (Vargas-Bernal, 2006). The mixing is realized through radial and convection diffusion with a reagent for a period of time before the sample reaches the detector. A study of fluid dynamics must be considered to determine the parameters required for controlling the quantity of solution delivered to the detector (Vargas-Bernal, 2007). Chemical reactions can be implicated during the mixing of chemical solutions; therefore a biochemical study also is associated to this design. In the same way, thermal study can be considered when a large time of mixing is implicated. The detectors used in FIA systems are colorimeter, fluorimeter, ion-selective electrode, amperometric, conductometric, potentiometric or capacitive. Pesticides such as paraoxon ethyl, monocrotophos and dichlorvos have been detected by this type of systems.

If multiple samples must be analyzed at the same time, then a microarray of biosensors is used. A microarray is defined as a 2D array on a solid substrate, which is normally a glass slide or silicon thin-film cell that distributes large amount of biological material through the use of a high-throughput screening. Millions of biochemical tests are realized by means of robotics, data processing and control software, liquid handling devices, and sensitive detectors. This process allows identifying pesticides and other chemicals in different samples extracted of the same biological entity to analyze. Each element in such array is called assay. An enzyme-linked immunosorbent assay (ELISA) can detect the presence of pesticide in a liquid sample or wet sample disposed in a site of the microarray of biosensors. Each assay can contain different types of enzymes or different quantity of them with the aim of obtaining an image that illustrates the differences achieved in biosensing of the analyte. This type of array is desirable when a calibration process will be realized by the characterization for developed biosensors. Enzymes with high turnover numbers are

preferred to obtain rapid response. The last asseveration is related with the increase of sensitivity of the assays and such rise in response can generate highly coloured, fluorescent or bioluminescent products during the catalytic reaction.

4. Advances in optical biosensors

Biosensors using optical detection are based in enzymatic reactions for altering the optical properties of some substances with the aim of allowing them either to change in light adsorption between the reactants and products of a reaction, or to measure the light output by a luminescent process. Generally, an optical biosensor is formed by several subsystems: a light source, a set of optical components used to generate a light beam with particular characteristics and to focus the beam to a modulating agent, a modified sensing head, and a photodetector (Jiang et al., 2008). Amongst the means employed for this type of detection are surface plasmon resonance (SPR), waveguides or evanescent wave, resonant mirrors, fluorescence, phosphorescence, and chemi/bioluminescence to analyze biomolecular interactions. These biosensors determine the affinity and kinetics of the molecular interactions in real time without requiring molecular tags or labels. The advantages of this type of biosensors are: 1) multicomponent detection due to intrinsic molecular specificity in near infrared range, 2) robustness, 3) remote sensing, and 4) absence of electromagnetic fields or surface potentials, which can modify the biological entities or the result of the detection.

Surface plasmon resonance is a method based on resonant collective oscillation of valence electrons in a solid stimulated by incident light, whose frequency matches the natural frequency of surface electrons oscillating against the restoring force of positive nuclei of the solid (see Figure 11). Such solid regularly is gold or silver, which must be coated with target analyte. These metals are placed as a wall of a thin microfluidic flow-cell, where an aqueous buffer solution containing biological entity with pesticide is induced to flow across of a tubular section by injecting it through this flow-cell. Next, light (visible or near infrared) is emitted through the glass slide and onto the gold surface to fulfill the condition of SPR, the optical reflectivity of the gold changes very sensitively with the presence of biomolecules on the gold surface or in a thin coating on the gold composed of biopolymers or another type of biological membrane. The high selectivity of the optical response is due to the fact that there is a very efficient, collective excitation of conduction electrons near the gold surface. The detection occurs only at a particular angle and wavelength of incident light (total internal reflection) and it is highly dependent on the surface of the gold used either as a thin film or a nanostructured material. The extent of binding between the solution-phase interactant and the immobilized interactant is easily observed and quantified by monitoring the reflectivity change between input and output. The optical signal obtained is proportional to the volume of pesticide bound near the surface. One of the most important advantages of SPR is its high sensitivity without any fluorescent or phosphorescent interactant. Plasmons are only originated at the interface between the metal and a dielectric material commonly glass. The minimum feature size is defined by the diffraction limit achieved in interface, and it is of the order of wavelength of the light, that is, in the range of nanometers to micrometers.

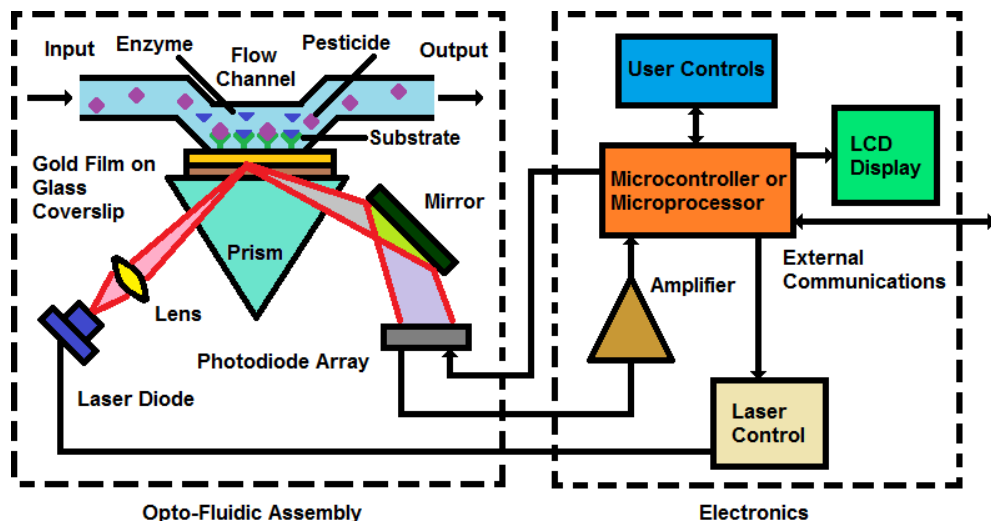


Figure 11. Optical biosensor based on surface plasmon resonance (SPR).

Amongst the advantages offered by this method are: 1) optical biosensors based on SPR render a label-free, fast, specific and sensitive alternative to develop laboratory analytical techniques, 2) SPR allows the study of macromolecules in real time and their interactions with the aim of determining specificity, interaction models, kinetic rates, equilibrium constants, thermodynamic constants, and epitope mapping, 3) SPR biosensors detect small (pesticides), medium, and large (proteins) biological analytes at different concentrations achieving minimum values such as 0.1 to 1 ng/ml, and 4) SPR biosensors can be applied to medicine, environmental protection, food and drug screening and security. Pesticides such as atrazine, chlorpyrifos, carbaryl, DDT, and simazine can be detected by this approach of biosensing. Nafion coated SPE biosensors using as inhibiting enzyme the butyrylcholinesterase can detect low levels of pesticides.

Prism in Figure 11 can be substituted with a single-mode or multi-mode optical fiber. The new system has additional advantages such as high sensitivity and it does not require the use of labels. These systems can be operated in two different modes: localized SPR (LSPR) and propagating SPR (PSPR). Optical fibers to be used must be side-polished to expose the core to the gold thin film and covered in the rest of the fiber. Fibers are susceptible to deformation, and hence, they can present changes in light polarization carrying to surface plasmon strength and do not diffuse resultant signal. A thin layer of ceramic material is used to adjust the refractive index of the wavelength desired.

An optical biosensor based on evanescent wave (EW) combines the use of molecular recognition by means of enzymes with the signal-transduction capability of a waveguide or optical fiber to detect changes in light intensity between their extremes, as shown in Figure 12.

Visible or near infrared light is emitted by a LED or a laser, and it is propagated through a waveguide or optical fiber with refractive index n_1 by multiple total internal reflections in

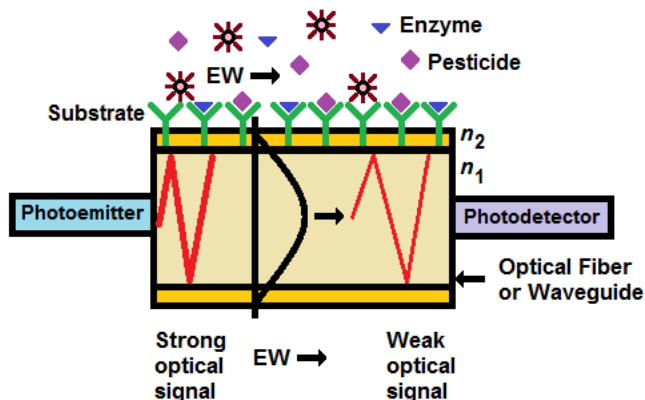


Figure 12. Evanescent wave biosensor for pesticides based on optical fiber or waveguide.

the optical fiber or waveguide axis, an electromagnetic wave (EM) called evanescent wave is produced in the optically less dense external medium with refractive index n_2 . If $n_1 > n_2$, then evanescent field penetrates into optical fiber or waveguide making that surface-bound molecules interact with the incident light. Energy optical absorption by the molecules favors the attenuation or reduction of light intensity reflected in the waveguide or optical fiber. An output optical signal is detected by means of a photodetector and a value of concentration of pesticide is indirectly obtained, which is proportional to the quantity of attenuation presented. An optical waveguide split to the input beam into two arms (sensing and reference), and after a certain distance they are recombined again. During the path of length of the waveguide, light traveling in the sensing arm will have a phase shift in comparison with guided light in the reference arm. Among the advantages of this biosensor are large surface area, and good optical, dielectric, thermal, and acoustic properties. In this type of biosensor, there exists a linear relationship between peak area of the absorption bands and input concentrations. Two different approaches can be distinguished: 1) high sensitivity and 2) fast time resolution. Pesticides such as parathion, fenitrothion, and paraoxon have been detected by this type of biosensor.

Chemiluminescence is the light emission produced by the excitation generated as result of an exothermic chemical reaction. In such reaction, reactants create an excited electronic state, which is associated with the maximum enthalpy. Generally, products generated have lesser chemical energy with respect to the reactants. The energy gained during the reaction when it is lost, then produces light emission and heat. The electronic state decays into an electronic ground state through either fluorescence or phosphorescence, in accordance with the spin state of the state formed during the reaction. Fluorescence is the property of a substance of emitting light, when it has previously absorbed light or another electromagnetic radiation. Commonly, emitted light by the substance has a longer wavelength and lower energy than absorbed radiation. The excitation is produced by the absorption of light of sufficient energy to carry one electron of the valence state to conduction state. Phosphorescence is a special case of the fluorescence where light is re-emitted at a lower intensity for up to several hours after the original excitation. This method offers the flexibility of controlling the amount of enzyme on a surface.

Chloroplast thylakoids can be used as biological units that detect herbicides such as atrazine and diuron in water samples or aqueous extracts of samples. Almost half of the herbicides actually used in agriculture inhibit the light reactions in photosynthesis. Thylakoids are disposed in form of membranes, which contain hundreds of molecules of chlorophyll that are used as light-trapping pigments required for photosynthesis. Enzymes and other molecules used in photosynthesis are added into thylakoid membranes. Two different types of photosensitive biological units can be designed through pigments and enzymes: Photosystem I and Photosystem II. Chlorophyll absorbs red and blue light, and scatters green light. In Figure 13, a fluidic optical biosensor can be used in the detection of herbicides, which is based on the inhibition of the hydrogen peroxide production generated by thylakoids, pesticide, and the light applied. Such production depends of the illumination applied by a LED into isolated thylakoids and whose activity is modified by the presence of different concentrations of pesticide. A chemiluminescence reaction is obtained when lysates containing luminol are oxidized by horseradish peroxidase (HRP). Chemiluminescence intensity is associated with the quantity of luminol, oxidant and catalyst, and any of these can be determined with this system. The presence of hydrogen peroxide reduces the photoemission generated by the system, which is detected by means of a photodetector and electronically quantified. This method makes use of superparamagnetic beads with the aim of reusing the biosensor and of regenerating the activity of the enzyme. Amongst the pesticides that have been detected by this method are triazinone, 2,4-Dinitro-6-isobutylphenol, diuron, atrazine, endosulfan, tebuconazole, paraquat, prometryn, etc.

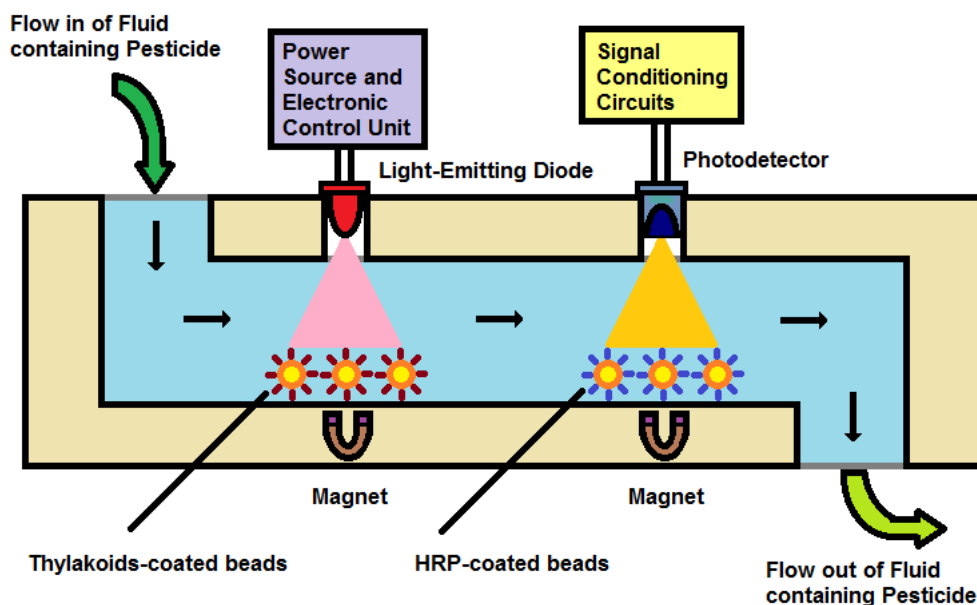


Figure 13. Optical biosensor based on chemiluminescence.

5. Advances in electrochemical biosensors

Electrochemical biosensors place suitable enzymes in their biorecognition layers to generate electroactive substances for the chemical detection through identification of similar compositions between the substrate and the analyte or the inhibition of the enzymatic activity in the substrate by the presence of analyte. Two main approaches are used: 1) enzyme inhibition (static detection or in repose), and 2) hydrolysis of pesticide (dynamic detection). Inhibition-based biosensors have been widely designed due to the simplicity of operation and a diversity of available enzymes. Pesticide enzymatic hydrolysis-based biosensors have a very fast catalytic reaction and exploit the use of flow injection analysis (FIA) to increase the response time. Electrochemical biosensors convert proportionally the chemical information obtained by the enzymes into a measurable electrical variable such as electrical current, voltage or resistance. Enzymes can be immobilized by four different methods adsorption, entrapment, covalent bonding, and cross-linking. Their efficiency is limited by the quantity of immobilized bioactive molecules on the sensor surface, inasmuch as these sensors have the characteristic of randomly adsorbing the analytes with bioefficiency and biofunctionality reduced. The actual trend consists in mitigating non-specific adsorption and creating tailor-made surfaces to control the immobilization of bioanalytes through specific receptor recognition interactions. Thanks to the technological advance, this type of biosensors offers the miniaturization of the detection systems with very attractive advantages with respect to other types of biosensors such as portability, rapid measurement, repeatability, robustness, compactness, excellent selectivity, high sensitivity, wider linear range, and reduction of the volume of sample to realize the recognition. This type of biosensors uses four different sensing modes potentiometry or voltammetry, amperometry, surface charge using field-effect transistors (FETs), and conductometry.

A potentiometric biosensor makes use of ion-selective electrodes to convert a biochemical reaction into an electrical signal. In the simplest terms, it consists of an immobilized enzyme membrane surrounding the probe from a pH-meter, where the catalyzed reaction generates or absorbs hydrogen ions. An amperometric biosensor generates an electrical current or changes in electrical current when an electrical potential is applied between a pair of electrodes positioned in an electroactive biological membrane. Any analyte capable of being oxidized or reduced chemically can be used as candidate for amperometric detection. The analyte is oxidized at the anode or reduced at the cathode. A conductometric biosensor is based on modulation of resistivity of the selective material. In this type of biosensor, the material changes its conductivity due to its interaction with chemical species. Such material is clamped between two contact electrodes and the resistance of the entire device is measured. Other version of the same biosensor consists in measuring the electrical resistance between a pair of electrodes that interconnects a biological medium: a suitable counter-electrode and one electrode immersed in a solution of electrolyte. A more exhaustive analysis of these types of biosensors can be found in (Vargas-Bernal, 2007).

During enzymatic catalysis of the biochemical reaction are produced ions. The substrate contains three electrodes which are used to determine the electrical parameters: a reference

electrode, an active electrode and a sink electrode. In addition, a counter electrode can be used as an ion source when the ions are not present by any another medium. The target analyte associated with the reaction produces ions in the active electrode surface. The electrical parameters can be measured between different electrodes by means of electrical conductivity, current, voltage or superficial charge.

In AChE-based biosensors, ACh is used as substrate for the detection of organophosphorus and carbamate pesticides. The product obtained during the catalytic reaction is called thiocholine, it is measured using spectrometric, amperometric or potentiometric methods. The enzyme activity is indirectly related with the pesticide concentration found in the analyte. When a mixture of pesticides is present in an analyte, precaution should be had due to a change in the whole activity of the pesticides why it can be disguised or not clearly distinguished. Thus, a more sensitive biosensor must be used in this case, which is obtained by means of two or more enzymes containing different functional groups and a set of two or more types of substrates in the same biosensor. The set of enzymes used in a multienzymatic biosensor must be carefully chosen so that the enzyme employed in the actual reaction generates subsequent products that will be used in the next reaction by the subsequent enzyme in action. In the static biosensors, the number of consecutive measurements with the same biosensor is limited to the quantity of enzyme used in each measurement. Therefore, inhibition-based biosensors are disposable biosensors and are designed with this orientation for a cheaper cost. The main drawbacks of the inhibition-based biosensors are slow and tedious, due to they require multiple steps of reaction such as measuring initial enzyme activity (before being used), incubation with inhibitor, measurement of residual activity (after being used), and regeneration and washing (i.e., add more substrate and remove biochemical residues found). Inhibition-based biosensors have detected pesticides such as sulfometuron methyl (herbicide), atrazin, diazation, dichlorvos, ziram, diram, zinc diethyldithiocarbamate, dithiocarbamates, malathion, parathion methyl, and paraoxon.

Biosensors containing a FIA system or dynamic biosensors have allowed to detect pesticides such as paraoxon, parathion, coumaphos, diazinon, methyl parathion, omethoate, dimethyl 2,2'-dichlorovinyl phosphate. The reactivation of the inhibited enzyme can be achieved by means of nucleophilic reagents which are oximes such as 2-pyridinealldoxime methiodide, 2-pyridinealldoxime methochloride, 1,1'-trimethylene bis 4-formylpyridinium bromide dioxime (TMB-4) and 2-pyridinealldoxime methiodide.

Gold nanoparticles (AuNPs o GNPs) can be used in biosensors for immobilizing biomolecules such as enzymes to provide better efficiency, environmentally amicable, and stability to them. Additionally, GNPs provide a conductive pathway that promotes electron transfer reactions to lower potentials and increases the surface hydrophilicity for immobilization of enzymes (Du et al., 2008). The formation of GNPs in layers with self-assembled monolayers (SAMs) of 11-mercaptopundecanoic acid (MUA) illustrated in Figure 14, allows a better electron transfer and a simple method to functionalize their surface by biomolecules. AChE has excellent activity to this substrate, since malathion, paraoxon, carbofuran, and phoxim can be detected in limits of detection very small using an

amperometric biosensor. Although, GNPs are competent to fabricate electrochemical biosensors, they are inherently instable and agglomerated by van der Waals forces. Therefore, protective agents must be added to them. Silk fibroin (SF) is a natural protein extracted from silkworm cocoon with thermal stability, nontoxicity, low cost and biocompatibility that can act as protective agent. GNPs and SF form a bioconjugated colloid that is used as substrate for the enzyme. Other chemicals used to generate SAMs with GNPs are alkanothiols, dialkyl disulfides and dialkyl sulfides.

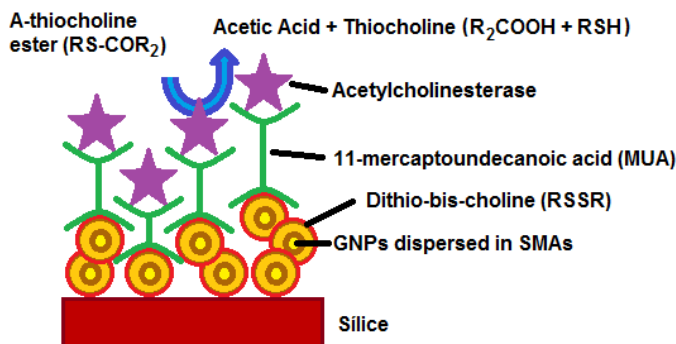


Figure 14. Electrochemical biosensor based on gold nanoparticles dispersed in SAMs.

The main drawback of amperometric biosensors based on AChE is related with the high potential that must be applied for maintaining constant the generation of enzymatic product, i.e. thiocholine, which has as consequence the instability of the biosensor. A solution has been applied to this problem, the use of mediators and highly conductive materials such as carbon nanotubes and other nanostructured materials. A mediator is an organic compound whose function consists in fixing a constant rate of enzymatic activity producing long-term stability.

A field-effect transistor (FET) is defined as a semiconductor device used for amplifying and switching electronic signals and power, which requires of an electric field to control the conductivity and tridimensional shape of a channel containing one type of charge carrier in a semiconducting material. These transistors have three terminals: source, drain and gate. Main electrical carrier can be an electron or a hole, which flows from the source to the drain. Source and drain terminals are connected to a semiconductor substrate called bulk through ohmic contacts. Between this pair of terminals is established a flow of current, which is controlled by the gate terminal. The conductivity of the channel is a function of the electric field applied to the gate terminal and the field applied between source and drain. Thus, gate terminal modulates the channel conductivity through the voltage applied to it. In a biosensor, gate terminal is modified with a substrate containing an enzyme with the aim of detecting very low concentrations of analytes containing pesticides which are made flowing through of it (see Figure 15); therefore, a change in the drain-source current is presented due to the changing of charge density on the gate surface. Such charge density is associated with the change of pH found in the gate terminal of the FET. A nonlinear relationship between the concentration of pesticide and

the current between source and drain is presented, but it can be linearized with the aim of quantifying the contents of pesticide in the sample. This device can be designed to detect multiple pesticides through of a multienzyme biosensor array with capacity of discriminating among different organophosphate compounds. Competitive adsorption behavior among pesticides depends of their diffusivity, inhibition potency, and concentration of pesticide present in the sample. The potency of an inhibitor depends of the stability of the enzyme-inhibitor complex and the velocity of formation of acylated enzyme (after of the hydrolysis). The reactivation percentage and stability of enzyme in the biosensor depend on the degree of inhibition and type of pesticide of the last use. The inhibition constant or degree of inhibition establishes a proportion between the quantities of enzyme hydrolyzed and enzyme inhibited.

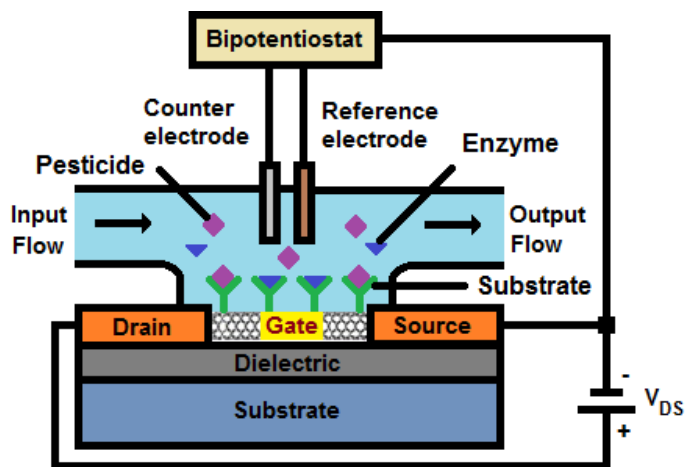


Figure 15. Biosensor based on FET with gate operating as detector of pesticide.

The design of the gate regularly implies that polymeric materials are placed to attach the biological substrate by means of covalent bonds, for example, the poly(3,4-ethylenedioxythiophene) (PEDOT) nanowires and amine-modified aptamer. PEDOT acts as mediator due to its water-soluble conductivity and it can be applied in mass production. Pesticides that can be detected with enzyme-modified field-effect transistors (ENFETs) are demeton-s, diisopropyl fluorophosphates, paraoxon, etc. Advantages of this type of biosensors are fast response time, reuse, less likelihood of generating false positive signal, and discrimination between different pesticides. A sol-gel modification to the gate terminal increases the enzyme immobilization thanks to the enhanced pH sensitivity and the reduction of the dependence between enzyme and the buffer capacity of the electrolyte in the solution that can be obtained.

Inasmuch as is very difficult to find a natural enzyme with all the features useful for a biosensor. Actually, there is a trend to design genetically engineered enzymes for electrochemical biosensors (Campàs et al., 2009). Such enzymes will be constituted of new biomolecules with tailor-designed physicochemical properties, where location and type of

mutations must be carefully selected to give to enzymes better properties: higher affinity toward specific analytes, higher stability, and higher electron transfer rates.

Electrochemical detection either catalytic or by affinity continues playing an important role in many clinical, environmental, industrial, pharmaceutical, defense, and security applications thanks to their high sensitivity and selectivity (Ronkainen et al., 2010). The novel developments in nanotechnology and materials science have allowed overcoming or minimizing their drawbacks by adding properties that bulk materials cannot offer.

6. Future trends

In spite of the years of research and technological development achieved in biosensors, there is still a lot of work that must be done with the aim of designing the most efficient and sophisticated biosensor for determining both qualitative and quantitatively the presence of pesticides in any type of biological entity. This section discusses the predictable future perspectives in pesticide biosensor research activities, which are illustrated in Figure 16.

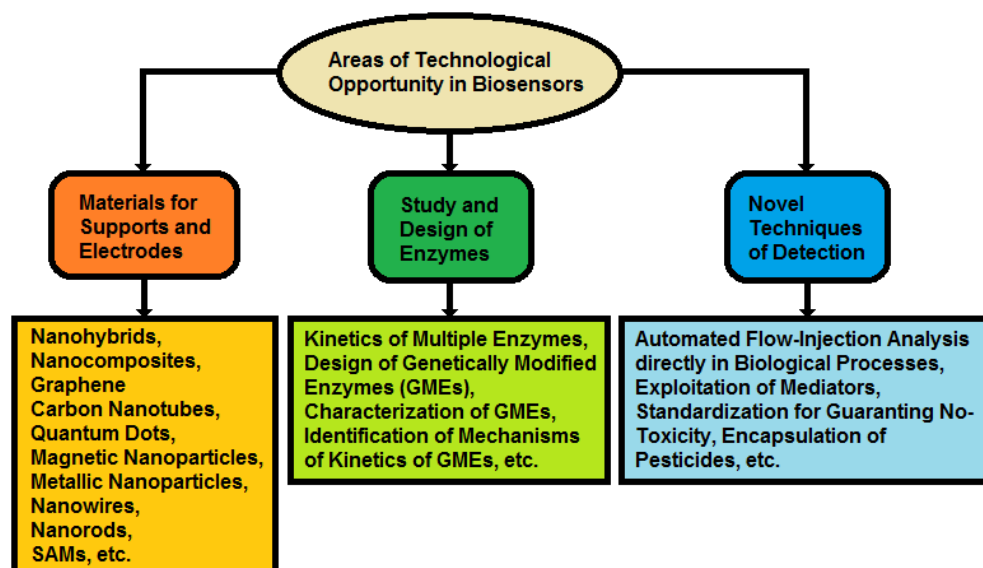


Figure 16. Future directions in scientific research related with biosensors.

A great diversity of electrical interfaces continues being developed with the aim of optimizing the performance of the biosensors. Amongst such interfaces are: field-effect transistor (FET) devices, nanotube arrays, nanoparticles, nanowires, electrodes, etc., as it was illustrated in Figure 9. With the reduction of size of the biosensors towards nanoscale, there is an urgent necessity of combining different methodologies to characterize and fulfill the performance desired through the manipulation of the interfaces linked in the recognition. Here, different strategies to increase such characteristics are studied as expectations of research and technological development towards novel biosensors.

6.1. Nanomaterials

One of the most important challenges is the extensive use of metals, ceramics and polymers both as individual nanomaterials as well as nanocomposites or nanohybrid materials. The advantage of these materials allows producing a synergetic effect derived of combining their individual properties, while are eliminated their particular drawbacks. The introduction of materials as the graphene will be strategic to design nanohybrids materials with fast detection due to the excellent electron-transfer, acceptable stability, good reproducibility, and higher affinity to the substrates used for placing the enzyme (Wang, K. et al., 2011). Therefore, a new generation of biosensors to fulfill the parameters of performance required will be developed as for example, must search that the detection of levels can be realized in smaller periods. In the design of reusable biosensors, it is indispensable the whole integration of subsystems for their strategic miniaturization. A biosensor will contain all blocks associated with the process: sampling of analyte, mixing of analyte and biochemical substances used in the reaction of detection such as buffers; separation of substances involved, delivery of substances for the catalysis, detection of pesticides, readout of levels, and regeneration of the substrate containing the enzyme. The use of microfluidics will allow the design of highly intelligent, integrated and high-throughput biosensors. The separation of substances could be realized by means of the integration of micro high-performance liquid chromatography (μ HPLCs).

The use of nanostructured materials as labels, signal enhancers or immobilizations supports is helping to obtaining highly sensitive analysis systems in pesticides biosensors. However, all their special properties have not been exploited at their maximum (Campàs et al. 2012). A lot of study must be realized with the aim of improving their stability and applicability. The use of magnetic particles as immobilization carriers in flow-injection analysis is in a first stage, since they will be strategic to give biosensors capacity of reuse.

A nanomaterial must be functionalized to be exploited as catalytic support, immobilization platform or as label to improve the quality of biosensing (Pérez-López & Merkoçi, 2011). The quality of biosensing is associated with three main parameters: sensitivity, stability and selectivity. Until now, different options of nanomaterials for biosensors have been proposed: carbon nanotubes (CNTs), metal nanoparticles (for example, gold nanoparticles (Au NPs)), magnetic nanoparticles (MNPs), nanowires (NWs), nanorods (NRs), nanocomposites (NCs), and quantum dots (QDs). Nanomaterials must be completely reproducible to guarantee their application in enzymatic pesticide biosensors, since any variation in their shape, dimension, or composition modifies the physicochemical properties required by each application (Hu & Li, 2011). The main applications of nanomaterials in biosensors are related with the supporting matrix or amplification tags. The processes of synthesis and bioconjugation of nanomaterials must be standardized to be applied in a mass production scale for the complete commercialization. In AChE biosensors, the use of nanomaterials such as carbon nanotubes, gold nanoparticles, zirconia nanoparticles, titania nanoparticles, and quantum dots of cadmium sulphide will enhance the OP pesticides determination due to that they require lower oxidation potentials, and will have rapid response and long storage stability (Periasamy et al., 2009).

6.2. Carbon nanotubes and their composites

The introduction of carbon nanotubes will have two strategic advantages: highly specific recognition and quasi-direct detection of biomolecules. Pristine carbon nanotubes cannot be applied directly in the detection; they must be functionalized either in the side walls or extremes to create active sites where chemical reactions with pesticides can be identified (Lei & Ju, 2010). Amperometric and voltammetric biosensors can be implemented by glassy carbon electrodes (GCE) covered with carbon nanotubes (Balasubramanian & Burghard, 2006). Properties such as efficient electron transfer from electrode surfaces to the redox sites of enzymes in an amperometric biosensor can be achieved by means of carbon nanotubes (Wang, J., 2005). When nanocomposites containing single-walled carbon nanotubes and Co phthalocyanine are used, properties such as high efficiency of sensing and minimal steric hindrance of the AChE active site can be obtained (Ivanov et al., 2011). In addition, lower working potential and minimal interferences from electrochemical active components are achieved. A great prospect for automatic monitoring of pesticides in water, have been proposed based on amperometric biosensor implemented by a Layer-by-Layer (LbL) assembly (Firdoz et al., 2010). This topology presents excellent electrocatalytic activity towards pesticides in water, due to the higher electrical conductivity of the composite material used. Poly(diallyldimethylammonium chloride)-Single-Walled Carbon Nanotubes/Acetylcholinesterase (PDDA-SWCNTs/AChE) multilayer films were deposited on glassy carbon electrode. This biosensor presents stability, good precision, low detection limit, and requires much lesser time to the determination.

The use of nanocomposites such as carbon nanotubes/Nafion could provide properties such as low potential of detection, high sensitivity, formation of a stable and highly conductive CNT surface, and high stability to the enzyme (Musameh et al., 2011). Nanocomposites such as Au-MWCNTs can also be applied to amperometric biosensors for achieving properties such as low detection limit, high electron transfer rates, high sensitivity, stability, wide linear dependence of concentration, large immobilization sites for the enzymes, and excellent anti-interference (Jha & Ramaprabhu, 2010; Ma et al., 2011). They have allowed detecting pesticides such as paraoxon and methyl parathion.

6.3. Ceramic materials

Ceramic materials such as zinc oxide (ZnO) can be used as a matrix for immobilization of AChE for a large period of time, under saline media and very low temperatures (Sinha et al., 2010). ZnO matrix designed in a sol-gel method offers stability, reproducibility, reduction of enzyme used, and operation at lower potentials. New options of substrates such as layered double hydroxides (LDHs) are being used for the immobilization of AChE distributed as insulator/semiconductor solid supports (Hidouri et al., 2011). A LDH is a hydrotalcite-like material with two-dimensional nanostructured anionic clays. Due to their lamellar structure formed by two types of metallic cationic species and interlayer domains occupied by anionic species, they have a large surface area, high anion exchange capacity, biocompatibility, tunable surface, and porosity properties. These advantages are exploited as host structures

for biomolecule immobilization. Combination between enzymes and LDHs is generating biohybrid materials, which are convenient for the development of electrochemical biosensors.

6.4. Genetically modified enzymes

The use of genetically modified enzymes will be extended with the aim of: 1) reducing the cross-reactivity, 2) developing simple, stable, regenerable, reliable and sensitive detection methods, 3) discriminating between the contributions to the response signal of other nonspecific interactions and transport phenomena of the real monitoring of the biointeraction among detection, kinetic and affinity analysis, and 4) searching the development of mimic systems (Puiu et al., 2012). Due to the high affinity between mutant enzymes and pesticides, detection methods in water or liquid foods are being developed (Febbraio et al., 2011). The detection of pesticides in this type of media implies high stability toward temperature, organic solvents, and pH. Such advantages will must to be exploited to design multienzymatic biosensors for real-time, qualitative, and quantitative identification of a wide range of pesticides. The use of artificial neural networks (ANNs) can be a very useful tool for the design of electronic tongues (containing biosensors for multiple concentrations of pesticide or biosensors for different pesticides).

6.5. Quantum dots

Actually, water-soluble bioconjugated quantum dots (QDs) are being investigated as an option to detect pesticides in food formulations and drinking water (Vinayasa & Thakur, 2010). A quantum dot is a portion of semiconductor material whose excitons are confined in all three spatial dimensions. Their electronic properties are intermediate between those of bulk semiconductors and those of discrete molecules. Generally, the smaller the size of the crystal, the larger the band gap, the greater the difference in energy between the highest valence band and the lowest conduction band becomes, therefore more energy is needed to excite the dot, and concurrently, more energy is released when the crystal returns to its resting state. When they have different sizes, a gradient multi-layer nanofilm can be assembled with them. QDs are used as labels due to their fluorescence, photostability and prolonged excitation for image observation. In addition, the detection of multiple analytes is possible given that they have a wide spectral range (QD emits white light). Biochemical such as 3-phenoxybenzoic acid (PBA) and atrazine mercapturate (AM) placed on quantum dots operate as biomarkers of the pyrethroid insecticide and/or herbicide atrazine. The quantification of pesticides depends on competitive binding between the free particular pesticide and a known and well-established concentration of QD-bioconjugated pesticide toward the immobilized substrate of the biosensor. Materials commonly used to design QDs are cadmium sulphide, cadmium selenide, cadmium telluride, indium phosphide or gallium arsenide. QDs are more stable than enzymes or fluorescence dies, hence the use of this type of supports will provide a more efficient biosensing system in a near future.

6.6. Metals

The use of metal-based nanostructures for electroanalytical applications is being consolidated thanks to the diversity of shapes synthesized: spheres, cubes, prisms, dendrites, stars, spikes, rods and flowers (Plowman, B.J. et al., 2011). The use of self-assembly monolayers (SAMs) is not restricted to applications of masking or inhibiting biological entities. They can be useful to impede electron transfer from the surface to the solution used for biodetection. Thus, SAMs allow a reduction of non-specific binding in the supports and therefore, a more efficient operation of the biosensor not only with respect to response time, and likewise sensitivity, selectivity and stability.

6.7. Paper

In search of alternative technological solutions, bioactive paper-based materials are being developed with the aim of detecting toxins in food packaging (Luckman & Brennan, 2010). The operating principle of this material consists in producing rapid colorimetric detection of one or more analytes simultaneously. A pesticide biosensor to realize the detection requires the encapsulation of biomolecules in sol-gel derived materials. Such encapsulation can be obtained by means of silica, where even gold nanoparticles can be grown to operate as indicators of bioactivity and a mechanism of signal generation. Silica allows the development of portable and recyclable solid-state analytical devices for detecting target molecules. Since sol-gel materials can be deposited on paper substrates by ink-jet printing, thus new enzymatically active paper materials suitable for biosensing of pesticides are obtained. The colorimetric biosensing platform by means of biocatalytic growth of sol-gel entrapped gold nanoparticles within a thin silica film coated onto a paper substrate is another viable solution. This type of biosensors is inexpensive, disposable, capable of transporting liquids via capillary action without external power, and environmentally friendly.

6.8. Novel techniques

Amongst novel techniques that will be exploited in a near future are: 1) the detection of pesticides during water cleaning through catalytic processes by means of biosensors based on nanomaterials, 2) the exploitation of properties derived of the mediators that produce non-enzymatic factors implicated in the inhibition quantification such as inclusion of polar functional groups and the use of surfactants, 3) the design of controlled methods to generate genetically modified enzymes with well-established properties, 4) the toxicity of nanomaterials used in biosensors must be widely studied with the aim of fulfilling with technical standards associated with each application, 5) the application of self-assembled monolayers (SAMs) to develop uniform supports for enzymes, and 6) the possible encapsulation of pesticides including biosensors to this scale of integration with the aim of optimizing their application.

7. Conclusions

The presence of pesticides in food, water, air and soil is of serious concern, since a minimum quantity of them can have catastrophic consequences in human beings. Enzymatic biosensors follow offering an excellent technical option to determine qualitative- and quantitatively the presence of pesticides. Definitively, the great progress achieved in last decade in materials science and biochemistry is impelling the development of novel biosensors capable of determining with high sensitivity, high selectivity, and long-term stability, the presence of pesticides. In accordance with the review proposed here, biosensors requires multiple physicochemical properties that a unique material used as mechanical and biochemical support of the analyte cannot provide by itself; hence a more sophisticated design will be the future trend to guarantee the efficiency and accuracy desired for each application and range of detection required. Although, limits of detection in the range nanomolar have been achieved until now; it is desirable that the newest biosensors can identify pesticides in the range of picomolar and attomolar. The introduction of nanobiosensors has allowed the development of user-friendly and in-field application devices. Due to the reproducibility problems, biosensors based on nanostructured materials will be introduced in the market until that their stability can be guaranteed. This chapter reviewed the advances that with respect to biosensors for detecting pesticides have been achieved and the future trends for coming related with their research and technological development.

Author details

Rafael Vargas-Bernal and Gabriel Herrera-Pérez
Instituto Tecnológico Superior de Irapuato, México

Esmeralda Rodríguez-Miranda
Universidad de Guanajuato, México

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Relationship Between Biomarkers and Pesticide Exposure in Fishes: A Review

Nédia de Castilhos Ghisi

Additional information is available at the end of the chapter

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1. Introduction

1.1. Human population growth and sustainability

In the modern world, small livelihood-based farms that grow multiple crops have been mostly replaced by large, agricultural conglomerates, in which food is grown in monocultures. This shift has allowed for the expansion of suburbs and new cities, but it has also made these places isolated, dirty, degraded, abandoned, and depersonalized. Owing in no small part to pesticide use, one of the legacies of the twentieth century is the potential for severe anthropogenic ecological damage. It has been recognized since the 1970s that byproducts and waste from technical-industrial development degrade the biosphere and threaten to irretrievably poison the environment to which we, as humans, also belong [1].

The term “sustainable” means that an activity can be continued and repeated indefinitely and predictably into the future. It is concerning that, in large part, human activities are logically unsustainable: global human population cannot continue to increase in size forever. We cannot continue to take fish out of sea faster than their populations can recover if we want to have fish to eat in the future. We cannot continue to develop crops in forests if soil quantity and quality deteriorates and water supplies become eutrophic or toxic. We cannot continue to use the same pesticides if an increasing number of pests and pathogens become resistant to them. We cannot maintain nature’s diversity if we continue to cause species extinction [2].

The source of many environmental problems, not to say of all, is simply our current level of rapid human population growth [Figure 1]. More people means more demand for energy, more consumption of non-renewable resources such as oil and minerals, more pressure on the renewable ones like forests and fisheries, more need for crops and food production, and so on. Surely, this cannot continue sustainably [2]. In addition to—or as a consequence of—this population growth, environmental pollution is also increasing.

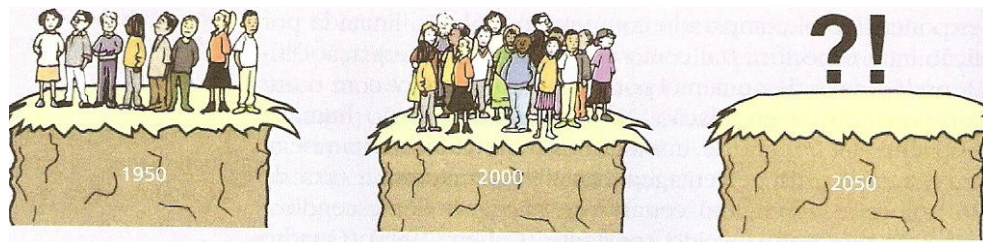


Figure 1. Representation of human population growth – before agricultural revolution [18th century] human population took around 1000 years to double in size. Currently, total population has been doubling in the last decade. Source: [2]

While world population [more than seven billion people] has doubled in the last decade, the demand for water supply increased by a factor of six according to United Nations data for the year 2000. Water demand grows faster than the population, mainly due to the introduction of more hygienic habits globally and the omnipresent desire to increase the productivity of food and industrialized products, the latter of which are generally also intended to maximize agricultural production. However, this increase in per-capita consumption exacerbates the imbalance between the distribution of water on Earth and its centers of human population density. Thus, the cheapest and most viable way to supply a growing world population is to learn how to use the available water in a more efficient manner [3].

1.2. Water sustainability as a resource

Water, when taken on the whole, can be considered an abundant resource on the planet. Total reserves sum up around of 1,265,000 trillion m³, distributed among solid [ice], liquid [rivers, lakes, oceans, water tables] and gaseous [atmosphere] phases [Figure 2].

However, out of the proportions shown in Figure 2, the relatively small parcel of water present in freshwater courses stands out. These waterways constitute the main source of water supply and are also the most common recipients of sewage discharge. Total world water demand is only around 11% of the mean discharge of rivers. Of this, 70% is used in agricultural activities, 20% in industrial activities and 10% goes to domestic and municipal use [3]. Therefore the actual crisis is not in terms of global water scarcity, but in its heterogeneous distribution. This is aggravated by the disorderly growth of local demand and, above all, by the fact that water degradation has reached unprecedented levels, not just in urban areas, but in rural areas as well [3].

In the same manner, water scarcity is not an issue that is exclusive to arid and semi-arid regions. Many areas with abundant water supplies, although insufficient to attend excessively high demands, have also experienced conflicts regarding water use and suffered consumption restrictions that have affected economic development and quality of life [4]. In addition, there has been severe extraction pressure on many aquifers to the point where many have been degraded nearly to extinction, especially near big cities or metropolises.

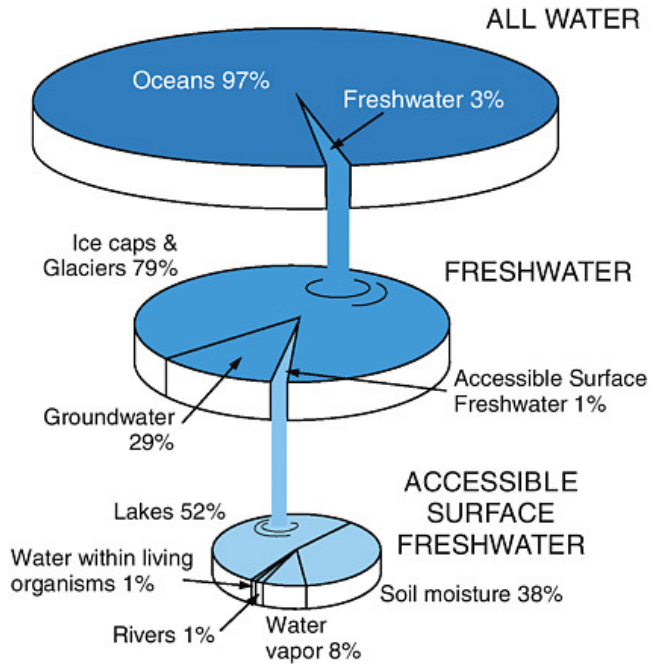


Figure 2. Distribution of the world's water. Source: Courtesy "Earth Update" CD-ROM, Rice University and the Houston Museum of Natural Science; used with permission.

The physical expansion of cities toward wellsprings has been causing serious damage, often forcing their relocation.

The demand for a clean and safe water supply for human consumption, agriculture, and recreational purposes has been rising rapidly in the last few years. Water has become a limiting factor for agricultural, urban and industrial development. Recipient waterways, such as lakes, rivers and coastal areas receive great amounts of industrial, agricultural and urban waste directly via inputs and conveyance as well as indirectly through atmospheric deposition of aerial emissions. A complex mixture of toxic substances with an increasing number of contaminants has been deposited in these waters, posing a threat to aquatic ecosystems as much as to the health and well-being of human populations [5].

Regrettably, the waterways that are becoming contaminated are otherwise especially valuable resources. Unfortunately, contamination is very easy, but decontamination is often very costly and in some cases impossible to achieve [2]. In contrast with waste disposed in terrestrial environments that has more-or-less local effects, toxic waste in aquatic environments can be easily transported by currents and dispersed over large areas. Toxic chemicals in water, even in low amounts, can be concentrated to lethal levels by filtering aquatic organisms and top predators [6]. Pesticides, herbicides, oil waste and leakages, heavy metals [such as mercury, zinc and lead], detergents, and industrial waste can harm and kill organisms that live in or use this contaminated water. The potential risks of

contamination in aquatic biota and humans can to be evaluated through biomonitoring programs. The relevance of these studies rises with the growth of urban, industrial, and agricultural activities around water sources [*i.e.*, rivers, lakes and reservoirs] associated with frequently inadequate or insufficient water treatment. This is reflected in attempts to establish rules concerning the safety of water supplies in reservoirs and possible risks to environmental and human exposure [7]. These kinds of studies fits in a discipline called ecotoxicology.

2. Ecotoxicology

The term ecotoxicology was introduced by Truhaut in 1969 and was derived from the root words ecology and toxicology. The introduction of this term reflected a growing interest in the effects of chemicals in other, non-human species. Truhaut identified a field of study that was interested in the harmful effects of these substances within the concept of ecology. Ecotoxicology can be defined as *the study of the harmful effects of chemicals on ecosystems, including effects on individuals as well as consequences in populations and higher levels of organization* [8].

Despite the definition above, many of the first works in ecotoxicology had little to do with ecology or ecotoxicology. At the time, principal importance was placed on the detection and determination of pollutants in animal and plant samples, although the analytical results could sometimes be related to the effect on populations and communities. Analytical techniques such as chromatography, thin layer chromatography and atomic absorption facilitated the detection of very low concentrations of chemicals in living organisms, but establishing the biological significance of their presence or the organismal response to a specific dose of these substances remained difficult [8].

A substance is considered a pollutant when it is detected in levels above those that would normally occur in a particular environment. This immediately brings up the question “what level is considered to be normal?” For most synthetic organic chemicals, such as pesticides, the answer is simple: no detectable level is “normal,” because these substances do not exist in the environment until they are introduced by humans. On the other hand, substances such as metals, sulfur dioxide, nitrogen oxides, polycyclic aromatic hydrocarbons [PAHs] and methyl mercury naturally occur and their presence in the environment pre-dates humans. Naturally, there is variation in the concentration of these compounds across different sites and through time. This complicates the assessment of what is normal [8].

There is a conventional distinction between the definitions of the terms “pollutant” and “contaminant”: a pollutant is a substance that causes real environmental damage, while the term “contaminant” does not necessarily imply that the chemical is harmful. Still, it is difficult to deal with this distinction. First, there is the general toxicological principle that toxicity is related to dose. In this way, a pollutant can fit the description of pollutant in one situation [at high doses] but not in others [low concentrations]. Second, there is no general agreement on what constitutes environmental damage. Some scientists consider deleterious biochemical changes in organisms to be an environmental damage; others apply the term to

population decrease. Third, the effects of the levels of chemicals measured in living organisms – or in their environment – are frequently unknown, even though the term pollutant is applied to them. This subject becomes even more complicated due to the possibility of toxicity increasing when organisms are exposed to a suite of environmental chemicals; such synergistic effects may result in chemicals having more a deleterious influence on organisms when in a mixture than they would otherwise in isolation [8].

Determining whether a contaminant is a pollutant also depends on its concentration in the environment, on the organisms to be considered, and on the possible damages of the contaminant to the organism. Thus, a compound can fit the description of a pollutant for one organism but not for another [8]. In order to minimize these problems of terminology, the term “pollutant” is used for environmental chemicals that exceed normal levels and cause damage. And *environmental damage* includes biochemical and physiological changes that adversely affect individual organisms, birth, growth and mortality rates[8], and reproduction.

An exciting aspect of ecotoxicology is that it represents an approach that extends from molecules to ecosystems, from genes to physiology [8]. This is further explored in the discussion of response levels in biomarkers.

3. Pesticides

When pesticides began to be developed on an industrial scale, manufacturers were not very concerned about the specificity of their products. These chemicals could damage anything, so long as they did not harm the crop, human beings, or their animals. A good example of this was when P. H. Müller received the Nobel Prize in Physiology or Medicine in 1948 “for his discovery of the high efficiency of Dichloride-Diphenyl-Trichlorethane [DDT] as a contact poison against several arthropods” [9]. This insecticide was widely used after World War II to exterminate mosquitoes that caused malaria and typhus. It is cheap and very effective in the short run, but in the long run it is harmful to human health and possibly carcinogenic. It also interferes with animal life, leading to, for example, higher mortality in birds. In less than two decades DDT was banned in many countries, and today its use is forbidden almost worldwide.

The study of the impacts caused by pesticides gained attention from 1979 on, inspired by discoveries of pollution by nematicides in aquifers of many north-American states. Following this, many other cases of pesticide contamination of soil, water resources, animals and, more critically, human beings were diagnosed in the temperate regions, but little investigation was carried in tropical regions [10].

Many chemicals used to kill plagues have become important environmental pollutants. These pesticides are pulverized or released above plague areas, but only a small amount reaches the target, with most of it falling over resident crops or bare soil. Therefore, such pesticides are used in excessive quantities. This occurs especially with herbicides because they are cheaper than insecticides and fungicides [2]. The real problem emerges when the

pesticide is toxic to species other than the target ones and, in particular, when they are transported outside the areas where they were applied and persist in the environment longer than expected.

Agricultural production is currently highly dependent on the use of pesticides, and the abandonment or reduction in their use would lead to a decrease in production, a rise in production costs, higher consumer prices, and, in some places, hunger and malnutrition[11].

The sale of pesticides involves billions of dollars a year. Current transgenic technology has increased the commercialization of certain kinds of pesticides, which can be deliberately used on resistant crops. With many of these substances reaching the environment, especially aquatic ecosystems where they are most concentrated, it is natural to think that some could be accumulating in individuals that compose food chains [12]. Due to their biological activity and to the huge quantity in which they are spread annually in the environment, pesticides can harm human health and the environment; for example, the induction of DNA damage can lead to adverse reproductive reactions, cancer and many other chronic diseases [13]. Recent evidence of negative effects of herbicides in amphibians, reptiles, fish and many other organisms continue to elucidate the fact that we are still discovering the extent to which populations can be affected by the current use of pesticides [14–18].

Therefore, the goal of this chapter is to discuss the methodologies and results of experiments and field surveys that analyze the effects of pesticides, primarily on aquatic communities and especially in fish.

4. Biomarkers

In ecotoxicology, there are many levels of response that can be evaluated.

The presence of a pesticide or other xenobiotic compounds in a portion of the aquatic environment does not, by itself, indicate a deleterious effect. Connections must be established between external levels of exposure, internal levels of tissue contamination and early adverse effects. The evaluation of these adverse effects—particularly if it is based upon only one level of response—can be affected by the ability of various pollutants [and their derivatives] to mutually affect toxicity, or even to act synergistically [19].

Deleterious effects on populations are often difficult to detect in feral organisms because many of these effects tend to be made manifest only after longer periods of time. When the effect finally becomes clear, the destructive process may have gone beyond the point where it could be reversed by remedial actions or risk reduction. In these sceneries the importance of early-warning signals, or biomarkers, that detect adverse biological responses towards anthropogenic environmental toxins become critical. A biomarker is any biological response to a chemical agent present in the environment that can be measured in the organism [or in its cells], in its metabolic products [urine, feces], or in hair, feathers, etc., that is indicative of some deviation in the standard pattern found in non affected organisms [20].

Pollutant stress generally triggers a cascade of biological responses, each of which may, in theory serve as a biomarker [21]. In the established reference response levels [22][Figure 3],

biomarkers evaluate the precocious responses to pollutants. The responses in higher hierarchical levels are late response measures, frequently when the entire environment is already impacted. Biomarkers are important because they give us much more information on the biological effects of a certain pollutant than simply its quantification. Moreover, by the use of multiple biomarkers important information can be obtained. Biomarkers can be used after trophic, environmental, or occupational exposure, to elucidate the relation of cause-effect and dose-effect in health risk assessment, and in clinical diagnoses and for monitoring purposes [19].

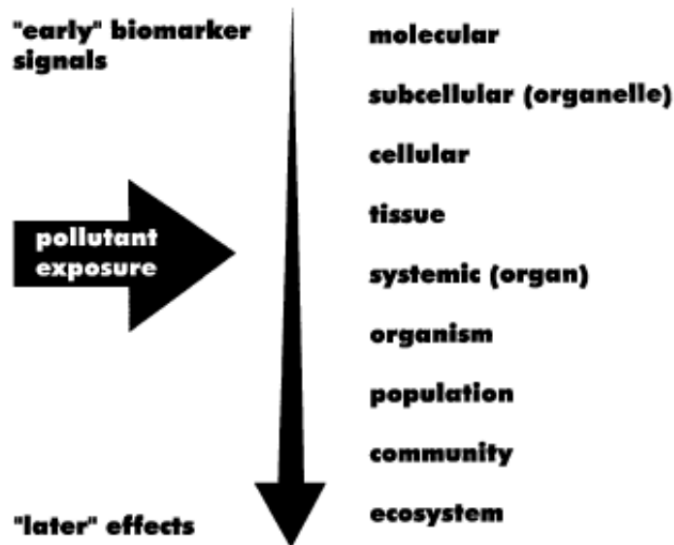


Figure 3. Schematic representation of the sequential order of responses to pollutant stress within a biological system. Modified from [22].

In developing a better understanding of the toxicity of contaminants, two kinds of studies can be carried out: *bioassays*, which are laboratory experiments, or *biomonitoring*, with direct field surveys. Although bioassays generate complementary data, it is important to note that experimental conditions do not always entirely reflect the natural environment [23].

On the other hand, we should not confound the term *biomarker* with *bioindicator*. A bioindicator is defined as an organism whose presence or absence, behavior, or some other characteristic gives information on the environmental conditions of its habitat. Fish species have attracted considerable interest as bioindicators in studies assessing the biological and biochemical responses to environmental contaminants. Fish can be found virtually everywhere in the aquatic environment and they play a major ecological role in the aquatic food-web because of their function as carriers of energy from lower to higher trophic levels [24]. Well like mammals, fish can suffer bioaccumulation, and have the advantage as bioindicators because they can respond to mutagenic agents in low concentrations by

activating the P450 cytochrome enzymatic system, a system of monooxygenase enzymes with a heme group and different specificities per substrate. These enzymes play a fundamental role in the metabolism of xenobiotic substances and of endogen compounds [25].

Genetic, biochemical, and histopathological biomarkers are among the most common biomarkers in ecotoxicological studies with fish.

4.1. Genetic biomarkers

Genetic biomarkers evaluate the most precocious level of response: at the molecular level. DNA is a molecule that contains all the necessary information for the survival and perpetuation of an organism [26]. The exposure of an organism to genotoxic substances can lead to a sequence of events [27] that affect higher levels of response. *Genetic ecotoxicology* can be defined as the study of pollutant-induced changes in the genetic material of biota in nature and has two components: first, initially, the genotoxicity of pollutants, such as structural alterations in the DNA, and second, consequently, the procession and expression of DNA damage in mutant gene products, resulting in long-term heritable effects, such as changes in gene frequency within exposed populations, mutational events, etc. [28].

Many biomarkers have been used as tools for exposure detection and for the evaluation of the effects of genotoxic pollution. These biomarkers consist of tests such as the evaluation of chromosomal abnormalities, DNA adducts and breaks, the measurement of micronucleus frequency and other chromosomal anomalies, and the Comet Assay [29]. Here we will discuss surveys that used the Piscine Micronucleus Test [in conjunction with nuclear morphological alterations] and the Comet Assay.

Among many mutagenicity assays, piscine micronucleus and nuclear morphological alterations test [Figure 4] has been applied successfully because it is simple, safe, sensitive and it does not depend on the karyotypic characteristic of the study animal [30]. This last point is important because most fish have a relatively large number of small chromosomes, which are hard to visualize [31]. When fish erythrocytes are used there is also no excessive time consumption or animal suffering. Thus the micronucleus test in fish erythrocytes has been shown to be a promising technique in the investigation of environmentally-caused mutagenesis [32].

Micronuclei are small cytoplasmic chromatin masses present outside of the main nucleus of the cells that can originate from a chromosomal break as well as from a dysfunction in the mitotic spindle apparatus [33]. They are whole or partial chromosomes that were incorporated inside the nucleus of the daughter cell during cellular division and appear with a small dark round structure identical in appearance to the cellular nucleus [29]. Although there is a measurable basal level of spontaneous formation of micronuclei in most fish species [32], broad scale exposure to environmentally-relevant levels of clastogenic compounds in the laboratory [29,34,35] has been shown to elevate the frequency of micronuclei.

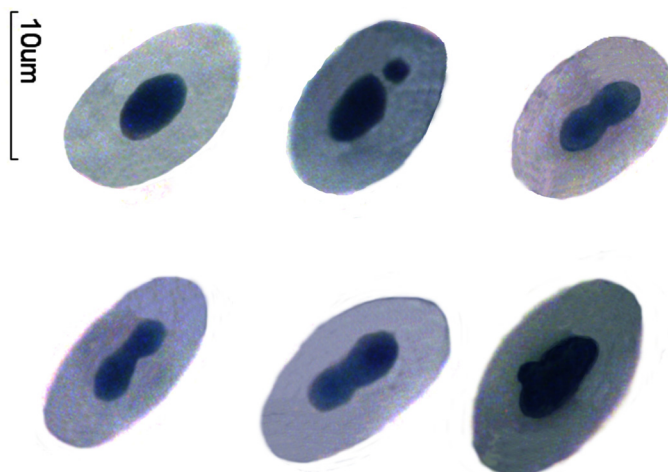


Figure 4. Photos of a micronucleus and nuclear morphological alterations found in erythrocytes of neotropical fish *Astyanax altiparanae*. The first cell represents a normal nucleus, the second cell has a micronucleus and all other cells show nuclear morphological alterations.

In addition to the presence of micronuclei, nuclear morphological alterations can also occur, such as when the nucleus does not show a regular oval shape, but has a projection or an invagination of chromatin. In reference [36], they showed that these alterations are induced by well known genotoxic compounds, even when the micronucleus has not been formed. It is believed that these nuclear anomalies are due to problems with the nuclear lamina, because this structure confers the regular oval shape and stability on the nucleus [37].

Tests that directly evaluate breaks in DNA strands or chain alterations followed by DNA damage are commonly used to analyze the genotoxic impact in aquatic animals [38]. The Single Cell Gel Electrophoresis [SCGE], or Comet Assay, was firstly applied in ecotoxicology fifteen years ago, and has become one of the most popular tests for the detection of strand breaks in aquatic animals under *in vitro*, *in vivo* and *in situ* exposure [39].

The Comet Assay is a rapid, quantitative technique in which visual evidence of DNA damage in karyotic cells can be measured [Figure 5]. It is based on the quantification of denaturated DNA fragments that migrate out of the cell nucleus during electrophoresis. This method has been broadly used in many areas, including biomonitoring, genotoxicity, ecological monitoring, and also as a tool for DNA damage research or reparation in many kinds of cells in response to a variety of DNA-damaging agents [40].

There are many advantages to the Comet Assay: [a] genotoxic damage is detected at the individual cell level; [b] most eukaryotic organisms can be used in the Comet Assay; [c] a small number of cells is required; [d] it is usually easier to perform and more sensitive than other methods for the evaluation of strand breaks; [e] DNA strand breaks form quickly after a genotoxic exposure, so the essay provides an early evaluation of biota's response [38].

The Comet Assay is usually done with erythrocytes because they are easily obtained through non-destructive methods and do not require the additional cellular isolation step. However, other tissues have also been tested for the genotoxic effects of contaminants because genotoxic effects can be tissue-specific [41].

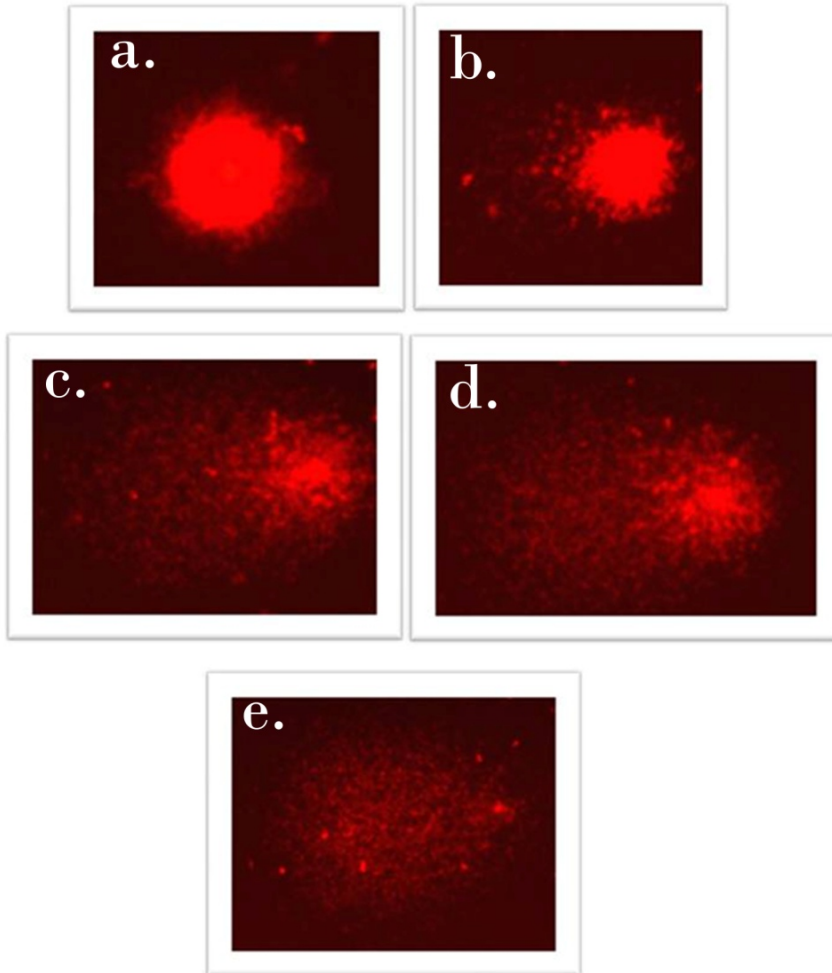


Figure 5. Pictures of five different damage rates in the Comet Assay through an immersion lens. a. zero damage; b. damage one; c. damage two; d. damage three; e. damage four [possibly in apoptosis]. Source: the author [2009].

4.2. Biochemical biomarkers

According to the Central Dogma of Molecular Biology [DNA → mRNA → protein], DNA is indirectly responsible for protein production [26]. Therefore, DNA alterations can lead to

damage in proteins [and the resulting enzymes]. These alterations can be quantified through biochemical biomarkers.

The most sensitive biomarkers are usually changes in the level and activity of biotransformation enzymes. Biotransformation is the conversion, catalyzed by enzymes, of a xenobiotic compound into a more water-soluble form, facilitating its excretion [19]. The enzymes responsible for biotransformation reactions are found throughout an organism [blood, kidneys, lungs, skin, nervous tissue, small intestine, and liver], but the liver is undoubtedly the organ in which they are most concentrated [42]. The biochemical biomarkers to be highlighted in this chapter are the activities of GST [Glutathione S-transferase], CAT [Catalase], lipoperoxidation [LPO], and Acetylcholinesterase [AChE].

The enzyme Glutathione S-transferase [GST] belongs to phase II of metabolism, and is responsible for the conjugation of electrophilic components or those that come from phase I with GST. The conjugation reaction started by GST is important to cells because it acts in the hydrolysis of lipophilic substances, which can then be excreted as inert substances in the organism. This super family of enzymes occurs in prokaryotes, plants, mollusks, crustaceans, insects, amphibians, reptiles, fish, and mammals [19].

Catalases are intra-cellular enzymes located in the peroxisomes that facilitate the removal of hydrogen peroxide, which is transformed into molecular oxygen and water [43]. Catalases are also cited as detoxication enzymes on some substrates, such as phenols, alcohols, formic acid and formaldehyde [44,45].

The lipid peroxidation or oxidation of polyunsaturated fatty acids is a regular physiological process that is important in cellular maturation [46–48] and lipid mobilization [49,50]. Some classes of contaminants, however, can have detrimental effects on this process [51,52] and can lead to damage in cellular function [52,53] and malfunction of cellular membranes and essential organelles, in addition to potentially affecting transporting processes, metabolites and ion gradient maintenance, and receptor-mediated signals transduction [54], with subsequent structural modification of the lipoproteic complexes in cellular membranes [55,56].

Lipoperoxidation has been used successfully as a xenobiotic-induced oxidative stress measurement in organisms exposed *in vivo* to myriad chemicals such as metals [iron, cadmium, mercury and lead], paraquat, malathion, deltamethrin, and glyphosate [19,57–61].

The term cholinesterase [AChE] usually refers to the sum of the activities of pseudo-cholinesterase, or butyrylcholinesterase, and acetylcholinesterase, or real cholinesterase, both of which are present in muscles [62]. The measurement of the AChE activity is often used to diagnose the exposure to anticholinesterasic toxins in fish, and can be considered one of the most ancient biomarkers [62,63]. Some authors [19] indicate that fish exposed to pesticides can show a reduction in acetylcholinesterase activity that is proportional to concentration and exposure time. The enzymatic measurement of cholinesterase allow the detection of sub-lethal toxicological effects, mainly of organophosphate compounds and carbamates, even without the presence of clinical symptoms.

4.3. Histopathological biomarkers

We can also observe damage in higher, cellular and tissue, response levels, which are detected through histopathological techniques. Morphological techniques such as light microscopy have been used in toxicology because they allow an evaluation of the possible effects of xenobiotics on target organs and tissues. According to [64], the effects in cell and tissue structure are important parameters to be considered in the evaluation of the potential toxicity of contaminants in living organisms.

Some authors [65] report that, through morphology, it is possible to reveal the most-affected target organs as well as to detect an organism's sensitivity to the toxicity level of the compounds to which it was exposed. Histopathology also permits the differentiation of injuries promulgated by disease from those caused by environmental factors, such as the exposure to pollutants [66].

The advantage of histopathology as a biomarker lies in its use at intermediate levels of biological organization. Histological changes appear as a medium-term response to sub-lethal stressors, and histology provides a rapid method for detecting effects from xenobiotic compounds, especially chronic ones, in various tissues and organs [67]. For example, fish exposure to chemical contaminants is likely to induce a number of lesions in different organs [68,69]. Gills [70], kidneys [71,72], liver [73,74] and skin [75] are suitable organs for histological examination in order to determine the effect of pollution.

The article [67] propose an index of histopathological tests for any given organ, which leads to standardized quantification and allows legitimate comparison between different studies and, with restrictions, between different organs. This tool leads to a better understanding of the significance of histological findings after exposure to contamination.

Certain organs are the primary markers for aquatic pollution. For example, gills and skin have large surfaces that are in direct and permanent contact with potential irritants, and both have mucous cells. The liver plays a key role in metabolism and subsequent excretion of xenobiotics and is also the site of vitellogene production. The kidneys are very important for maintenance of a stable internal environment with respect to water and salt, excretion, and partially for the metabolism of xenobiotics [67].

5. Relationship between biomarkers and pesticide exposure in fishes

Every week, new articles are published showing the detrimental effects of many pesticides. These effects can be seen at all response levels: molecular, cellular, histological, individual, or even at higher ecological levels such as population, community, or ecosystem.

It is important to evaluate the effect of pesticides at lower response levels for the purposes of early damage detection, before they affect higher levels and decimate an entire community or ecosystem. Hence, we will focus on lower level responses at the molecular, cellular and histological level.

Mutagenic chemicals have a high probability of inducing carcinogenic effects in various fish species. A majority of these chemicals have been found to cause tumors at specific or multiple sites in fish [76]. Herbicides and pesticides comprise a large group of mutagenic chemicals, but information on herbicidal genotoxicity is lacking. Pentachlorophenol [PCP] and 2,4-dichlorophenoxyacetic acid [2,4-D] are chlorinated phenols widely used in agriculture. Chlorinated phenols in general are noted for exhibiting strong biological effects. For example, 2,4-dinitrophenol decouples oxidative phosphorylation; intervening in the oxidative pathways of metabolism. A clinical manifestation of this effect is the very rapid onset of rigor mortis in victims of pentachlorophenol poisoning [77]. Another study with humans revealed a significant increase in chromosomal abnormalities observed in the lymphocytes of workers exposed to PCP, leading to possible carcinogenic effects [78]. [79] surveyed the mutagenic effect of these two pesticides [PCP and 2,4-D] in the fish *Channa punctatus*. Using the Piscine Micronucleus Test [PMT], which evaluates the rate of permanent DNA damage, they observed that an increase in the dose and exposure time to both pesticides increased the rate of mutation in fish erythrocytes. In this work, they concluded that PCB was more toxic than 2,4-D in terms of Micronucleus induction.

The same fish species was used to evaluate the acute genotoxic effect of the insecticide Endosulfan [80]. Endosulfan is one of the most abundant organochlorine pesticides in the global atmosphere and is capable of undergoing long range transport to remote locations such as the Arctic [81]. Using the Comet Assay with liver and gill tissue, the authors observed a dose-dependent response; that is the higher the dose, the higher the rate of DNA breakdown in *C. punctatus*. In this case, there is evidence that gill cells are more sensitive than hepatic ones.

We reported on the case of an accidental spill of about 8000 liters of endosulfan in Paraíba do Sul River in the state of Rio de Janeiro State [Brazil], in November 2008 [7]. In this study, we analyzed the fish *Pimelodus maculatus* before [dry season] and after [rainy season] the spill in two affected drinking water reservoirs [Ilha dos Pombos and Santa Cecília] and in one that was unaffected [Santa Branca] These reservoirs are destined for human water provision. Fish from the Ilha dos Pombos reservoir [rainy season] that had been affected by the endosulfan spill showed several histopathological alterations in the gills and liver. Gill alteration index was similar in the fish from the three reservoirs in the dry season, but increased in the affected reservoirs during the rainy season, probably due to the endosulfan spill that occurred two months before this sampling. Figures 6 and 7 show the alterations found in the Paraíba do Sul River fishes. With regard to biochemical biomarkers, Cholinesterase activity in axial muscle was higher in *P. maculatus* from Ilha dos Pombos [33X] and Santa Branca reservoirs [11X] during the rainy season sampling after the endosulfan spill. Although we do not have normal values for acetylcholinesterase activity in this fish species, based on studies with other fish species the activity in the muscle showed a decrease in the dry season in all the reservoirs. Several pollutants such as organophosphates, carbamates [82], metals [83], hydrocarbons, and endosulfan [84] can decrease cholinesterase activity through inhibition or reduced expression, although an increase in activity has also been reported for fish muscle [85].

A similar study to [7] was also carried out in polluted and unpolluted areas in Estuarine Lakes at Santa Catarina Coast in southern Brazil, using the bioindicator fish *Geophagus brasiliensis* [86]. Estuaries are important sinks of pollutants derived from anthropogenic activities. The lakes in Santa Catarina are of great economic importance to the surrounding areas, enabling cultivation of rice crops and pig farming by various irrigation and drainage channels, as well as providing fish and shrimp to support ~10,000 artisanal fishermen. The results showed that both studied lakes are impacted by potential genotoxic substances. Severe lesions in the livers of *G. brasiliensis* were also observed. The inhibition of acetylcholinesterase activity suggested the presence of pesticides or metals in the study sites. The presence of large areas of rice crops around Santa Marta Lake [one of the studied lakes] may provide an explanation for the occurrence of substances with neurotoxic potential in the lakes. Three pesticides widely used on rice crops in southern Brazil [Clomazone, Quinclorac and Metsulfuron-methyl] have been shown to inhibit AChE activity in another species of fish, *Rhamdia quelen* [87]. The inhibition of AChE activity in fish can have adverse consequences for the animal itself, mainly by affecting its swimming ability and therefore its ability to find food and escape from predators [88].

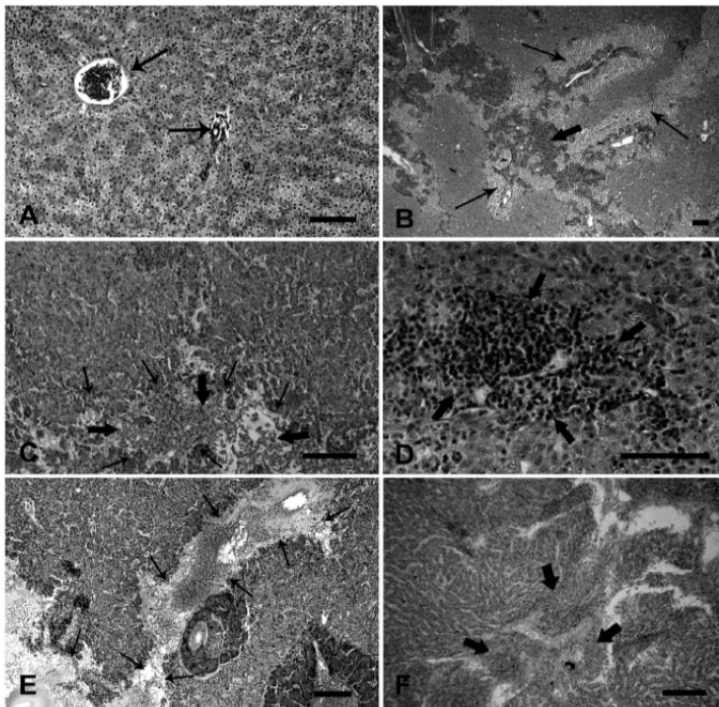


Figure 6. Histopathological findings from the liver of *Pimelodus maculatus* in Paraíba do Sul River. [A] Normal tissue. Arrows show vessels. [B] The presence of pancreatic tissue [large arrow] and the high incidence of melanomacrophage centers [small arrows]. [C] Occurrence of differentiated tissue [small arrows]. [D] Arrows show leukocyte infiltration. [E] Large necrosis area [arrows]. [F] Arrows show a large differentiated area of tissue. Scale bar=100 mm. Font: [7]

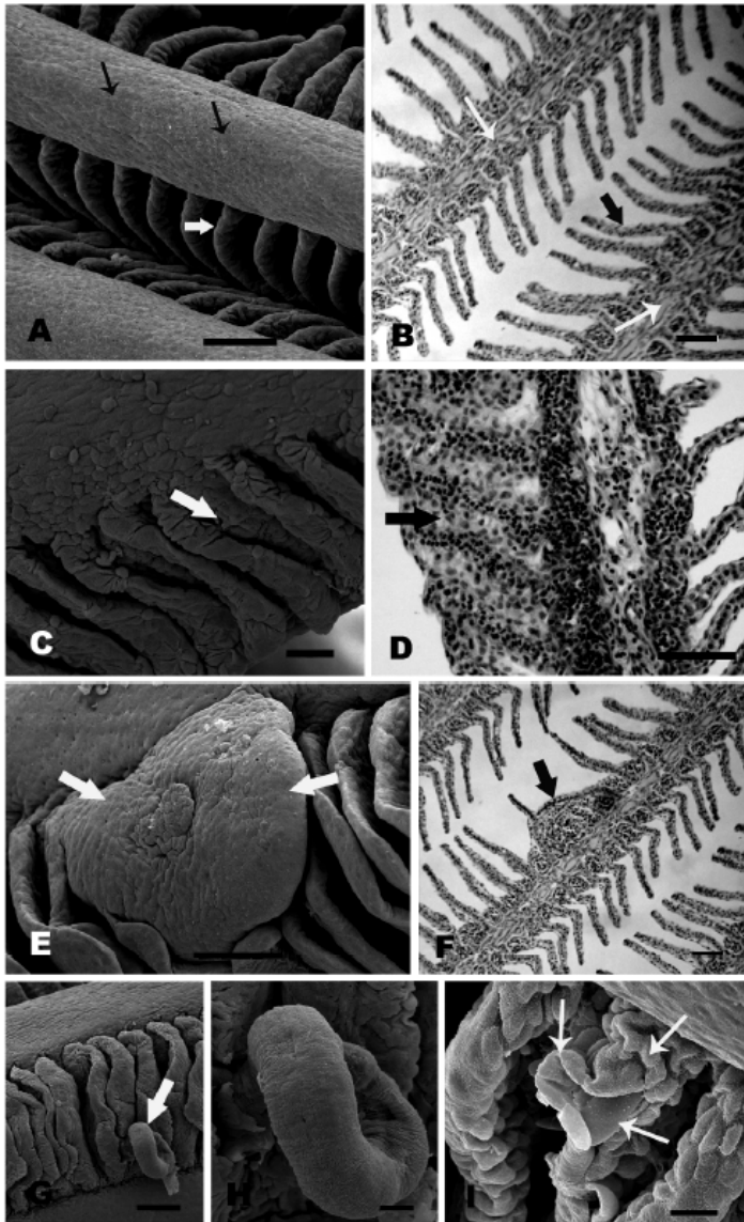


Figure 7. Gills of *Pimelodus maculatus* from Paraíba do Sul River. A and B: Normal aspect of gills showing primary [small arrows] and secondary lamellas [large arrows]. Scale bar = 50 and 100 μ m respectively. C and D: arrows: fusion among secondary lamellas. Scale bar = 20 and 50 μ m respectively. E and F: neoplasia. Scale bar = 20 and 100 μ m. G and H: arrow= ectoparasite. Scale bar = 50 and 10 μ m. I: Observe the epithelial cells alterations [arrows]. Scale bar = 10 μ m. Font: [7].

Another field study compared two areas in southern California with records of chlorinated hydrocarbon [DDTs and PCBs] contamination to one less contaminated site. The frequency of micronuclei in circulating erythrocytes of two sea fishes was much higher in the contaminated areas. The DNA damage rate was up to four times lower in the uncontaminated site [89]. Organochlorine compounds such as in the DDT family, used as pesticides in agriculture, and polychlorinated biphenyls or PCB, which are important industrial chemicals and are used as non-flammable oils in many commercial products, are extremely persistent and difficult to degrade. Despite the fact that these compounds have been forbidden in many developed countries and their worldwide production and use have drastically decreased in recent years [90], at present they are widespread and have become ubiquitous contaminants of natural systems. PCBs are currently the most abundant chlorinated aromatic contaminants in the environment.

It was not until after DDT use had become widespread that the impacts of pesticides started to gain world's attention and an environmental revolution began. This happened in 1962, with the release of the famous book *Silent Spring*, by Rachel Carson [91]. She described the process known as biomagnification, through which DDT and other organochlorine insecticides become more concentrated in higher levels of the food chain, being detected in the breast milk of women around the world and in the fatty tissues of Eskimos, inhabitants of isolated lands in Arctic. DDT is responsible for making bird's egg shells thinner, particularly in birds of prey; this compound nearly drove the Peregrine falcon to extinction. DDT blocks calcium absorption, which makes the eggs easily broken and interrupts incubation, consequently undermining reproduction.

Currently, the pesticides with the highest sales rates worldwide are those based on glyphosate. Their sales have risen 20% a year, mainly due to the advent of biotechnology, which has provided plants that are resistant to this herbicide. Described by the manufacturers as pesticides low in toxicity and with good environmental compatibility, the glyphosate-based herbicides can seem like a silver bullet to those dealing with unwanted vegetation. However, there is public interest in the ecological, safety, and health concerns that may arise through the use of products from transgenic harvests [92].

There is some literature on the undesirable effects of glyphosate. Laboratory studies have detected adverse effects in every toxicological test category: medium-term toxicity [salivary gland lesions], long-term toxicity [inflammations of the mucous membranes of the stomach], genetic damage [human blood cells], reproductive effects [reduction in the number of spermatozoa in mice; higher frequency of abnormal spermatozoa in rabbits], and carcinogenicity [higher frequency of liver tumors in male mice and thyroid cancer in female mice] [93].

The author [94] cites many positive results for the mutagenicity of glyphosate for a variety of test systems [e.g. *Salmonella typhimurium* – reverse mutation test, *Drosophila melanogaster* - induced sex-related lethal recessive mutations, and chromosomal aberrations in *Allium cepa* and cultures of human lymphocytes].

The most popular commercial product based on glyphosate is Roundup®. Its active ingredient is the 48% acid equivalent of the isopropylamine salt of N-[phosphonomethyl] glycine [C₃H₈NO₅P; Monsanto Agricultural Co, St. Louis, MO, USA]. Roundup is a broad-spectrum, nonselective, postemergent herbicide that is used to kill unwanted plants in a wide variety of agricultural, lawn and garden, aquatic, and forestry situations. Despite its long and extensive use, the ecotoxicological data for Roundup are scarce.

A study by [95] evaluated the genotoxic potential of Roundup® in blood cells of the European eel [*Anguilla anguilla*]. In a bioassay, they subjected the fish to realistic exposure concentrations of 58 and 116 µg/L for 1-3 days, and also addressed the possible association with oxidative stress. Comet and erythrocytes' nuclear abnormalities assays were used as genotoxic end points, reflecting different types of genetic damage. The authors showed higher rates of DNA damage in the contaminated fish than in the control group after 3 days of exposure [the same result was obtained in the Piscine Micronucleus Test]. The biochemical markers were assessed through enzymatic [catalase, glutathione-S-transferase, glutathione peroxidase and glutathione reductase] and non-enzymatic [total glutathione content] antioxidants, as well as by lipid peroxidation [LPO] measurements. Antioxidant defenses were unresponsive to Roundup. LPO levels increased only for the high concentration after the first day of exposure, indicating that oxidative stress in blood caused by this agrochemical was not severe. Overall results suggested that both DNA damaging effects induced by Roundup are not directly related with an increased pro-oxidant state.

Another study [96] showed different results. These authors evaluated the effects of Roundup Transorb® [RDT] on the Neotropical fish *Prochilodus lineatus*. Juvenile fish were acutely exposed [6, 24 and 96 h] to 1 mg/L of RDT, 5 mg/L of RDT, or only water [control]. They performed antioxidant analysis in the liver and acetylcholinesterase [AChE] determination in brain and muscle. After 6 h of exposure fish showed a transient reduction in superoxide dismutase and catalase activity. RDT also inhibited glutathione-S-transferase after 6 and 24 h of exposure. The reduction in these enzymes is probably related to the occurrence of lipid peroxidation [LPO] in fish exposed to the herbicide for 6 h. LPO returned to control levels after 24 and 96 h exposure to RDT, when fish showed an increased activity of glutathione peroxidase. The content of reduced glutathione also increased after 96 h exposure. Thus, after 24 and 96 h the antioxidant defenses were apparently enough to combat ROS, preventing the occurrence of oxidative damage. The exposure to RDT for 96 h led to an inhibition of AChE in brain and muscle at rates, which may not be considered a life-threatening situation.

The contradictory results of these studies warrant closer inspection. First, the concentration used in [96] was up to 86 times higher than the one used by [95]. Some studies show that biomarker responses are dose-dependent [19]. Second, the sensitivity of the fish must be taken into account. Not all fish have the same response to the same contaminant. The exposure time to the contaminant is also a factor that can be responsible for the differences in the results. Finally, the products used had different commercial names and different surfactants in their composition. Virtually all pesticides have other ingredients other than the *active* one, which actually has the exterminating action. Such ingredients are mistakenly

called *inert*. Their purpose is to facilitate the use of the product or to make it more efficient. Usually the inert compounds are not identified in the pesticide's label. In the case of glyphosate-based products, many "inert" ingredients were identified [93]. Differences in the test-organisms' responses to glyphosate and to Roundup, its commercial formula, can be attributed to the toxicity of different compounds and surfactants in the commercial formula. Research has revealed that Roundup can be up to 30 times more toxic to fish than the pure glyphosate, due to the so-called *inert* compounds in the formula [94].

Some studies report pathological damage in fish exposed to glyphosate. The author [97] exposed *Oreochromis niloticus* to sub-lethal concentrations [5 and 15 mg/L] of Roundup for 3 months, and the organs exhibited varying degrees of histopathological change. In the gills, filament cell proliferation, lamellar cell hyperplasia, lamellar fusion, epithelial lifting, and aneurysm were observed. In the liver, vacuolation of hepatocytes and nuclear pyknosis occurred. Kidney lesions consisted of dilation of Bowman's space and accumulation of hyaline droplets in the tubular epithelial cells. The results indicated that long-term exposure to glyphosate at sub-lethal concentrations had adverse effects stemming from histopathological and biochemical alterations in the fish. [98] has exposed *Cyprinus carpio* to immersion in Roundup [205 mg of glyphosate/L and 410 mg of glyphosate/L] in concentrations of 40 to 20-fold lower than those used in practice. Electron microscopy revealed that Roundup caused appearance of myelin-like structures in carp hepatocytes, swelling of mitochondria and disappearance of the internal mitochondrial membrane at both exposure doses. In this case, both studies, even though with different concentrations and species, confirmed that glyphosate can cause damages to fish tissues.

A study with the neotropical fish *Corydoras paleatus* contaminated with 3.20 µg/L glyphosate [6.67 µg/L Roundup®] showed that this pesticide might have genotoxic effects even at very small concentrations [99]. In this work, we performed PMT and Comet Assays with blood and liver cells, after the fish had been exposed to herbicide for 3, 6 and 9 d. A similar study [100], evaluated the sublethal effects of Roundup on the fish *Astyanax sp.* for 4 days. They tested two concentrations of Roundup: 3 µL/L and 6 µL/L. The PMT outcome was that only the highest dose showed any difference in response compared to the control group. Both works used the same commercial product, tested similar doses, and had similar responses, even though they were conducted with different fish.

The study [101] observed that Roundup® could affect cellular function [e.g., DNA] and that Roundup® and several glyphosate-based products interfered with cell-cycle regulation. In this work, the dose-response curves of the formulation products indicated a threshold for cell cycle induction even at very small concentrations, in agreement with other studies cited above. Failure in the cell cycle checkpoints leads to genomic instability and subsequent development of cancers from the affected cell [102,103]. Several lines of evidence have shown the highly conserved molecular basis of the cell cycle, from simple unicellular eucaryotes such as yeast to complex metazoans such as fishes or humans [104].

As discussed in the first pages of this chapter, a substance is considered harmful when it is detected in the environment at a higher concentration than it would *normally* occur. But

what is the normal level for each substance? For many synthetic organic chemicals, such as pesticides, the answer is quite simple – no detectable level is normal because these compounds do not exist in the nature unless they are introduced by humans [8]. However, considering the current worldwide dependence on pesticides, it is impossible to avoid their entering natural environments, reaching animals, contaminating our food supplies and drinking water, etc. For this reason, countries try to establish a maximum tolerance limit for each pesticide in each component of the environment. One of the lowest limits is the one established by the European Union legislation, which is 0.10 µg/L [or 0.0001 mg/L] for all pesticides [individually] in water designated for human consumption [105]. Many studies have shown that this limit is safe [106]. In Brazil, Ministry of Health law 518 establishes the limits of some agrochemicals in drinking water, such as atrazine [0.002 mg/L or 2 µg/L], 2,4 D [0.03 mg/L], DDT [0.002 mg/L], Endosulfan [0.02 mg/L] and glyphosate [0.5 mg/L][107].

In 1974, the US Congress passed the Safe Drinking Water Act. This law requires the US Environmental Protection Agency [EPA] to determine the level of contaminants in drinking water at which no adverse health effects are likely to occur. These non-enforceable health goals, based solely on possible health risks and exposure over a lifetime with an adequate factor of safety, are called maximum contaminant level goals [MCLG]. Maximum contaminant levels [MCLs] are set as close to the health goals as possible, considering costs, benefits, and the ability of public water systems to detect and remove contaminants using suitable treatment technologies. The MCLG for glyphosate is 0.7 mg/L, or 700 ppb. EPA has set an enforceable MCL regulation for glyphosate at 0.7 mg/L, or 700 ppb. The MCLG for 2,4-D is 0.07 mg/L, or 70 ppb. For atrazine, the MCLG is 0.003mg/L; and for PCBs [Polychlorinated biphenyls] the MCLG is zero, and the MCL is 0.0005mg/L [108].

Canada has the Guidelines for Canadian Drinking Water Quality, which are intended to protect freshwater and marine life from anthropogenic stressors such as chemical inputs or changes in physical components. In this, the Maximum Acceptable Concentration [MAC] for atrazine and its metabolites is 0.005 mg/L; for 2,4-D is 0.1 mg/L, and for glyphosate the MAC is 0.28 mg/L [109].

6. Conclusions

In this chapter, we make explanations about some pesticides, and the effects of these on fishes, in field or laboratory assays. In addition to the pesticides cited above, many others are spread daily in the environment. However, little is known about the individual or synergistic effects that these products may have at the various levels of biological systems [in the short or long run]. Thus, many efforts have been made to explore to the deleterious effects of pesticides on non-target species, but there is still a lot to be done. These efforts are of great importance in understanding the impacts of pesticides in organisms and in the environment as well as in establishing of safe limits on the use of these products in the environment.

Author details

Nédia de Castilhos Ghisi

Programa de Pós-Graduação em Ecologia de Ambientes Aquáticos Continentais (PEA), Universidade Estadual de Maringá, Paraná, Brazil

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