University of Southampton

Faculty of Environmental and Life Sciences

School of Biological Sciences

Risk assessment and risk management options for neonicotinoids

Volume 1 of 1

by

Brekhna Faheem

Thesis for the degree of Doctor of Philosophy

October 2019

University of Southampton

Abstract

Faculty of Environmental and Life Sciences
School of Biological Sciences

Thesis for the degree of Doctor of Philosophy

Risk assessment and risk management options for neonicotinoids

by
Brekhna Faheem

Organisms in the environment are exposed to a cocktail of chemicals that may be detrimental to their health. The risk assessments of pesticides is an integral part of the decision-making process which usually evaluate the effects per individual pesticide, rather than considering their effects when combined. It is only recently that the interactions between agrochemicals have been considered, predominantly at ecologically relevant levels. This is an important step towards understanding the impact of these chemical combinations on the environment. A more streamlined investigation is still required to provide scientifically factual data on improved use of pesticides that can help control pests proficiently as well as contribute towards effective decision-making. The aim of this study was, therefore, to address the risk assessment process of pesticides in mixtures on target and non-target organisms to understand how these risks could be managed in the future. Lethal and sublethal endpoints were assessed for acetamiprid and thiacloprid neonicotinoid insecticides, and tebuconazole fungicide. *Chrysoperla carnea*, was taken as a model non-target insect and *Plutella xylostella*, as a model target insect. In *Plutella xylostella*, the lethal toxicity of pesticides was age dependent with younger instars being more susceptible and their mortality rate increased with increasing the dose of pesticides both individually and in mixtures. Furthermore, the toxicity of mixtures of acetamiprid and tebuconazole was greater for *Plutella xylostella* in comparison with *Chrysoperla carnea*. Low LC_{50} values of acetamiprid for *Plutella xylostella* indicated its high toxicity to target relative to *Chrysoperla carnea*. Tebuconazole however, was found to be highly toxic to both insects' larvae. Moreover, for *Chrysoperla carnea*, lethal toxicity of thiacloprid and thiacloprid-tebuconazole mixtures was not significantly different. Sublethal endpoints such as the avoidance rate of *Plutella xylostella* larvae was affected by higher concentrations of pesticides because it declined due to a higher mortality rate at increased concentrations and remained higher at lower concentrations. In *Chrysoperla carnea*, it increased with the increasing concentrations of the pesticides treatments. Speed of *Plutella xylostella* declined and number of stationary periods increased with rise in concentrations of acetamiprid, tebuconazole and acetamiprid – tebuconazole mixtures whereas, only the feeding rate declined with increasing concentrations of tebuconazole and acetamiprid – tebuconazole mixtures. In *Chrysoperla carnea*, only its feeding rate declined with exposure to tebuconazole and thiacloprid – tebuconazole mixtures. Exposure time to these pesticides and mixtures mostly affected the lethal and sublethal parameters of *Plutella xylostella*. The maximum difference (53.3%) in mortality rate existed between *Chrysoperla carnea* and second instar larvae of *Plutella xylostella* in 24 h of exposure to mix3 (0.5 mlL^{-1} tebuconazole + 0.25 gL^{-1} acetamiprid). However, this difference declined to 45% over 48 h due to a relative increase in the mortality rate of *Chrysoperla carnea* larvae. Based on these findings, recommendations on optimizing the use of pesticides mixtures have been outlined in this study. This study highlights the importance of timing the spray mixtures accurately to optimise their impact on target insects while maximizing the conservation of non-target insects.

Table of Contents

Table of Tables

Table of Figures

Dedication

I dedicate this PhD thesis to my three years old son whose presence around me has been a source of positive energies and motivation for me.

Research Thesis: Declaration of Authorship

Print name: Brekhna Faheem

Title of thesis: Risk assessment and risk management options for neonicotinoids

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

Signature: Date: 31 October 2019

Acknowledgements

This PhD progression has been a life-changing experience for me, which would not have been possible without the help and support of many people.

My sincere gratitude goes to my supervisors, Prof. Guy M. Poppy and Prof. Philip L. Newland, for their motivation and encouragement through my entire PhD study. Their immense knowledge provided me with the opportunity to make the best use of time and facilities. Their continuous guidance and patience truly have enabled me to write and complete this thesis on time.

I also gratefully acknowledge Abdul Wali Khan University Mardan, Pakistan for funding my PhD. I am much thankful to the whole HRD, AWKUM, staff for their help and support on financial part of my PhD program.

Thanks to FELS technical staff whose support during my laboratory work was also one of the key factors that enabled me to conduct my laboratory tasks smoothly and efficiently.

I would like to extend my thanks to my friends. Their enormous help and support in my work mean a lot to me.

And lastly, my heartfelt gratitude to my family, parents and husband who always believed in my potentials and encouraged me to follow and live my dreams. Their love and encouragement during these challenging years have given me tremendous strength to complete and finish what I started.

Definitions and Abbreviations

Acet - Acetamiprid
DBM – Diamondback Moth
DMI – Demethylation Inhibitor
EBI – Ergosterol Biosynthesis inhibitor
IPM – Integrated Pest Management
Mix – Mixtures
MRA – Field Recommended Application rate
PPP – Plant Protection Products
SPn – Number of stationary periods
SBI – Sterol Biosynthesis Inhibitor
Teb – Tebuconazole

Chapter 1 Introduction

The use of pesticides, predominantly the insecticides, is arguably the most influential pest management tool around the globe. Many agrochemicals are used on crops in agriculture, and each of these chemicals has been subject to a risk assessment before being put on the market for use. Surprisingly, evaluating the effects of agrochemicals when applied in mixtures is not the part of conventional risk assessment. Moreover, studies related to the non-target impacts of mixture applications are limited. This subject has only recently gained attention; little research has been conducted to investigate the impact of pesticide mixtures on behaviour, survival and population of various non-target species. Some recent studies have reported the interactions between pesticides such as fungicides and neonicotinoids (Thompson et al., 2014; Goulson et al., 2015; Tomé et al., 2017). However, the next objective should be to optimize the use of these mixture so as to have maximum pest control while minimally affecting the non-target beneficial insects. This objective forms the basis of this study.

Pesticide use remains a contentious subject which occupies the pole position in regulatory processes in majority of countries (Guedes et al., 2014). Studies over many years have revealed that the major issue related to pesticides use is the risk that they pose to non-targets by their direct exposure. Recently however, there has been an increased controversy surrounding the use of neonicotinoid insecticides and the deteriorating health of the non-target populations of insects, especially natural pollinators like bumble bees and honey bees (Pisa et al., 2015; Sanchez-Bayo and Goka, 2014). Moreover, their toxicity to non-targets may increase up to a factor of as much as 1000 in the presence of other agrochemicals (Iwasa et al., 2004), especially fungicides which are applied widely to the crops (Johnson, 2015). The presence of these complex mixtures containing a wide array of pesticides, especially the neonicotinoids and fungicides has been confirmed (David et al., 2016; Krupke et al., 2012; Goulson, 2013b). However, understanding the effect of neonicotinoids-fungicides mixtures is still challenging and need further exploration. Neonicotinoid insecticides target the nicotinic acetylcholine receptors (nAChRs) in central nervous system (Taillebois et al., 2018). They bind efficiently to the insect's nAChRs in post-synaptic neuron thus acting as agonist, interfere with the signals sent by acetylcholine neurotransmitter. This produce a continuous activation of the receptors that leads to paralysis and eventually the death of the insect (Gibbons et al., 2015).

Scientists and stakeholders around the world have different opinions regarding the use of neonicotinoids. This led to the European Commission imposing a moratorium on the practices of

using thiamethoxam, clothianidin and imidacloprid as seed dressings on plants attractive to bees in 2013 (Carreck and Ratnieks, 2014). The major drivers for this step were the studies like that of Whitehorn et al. (2012) that showed acute and chronic sublethal effects of these neonicotinoids on bees (Ratnieks and Carreck, 2010). Neonicotinoids alone, however, cannot be held solely responsible for the decline of these beneficial insects, because many different chemicals like fungicides and herbicides might be producing various chemical effects when come together. Some of these chemicals are well known for their synergies with neonicotinoids (David et al., 2016; Schmuck et al., 2003). The regulatory risk assessment procedures on the other hand, typically involve the exposure of organisms to only a single pesticide (active ingredient and formulation) for a shorter period (EFSA, 2013b). Therefore, there is a need for further investigation and to carry out a risk assessment for pesticides when they are used in mixtures.

1.1 Pesticides use as mixtures

A pesticides mixture is defined as when two or more individual compounds are blended and applied simultaneously. These mixtures involve the concurrent exposure of the pest population to different pesticides. Relative to the individual applications, the combinations may show more pronounced effects at specific life stages such as ova, nymphs, larvae or fully grown adults (Moniski, 1998). The effectiveness may vary however, depending on the formulation and rate at which pesticides are combined (Das, 2014).

The practice of using pesticides mixtures is widespread throughout the world, partly because the combined application of pesticides may be an effective measure to cope with various pest populations present in the crop at a particular instant of time. In this way, more than one pest species can be controlled with a lower quantity of pesticides and at a lower cost, as well as saving time and labour by reducing the number of sprays farmers have to apply (Ahmad et al., 2009; Das, 2014). One common practice in agriculture is the use of binary mixtures; however, it is also likely to pool several pesticides into a single mix in a situation where multiple pests need to be targeted in a single crop.

A mixture may augment the suppression of pest population through either synergism between the component pesticides or via additive effects. Synergistic interaction involves the enhancement of the effect of given pesticides (or sometimes, other synergist compounds) after fetching them together in a mixture. In this case, the component compounds are usually relatively less toxic (Ahmad et al., 2009). In additive action, the joint effect of two compounds is equal to the sum total effect of components alone. Alternatively, there may be independent or

antagonistic action. The former means the component chemicals work independently, in the later case, one component of the mixture interferes with the other by reducing its activity (Parmar and Tomar, 2004; Lash et al., 2007; Das, 2014).

Mixtures of pesticides have many advantages however, in the last two decades, interest over chemical mixes has increased, the major concern of which is their impact on non-target entities (Monosson, 2005; Carpenter et al., 2002; Botias et al., 2017). There have been studies on synergistic toxicity of some mixtures of insecticides and other pesticides. For example, the toxicity of pyrethroid insecticides cypermethrin and deltamethrin was significantly enhanced against *Spodoptera litura*, when combined with the organophosphates chlorpyrifos and profenofos (Ahmad et al., 2009). In addition, various studies have demonstrated the enhanced toxicity of some fungicides and insecticides mixtures for honey bees or their larvae as a result of synergistic interaction between these pesticides (Chauzat et al., 2006; Williamson and Wright, 2013). The level of synergism may vary subject to the combination of different pesticides (Sanchez-Bayo and Goka, 2014). For instance, the toxic effect of some neonicotinoids, multiply by a factor of as much as 1000 with concurrent application of some fungicides like demethylation inhibitors (DMI) or ergosterol biosynthesis inhibitors (EBI) (Iwasa et al., 2004; Schmuck et al., 2003). These types of fungicides exert their effect by obstructing Cytochrome P450 (CYTP450) dependent ergosterol biosynthesis in fungal cell and are also believed to disrupt Cytochrome P450 enzymes in insects that are essential to detoxify the insecticides, including neonicotinoids (Schmuck et al., 2003). Another such example is an increase in the degree of toxicity of pyrithroid to honey bees in the presence of fungicides belonging to EBI group, responsible for the inhibition of microsomal monooxygenases (P450s) which is necessary for oxidative detoxification of insecticides (Johnson et al., 2012; Pilling and Jepson, 1993).

Likewise, seeds treated with systemic insecticides give rise to their residues in pollens, nectar and the exudate produced in the process of guttation by those plants. When combined with other pesticides applied as spray, these residues result in chemical cocktails that have the potential to adversely affect the natural pollinators which come in contact with them while foraging on these plants (Iwasa et al., 2004). There is concrete evidence that bees are experiencing prolonged exposure to many cocktails of pesticides during the course of their lives (Paradis et al., 2014; Sanchez-Bayo and Goka, 2014). Despite strong evidence from recent research (Thompson et al., 2014; David et al., 2016; Zhu et al., 2014) the risks associated with the mixtures of different agrochemicals are not addressed by current regulatory authorities (Thompson et al., 2014; Williamson and Wright, 2013).

To determine the specific gaps in our understanding, the primary emphasis of this study is to analyse the toxicity of neonicotinoids in the presence of fungicides, on both the target insect *Plutella xylostella* (Diamondback moth) and the non-target *Chrysoperla carnea* (green lacewing).

1.2 Role of neonicotinoids in agriculture

Among the vast majority of pesticides which have been used for many decades, neonicotinoids is a moderately new group of broad-spectrum insecticides brought into the market in the early 1990s (Elbert et al., 2008). These neurotoxic, nicotine mimics, principally interfere with nicotinic acetylcholine receptors (nAChR) in the central nervous system (Tomizawa and Casida, 2005). Like nicotine, neonicotinoids bind to these target receptors on post-synaptic membrane in insect nerve cells. The binding being irreversible, leads to an excessively high influx of K^+, Na^+ and Ca^{2+} through the cell membranes. Consequently, a prolonged action potential is build up which causes over-excitation and paralysis that ultimately ends in death of the organism (Millar and Denholm, 2007; Goulson, 2013b). Neonicotinoids are extensively utilized as systemic insecticides in plants to kill pest insects that are likely to cause damage to crops. Besides, some of these compounds are utilized as a part of veterinary pharmaceuticals control of parasites such as worms, ticks and fleas in pets and against non-agricultural pests (Simon-Delso et al., 2015).

Among all the currently available neonicotinoids, imidacloprid was the first that was launched commercially by Bayer Crop Sciences in 1991 (Elbert et al., 2008). Following its outstanding success and efficacy, accompanied by its increased market demand, the agrochemical companies extended their efforts to explore and develop similar plant protection products. As a result, six additional neonicotinoids were brought onto the market for commercial use within the following decade (Reynard, 2012). These included acetamiprid by Nippon Soda (1995), nitenpyram by Sumitomo Chemical Takeda Agro Company in 1995, thiamethoxam by Syngenta in 1998, thiacloprid by Bayer CropSciences in 2000, clothianidin from Sumitomo Chemical Takeda Agro Company, Bayer CropScience in 2001 and dinotefuran by Mitsui Chemicals in 2002 (Elbert et al., 2008).

Relatively small molecules with good water solubility, neonicotinoids are readily taken up by the plants (Krupke et al., 2012; Richmond and Patton, 2014). Once absorbed, they are systemically incorporated in plant foliage and vascular tissues which enables them to act vigorously and protect the plants against the herbivorous pests (Wood and Goulson, 2017). In addition, neonicotinoids have demonstrated a higher affinity for insect nAChRs than those of mammals or other vertebrates. Therefore they are relatively less toxic to vertebrates (EPA, 2003; Gibbons et

al., 2015). Based on their efficacy and systemic activity against many insect pests and relatively low risk of adverse effects to vertebrates, they are at present certified for controlling and managing insect pests in over 120 countries around the world on a wide variety of vegetables, fruits and grain and oilseed rapes (Reynard, 2012). They are widely used in agriculture, to the extent that by the end of 2008, neonicotinoids represented one fourth of the world's insecticide trade (Jeschke et al., 2011), whilst the proportion is still expanding (Simon-Delso et al., 2015). By 2010, it reached up to 27 % of global insecticide usage (Casida and Durkin, 2013). They are applied using different methods such as seed dressing, foliar spray, stem injections and soil drench application on roots, however it is estimated that nearly 80% of neonicotinoids application is carried out via seed dressing (Jeschke et al., 2011).

Whilst neonicotinoids have many advantages, they are responsible for environmental contamination which either directly or indirectly affects the non-target fauna (Pisa et al., 2015). Ghananand et al. (2011) conducted a higher tier study to assess the impact of insecticides, bio-pesticides and botanical extracts on a population of braconid wasps, coccinellid beetles and predatory spiders, the common natural enemies in aubergine ecosystems. Their results revealed that cypermethrin and imidacloprid were relatively more toxic to natural predators compared to neem based botanical extracts and pesticides of biological origin.

Kumar et al. (2012), compared the effects of various pesticides on the pests and arthropod predators in cotton fields. They observed this using two different application methods; foliar spray and stem application. Their results showed that when applied as foliar spray, pesticides were effective against different classes of pests of the cotton crop. However, the five neonicotinoids, imidacloprid, acetamiprid, clothianidin, thiacloprid and thiamethoxam were also identified as extremely noxious to natural predators and parasitoids of the cotton crop pests compared to fipronil, spirotetramat and buprofezin. By contrast, a study by Carvalho et al. (2010) on residual and sublethal effects of different classes of insecticides on *Trichogramma pretiosum* showed that imidacloprid, acetamiprid, triflumuron and lufenuron were found harmless and recommended as a part of integrated pest management programme. Similarly, Ahmed et al. (2014) found that nitenpyram, imidacloprid and thiacloprid were effective against sucking pests and safer to naturally occurring enemies and predators when contrasted with other traditionally used insecticides.

Neonicotinoids are of significant concern due to their low LD_{50} and high oral toxicity that range between 4 to 5 ng/honey bee (Suchail et al., 2000). Bees pollinate more than 66% of the total crop cover in the world, and therefore, their role as natural pollinators is crucial in maintaining a

healthy ecosystem (Blacquiere et al., 2012; Kremen et al., 2002). Studies on honey bees food stores have demonstrated that they face chronic exposure to neonicotinoids and their metabolites (an average range of 1–100 ppb) (Bonmatin et al., 2015). Even with concentrations lower than 0.25 ppb, long-term chronic exposure to these compounds increase the mortality rate of honey bees, especially when feeding on contaminated food in their overwintering period (Rondeau et al., 2014). Sublethal effects of neonicotinoids in honey bees and bumble bees include reduced learning, homing and foraging capability, each of which is vital for bees to survive and exist (Yang et al., 2008). By contrast, some studies are in disagreement with the above debate. As an example, a field realistic study on honey bees conducted by Schmuck et al. (2001), showed no significant mortality of worker bees when they were fed on imidacloprid contaminated sunflower nectar in hives (2.0 to 20µg/kg) for 39 days. Cresswell (2011), and Faucon et al. (2005), reported similar results. In both of these studies, the exposure to food contaminated with imidacloprid had no significant effect on the mortality rate of worker bees.

A recent study by Moffat et al. (2016) exploring the relative toxicity of three types of neonicotinoids; thiamethoxam, clothianidin and imidacloprid (disqualified by EU in 2013) on bumble bees (*Bombus terrestris audax)*, suggested that not all the banned neonicotinoids were equally harmful to bees. They found that individually, imidacloprid induced immobility in bees while thiamethoxam altered their sex ratio within the colony, and together they were responsible for a decline in colony strength. By contrast, clothianidin increased the queen production in the colony and was not found harmful to bees at field-relevant levels. From their findings, they suggested that the risks accompanying the use of neonicotinoids should be considered individually and separately to different target species, because a compound may show different behaviour to a diverse group of insects.

1.3 Analysing ecological risk: risk assessment and risk management

The ecological risk analysis process encompasses two interdependent elements that are 1) risk assessment, and 2) risk management. This collective set of analysis aims to evaluate information on the impact of a newly developed substance (different types of agrochemicals, GM crops etc.) on various components of ecosystems and how to manage any risks associated with them. The decision-making process about the situation follows the integration of knowledge from the two. More precisely, risk assessment forms the scientific part which is more of objective nature while risk management is the political and subjective fraction of the process (van Leeuwen and Vermeire, 2007).

Risk assessment provides scientific data on potential risks in the food chain (EFSA, 2014). It describes the type, characteristics and the degree of harm to humans or the environment if exposed to a hazard (van Leeuwen and Vermeire, 2007). In other words, risk assessment focuses on the adverse ecological effects of one or more stressors introduced into the environment through human activities on anthropogenic communities and ecosystems. The stressors could be a new chemical, an invasive species, disease or climate and land-use changes (EPA, 1992).

A risk assessment is generally comprised of three main phases. The first phase is 'Problem formulation', which involves the identification of potential harm associated with a particular stressor and formulating a plan of study accordingly. This is usually done by integrating the information already available from the previous studies regarding the stressors, their receptors and effects on ecosystems. The second phase is the 'Analysis phase' during which data obtained from exposure to, and effect of, a stressor is evaluated. In the third phase, 'Risk characterization', information gained from the exposure and response, is collated to describe and categorize the risks associated with a particular set of stressors (EPA, 1998). Chapter 2, 3 and 4 of this thesis have their focus on risk assessment part of the study.

Risk management, on the other hand, entails choosing an action pathway and taking effective measures in response to an identified risk. Moreover, along with the results fetched by risk assessment, risk managers contemplate many other factors, such as political, social, economic, constitutional and statutory issues, before making a decisions on ecological risks, (EFSA, 2014). For instance, the risk assessment results may be employed to analyse the ecological cost-benefit ratio of stressors, which may involve the transformation of tools identified via risk assessment goals, to their monetary worth. On the other hand, risk managers may equally think about methodologies for decreasing dangers, for example, hazard alleviation alternatives or substitutions depending on relative hazard correlations. For example, the risk assuagement methods, like decreasing the pesticides application rates, can be utilised to lower down their exposure and associated risks. Furthermore, during the registration of a newly developed pesticide, the comparison of their risks to those already in the market can result in an overall lower risk. Finally, the general public attitude, as well as political requirements are considered and incorporated in the decision. Jointly, all these elements may render high dangers adequate or extremely low risk as suitable (EPA, 1998).

van Leeuwen and Vermeire (2007) have described the difference as *"Risk assessors ask 'How risky is this situation?' and risk managers then ask 'What are we willing to accept?' and 'What shall we do about it?"*.

The whole process can be explained with an example. As a risk assessor, the European Food Safety Authority (EFSA), evaluates the safety of applying a chemical or a GMO in the environment by collecting and analysing the existing data and thus provide scientific advice to support decision-making. Their role ends at this point and the process taken over by risk managers such as the European Commission (EC) and the member countries, who then decide whether to authorise the use of the substance. Chapter 5 of this thesis makes recommendations on the risk management options of pesticides mixtures.

All of these processes assist regulatory authorities in making effective decisions to regulate pesticides, industrial chemicals, high-risk wastes or any other non-chemical stressor that may be affecting the ecosystem (EPA, 1998).

1.4 Tiered approach used in ecological risk assessment

Regulation of plant protection products (PPPs) requires the regulatory bodies to evaluate their potential risks in an ecological regime before they are registered and placed in the market. Typically these risk assessments are carried out by adopting tier testing approach. The first tier also known as the lower tier or screening level test, involve the utilization of a limited volume of data and simple models which often requires low cost. Therefore, it is regarded as a relatively conservative tool for analysis. It is however, valuable because it serves to identify the pesticides that do not require any further assessment or those with potential ecological concerns and need further elaborative investigation. This is followed by producing more advanced level information via more progressive techniques in higher tier evaluations (second and third tier testing) (Levine et al., 2019). For instance, in tier 2 or second tier assessment a more in-depth evaluation of pesticides is carried out in semi-field conditions using the model organism or populations identified to be at risk in screening level testing. However, if tier 2 assessment is not sufficient to deliver the required level of information, the process is escalated to the third tier or tier 3 evaluation which is a more intricate level, examining the effects of a stressor in real field scenario (Money, 2018). As this involves the exposure of organisms to more realistic field conditions, the data obtained in higher tier risk assessments is environmentally more relevant and offers reduced ambiguity. Besides, the outcomes of higher tier tests do not necessarily refute the inferred risks from screening level tests but mostly provide further clarity of information (Levine et al., 2019).

In an ideal world, information on the exposure of almost all organisms of ecological interest and their populations, to every stressor in the environment should exist. This goal however, is not possible except for a few substance of high ecological concern. Therefore, the tier level approach used in ecological risk assessment is an effective tool for fetching meaningful and important information in an efficient and cost-effective manner that can help in making informed decisions (Money, 2018; Meek et al., 2011). In this study, the first tier risk assessment of pesticides in laboratory has been carried out to analyse their effects on target and non-target insects. Lower tier assessment is more profitable because it can serve to determine whether higher tier investigation is required at all. In this way, saving time, money and labour otherwise required for conducting higher tier evaluation. Therefore, it is the most popular and relatively more adopted tool in various scientific examinations (Rohr et al., 2016).

1.5 Risk assessment of pesticides mixtures

The subject of agrochemical mixtures and their effects on non-target insects in various agricultural systems has now gained considerable attention (David et al., 2015; Tian et al., 2018). The application of chemicals in mixtures is common, and many recent studies have highlighted the effects of chemical combinations on non-target fauna in various taxonomic groups. However, chemical risk assessments are still carried out element by element, which often leads to risk underestimations (Backhaus and Faust, 2012). Therefore, risk assessments of chemical mixtures are now indispensable in acquiring factual risk assessment methodology for them (Tian et al., 2018).

Recent studies have suggested the Concentration Addition (CA) also known as Dose Addition (DA) approach for mixture risk assessment. This approach is based on the idea that the combined risk of a chemical mixture can be the outcome of adding up the risks associated with the individual components of that mixture (Bundschuh et al., 2014). Another approach is the Independent Action (IA) model which aims to assess the mixtures that have components with different modes of action, each of which acts independently (Backhaus and Faust, 2012). This is different from concentration addition model which relies on the chemical combinations, the components of which have analogous mechanism of action. Both of these concepts assume that the constituents of a mixture do not interact with each other and thus have no influence on each other's absorption, distribution and breakdown. These models however, lose their strength when mixtures with non-CA effects need to be assessed. For example, the mixtures giving rise to either more than additive/synergistic effect or lower than additive/antagonistic effect (Tian et al., 2018).

For example, the synergistic toxicity of neonicotinoids in the presence of triazole fungicides that have been documented in many previous studies. Therefore, it is still not clear how the risks associated with different types of mixtures can be evaluated.

1.6 Intensification of chemical farming and the driving forces behind it

Today global agricultural system is tending to adopt intensive farming practices, and pesticides make a significant contribution to this intensive agricultural system. The prime goal is to obtain maximum yield from relatively limited land area available. This is achieved by high proportion input in terms of capital, labour, chemicals fertilizers and pesticides, fossil fuel and modern heavy machinery (Jackson et al., 2007).

The custom of excessive pesticides application however, has been detrimental to overall environmental health, affecting both its biotic and abiotic components. In the agricultural system, pollinators and natural enemies of pests are of vital concern (Whitehorn et al., 2012; Chauzat et al., 2006; David et al., 2016). Numerous reports have documented the harmful effects of pesticides on insects, with a considerable focus on the direct acute and chronic sublethal toxicities (Laycock et al., 2012; Brittain et al., 2010). A big chunk of these studies focus on honey bees and bumble bees (Pisa et al., 2015), due to their role as natural pollinators, which is crucial in maintaining a healthy ecosystem. For instance, in the USA, pollinator-dependent crops in 2010 were estimated to worth up to $30 billion annually (Stevens and Jenkins, 2014). The Almond industry of California, which generates an annual revenue of $2 billion, relies on approximately one million honey bees for cross-pollination (Ratnieks and Carreck, 2010). Nevertheless, since the late 1990s, there has been a marked reduction in the cost of pesticides relative to labour, fuel costs and the value of crops (Stevens and Jenkins, 2014). This has led to the current unjustifiably high levels of pesticides use to maintain the high yield, that is adversely affecting the non-target insects (Goulson, 2013b; Stevens and Jenkins, 2014). Goulson et al. (2015), in their review of causes and factors of the decline of honey bees, pointed out that there are several interacting factors causing a significant drop in honey bees population. These primarily include a shortage of floral diversity and abundance, increased pressure of parasitic and disease-causing pathogens and contact with large volumes of pesticides due to agricultural intensification.

The benefits of using these chemicals for agricultural purposes are undeniable. However, living organisms belonging to almost every taxonomic group are being adversely affected either via

direct or indirect exposure to most of these ecologically toxic substances (Millennium Ecosystem Assessment, 2005). Agricultural practices that involve the mass application of fertilizers and pesticides are the major aggravating factors in the degradation of soil quality because they increase both the nutrient content as well as the volume of chemical toxins in the soil (Udeigwe et al., 2015). From there, nutrients leach into ground and surface water, which in turn contaminates of aquatic habitats (Zhang et al., 2018). This contamination of aquatic ecosystems not only influences the fishery and aesthetic value of waterways but also make the water unfit for drinking that incur additional health and water purification cost (Cassman, 1999; Rother, 2008). Moreover, the rate of occurrence of soil-borne pests and disease-causing pathogens is much higher in agroecosystems compared to natural ecosystems. This may be due to the disturbance caused by extensive use of pesticides that leads to lowering down the biodiversity in farmlands rendering them vulnerable to the detrimental effects of pathogens (Brussaard, 1997; Gill and Garg, 2014). Millennium Ecosystem Assessment (2005) has also reported that natural ecological relationship between biotic and abiotic factors usually constrain the transmission and the subsequent dissemination of an infectious agent into the human body. This balance, is often altered by human-induced changes in the environment which leads to detrimental effects on non-targets, sometimes at population levels (Ciach and Peksa, 2019). McArt et al., (2017) investigated the factors contributing towards bumblebee decline, principally due to pathogen infestation and range contraction. From their study they revealed that the greater use of chlorothalonil, a broadspectrum fungicide, was the major cause of pathogen prevalence (*Nosema bombi*) in four declining species of bumblebees i.e *Bombus terricola*, *Bombus pensylvanicus*, *Bombus affinis*, and *Bombus occidentalis*. Moreover, the fungicide was also identified as the strongest factor contributing in range contraction in these bumblebee species.

In the due course of maximizing the food production driven by the motivational force of growing poverty and lack of food security, the long term environmental consequences are usually overlooked (Powlson et al., 2011). For a sustainable agricultural system, which is fundamental for the well-being of agriculture and society as a whole (Millennium Ecosystem Assessment, 2005), it is essential to avoid the practices that may lead to irreversible impairment in the environment (Powlson et al., 2011).

1.7 Use of pesticides as a part of sustainable intensification of agriculture

In a broader sense pesticide is a term that may refer to any substance, pure or blend used for eradicating, impeding, deterring or relieving a pest (Ragsdale, 2006). Pesticide is a wide term encompassing a variety of chemical compounds such as herbicides, fungicides, nematicides, molluscicides, insecticides and many others. Among these the introduction of synthetic insecticides organophosphates in 1960s, carbamates, pyrithroid, herbicides and fungicides in 1970s and 1980s and neonicotinoids, a more advanced and potent class of insecticides, in 1990s, considerably improved the pest management and agronomic productivity (Aktar et al., 2009). These pesticides may either act against a pest by completely killing them or interfering with various physiological processes (Ragsdale, 2006).

Sustainable intensification on the other hand, is a term brought very recently into discussions mainly concerning the future of agriculture and food security (Garnett and Godfray, 2012). It can be defined as a framework of productivity in which the percentage yield is enhanced without harming the environment and without extra land usage (Royal Society, 2009).

The ability of an ecosystem to provide its valuable services largely depends on the abundance and diversity of species. Practices which reduce or alter the species composition may diminish the ecosystem goods and services (Tilman et al., 2002). Whilst intensive farming has greatly increased food production on global scale in the past few years, this type of cropping system, chiefly relies on high yielding genotypes which in turn require intensive input of fossil fuel, irrigation water, synthetic fertilizers and pesticides for yield and pest management. The payoff in terms of damage caused to the environment and the subsequent loss of biodiversity, both in agroecosystems and wildlands has been high (Altieri, 2004; Jackson et al., 2007; Brush, 2008). Studies have shown a greater diversity and abundance of arthropods in chemical free areas relative to pesticides treated fields (Amalin et al., 2009). Fountain et al. (2007) showed a significant reduction in species diversity, abundance and uniformity of springtails and number of spiders in the plots treated with chlorpyrifos.

Theoretically, in an ideal world, the toxicity of pesticides should be confined to the target organism only. Moreover, they should be environmentally green and biodegradable (Rosell et al., 2008). Unfortunately, in practice this seldom occurs as most of the pesticides are rarely selective and therefore, cannot be regarded as completely harmless to non-target and beneficial organisms of an ecosystem (Carriger et al., 2006). Most of the pesticides used today are persistent in nature

as well as not biodegradable, so their use and abuse spark a big controversy (Aktar et al., 2009). In general, only about 0.1% of the total pesticides applied, reach and influence the target individuals while the rest remains as a major contaminant in the environment (Carriger et al., 2006). For instance, in woody plants neonicotinoid can persist upto as long as one year. Similarly, in soil the half lives of most neonicotinoids has been identified as greater than a thousand days which means the residues accumulate over time with their repetitive applications (Bonmatin et al., 2015).

The repetitive, indiscriminate use of these chemicals, not only make the pest species resistant and increase the incidents of pest resurgence, but also lead to their bioaccumulation in various trophic levels of a food chain (Tabashnik et al., 2009). This results in serious complications among various groups of non-target organism, such as the natural predators of pests, pollinators, aquatic organisms, birds and mammals, including humans (Mostafalou and Abdollahi, 2012; Aktar et al., 2009).

A significant concern over the toxicity of pesticides on non-target species has aroused, particularly in the recent decades which has led to many reports being complied (Desneux et al., 2007; Carreck and Ratnieks, 2014; Goulson et al., 2015). Natural enemies and predators play a vital role by keeping pest populations in check. The indiscriminate use of pesticides however, frequently results in high rates of mortality of natural predators and parasitoids which in turn exacerbate pest problems (Pelosi et al., 2014). Likewise, pesticides can influence the predator's general behaviour and their life-history parameters. Such as exposure to pesticides may alter the behaviour of non-target organisms, which subsequently can influence their growth, development and reproductive success. For instance, a study conducted by Evans et al. (2010) in eastern United States investigated the effect of glyphosate-based herbicides on the survival and activity of three predatory arthropods: the ground beetle (*Scarites quadriceps*), larger wolf spider, (*Hogna helluo*), the wolf spider (*Pardosa milvina*). Their results showed that exposure to these herbicides adversely affected their performance and long-term survival, which indirectly influenced the natural biological control mechanism of an agroecosystem. A similar study by Giglio et al. (2011) showed that dimethoate insecticide, which acts as cholinesterase inhibitor of nervous systems, was responsible for significant reductions in morphometric parameters such as body size as well as hematocyte count of the carabid beetle (*Pterostichus melas italicus*). As an important generalist predator of slugs, aphids, lepidopterans (caterpillars of moths) and dipteran (fly maggots), reduction in body size or haemocyte count of carabid beetles, may affect their predatory proficiency over long term, that may lead to an upsurge of the pest population.

Similarly, soil inhibiting fauna such as mites, springtails, nematodes, earthworms, micro-arthropods, insects, spiders and other minor invertebrates are also adversely affected by pesticides. These organisms essentially decompose the organic matter like leaves, manure and plant residues to humus for maintaining and improving soil structure and fertility, (Paoletti, 1999). Previous studies have outlined the evidence concerning the disadvantages associated with the pesticides use, particularly their wide scale chronic impact on natural pollinators (Sanchez-Bayo and Goka, 2014; Mullin et al., 2010; Krupke et al., 2012; Paradis et al., 2014; Bourguet and Guillemaud, 2016). To minimize these hazards, it is essential that they should be addressed adequately. Effective strategies and rigorous regulations for trained use of pesticides should be adopted in order to have more sustainable practices in agriculture and pest control (Powlson et al., 2011). Despite of the continued debate, pesticides are still widely used because of their availability at low prices thus being supportive to ensure the economic survival of farmers through minimal loss of crop yields. Moreover, at some point, the high cost of reversion to a previous control system may become impossible and the farmers will be thus 'locked' into unsustainable agricultural practices.

There are however, some other positive impacts of pesticides which are mostly overlooked. For instance, researchers and producers across the world are developing novel formulations to improve and enhance the quality and performance of pesticides (Rosell et al., 2008). Therefore, their use has been advantageous to a considerable extent as they have played a critical role in the achievement of the Green Revolution by increasing the overall agricultural output throughout the world (Dubey et al., 2010). Moreover, their contribution to safeguarding the crop yield against various pests has been momentous. About one fifth of the total produce of principal crops such as soybean, maize, cereals and many important vegetables and fruit is preserved by them (Oerke and Dehne, 2004). On the other hand, despite the use of pesticides, about 20 to 40% of global food crops are destroyed each year due to severe damage caused by some 30,000 weed species, 10,000 species of plant-eating insects, and 3000 species of nematodes (Gavin, 2015). This means food crops face severe threats for their survival (Pretty, 2008). If crop protection product were excluded from any agricultural system, the production loss would be intensified even further. Consequently, to compensate for these losses, the land use for agriculture would be extended at the cost of altering the natural habitats.

Likewise, the introduction of pesticides has brought a dramatic increase in productivity in many countries. In United Kingdom, for example, the production of wheat went up to 7.5 tons/ha from 2.5 tonne/ha between 1948 and 1997 (Austin, 1999). Similarly, in United States, maize production

improved from 30 to 100 bushels/acre during between 1920 and 1980. (Kucharik and Ramankutty, 2005). Larger yields in relation to the amount and quality of crop has not only improved the quality of life, but also reduced the pressure of cultivating spare land areas which in turn is advantageous for conserving biodiversity and the environment (McNeely and Scherr, 2003). Apart from raising revenue and improving the food quality, insecticides use has proven effective in controlling vector-borne diseases such as malaria, trypanosomiasis, (sleeping sickness), river blindness and a variety of severe illnesses which had been causing devastating effects on human health in the past (Cooper and Dobson, 2007). For example, Yadav et al. (2001), found that deltamethrin treated bed nets reduced the incidence of malaria by up to 59% in areas being investigated. Studies conducted by Townson et al. (2005), Lindblade et al. (2004), Curtis et al. (2003) and Kamuanga et al. (2001) have highlighted the significance and effectiveness of insecticides in controlling vectors, thereby minimizing the incidence of diseases caused by them. Furthermore, the use of herbicide is indispensable for clearing the unwanted vegetation from waterways, roads and railways to ensure smooth and safer transportation. These benefits are substantial and cannot be overlooked however, they could still be achieved by integrating a careful and rational use of pesticides with integrated pest management programs and other modern complementary technologies (Cooper and Dobson, 2007).

1.8 The insects

Insects comprise the major taxonomic group of Kingdom Animalia, which include approximately 80% of animal species, identified so far. Of these, some species are considered pest, either due to their direct pathogenic effect on man or by devastating the agricultural production. Insect control is therefore, one of the oldest and most central concern of the mankind (Sarwar and Salman, 2015; Khalid et al., 2015). Another category of insects is that of beneficial species, a major proportion of which helps protect crops from pests by acting as biological control agents (Carvalho, 2017).

1.8.1 *Plutella xylostella*

Plutella xylostella (Diamondback Moth or DBM), is a destructive pest that targets crucifers, sometime causing more than 90% of crop loss (Grzywacz et al., 2010; Machekano et al., 2017). It is cosmopolitan in distribution and can withstand a wide variety of climates, thus capable of establishing itself and causing severe economic losses worldwide (Jankowska and Wiech, 2006). Common belief is that it first originated in either South Africa or Mediterranean region. From

there, it migrated to various parts of the world and established itself successfully in Europe, Australia, Americas, Newzealand as well as Hawaiian regions Talekar and Shelton1993; Wei 2014). The evidence of its origination in Southern Africa is chiefly based on the occurance of a variety of its parasitoids and abundance of a great number of cruciferous plants which are native to that area. Its common natural enemies include *Cotesia plutella* and *Trichogramma chilonis*, which parasitse its eggs, *Diadegma insulare* that is a larval parasitoid while *Chrysoperla carnea* feeds on its eggs and larval stages (Sarfaraz etal., 2007).

The host range is restricted to crucifers only; however, they can attack every species of *Brassica* vegetable crops and weeds. The weedy species usually serve as essential hosts maintaining the DBM populations in the early season before the availability of cultivated plants. It is a highly dispersive insect and is often expected to be present in areas where it can successfully overwinter (Machekano et al., 2017). Nguyen et al., (2014) investigated the thermal sensitivity of *Plutella xylostella*. They reported an upper lethal temperature as 42.6°C and lower kethal temperature as 16.5°C. While the upper and lower temperature range for 25% survival was recordes as 41.8°C and 15.2°C. from their results they suggested that the rapidly changing climatic condition would have a minimal effect on the physiology of Plutella xylostella.instead the presence and accessibility of this pest to the host plants in maginal locales have favoured them to expand their geographical distribution.

The larvae attack and play havoc to (more than 42.2 million tons) important cruciferous vegetable crops like broccoli, cauliflower and cabbage thus posing the most significant challenge to worldwide crucifers production (Talekar and Shelton, 1993; Furlong et al., 2013). The global annual cost of DBM control and management has been estimated as $ 5 billion (Zalucki et al., 2012). A recent high-grade influx of the pest was reported in the UK, which was considered alarming for the growers as in large numbers it has the potential to cause devastating damages to cabbage, oilseed rape, cauliflower and broccoli across the country (Pallab, 2016). Chemical control generally involves exposure to broad-spectrum synthetic insecticides by growers (Wright, 2004), which are also lethal to natural enemies. Moreover, the frequent and inconsistent use of these insecticides has made the pest resistant to most of them. Resisitance to insecticides as well as the absence of efficient natural biological control agents have been identified as the major causes of Its outbreaks throughout te world (Philips et al., 2014). As a result, the control of DBM in a sustainable manner has become very challenging (Grzywacz et al., 2010).

1.8.2 *Chrysoperla carnea*

Chrysoperla carnea (Green lacewings) (Neuroptera, Chrysopidae) has long been recognised as a polyphagous natural enemy of a wide variety of herbivorous insect (McEwen et al., 2007; Loru et al., 2014). This natural predator occurs ubiquitously on foliage over an extensive range of agrarian abodes and is capable of mass reproduction easily. It has been reported to exist in various habitats from Africa to Asia, Europe, Ociana, North and South America. They are predominantly well known for their effectiveness against aphids. Their larvae, however, also feed voraciously on many soft-bodied insects like whiteflies, thrips, mites, caterpillars of moths and their eggs (Shankarganesh et al., 2016; Pasini et al., 2018). The ideal temperature tolerance range for this natural predator has been identified as between 25°C to 30°C. Saljoqi et al., 2015 investigated the impact of changing temperature on various biological parameters of *Chrysoperla carnea*. Thei results showed that chrysoperla carnea permormed ideally in terms of percentage survival, egg to adult emergence as well as femal fecundity at 28 ± 1°C when fed on cabbage aphids (*Brevicoryne brassicae*). Due to their higher tolerance to various temperature they has established their population successfully in various habitats. In addition, it has a wide range of tolerance to many insecticides relative to other natural enemies of different pests (Shankarganesh et al., 2016). For these reasons, it is one of the primary choices for Integrated Pest Management (IPM) and biocontrol programmes, both indoors in greenhouses as well as open fields in many countries throughout the world (McEwen et al., 2007). Moreover, *Chrysoperla carnea* is indicator species for pesticides testing under many scenarios such as effects of the Bt toxin as well as registration of many pesticides in European Union (Romeis et al., 2008; Maia et al., 2016). The practice of using *Chrysoperla carnea* in biocontrol programmes is becoming more widespread due to its many advantages over other natural predators (Pappas et al., 2011; Rugno et al., 2015). Since January 2014, when the EU 2009/128/EC directives on sustainable use of pesticides came into implementation, the value of biological control has increased (Rotteveel, 2013). This has entailed the integration of useful insects into the agricultural crop systems, in such a way that provides a comprehensive strategy for pest management while at the same time conserving their higher contribution in agricultural economics (Messelink et al., 2014).

1.9 Host plant

Brassica rapa var. *pekinensis* (chinese cabbage), a member of family Brassicaceae was the host plant of choice in the current study. At present China ha sthe highest acreage forthis economically important crop however, it has now acquired a large scale acceptance and popularity by people in

countries throughout the world. This is primarily due to its nutritional value. The leaves of chinses cabbage are a rich carotinoid and anthocyanin (Wang et al., 2008). The former has numerous health benefits to human health as it is primarily converted into viamin A. Likewise, carotinoid possess antioxidant properties and can help prevent many cardiopathies and other malignancies. Similarly, anthocyanin is an essential pharmaceutical ingredient which is used as antimicrobial, anticancer, antidiabetic and anti-inflammatory drugs (Li et al., 2012). Wang et al., 2019 investigated the uptake and distribution of four systemic pesticides in *Brassica rapa* var. *Chinesis* in controlled laboratory conditions. They found that the neonicotinoid thiamethoxam was more readily absorbed and translocated from roots through the stem while avermectin, fenbuconazole and spirotetramat tended to accumulate in its root. Studies like these are important as they inform about the behaviour of host plant towards pesticides and thus crucial when selecting a host plant for experimental purpose.

In general, family Brassicaceae includes many other economically important species of plants such as *Brassica oleracea*, *Brassica napus*, and *Brassica rapa*. All of these serve as host plants for DBM (Canico et al., 2013; Imran, 2017). These are of great economic and commercial importance as they represent a significant part of the human diet, as well as animal fodder and weeds, worldwide (Onyilagha et al., 2003; Verkerk et al., 2001; Thiyam et al., 2004). People from all over the world consume various varieties of these vegetables and they are considered as major food crops in Asia and Europe (Sasaki and Takahashi, 2002; Kusznierewicz et al., 2008). *Brassica* crops are used for a variety of purposes. A single species can exhibit several uses, subject to various forms or types, using their seeds, bud, inflorescence, leaves, roots and shoots. One of the primary reasons for their global consumption as mentioned earlier is their nutritional value. They are a rich in vital nutrients such as vitamins, minerals, carotenoids, fibre and sugars which have a pivotal role in promoting health and fitness (Sanlier and Guler, 2018; Kapusta-Duch et al., 2012). *B. oleracea* is the chief vegetable species, which encompass many varieties and cultivars, such as cabbage, cauliflower, kale, brussels sprouts, broccoli and others. *B. rapa* comprises of cultivars like Chinese cabbage, pak choi and turnip, along with fodder and oilseed varieties. *B. napus* species are mostly utilised as oilseed, even though vegetable and forage forms are also present (Cartea et al., 2011). The last is mustard, which consist of three species, *Brassica nigra*, *Brassica juncea* and *Brassica carinata*. *B. juncea* leaves, although are well known as a vegetable, the species are chiefly used as a condiment for seasoning, flavouring and spices. In most Asian countries, they are extensively marketed as both, fresh and processed foodstuff (Cartea et al., 2010).

A significant part of the *Brassica* crops grown is often for local consumption in countries like India and China (Rakow, 2004). Many countries with production centres for different species and varieties of *Brassica* however, export these crops. For instance, Southern California, the main centre from where these vegetables are supplied to other regions of the state and Canada all the year around. Similarly, Brittany (France) is the chief production and research region on *Brassica* vegetables (Kimber and McGregor, 1995). Likewise, in Southeast Asian countries, various varieties such as *B. juncea* (leaf mustard), *Brassica rapa* var. *pekinensis* (Chinese cabbage) and *Brassica rapa* var. *chinensis* (Pak choi) are grown extensively. *Brassica alboglabra* (Chinese kale) is among the top 10 key market garden vegetables in countries like China and Thailand (Rakow, 2004).
In addition, about 12% of the world's edible vegetable oil is obtained from *Brassica* oilseed rape. Over the last four decades, its production has expanded incredibly (Paterson et al., 2001), making it the third most important source of vegetable oil after soybean and palm oil (Thiyam et al., 2004). Although India, China and Canada have the most extensive acreage of *Brassica* oilseeds, the total seed production, is highest for Europe (UK, Germany and France) with an average output of approximately 3.0 tonnes/ha. This is almost double the amount of seeds produced in Australia and Canada. Canada and Australia are the chief rapeseed exporters, while China and Japan are the major importing countries (Kimber and McGregor, 1995). European Union contributes to the refining sector, refining almost one half of the total rapeseed oil on the international market (Rakow, 2004).

The two main pests of Brassica crop that put major constraints on their production are *Plutella xylostella* and *Pieris rapae*. The later is less widely distributed relative to *Plutella xylostella* however, it can prove as a destructive pest species in cooler and temperate regimes. On the other hand, *Plutella xylostella* has attained a global status of one of the most difficult to manage pest which has played havoc to many Brassica crops in the past (Furlong et al., 2008).

1.10 Aim of the study

The emphasis on interactions between synthetic chemicals, principally at ecologically relevant levels, is an important step towards improving our knowledge of the impact of these chemical combinations on human and environmental health (Monosson, 2005). Nevertheless, there are still many gaps in our understanding and therefore, there is a pressing need for more streamlined studies to provide scientifically factual data enabling improved application of pesticides that helps control pests efficiently and contribute towards effective decision-making. Moreover, while the

study of the toxic effect of pesticides is comparatively more comprehensive on bees, non-bee pollinators, as well as other beneficial insects are equally as important in agriculture because they all serve their respective vital roles. The main aim of this study was to carry out a tier one risk assessment of pesticides in controlled laboratory conditions to understand the effects of neonicotinoids and fungicides applied simultaneously to the target and non-target insects and how to manage these pesticides mixtures. The hypothesis of the study was that pesticides can be safer for non-target and perform better against pests when applied together in mixtures at levels lower than their normal recommended application rates.

The current study was conducted with the following objectives:

1. To analyse the effects of insecticides and fungicides on pest (target) insects and their natural enemies (non-target) individually (chapter 2, 3 and 4).
2. To determine whether the combination of pesticides (insecticides and fungicides) have a greater impact on target and non-target insects (Chapter 2, 3 and 4).
3. To provide risk management options based on risk assessments results to see whether these effects can be mitigated by making variations in the rate of application of these pesticides in mixtures ------ Chapter 5.

Chapter 2 Lethal effect of acetamiprid, tebuconazole and their mixtures on target insect *Plutella xylostella* and non-target insect *Chrysoperla carnea*

2.1 Introduction

To control the pest populations and increase the agricultural production, many different classes of pesticides have been developed and the addition of newer, more effective classes of these pesticides is still going on (Carvalho, 2017; Aktar et al., 2009; Rodriguez-Saona et al., 2016). Among them, the use of newly developed neonicotinoids is widespread throughout the world due to their promising results in pest control (Furlan and Kreutzweiser, 2015). The growing use of pesticides principally over the last decade, however, has given rise to an increased concern on the decline of non-target insects (Brown and Paxton, 2009; Burkle et al., 2013; Cook et al., 2016; Francesena et al., 2017). Neonicotinoids, have been implicated as a major contributor towards this decline (Vanbergen, 2013; Goulson, 2015; Tappert et al., 2017). Many studies however, have also shown the presence of complex mixtures of chemicals, to which these non-target insects are routinely exposed and victimised (David et al., 2015; David et al., 2016; Willow et al., 2019). Although effective against target insects, it is still not clear that whether it is the neonicotinoids alone or their mixtures with other chemicals, that makes the situation worst for non-target insects in the environment. Hence, it is essential to investigate this issue by carrying out the risk assessment of chemicals in mixtures so that the subject is carefully addressed by the risk assessment authorities.

Pesticides are mixed, very often to reduce labour and cost as well as to multiply the spectrum of its activity against a diverse group of organisms. In most cases however, the primary focus is a single target species (Cloyd and Raudenbush, 2014; Das, 2014). Mixtures of two or three pesticides expose the pest population simultaneously to various pesticides. At the same time their application increases the chance of affecting multiple pest populations. Likewise, these mixtures can be effective against different life stages of pests. (Cloyd, 2009). The most commonly used components of chemical mixtures to which organisms in the environment are routinely exposed are fungicides, insecticides and herbicides (Johnson et al., 2012; Mullin et al., 2010; Lambert et al., 2013; Mullin et al., 2015). For example, the efficacy of mixing the fungicide propiconazole and epoxiconazole with pyrithroid insecticides against aphids and fungi on winter wheat (*Triticum*

aestivum) has been demonstrated by Norgaard and Cedergreen (2010). Nonetheless, due to their effects on non-target populations, the synergistic interaction of these mixtures and their cocktail effect are the areas of major concern for public as well as regulatory authorities in both Europe and US (Cedergreen, 2014; Backhaus et al., 2010). This has led to the intensification of research on chemical mixtures. The focus on chemical mixtures and interaction between their chemical components is important to take forward our knowledge and understanding of their impact on man and the environment (Monosson, 2005). In Europe, the regulatory bodies usually assess the toxicity of both active ingredients and formulation of a pesticide (EFSA, 2013a). The combination of pesticides at lower doses is expected to produce a more profound toxicity through additive or synergetic action (Vidau et al., 2011; Yi et al., 2012). In literature, there exists an evidence of synergism as well as additive toxicities when pesticides are applied in mixtures (Johnson et al., 2012; Taillebois and Thany, 2016). The sequential use of pesticides and their tank-mixes as well as the risks associated with them, however, are not addressed to the required extent.

In addition, despite of being held responsible for many adversities to the environment specially with regards to harming non-target insects (Whitehorn et al., 2012; Gill et al., 2012), the use of neonicotinoids has increased significantly since their introduction onto the global commercial market in early 1990s. In 2006, this group of insecticides accounted for an annual sale of $US 1.5 billion, making a virtual share of 17% in global commercial market of agrochemicals. By the end of 2009, it further raised to $US 2.6 billion, making nearly a quarter of the total insecticides market (Jeschke et al., 2011). The UK alone accounted for a substantial rise in their use from 3 tonnes to approximately 80 tonnes between 1994 and 2011 (Goulson, 2013a). Part of this success lies in their systemic nature. They possess high water solubility due to which they are readily taken up by roots or leaves of plants and incorporated all the way through plant tissues providing them protection against various pests (Goulson, 2013a; Simon-Delso et al., 2015).

Efficacy of neonicotinoids against a variety of pests has been established by many previous studies (Ahmed et al., 2014; Anjum and Wright, 2016). However, contact with neonicotinoids like imidacloprid, thiacloprid, acetamiprid, clothianidin, thiamethoxam and dinotefuran are extremely toxic to both terrestrial as well as aquatic non-target insects, which include pollinators, parasitoids, predators and natural enemies of the pests (Brandt et al., 2016; Beloti et al., 2015; Bhojani et al., 2018; Chandran et al., 2018; Cloyd and Dickinson, 2006; Yu et al., 2014; Youn et al., 2003). These can be lethal to almost all life stages of these beneficial insects which serve a vital role as biocontrol agents against a vast array of pests (Quarles, 2014). For instance, feeding on imidacloprid sprayed aphids, can not only increase the mortality in predatory lady beetles

(*Hippodamia undecimnotata*) but also affect the their longevity and fecundity (Papachristos and Milonas, 2008). Many other beneficial insects such as many parasitic wasps are also killed either through direct acute exposure to neonicotinoids sprays or chronically by consuming contaminated food and poisoned prey (Hopwood et al., 2012). Honey bees and bumble bees however, are the most comprehensively studied insects for the toxicity of pesticides (Pisa et al., 2015), due to their fundamental role as natural pollinators and value in integrated pest management (IPM), for conserving a healthy ecosystem (Potts et al., 2010; Winfree et al., 2008). Yet, the services provided by other non-target beneficial insects such as parasitoids and natural predators of pests, as a biological control are not of least significance. Their annual worth in agriculture has been estimated up to 4 billion US Dollars.

Fungicides are the third most used category of pesticides after herbicides and insecticides. A greater proportion of these are used in crops of economic importance like soybean. They are gradually becoming widespread throughout the world with an estimated trade of $US 8 billion and $US 21 billion between 2005 and 2017 (Brandon, 2015). Neonicotinoids when co-applied with some fungicides, (predominantly DMI or EBI fungicides), have been reported to show high degree of synergism in their activity (Thompson et al., 2014; Cedergreen, 2014).

In this study, acetamiprid a neonicotinoid insecticides and tebuconazole, a demethylation inhibitor fungicide (also known as EBI, ergosterol biosynthesis inhibitor), are used to determine their combinatorial effect on mortality of *Plutella xylostella* and *Chrysoperla carnea* larvae. Acetamiprid is an authorised neonicotinoid for use in Europe, North America and in many countries in Asia and the Pacific (Reynard, 2012) both indoors and outdoors, formulated both as foliar and soil applications. Their efficacy is predominantly high against many lepidopterans pests (Takahashi et al., 1999). In countries like Japan acetamiprid has now became a requisite part of DBM (Diamondback Moth: *Plutella xylostella*) control management programmes (Ninsin et al., 2000). Moreover, it has been reported as less toxic to natural predators and enemies of pests as compared to other neonicotinoids such as imidacloprid, thiamethoxam and clothianidin (Iwasa et al., 2004; Shankarganesh et al., 2016; Tsvetkov et al., 2017). In addition, it has been used in the presence of triazole fungicides, which act as synergists to increase the potential toxicity of acetamiprid to natural pollinators (Iwasa et al., 2004). Furthermore, this neonicotinoid is not a part of EU moratorium that was set on outdoor application of clothianidin, imidacloprid and thiamethoxam in 2018 on plants attractive to bees https://ec.europa.eu/food/plant/pesticides/approval_active_substances/approval_renewal/neoni

cotinoids). Thus, this neonicotinoid was found suitable to evaluate its toxic activity in the presence of a fungicide in mixtures.

Tebuconazole, the second agrochemical of choice, is an important fungicide belonging to triazole family. It has been identified as one of the most frequently used fungicide in the UK in the presence of neonicotinoids, which together has a synergistic effect against insects (Botias et al., 2017). It acts by inhibiting the biosynthesis of ergosterol in fungal cell membrane by interfering with CYTP450 protein, offering high efficacy against numerous fungi (Martinez et al., 2015). Ergosterol although is not a component of animal cell membrane, DMI fungicides when come in contact with insects, disrupt the synthesis of Cytochrome P450 enzymes (such as monooxiginases, carboxylases). Due to the inhibition of these enzymes insects' ability to efficiently metabolize the xenobiotics decline. Consequently their susceptibity to insecticides increase many fold (Manning et al., 2017). In oilseed rape it is commonly used for protection from light leaf spot, white mold and phoma (Coules et al., 2002). Moreover, along with other fungicides, it has been used effectively as systemic and foliar sprays against important fungal diseases such as *Alternaria* leaf blight and *Alternaria* black rot in carrots (Survilienė and Dambrauskienė, 2006). Similarly Basallote-Ureba et al. (1998) found tebuconazole effective for the control of leaf spot disease in garlic (*Allium sativum*). As indicated by Akhtar et al. (2014) in their study, tebuconazole caused inhibition of more than 90% of fungal growth, hence was recommended as an effective control for canola spot disease by *Alternaria* species. In Eastern Spain its use in paddy fields is widespread, chiefly as a replacement of prochloraz and tricyclazole treatments to restrain rice blast disease that mainly occurs during the month of July and August (Sancho et al., 2010).

Due to its large scale usage, it is one of the most repeatedly recovered pesticides from pollens collected by honey bees and bumble bees as well as wild bees (Johnson, 2015; Thompson et al., 2014; David et al., 2015; David et al., 2016; Botias et al., 2017). In addition, this fungicide possess compatibility with cyano group neonicotinoids acetamiprid and thiacloprid (having cyano (-C=N) functional group instead of nitro ($-NO_2$) functional group in imidacloprid, thiamethoxam and clothianidin) in tank mixes and has been used in mixtures in a few recent studies (Ostyn, 2017; Vanderhaegen, 2017). Compatibility among the component chemicals is one of the most important factor taken into account when applying pesticides mixtures because any conflict among the components of a mixture might lead to the formation of lumps, clogs or precipitates which may not only effect the chemical distribution of the spray mixture but can potentially damage the spraying equipment. Besides, such incompatibilities can potentially increase the loss

of product effectiveness, phytotoxicity and consequently the decreased crop yield (Petter et al., 2012; Gazziero, 2015).

Tebuconazole has been categorised as toxic towards aquatic life and can cause strong and long established adversities in the aquatic domain (Bayer CropScience Limited, 2016). For instance, a study conducted by Sancho et al. (2009) showed a drop in energy content of the *Daphnia magna* upon exposure to tebuconazole concentrations of 0.52 mgL^{-1} or higher, especially after 96 to 120 hours. The study suggested that tebuconazole could cause serious impairment of metabolic processes due to the resultant alteration in biochemical constituents, caused by tebuconazole. In addition, exposure to fungicide also inhibited the algae feeding rate of *D. magna*, such that feeding rate dropped just after 5 hours of the treatment. Likewise, in a study by Heath (1995), Zebrafish subjected to subacute concentrations of tebuconazole showed an alteration in intermediary metabolism as a stress response to cope with changes caused by the toxicant and to maintain the homeostasis.

Keeping in view the previous literature, the hypothesis of this study was that, the combination of various pesticides in an agricultural system has a greater impact on the mortality rate of insects. The focus of this chapter was therefore, to carry out the risk assessment of acetamiprid and tebuconazole individually and in combinations to compare their effects individually and most importantly when applied together, on the mortality rate of target and non-target insects. For this purpose, a tier one risk assessment of these pesticides was conducted in laboratory where a model system of *Plutella xylostella* as target and *Chrysoperla carnea* as non-target organisms were used.

2.1.1 Study objectives

The overall aim of this study was to determine the toxicity of acetamiprid and tebuconazole on different developmental stages of *Plutella xylostella* and *Chrysoperla carnea* when applied in mixtures. To accomplish this, the study was designed with the following objectives:

1. To determine the lethal effect of acetamiprid, tebuconazole and their mixtures on larvae of *Plutella xylostella*.
2. To determine the lethal effect of acetamiprid, tebuconazole and their mixtures on larvae of *Chrysoperla carnea*.
3. To determine the LC_{50} for *Chrysoperla carnea* and *Plutella xylostella* on acetamiprid, tebuconazole and their mixtures as an indicator of extent of their toxicity.

4. To determine LC_{50} for 24 h and 48 h of exposure in order to know whether time has any effect in raising or lowering the toxicity. The 24 h and 48 h intervals were chosen as these are commonly used in the literature and are good for comparison (Nasreen et al., 2003; Roubos et al., 2014).

2.2 Materials and methods

2.2.1 Plant culture

To maintain a continuous source of food supply for raising the *Plutella xylostella* culture and to obtain the fresh leaves for leaf dip bioassays, Chinese cabbage, Wong Bok (*Brassica rapa* var. *pekinensis*) plants were grown at mean temperature of 25 ± 2^{o}C in the glasshouse of Faculty of Environmental and Life Sciences, University of Southampton.

The seeds of Chinese cabbage were obtained from King Seeds, UK. The growth medium used was Levington ® F2+S (Seed and Modular Compost plus Sand) by Everris Limited with N144 P73 K239 mgL^{-1} added fertilizer. Growth medium is of paramount importance when growing plants in soilless system. An effective growth medium in its physical shape should be capable of sustaining an efficient balance between water holding capacity and and air porosity. To preclude draught stress or root asphyxia. Moreover,it should provide a favourable chemical and biological environment where a desirable access to the nutrients can be accessable by plant roots (Barrett et al., 2016). The compost used in this study contains all the essential nutrients thus providing a perfect growth media for plants to maintain their quality and integrity. Seeds were sown in separate pots in compost with no further addition of fertilizer, on weekly basis (Anjum and Wright, 2016), such that 15 to 20 pots of approximate size 8 cm height and 7.5 cm diameter were partially filled with compost. In each pot, 3 to 4 seeds were added at a time to maximise the chance of plant growth in each pot. A small amount of compost was then added to each pot to cover the seeds. The pots were kept in the growth room and watered manually with a hand-held water sprinkler. Each weekly batch of pots was labelled with date of sowing. Subsequently, the pots containing seeds or plants were watered through an automated watering system at the glasshouse twice every day. Any chemical spraying was avoided in the growth room to protect the plants from chemicals prior to the experimental treatments.

2.2.2 Insects supply

2.2.2.1 *Plutella xylostella*

To start a basic culture of the test insect, 2 to 3 potted Chinese cabbage plants with freshly deposited eggs of *Plutella xylostella* were obtained from a primary stock culture of the invertebrate facility of the Faculty of Environmental and Life Sciences, University of Southampton, UK. The pots with eggs were kept in ventilated fabric cages under controlled conditions of 25 ± 2^{o}C

temperature, 60% relative humidity (RH) and 16:8 light and dark period (Anjum and Wright, 2016). The eggs hatched in 5 to 7 days after which fresh cabbage plants were provided to the growing larvae as required. Adults, which feed on sugar and carbohydrates, were supplied with the food through a cotton swab soaked in 10% honey solution and kept in a small plastic vial inside the cages (Rodriguez-Saona et al., 2016). The plant residues after consumption by larvae and old cotton swab for adults were renewed when required.

2.2.2.2 *Chrysoperla carnea*

To ensure the test insects come from a pesticides free environment, the larvae were purchased from Agralan Limited. To acclimatize, these larvae were kept in the fabric cages providing them with aphids as their food for 2 to 3 days before subjecting them to experimentation. The larvae were maintained under the same condition as were *Plutella xylostella*, at 25 ± 2°C, 16:8 h photoperiod and 60% relative humidity.

2.2.3 Pesticides used in the experiment

Two types of pesticides were used, including an insecticide and a fungicide. Details of these compounds are given in the Table 1.

Table 1 Details of pesticides used in the present study.

S. No	Pesticide Trade Name	Group	Active Substance	Manufacturer	Supplier
1.	Gazelle® SG 20%	Neonicotinoid	Acetamiprid	CERTIS-Solution for Crop Protection	Fargro® Ltd
2.	Folicur® 250 EW	Fungicide	Tebuconazole	Bayer CropSciences	Fargro® Ltd

2.2.4 Bioassay

A leaf dip bioassay technique adapted by Anjum and Wright (2016) with slight changes, was followed during the present study to assess the toxicity of the given pesticides on *Plutella xylostella* and *Chrysoperla carnea*. Dipping techniques are generally employed when topical or injection application is impractical. This method allows a uniform and even distribution of a chemical compound on the leaf surface and therefore, the efficacy of the field doses for pest eradication can be investigated efficiently (Paramasivam and Selvi, 2017).

2.2.4.1 Solutions for bioassay

Gazelle acetamiprid and Folicur tebuconazole were dissolved in deionized water to prepare solutions of different concentrations. Various treatments used in the bioassay are given in Table 2. The recommended rate of application of Folicur is 1 Lha^{-1} in a water volume of 200 Lha^{-1} whereas, Gazelle SG acetamiprid is recommended at a rate of 250 gha^{-1} in a total water volume of 1000 Lha^{-1}. Three levels of application were used in this study, which were 0.025 gL^{-1}, 0.05 gL^{-1} and 0.25 gL^{-1} for Gazelle® whereas, 0.5 mlL^{-1}, 1 mlL^{-1} and 5 mlL^{-1} for Folicur®. The lower two values represent 10X and 5X lower concentrations than field application rates of the two pesticides (Chandler and Davidson, 2005). To obtain the desired level of concentrations a fresh stock solution of 250 ml for each treatment was prepared at the time of experimental procedure.

Table 2 Chemical treatments used in leaf dip bioassay against *Plutella xylostella* and *Chrysoperla carnea* larvae.

Treatments	**Pesticide Active substance**	**Concentration Relative to recommended file rates**
0.025 gL^{-1}	Acetamiprid	10 X lower
0.05 gL^{-1}	Acetamiprid	5 X lower
0.25 gL^{-1}	Acetamiprid	Field rate
0.5 mlL^{-1}	Tebuconazole	10 X lower
1 mlL^{-1}	Tebuconazole	5 X lower
5 mlL^{-1}	Tebuconazole	Field rate
0.5 mlL^{-1} + 0.025 gL^{-1} (mix1)	Tebuconazole + Acetamiprid	10 X lower TEB + 10 X lower ACET
0.5 mlL^{-1} + 0.05 gL^{-1} (mix2)	Tebuconazole + Acetamiprid	10 X lower TEB + 5 X lower ACET
0.5 mlL^{-1} + 0.25 gL^{-1} (mix3)	Tebuconazole + Acetamiprid	10 X lower TEB + Field rate of ACET
Control	Deionized water	0

2.2.4.2 Gazelle ® SG 20% acetamiprid

To achieve the three desired concentrations of Gazelle acetamiprid for experimental purpose (given in Table 2), originally 6.25 mg, 12.5 mg and 62.5 mg of acetamiprid each in 250 ml of deionized water was dissolved. While in a separate beaker, 250 ml of deionized water was taken as a control treatment. All the solutions were made in such a way that the insecticide granules were first correctly weighed with the help of an electronic balance. The measured quantity of granules was then added to an empty beaker. Half of the total volume of water was poured in beaker and constantly agitated until all the granules were dissolved. The remaining water was then added to make up to the required level of dilution.

2.2.4.3 Folicur® 250 EW tebuconazole

For the three oncentrations of tebuconazole, 0.125 ml, 0.25 ml and 1.25 ml of Folicur® was pipetted out from its container and transferred into three separate beakers. Deionized water was then added slowly with constant agitation to make an even solution until the final volume reached to 250 ml.

2.2.4.4 Mixtures of acetamiprid and tebuconazole

In these mixtures, the amount of tebuconazole was kept constant while it was mixed with three quantities of acetamiprid i.e. 0.025 gL^{-1}, 0.05 gL^{-1}, 0.25 gL^{-1}. These mixtures were prepared such that in three separate 400 ml capacity glass beakers 0.125 ml + 6.25 mg, 0.125 ml + 12.5 mg, 0.125 ml + 62.5 mg tebuconazole and acetamiprid respectively were added. By adding a small amount of deionized water into each beaker, the pesticides were mixed to make a dense solution. Subsequently more water was added to make up to the desired level of dilution.

2.2.5 Leaf disc preparation

For leaf dips, 6 to 8 weeks old, Chinese cabbage plants were taken and the large leaves were cut from the main stem with scissors at the leaf stalk. The leaves were then cut into approximately 45 mm round leaf discs using a sharp round-shaped cutter of size approximately 4.5 cm diameter (Fig. 1a). Leaf discs were then individually dipped in prepared solutions of acetamiprid and tebuconazole for 10 s making sure the entire leaf surface was immersed in the liquid (Fig. 1b and c). The leaf discs after treatment were taken out of the solutions using forceps and placed on paper towel to surface dry inside a fume hood. The treated leaf discs were placed in such a manner that their abaxial surface was facing upward (Fig. 1d). Equal number of leaf discs were treated with each treatment at the time of bioassay. Once dry, individual leaf discs were placed on the surface of a wet filter paper (Whatman no. 1, 70 mm) in a 100 mm petri dish. The leaf disc was placed in such a manner that the leaf abaxial surface was facing upward.

2.2.6 Insects treatment

A total of 12 replicates (represented by 12 Petri dishes containing the pesticide treated leaf disc and larvae of the insects) for each concentration and each insect were carried out

in this study. Thus in total 48 Petri dishes, each with a leaf disc treated either with the pesticide or deionized water (as control) were used for each developmental stage of the insects. The Petri dishes were labelled on the basis of the treated leaf disc and insect type and/or developmental stage. Transfer of insects larvae was carried out in the insectary where *Plutella xylostella* and *Chrysoperla carnea* were maintained for experimental purpose on Chinese cabbage (*Brassica rapa* var. *pekinensis*) plants and aphids respectively. Because setting up and performing all these replicates simultaneously was not possible for a single PhD student due to the amount of time it would take in laboratory as well as its handling accuracy, these experiments were carried out in overlapping time, seasons and batches of the insects. Such that, at a given time 3 replicates for each treatment and each insect and their developmental stages was performed.

For *Plutella xylostella,* the cabbage plants with highest larval infestation were picked from the cages and collected in a separate tray. Using a fine paintbrush with pointed tip 10 randomly selected second, third and fourth instar larvae were gently transferred on the surface of leaf disc in each of the Petri dish. Similarly, the treated leaf disc kept on the surface of wet filter paper in a 100 mm diameter Petri dish served as a substrate for lacewing larvae. Ten larvae per each pesticide infused leaf disc were released. Thus a total of 120 larvae for each insect and/or their developmental stages were utilised in this study. As the larvae of *Chrysoperla carnea* were coming from an outer source, majority of the larvae received were in early stages of their life cycle. Secondly their role as biological control, is important at all life stages, therefore, these were not segregated on the basis of their developmental stages. Aphids were supplied *ad libitum* to the predacious lacewing larvae during the bioassay period. The leaf discs wet with deionized water only, served as control. Each Petri dish with larvae were then covered with a plastic wrapping foil to prevent the insects from escaping the experimental unit. Each Petri dish was considered as a replicate and 12 replicates of each concentration and each insect were carried out in the experiment. The Petri dishes were then kept under controlled conditions as provided for *Plutella xylostella* culture for 24 h until the first mortality count was recorded.

2.2.7 Mortality counts

Mortality counts and activity of the treated larvae were recorded after 24 h and 48 h of treatment. This interval was chosen because from a farmers perspective allowing 48 h time to eradicate the pest is pretty decent interval. Secondly, this time interval has been adopted by previous studies (Roubos et al., 2014). To achieve this, individual replicates of each treatment were examined for the activity of larvae subjected to pesticide treated leaf discs. A larva was considered dead when it remained motionless for 10 s after repeatedly prodding with a fine paintbrush at different parts of its body. The process was repeated after 48 h of treatments to record the rate of mortality after 48 h.

2.2.8 Statistical analysis

All the data was analysed using SPSS version 22 and Graph Pad Prism version 6. Normality test was conducted for data and where required log transformation was applied. The effects of different concentrations and exposure time of the pesticides on mortality rate of larvae was determined by generalized linear model. Differences in mortality rate of an insect larvae by various concentration of a pesticide was identified using a pairwise comparison of treatments in a generalized linear model with a *post-hoc* Bonferroni test. The difference in mortality rate was significant if the p-value was below 0.05. Median lethal toxicity LC_{50}, with 95% Fudicial limits was carried out through Probit - analysis. The LC_{50} values of larvae after 24 h and 48 h of exposure to the pesticides were significant if their 95% fuducial limits were non-overlapping (Crawley, 2007).

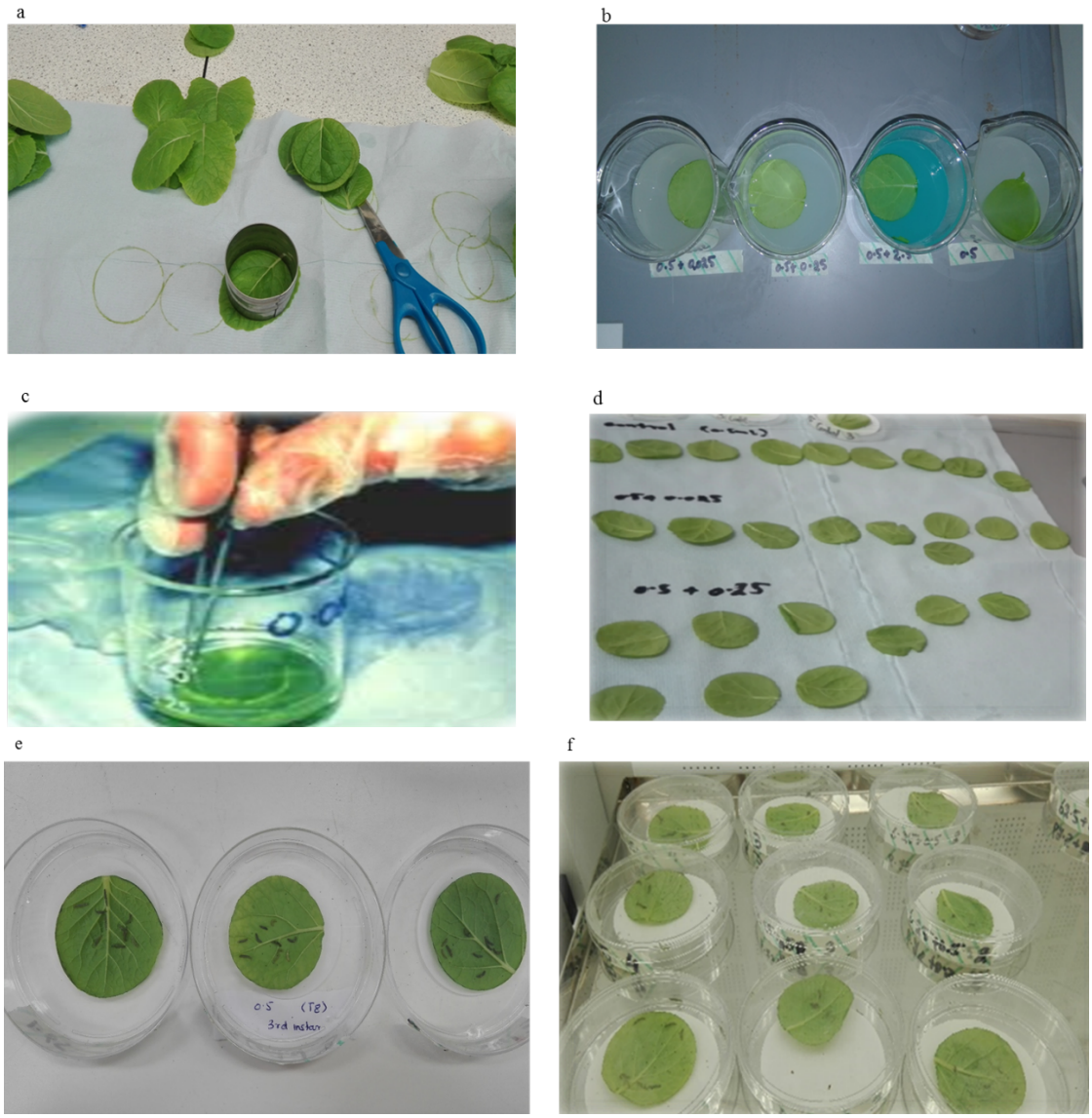

Figure 1 Leaf dip bioassay technique used in this study. (a) Leaf disc preparation (b) Preparation of solutions with different concentrations (c) Individual leaf dip in solution (d) Drying of treated leaves. (e) 3 replicates of single concentration – petri dishes containing larvae on the surface of treated leaves (f) Replicates of larvae of insect.

2.3 Results

2.3.1 Effect of acetamiprid on mortality of target insect, *Plutella xylostella*

2.3.1.1 Mortality of *Plutella xylostella* at second instar stage

The mean percentage mortality increased significantly with increasing concentrations of acetamiprid ($F_{(3, 88)} = 105.152$, $p = 0.000$) as well as its exposure time ($F_{(1, 88)} = 8.801$, $p = 0.004$) (Fig. 2a). Pairwise comparison showed no significant difference between mortality of control and 0.025 gL^{-1} of acetamiprid concentration. However, 0.25 gL^{-1} caused significantly higher mortality in larvae than 0 gL^{-1}, 0.025 gL^{-1} and 0.05 gL^{-1} of acetamiprid. Likewise, the mortality rate of larvae with 0.05 gL^{-1} acetamiprid was significantly greater than 0 gL^{-1} and 0.025 gL^{-1} acetamiprid concentration.

2.3.1.2 Mortality of *Plutella xylostella* at third instar stage

For third instars, a slightly less number of deaths were observed than in second instars. A statistically significant difference was found in the mean percentage mortality across the different concentrations of acetamiprid ($F_{(3, 88)} = 80.505$, $p = 0.000$) whereas, the effect of exposure time was not significant ($F_{(1, 88)} = 0.439$, $p = 0.508$) (Fig. 2b). Mortality rate recorded among the four treatments was significantly higher for the field recommended dose of acetamiprid (0.25 gL^{-1}) at $p < 0.05$ as compared to the rest of treatments. Lowest mortality was found with 0.025 gL^{-1} concentration, which was not different from that in control. At 0.05 gL^{-1}, although mortality rate was higher than 0.025 gL^{-1} but lower than 0.25 gL^{-1}.

2.3.1.3 Mortality of *Plutella xylostella* at fourth instar stage

A significant difference in mean percentage mortality was observed with increasing concentrations ($F_{(3, 88)} = 59.556$, $p = 0.000$) (Fig. 2c). Similarly, the effect of exposure time was significant which means the mortality rate of these instars increased with increased duration of exposure to acetamiprid ($F_{(1, 88)} = 23.785$, $p = 0.000$). Here again the mortality with 0.25 gL^{-1} was significantly high than the two lower concentrations as well as control. No significant difference was observed between mortality rates of control and the two lower concentrations.

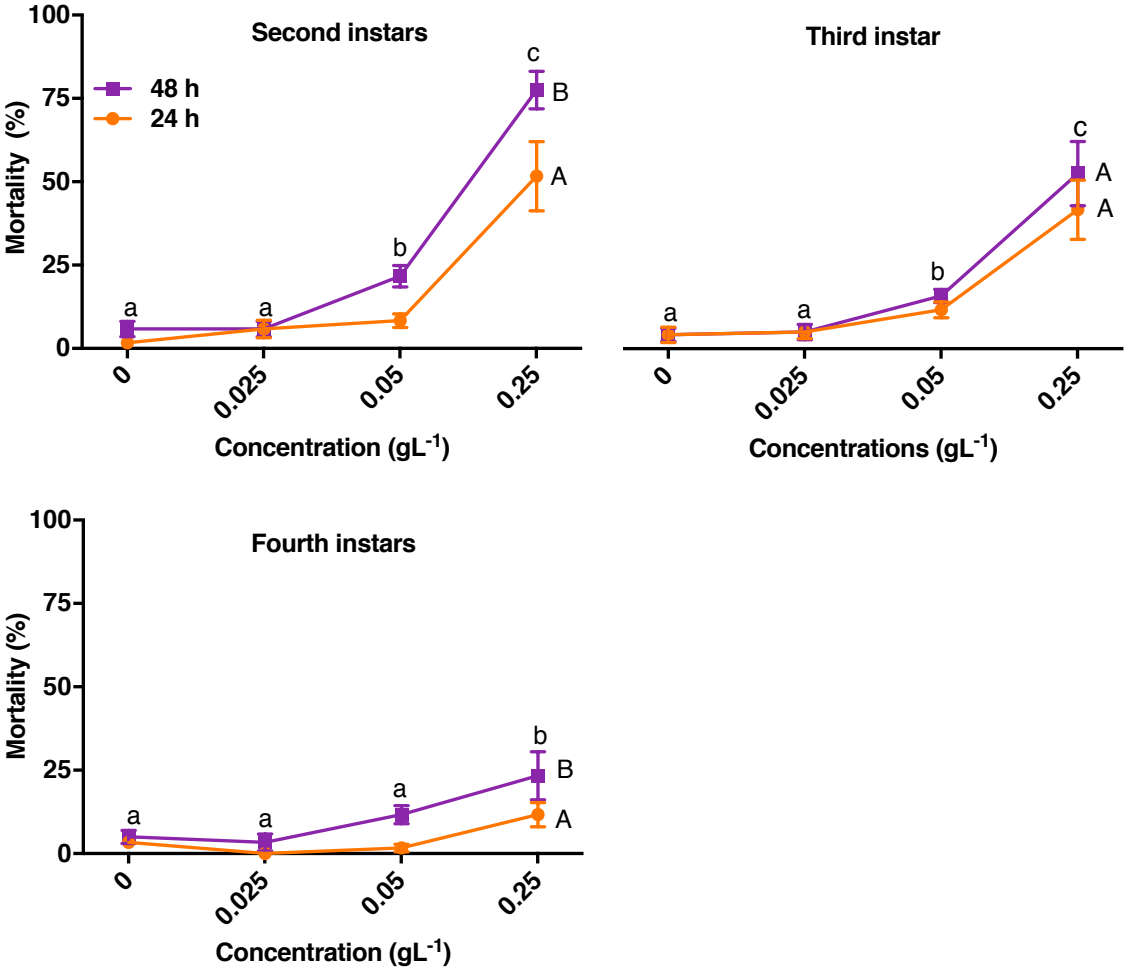

Figure 2 Effect of acetamiprid on mean mortality of three developmental stages of *Plutella xylostella*. Error bars sharing the same lowercase letters show no significant difference in mortality over the four treatments for an instar group ($p > 0.05$). Similar uppercase letters between the two lines show no significant difference of exposure time ($p > 0.05$).

2.3.2 Difference between mortality of three developmental stages of *Plutella xylostella* over time on acetamiprid

Of all the instar stages, the fourth instars showed significantly lowest mortality rate with the three concentrations of acetamiprid. After 24 h of exposing the three instar groups of *Plutella xylostella*, a significant difference in mortality rate of the three instars was found ($F_{(2, 136)} = 33.175$, $p = 0.000$) (Fig. 3a). This difference was such that mortality rate of second and third instar was similar ($p > 0.05$) however, it was significantly greater than fourth instar larvae ($p < 0.05$). Over 48 h duration of exposure the difference in mortality rate was again significant ($F_{(2, 136)} = 8.488$, $p = 0.014$) (Fig. 3b). Here mortality rate of second instar larvae was consistently and significantly

higher than fourth instars ($p < 0.05$) however, mortality rate of third instars remained non-significant from second instars as well as fourth instar larvae ($p > 0.05$).

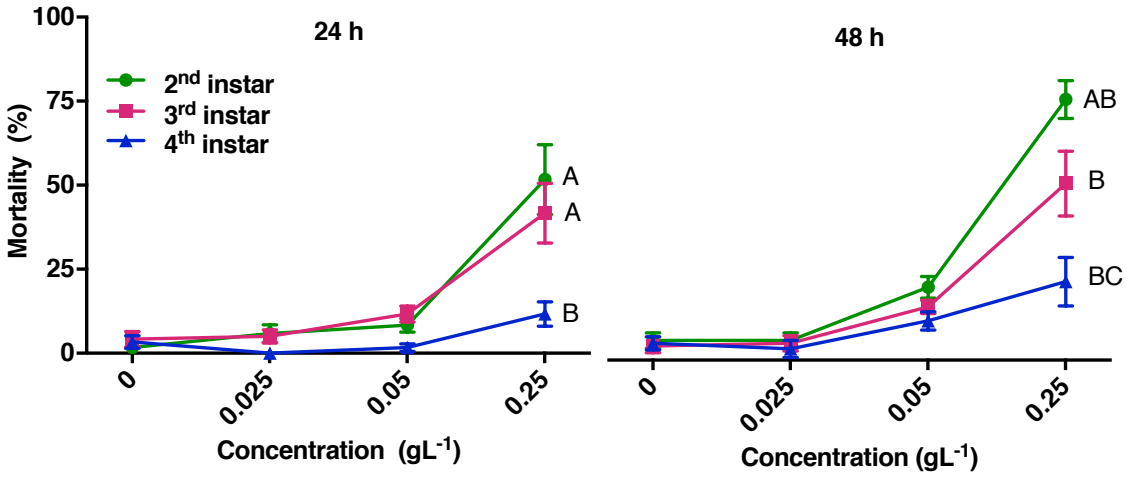

Figure 3 Summary of the mean mortality rate of three developmental stages of *Plutella xylostella* on acetamiprid over time. Similar uppercase letters across the three lines show no significant difference in the mortality rate of the three instar groups ($p > 0.05$).

2.3.3 Effect of acetamiprid on mortality of non-target insect, *Chrysoperla carnea*

The mortality of *Chrysoperla carnea* increased with increase in concentration of acetamiprid. A significant difference in mortality of larvae between lower and higher concentrations of acetamiprid was found ($F_{(3, 88)} = 24.427$, $p = 0.000$) (Fig 4). Mortality of lacewings larvae was significantly high with 0.25 gL^{-1} of acetamiprid as compared to the two lower concentrations used (0.025 gL^{-1} and 0.125 gL^{-1}) as well as control (0 gL^{-1}). In addition, mortality also increased significantly with increase in exposure time from 24 h and 48 h ($F_{(1, 88)} = 4.778$, $p = 0.029$).

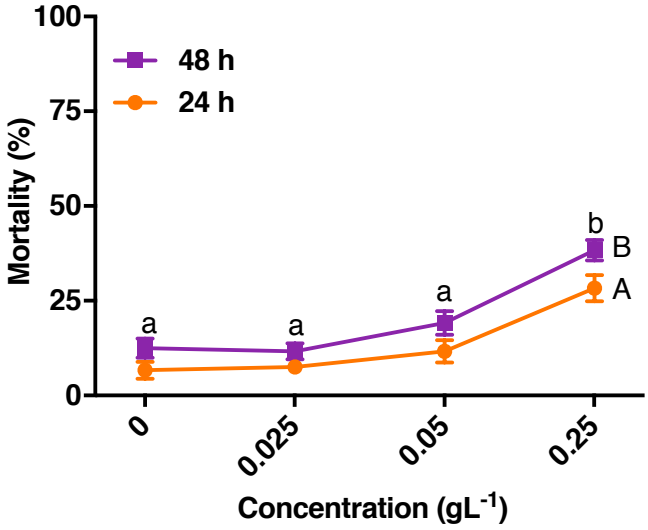

Figure 4 Effect of acetamiprid on mean mortality of *Chrysoperla carnea*. Error bars sharing the same lowercase letters show no significant difference in mortality over the four treatments ($p > 0.05$). Similar uppercase letters between the two lines show no significant difference of exposure time ($p > 0.05$).

2.3.4 Effect of tebuconazole on mortality of target insect, *Plutella xylostella*

2.3.4.1 Mortality of *Plutella xylostella* at second instar stage

The mean percentage mortality of *Plutella xylostella* second instar larvae was significantly different for tebuconazole ($F_{(3, 88)} = 117.752$, $p = 0.000$) (Fig. 5a). This difference in mortality however, was only significant between control and the three-tebuconazole treatments, while mortality among the three concentrations of tebuconazole had no significant difference. This means the lowest concentration of tebuconazole was as toxic as its manufacturers recommended application rate on second instars. Similarly, mortality of *Plutella xylostella* second instar larvae was significantly affected by increase in exposure time ($F_{(1, 88)} = 5.613$, $p = 0.018$).

2.3.4.2 Mortality of *Plutella xylostella* at third instar stage

The third instar larvae also showed a significant difference in mortality with increase in concentration of tebuconazole ($F_{(3, 88)} = 90.866$, $p = 0.000$) (Fig. 5b). The effect was such that mortality was significantly lowest in control. Among the three concentrations of tebuconazole however, mortality of larvae with 0.5 mlL^{-1} and 1 mlL^{-1} tebuconazole had no significant difference as well as between 1 mlL^{-1} and 5 mlL^{-1}. The mortality of larvae on 5 mlL^{-1} was significantly greater than 0.5 mlL^{-1}. Likewise, there was a significant effect of exposure time on mortality of third instar larvae ($F_{(1, 88)} = 6.126$, $p = 0.013$). The mortality of larvae raised significantly with increasing exposure time to tebuconazole.

2.3.4.3 Mortality of *Plutella xylostella* at fourth instar stage

Similar to the two early developmental stages, the mean percentage mortality of fourth instar larvae showed a significant difference over the four treatments including control ($F_{(3, 88)} = 56.520$, $p = 0.000$) (Fig. 5c). Mortality for fourth instar larvae remained similar within the three concentrations of tebuconazole. It was however, significantly greater than control group larvae. The effect of exposure time on the other hand, was not significant ($F_{(1, 88)} = 2.098$, $p = 0.148$) as the larvae experienced only a slight rise in the mortality over time.

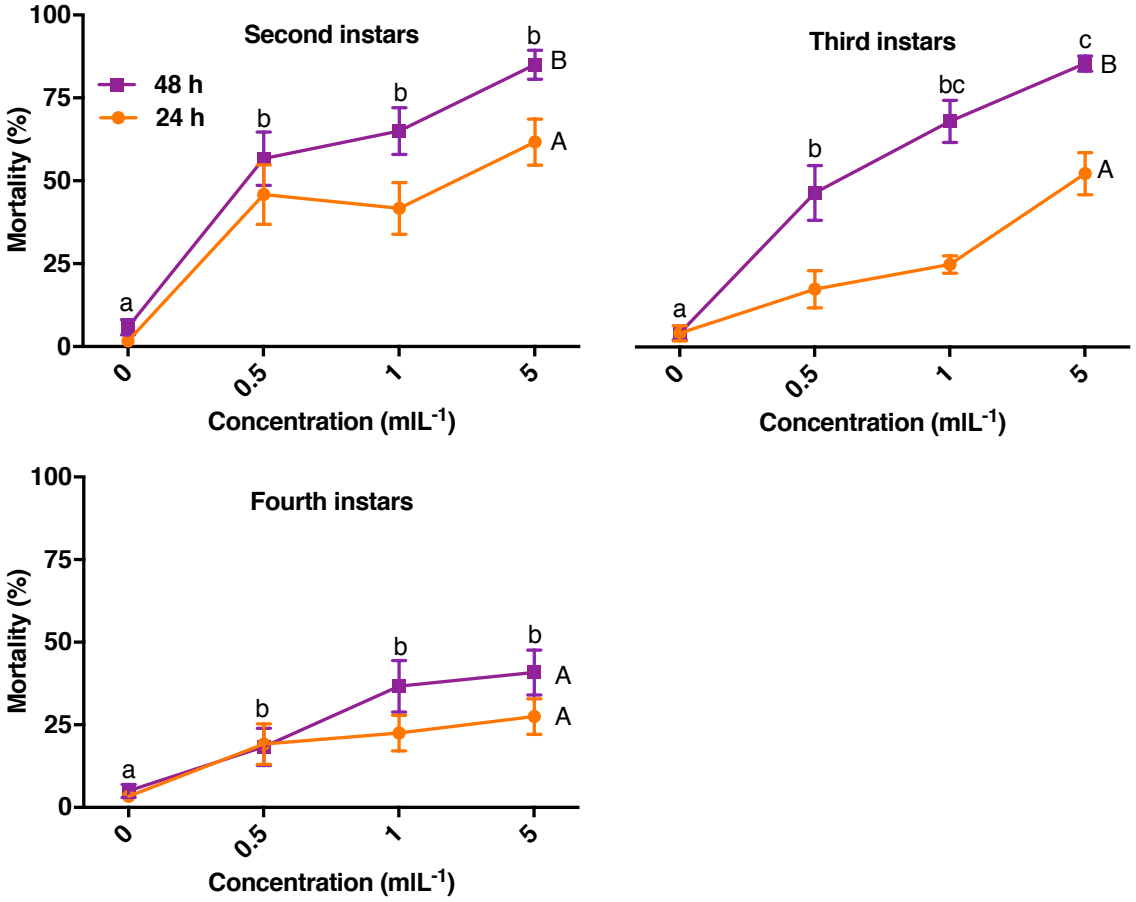

Figure 5 Effect of tebuconazole on mean mortality of three developmental stages of *Plutella xylostella*. Error bars sharing the same lowercase letters show no significant difference in mortality over the four treatments for an instar group ($p > 0.05$). Similar uppercase letters between the two lines show no significant difference of exposure time ($p > 0.05$).

2.3.5 Difference between mortality of three developmental stages of *Plutella xylostella* over time on tebuconazole

Similar to acetamiprid, mortality rate for fourth instar larvae of *Plutella xylostella* on tebuconazole was lowest among the three developmental stages. In 24 h of exposure, the difference in mortality rate was not significant ($F_{(2, 136)} = 5.808$, $p = 0.055$) (Fig. 6a). In contrast, the mortality rate of three developmental stages was significantly different in 48 h exposure ($F_{(2, 136)} = 10.542$, $p = 0.005$) (Fig. 6b). Here, second and third instars had no significant difference in their mortality rates however; mortality rate of fourth instars was significantly lower than the two younger developmental stage larvae.

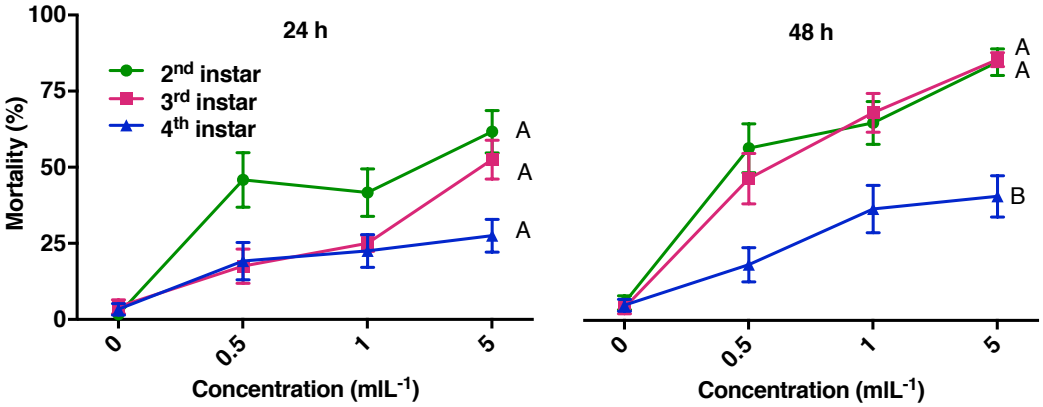

Figure 6 Summary of mean mortality rate of three developmental stages of *Plutella xylostella* on tebuconazole over time. a) 24 h exposure time b) 48 h exposure time. Similar uppercase letters across the three lines show no significant difference in the mortality rate of the three instar groups ($p > 0.05$).

2.3.6 Effect of tebuconazole on mortality of non-target insect, *Chrysoperla carnea*

Tebuconazole was found toxic to lacewing larvae. The mortality rate of green lacewings was significantly different with tebuconazole treatments ($F_{(3, 88)} = 34.571$, $p = 0.000$) (Fig. 7). Highest mortality of lacewings larvae was observed with the solution containing 5 mlL^{-1} of tebuconazole followed by 1 mlL^{-1} and 0.5 mlL^{-1}. The pairwise comparison showed the difference in mortality although increased with concentration, remained only significantly different between control group and those treated with 0.5mlL^{-1}, 1mlL^{-1} and 5 mlL^{-1}. On the other hand, increase in exposure time to tebuconazole had no significant effect on mortality of *Chrysoperla carnea* larvae ($F_{(1, 88)} = 3.003$, $p = 0.083$). This means toxicity of tebuconazole remained almost unchanged and did not increase over exposing the insect for a greater period.

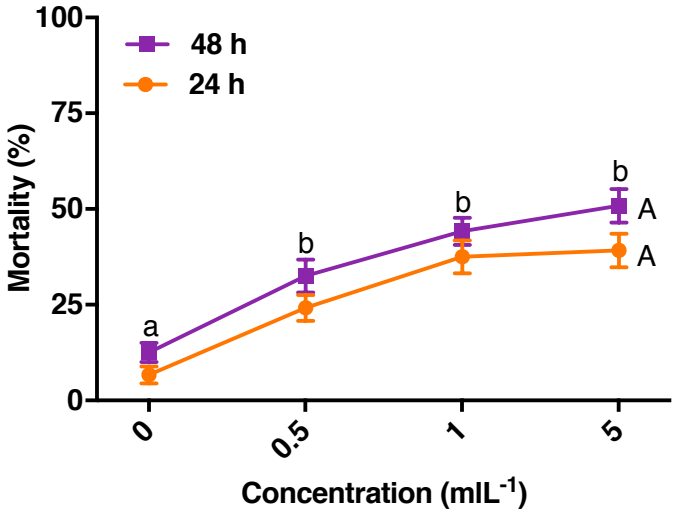

Figure 7 Effect of tebuconazole on mean mortality of *Chrysoperla carnea*. Error bars sharing the same lowercase letters show no significant difference in mortality over the four treatments ($p > 0.05$). Similar uppercase letters between the two lines show no significant difference of exposure time ($p > 0.05$).

2.3.7 Effect of mixtures of tebuconazole and acetamiprid on mortality of target insect, *Plutella xylostella*

2.3.7.1 Mortality of *Plutella xylostella* at second instar stage

Mortality of second instar larvae of *Plutella xylostella* varied significantly with different mixtures of tebuconazole and acetamiprid ($F_{(3, 88)} = 119.491$, $p = 0.000$) (Fig. 8a). Pairwise comparison showed a significant difference between control and the three mixtures ($p < 0.05$). Similarly, a significantly high mortality of larvae was observed with mix3 containing the highest level of acetamiprid (0.5 + 0.25) than the other two mixtures. In addition, there was also a significant effect of exposure time on their mortality which means the mortality rate of the larvae significantly increased after 48 h ($F_{(1, 88)} = 10.215$, $p = 0.001$).

2.3.7.2 Mortality of *Plutella xylostella* at third instar stage

For third instar larvae the mean percentage mortality at different concentrations of the mixture was significantly different ($F_{(3, 88)} = 103.52$, $p = 0.000$) whereas, no significant effect on their mortality was found with respect to exposure time ($F_{(1, 88)} = 2.776$, $p = 0.096$) (Fig. 8b). The difference in mortality among the treatments was such that mix3, which contained the highest concentration of acetamiprid, showed a significantly greater mortality than mix2 and mix1 which were made up of 5 times and 10 times lower acetamiprid concentrations respectively. Moreover, mortality rate of larvae exposed to mix2 was significantly higher than mix1 and control. However, mix1 showed a significantly greater mortality rate than control only.

2.3.7.3 Mortality of *Plutella xylostella* at fourth instar stage

The mean percentage mortality of fourth instar larvae at 24 h and 48 h was significantly different at different concentrations of mixture ($F_{(3, 88)} = 59.055$, $p = 0.000$) and exposure time ($F_{(1, 88)} = 15.889$, $p = 0.000$) (Fig. 8c). This means mortality rate increased with increase in exposure time to the mixtures. Moreover, at fourth instar stage, mortality rate of mix3 was significantly greater than all other treatments. On the other hand, mix2 showed a significantly higher mortality rate than mix1 and control only. No significant difference in mortality rate of control and mix1 existed for fourth instar larvae.

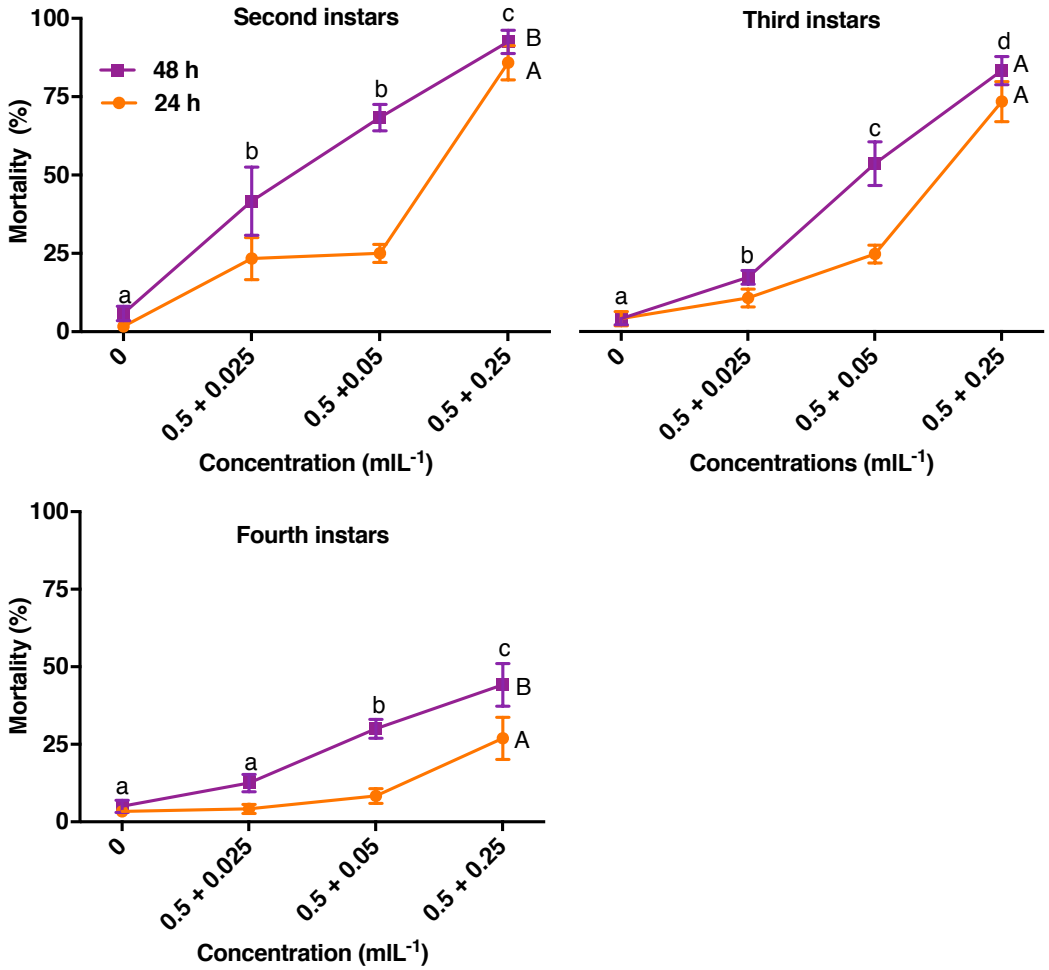

Figure 8 Effect of mixtures of acetamiprid and tebuconazole on mean mortality rate of three developmental stages of *Plutella xylostella*. Error bars sharing the same lowercase letters show no significant difference in mortality over the four treatments for an instar group ($p > 0.05$). Similar uppercase letters between the two lines show no significant difference of exposure time ($p > 0.05$).

2.3.8 Difference between mortality of three developmental stages of *Plutella xylostella* over time on mixtures

In 24 h, the difference in mortality rate between the three developmental stages of *Plutella xylostella* was significant ($F_{(2, 136)} = 20.817$ $p = 0.000$) (Fig. 9a). Similarly, mortality rate of the three instar groups was also significantly different after 48 h of exposure to the mixtures ($F_{(2, 136)} = 12.359$ $p = 0.002$) (Fig. 10b). The pattern of mortality was such that no difference in mortality rate of second and third instars was observed whereas, the mortality rate of fourth instar was significantly lower than second and third instar larvae, both after 24 h and 48 h.

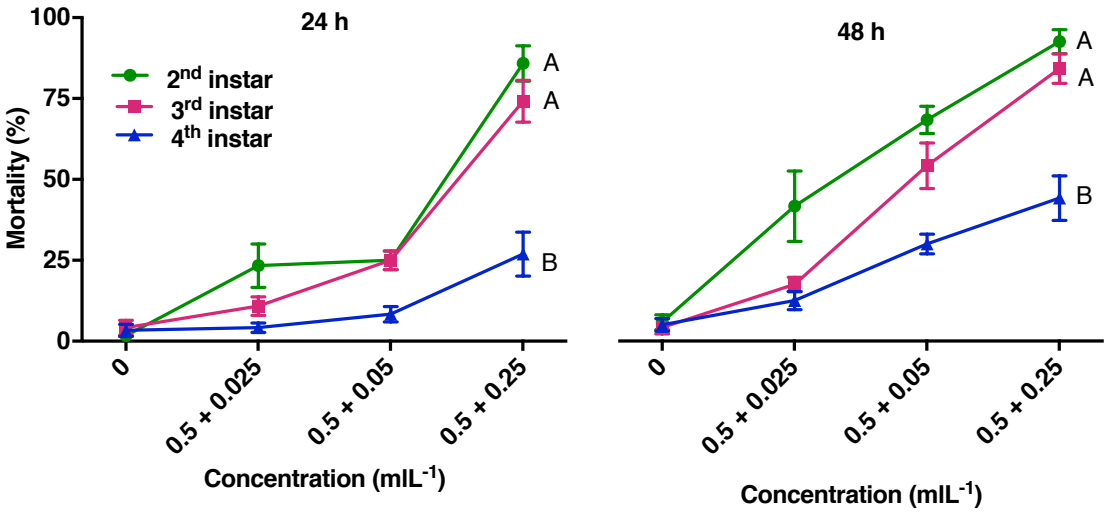

Figure 9 Summary of mean mortality rate of three developmental stages of *Plutella xylostella* on mixtures of tebuconazole and acetamiprid over time. a) 24 h exposure time b) 48 h exposure time. Similar uppercase letters across the three lines show no significant difference in the mortality rate of the three instar groups ($p > 0.05$).

2.3.9 Effect of mixtures of tebuconazole and acetamiprid on mortality of non-target insect, *Chrysoperla carnea*

In case of mixtures, the results showed that, mortality was dependent on concentrations of mixtures as well as exposure time. The larvae of *Chrysoperla carnea* showed a significantly increased mortality with increase in time of exposure ($F_{(1, 88)} = 4.299$, $p = 0.038$). Similarly, the mortality rate with the four treatments was also significantly different ($F_{(3, 88)} = 33.055$, $p = 0.000$). This significant difference however, was only caused by control. This means mortality caused by three mixtures was significantly high than control only and it remained non-significant among them. Consequently, it can be said that increase of acetamiprid concentration in mixtures had no significant effect on mortality rate (Fig. 10).

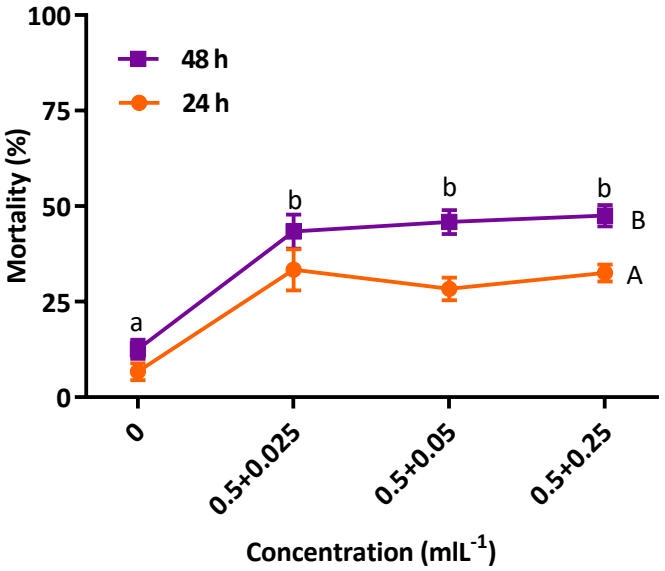

Figure 10 Effect of mixtures of acetamiprid and tebuconazole on mean mortality of *Chrysoperla carnea*. Error bars sharing the same lowercase letters show no significant difference in mortality over the four treatments ($p > 0.05$). Similar uppercase letters between the two lines show no significant difference of exposure time ($p > 0.05$).

2.3.10 Estimation of LC_{50}

LC_{50} is a term used to describe acute toxicity of a chemical where LC means lethal concentration and the subscript 50 indicates the concentration which is acutely fatal to 50% of the population of test organisms to whom the chemical was administered under controlled laboratory conditions. In other words, LC_{50} represents statistically derived single dose of a chemical that can kill 50% of animals under experimental conditions. The route of chemical exposure may be oral, skin contact, intravenous or inhalation, depending on the experimental system. LC_{50} shows the relationship between the dose of the chemical and the most extreme death response. Lower LC_{50} value indicates a higher toxicity of the chemical which in turn means a smaller quantity of the cehimcal is required to kill the organism and vice versa (Raj et al., 2013).

2.3.11 Effect of acetamiprid on LC_{50} of *Plutella xylostella* and *Chrysoperla carnea*

Acetamiprid was found significantly more toxic ($p < 0.01$; non-overlapping 95% FL) to *Plutella xylostella* than *Chrysoperla carnea* after 24 h as well as 48 h of exposure of the insects as evident from the difference in their LC_{50} values (Table 3). Moreover, toxicity of acetamiprid for *Plutella xylostella* developmental stages increased significantly with time as shown by the decline in their LC_{50} values over 48 h exposure. Furthermore, LC_{50} of third and fourth instar larvae was comparable to second instar larvae after 48 h. Among the three developmental stages of *Plutella xylostella* however, after 24 h of exposure, no significant difference was observed in their LC_{50}

values. On the other hand, after 48 h, LC_{50} of second instar remained significantly lower than the other two developmental stages.

Table 3 LC_{50} of *Plutella xylostella* and *Chrysoperla carnea* on acetamiprid after 24 h and 48 h exposure to acetamiprid.

Insect		**24 h** **LC_{50} (95% FL)**	**48 h** **LC_{50} (95% FL)**
Plutella xylostella	Instar 2	0.237^{ac} (0.208 – 0.270)	0.168^{b} (0.144 – 0.196)
	Instar 3	0.455^{a} (0.296 - 0.902)	0.247^{c} (0.216 - 0.285)
	Instar 4	1.073^{a} (0.586 - 2.949)	0.304^{c} (0.267 - 0.350)
Chrysoperla carnea		6.797^{d} (4.857 - 12.416)	4.807^{d} (3.685 - 7.628)

Values across the columns sharing the same lowercase letter have overlapping 95% FL and are not significantly different at $p > 0.01$.

2.3.12 Effect of tebuconazole on LC_{50} of *Plutella xylostella* and *Chrysoperla carnea*

Toxicity of tebuconazole for *Chrysoperla carnea* and second and third instars of *Plutella xylostella* was comparable due to their non-significant LC_{50} values in 24 h of exposure ($p > 0.01$; non-overlapping 95% FL) (Table 4). Likewise, no significant difference was found in LC_{50} of *Chrysoperla carnea* on tebuconazole at 24 h and 48 h of exposure period.

On the other hand, LC_{50} values of the three developmental stages of *Plutella xylostella* decreased significantly over time from 24 h to 48 h showing the aggravated toxicity of tebuconazole with rise in exposure time. Within 24 h however, no significant difference in LC_{50} of the three developmental stages was found. In contrast, after 48 h, LC_{50} value of fourth instar larvae was significantly greater than second and third instar larvae indicating the minimum toxicity of acetamiprid against older larvae. Similarly, LC_{50} of fourth instar after 48 h was comparable to that of *Chrysoperla carnea*.

Table 4 LC_{50} of *Plutella xylostella* and *Chrysoperla carnea* on tebuconazole after 24 h and 48 h exposure to tebuconazole.

Insect		**24 h LC_{50} (95% FL)**	**48 h LC_{50} (95% FL)**
Plutella xylostella	Instar 2	3.627^{ac} (2.665 - 4.866)	1.655^{b} (0.986 - 2.310)
	Instar 3	5.153^{acd} (4.034 - 6.801)	1.828^{b} (1.163 - 2.493)
	Instar 4	6.701^{d} (5.335 - 8.860)	4.369^{c} (3.665 - 5.286)
Chrysoperla carnea		5.381^{d} (3.448 - 17.079)	2.738^{cd} (1.996 - 4.902)

Values across the columns sharing the same lowercase letter have overlapping 95% FL and are not significantly different at $p > 0.01$.

2.3.13 Effect of mixtures of tebuconazole and acetamiprid on LC_{50} of *Plutella xylostella* and *Chrysoperla carnea*

In case of mixtures of acetamiprid and tebuconazole, toxicity for *Plutella xylostella* was significantly higher (lower LC_{50} values) as compared to *Chrysoperla carnea* over the entire exposure period ($p < 0.01$; non-overlapping 95% FL) (Table 5). This means the addition of 10 times lower than recommended quantity of tebuconazole to acetamiprid makes the mixture significantly more potent against the target insect *Plutella xylostella* as compared to non-target *Chrysoperla carnea*. Furthermore, the LC_{50} values of *Plutella xylostella* second instars after 24 h was within the range of mix2 (0.5 + 0.05) and mix3 (0.5 + 0.25), whereas, at 48 h it was almost closer to mix1 which contained (0.5 + 0.025) ml/L of tebuconazole and acetamiprid. Likewise, in case of third instars the LC_{50} at 24 h and 48 h, range between mix2 and mix3. On the contrary, for fourth instar larvae LC_{50} exceeded the range of all mixtures. Moreover, a significant difference in LC_{50} of third and fourth instar stages was observed over the exposure time of 24 h and 48 h however, it was not the case for second instar larvae.

Table 5 LC_{50} of *Plutella xylostella* and *Chrysoperla carnea* on mixtures after 24 h and 48 h exposure to mixtures of tebuconazole and acetamiprid.

Insect		**24 h** **LC_{50} (95% FL)**	**48 h** **LC_{50} (95% FL)**
Plutella xylostella	Instar 2	0.672^{abc} (0.550 - 0.830)	0.489^{b} (0.414 - 0.559)
	Instar 3	0.742^{a} (0.603 - 0.908)	0.609^{b} (0.539 - 0.682)
	Instar 4	0.942^{a} (0.782 - 1.273)	0.785^{c} (0.709 - 0.877)
Chrysoperla carnea		4.070^{d} (3.397 - 5.176)	2.758^{d} (2.287 - 3.439)

Values across the columns sharing the same lowercase letter have overlapping 95% FL and are not significantly different at $p > 0.01$.

2.3.14 Maximum efficacy and difference in mortality rate of target and non-target insects, based on concentrations of pesticides

The top five differences in the mortality rate of *Chrysoperla carnea* larvae existed with second and third instars of *Plutella xylostella* (Table 8). This was such that highest efficacy against target insect was demonstrated by mix3 at its second instar stage which resulted in a maximum difference in the mortality rate of the target and non-target insect after 24 h and 48 h. This was followed by the difference in mortality rate of *Chrysoperla carnea* larvae and third instars of *Plutella xylostella* on mix3 in 24 h and 48 h and second instar with mix2 after 48 h. Moreover, with one exception of teb3, the mortality rate of *Chrysoperla carnea* remained below 50% regardless of the chemical treatments, whereas, in *Plutella xylostella* it reached up to as much as 92.5% (Table 7).

2.3.15 Maximum efficacy and difference in mortality rate of target and non-target insects, based on developmental stages of insects

Maximum efficacy and difference in mortality rate was found between *Chrysoperla carnea* larvae and second instar larvae of *Plutella xylostella* with acetamiprid, tebuconazole and their mixtures (Table 9, 10). Second instar larvae are rapacious consumers of foliage (Talekar and Shelton, 1993). Their nonstop feeding on pesticides treated leaf discs, as well as the contact toxicity of pesticides together, may be the factors contributing to their higher mortality rate. Similarly, this difference in mortality rate and therefore, the efficacy of these pesticides and mixtures, declined as the larvae of *Plutella xylostella* progressed through their life stages (Table 6 and 7). For instance, relative to fourth instar larvae of *Plutella xylostella*, the mortality count of *Chrysoperla carnea* was always high regardless of the chemical treatment.

Table 6 Mean percentage mortality of *Plutella xylostella* and *Chrysoperla carnea* after 24 h of exposure to different concentrations of acetamiprid, tebuconazole and their mixtures.

Treatments	**Percentage mortality**			
	Plutella xylostella			***Chrysoperla carnea***
	2nd instar	**3rd instar**	**4th instar**	
Acet1 (0.025gL^{-1})	5.83	5.00	0.00	7.50
Acet2 (0.05gL^{-1})	8.33	11.67	1.67	11.67
Acet3 (0.25gL^{-1})	51.67	41.67	11.67	28.33
Teb1 (0.5 mlL^{-1})	45.83	17.50	19.17	24.17
Teb2 (1 mlL^{-1})	41.67	25.00	22.50	37.50
Teb3 (5 mlL^{-1})	61.67	52.50	27.50	39.17
Mix1 (0.5 + 0.025)	23.30	10.83	4.17	33.30
Mix2 (0.5 + 0.05)	25.00	25.00	8.33	28.33
Mix3 (0.5 + 0.25)	85.83	74.17	26.91	32.50
Control (0)	1.67	4.17	3.33	6.67

Table 7 Mean percentage mortality of *Plutella xylostella* and *Chrysoperla carnea* after 48 h of exposure to different concentrations of acetamiprid, tebuconazole and their mixtures.

Treatments	**Percentage mortality**			
	Plutella xylostella			***Chrysoperla carnea***
	2nd instar	**3rd instar**	**4th instar**	
Acet1 (0.025gL^{-1})	5.83	5.00	3.33	11.67
Acet2 (0.05gL^{-1})	21.67	15.83	11.67	19.17
Acet3 (0.25gL^{-1})	77.50	52.50	23.33	38.33
Teb1 (0.5 mlL^{-1})	56.67	46.67	19.17	32.50
Teb2 (1 mlL^{-1})	65.00	68.33	36.67	44.17
Teb3 (5 mlL^{-1})	85.00	85.83	40.83	50.83
Mix1 (0.5 + 0.025)	41.67	17.50	12.50	43.33
Mix2 (0.5 + 0.05)	69.33	54.17	30.00	45.83
Mix3 (0.5 + 0.25)	92.50	84.17	44.17	47.50
Control (0)	5.83	4.17	5.00	12.00

Table 8 Top five mixtures of acetamiprid and tebuconazole for maximum efficacy against the target insect.

Mixtures of acetamiprid and tebuconazole	**Mean mortality (%)**	**Developmental stage of *Plutella xylostella***	**Exposure time**
Mix3	92.5	Second instar	48 h
Mix3	85.5	Second instar	24 h
Mix3	84.8	Third instar	48 h
Mix3	74.8	Third instar	24 h
Mix2	69.3	Second instar	48 h

2.3.16 Maximum efficacy and difference in mortality rate of target and non-target insects, based on exposure time to the pesticides

The effect of time was such that in 24 h, *Plutella xylostella* showed a greater number of deaths as compared to *Chrysoperla carnea*, eventually giving rise to a relatively greater difference in 24 h (Table 9). Over time however, mortality rate of *Chrysoperla carnea* also increased which led to a drop in the mean difference between the two insects' mortality rate after 48 h. For instance, the highest maximum difference between *Chrysoperla carnea* and second and third instar larvae of

Plutella xylostella with mix3 in 24 h declined after 48 h. This was because the mortality rate of second and third instar DBM larvae increased from 85.83% to 92.50% and 74.17% to 84.17% between 24 h and 48 h respectively. Whereas, the rise in mortality rate of *Chrysoperla carnea* larvae was from 32.50% to 47.50% (Table 6 and 7).

Table 9 Top five mixtures of acetamiprid and tebuconazole based on maximum difference in mortality rate of the target and non-target insects.

Mixtures of acetamiprid and tebuconazole	**Mean difference in mortality (%)**	**Developmental stage of *P. xylostella* larvae at which the difference lies w. r. to *Chrysoperla carnea***	**Exposure time**
Mix3	53.3%	Second instar	24 h
Mix3	45.0%	Second instar	48 h
Mix3	43.3%	Third instar	24 h
Mix3	36.7%	Third instar	48 h
Mix2	22.5%	Second instar	48 h

Table 10 Top five mixtures of acetamiprid and tebuconazole with lowest mortality rate in the non-target insect and their effect on mortality rate of three instar stages of *Plutella xylostella*.

Mixtures of acetamiprid and tebuconazole	**Mean mortality of *Chrysoperla carnea* (%)**	**Exposure time**	**Mean mortality of *Plutella xylostella* (%)**		
			2nd instar	**3rd instar**	**4th instar**
Mix2	28.3	24	25	25	8.3
Mix3	32.5	24	85.8	74.5	26.9
Mix1	33. 3	24	23.3	10.8	4.17
Mix1	43.3	48	41.7	17.5	12.5
Mix2	45. 8	48	69.3	54.2	30

2.4 Discussion

The results of this study revealed a significant increase in mortality of insects' larvae with an increase in the concentration of the pesticides. Mortality rate was significantly higher in *Plutella xylostella* as revealed from the lower LC_{50} values than *Chrysoperla carnea* larvae. In addition to the toxicity of mixtures, the lower LC_{50} values of larvae with tebuconazole (below its recommended application rate) shows its toxicity to both *Plutella xylostella* and *Chrysoperla carnea* larvae under this study.

2.4.1 Target toxicity of acetamiprid

In this study, the field relevant concentration of acetamiprid demonstrated a significantly high mortality in second instars of *Plutella xylostella* larvae in 24 h and 48 h of exposure while fourth instars exhibited the lowest mortality rate. The LC_{50} values were significantly lower which also reduced significantly with increase in exposure time. In addition, the LC_{50} at 24 h was the same as its recommended application rate, however it went down after 48 h. These results suggests the increase in toxicity of acetamiprid over time as well as their efficacy against early developmental stages of the pest. These results are in line with Wallingford et al. (2012), who found LC_{50} values of imidacloprid, dinotefuran and clothianidin lower than their authorised application rate against *Murgantia histrionica* (Harlequin bug), an important pest of Cole crop. In contrast, Rodriguez-Saona et al. (2016) reported acetamiprid and thiamethoxam less effective against larval stages of *Choristoneura parallela and Sparganothis sulfureana* (two key Lepidopteran pests of cranberries), but effective against adults of these two pests. Likewise, contrasting to our results, Hill and Foster (2000) also reported a significantly lower mortality of *Plutella xylostella* larvae on field relevant dose of imidacloprid after exposure time of 24 h to 72 h as compared to spinosad and permethrin.

2.4.2 Non-target toxicity of acetamiprid

Acetamiprid was found significantly less toxic to *Chrysoperla carnea* larvae compared to *Plutella xylostella* larvae, such that the LC_{50} remained significantly above its application rate for this non-target insect. The lower non-target toxicity of neonicotinoids has been reported by Shankarganesh et al. (2016). By assessing the effect of different groups of pesticides on various developmental stages of green lacewing, they found thiamethoxam the least toxic to lacewings (*Chrysoperla zastrowi sillemi*) due to maximum number of pupation attained in larvae exposed to it. In addition, low toxicity of acetamiprid can be explained with the rapid metabolism of cyano-group neonicotinoids by insects (Iwasa et al., 2004; Manjon et al., 2018). Moreover, *Chrysoperla carnea* was exposed to contact toxicity, while *Plutella xylostella* fed on the treated leaf discs along

with its contact exposure, which is why mortality rate in target insect may be higher than non-target insect. Furthermore, Cloyd and Dickinson (2006) reported acetamiprid, clothianidin and dinotefuran highly toxic to *Cryptolaemus montrouzieri* adults (natural enemy of citrus mealy bug, *Planococcus citri*). With these three insecticides, mortality rate was high (up to 70%) as compared to pyriproxyfen, buprofezin and bonicamid, whose toxicity was much less i. e., up to 20% mortality after 48 h. In the same way, oral introduction of acetamiprid at the rate of 200 mgL^{-1} in water led to as much as 90% mortality in adult ladybirds (*Eriopis connexa*), after a period of 15 days. On the other hand, dropping the exposure rate of acetamiprid to half, caused only 15% mortality in pupae. However, 83% of the newly emerged adults were reported to have numerous abnormalities (Fogel et al., 2016). Likewise, in a semi- field experiment, Khan et al. (2015) have reported 80% mortality of *Trichogramma pretiosum*, an egg parasitoid, when exposed to acetamiprid at the rate of 429 ppm. In a similar way, mortality rate of adult *Tamarixia radiate* (parasitoid of *Diaphorina citri*) was 66% after three days of exposure to paper disc treated with 60 ppm of acetamiprid (Beloti et al., 2015). Bhojani et al. (2018) reported thiacloprid (a cyano-group neonicotinoid) and imidacloprid equally toxic to *Chrysoperla zastrowi sillemi* after 48 h of exposure. The differences in results from the current study and previous findings may be due to factors like variation in exposure method, life stage at which an insect is exposed to a certain chemical, dose of chemicals to which the insect were exposed as well as the strength of chemical formulations and structural differences of the pesticides formulations.

2.4.3 Target toxicity of tebuconazole

For *Plutella xylostella* larvae, a significant difference in mortality was found between control and the three concentrations of tebuconazole. No statistically significant difference in mortality however, was found among the three concentrations of tebuconazole. The LC_{50} of second instars over the entire exposure period as well as for third and fourth instars after 48 h of exposure, was below its manufacturer recommended rates. This means that tebuconazole was highly toxic to insects at its recommended application rate. Tebuconazole and other fungicides are primarily used against fungal infestations while their mechanism of action in insect bodies has not been extensively studied. However, triazole fungicides are known to disrupt Cytochrome P450 monooxygenase enzymes in insects. These enzymes, along with insecticides detoxification, are also necessary to detoxify phytochemicals such as flavonol quercetin. The inability of insects to efficiently metabolize these types of phytochemicals, found in their natural diet, in the presence of triazole fungicides may lead to a reduced ATP production (Mao et al., 2017). Thus lack of adequate energy to carry out normal functions like locomotion, foraging and escaping the unfavourable conditions may be the factors contributing to the high toxicity of tebuconazole.

A toxic effect of tebuconazole has been documented in aquatic organisms by Sancho et al. (2010). They reported a reduction in energy content, feeding rate and metabolic functions of *Daphnia magna* after exposure to as low as 0.52 mgL^{-1} of tebuconazole. Likewise, Shentu et al. (2019) demonstrated that propiconazole injections to *Nilaparvata lugen* (rice brown planthopper) can lead to reduced survival and fecundity of these insects by affecting the yeast-like symbiotic organisms in their abdomen. They suggested that by inhibiting these symbiotes under the effect of propiconazole, rice crop can be protected from *Nilaparvata lugens* infestation.

2.4.4 Non-target toxicity of tebuconazole

In *Chrysoperla carnea*, no significant difference in the mortality rate was observed with three concentrations of tebuconazole however, it was significantly greater relative to control. Moreover, the LC_{50} of tebuconazole was slightly more than its authorised application rate at 24 h but it lowered down after 48 h. The high mortality of insects found in the current study could be due to the disturbance in metabolism caused by the toxicant, which could lead to lack of homeostasis, eventually resulting in death of the insects. Additionally, feeding on prey (in this study the green aphids) that come in contact with fungicide on treated leaf discs could be another possible reason of this higher mortality rate of the insect larvae with tebuconazole. These results are in line with the findings of Ostyn (2017) who reported a significantly higher mortality of bumble bees upon oral exposure to tebuconazole contaminated food sources. Likewise, Tomé et al. (2017) showed that although commercial fungicides, chlorothalonil and thiophanate-methyl individually caused low mortality, their combination was as toxic as imidacloprid to *Apis mellifera* and *Partamona helleri* in their study. Similarly, Ladurner et al. (2005) also showed acute oral toxicity of propiconazole (a demethylation inhibitor fungicide) to *Osmia lignaria* (33.30 µg a.i./bee) and *Apis mellifera* (57.25 µg a.i./bee). Bernauer et al. (2015) exposed the colonies of bumblebees (*Bombus impatiens*) to chlorothalonil fungicide. Their results showed a reduced total bee biomass, smaller number of workers and lighter weight queens in the fungicide treated flower fed colonies than control. From their findings, they suggested that fungicides could have severe negative impact on insect foraging and colony success. Delpuech and Allemand (2011) from their study revealed a decreased abundance of fruit fly (*Drosophilla melanogaster*) and its parasitoids in high fungicide treated orchid than the orchid, which received low amount of fungicides. Likewise, a recent study by Chen et al. (2018) showed a higher contact toxicity of tebuconazole to earthworm, *Eisenia fetida*. All these studies confirm the toxicity of fungicides to different classes of terrestrial as well as soil fauna in different ways.

2.4.5 Target toxicity of mixtures

Toxicity of mixtures increased significantly to *Plutella xylostella* with increase in concentration of acetamiprid in mixtures as revealed from high mortality rates and lower LC_{50} values. In 24 h, the LC_{50} of larvae of *Plutella xylostella* was between the range of mix2 and mix3. This means the mixtures with high level of acetamiprid was effective against larvae in first 24 h. However, with increase in exposure period, the mixture containing lower concentration of acetamiprid became equally toxic as the LC_{50} for larvae shifted between mix1 and mix2.

2.4.6 Non-target toxicity of mixtures

Mortality of *Chrysoperla carnea* was not significantly affected by the three binary mixtures of acetamiprid and tebuconazole however, The LC_{50} for *Chrysoperla carnea* larvae was significantly above that of *Plutella xylostella.* Moreover, during the whole exposure time, it was above the range of mixtures applied to them under current experiments. In other words, the toxicity of mixtures was far less for non-target insect under this study. These results are in line with findings of Ostyn (2017) who also reported a lower toxicity of combination of thiacloprid and tebuconazole to bumble bees. These results suggest that the mixtures can be used effectively against pests species with minimum harm caused to non-target green lacewings.

In addition, the resulting mortality rate with mixtures was although higher for both *Chrysoperla carnea* and *Plutella xylostella* larvae, but still lower than the sum total effect of individual applications of the two. Thus, the combination of the two pesticides was not indicating any synergism or additive effect. This may be because acetamiprid is a cyano-guanidine neonicotinoid that are rapidly metabolized by insects (Mommaerts et al., 2010) as compared to nitro group neonicotinoids and the concentration of tebuconazole in mixtures was kept constant while change was made in acetamiprid concentration. Thus, rapid metabolism of one component of mixture could possibly leave little room for its enhanced toxicity. Moreover, cyano-group neonicotinoids have been reported as least toxic (Ostyn, 2017; Mommaerts et al., 2010), as compared to nitro group neonicotinoids, which are highly toxic to insects even in their individual applications. For instance, Tomé et al. (2017) demonstrated high synergies of the individual mixtures of imidacloprid and deltamethrin with thiophanate-methyl and chlorothalonil fungicide against *Partamona helleri* and *Apis mellifera*. Furthermore, they also showed the combination of the two fungicides equally toxic as imidacloprid for both the species and more than 400 times more toxic than deltamethrin for honey bees. Thompson et al. (2014) also reported an increased toxicity of clothianidin with increasing dose of fungicide. Furthermore, Zhu et al. (2017) studied the synergistic toxicity of imidacloprid separately, as well as in combination with seven other

pesticides that represented different chemical groups, in honey bees (*Apis mellifera*). In their study, they found a significant increase in toxicity of imidacloprid when mixed with Domark, (active ingredient = tetraconazole). Tetraconazole belongs to triazole family of fungicides, which are demethylation inhibitors (DMI). Taillebois and Thany (2016) from their study demonstrated similar results of enhanced additive and synergistic toxicities of various combinations of four insecticides acetamiprid, deltamethrin, chlorpyrifos and fipronil on pea aphid*, Acyrthosiphon pisum*.

2.4.7 Effects of exposure time on mortality of target and non-target insect

An increase in exposure time to pesticides showed a significant effect on toxicity of pesticides. For instance, LC_{50} values of *Plutella xylostella* larvae were significantly different after 24 h and 48 h when exposed individually to acetamiprid, tebuconazole and its mixtures. On the other hand, change in exposure time had no effect on LC_{50} of *Chrysoperla carnea* on acetamiprid and tebuconazole alone as well as their mixtures. Overall, however, the toxicity of the pesticides and their mixtures was significantly high to target insect than non-target. For second instar of *Plutella xylostella,* the LC_{50} after 24 h was between the range of mix2 and mix3, which contained 10 times lower than field recommended rate of tebuconazole and 5 times lower and field recommended application rate of acetamiprid respectively. At 48 h, its LC_{50} declined and reached between mix1 and mix2 which contained 10 and 5 times low rates of acetamiprid. The increased toxicity with exposure time is consistent with previous findings. Hill and Foster (2000) found an increased mortality of *Plutella xylostella* larvae with permethrin and spinosad after 4, 8, 24 and 48 h of leaf dip bioassay treatments, as compared to imidacloprid. In addition, they also found an increase in mortality of imidacloprid to *Diadegma insulare* (a common predator of *Plutella xylostella*) after 24 h as compared to mortalities after 30 mins and 8 h. For example, Suchail et al. (2001) in their study on *Apis mellifera* also reported a reduction in LC_{50} value of imidacloprid from 60 ng/bee at 48 h to 40 ng/bee after 72 h and 96 h of exposure. Similarly, Ladurner et al. (2005) in their study showed a reduction in LD_{50} for the fungicides used in their study. For instance in contact toxicity test, LD_{50} of dimethoate reduced from 1.96 in 24 h to 1.02 (µg a.i./bee) at 72 h. Likewise, Mullin et al. (2010) exposed 18 different carabid species to corn seedlings treated with field-relevant doses of either imidacloprid, thiamethoxam or clothianidin. For all the species, mortality was initially low but reached to as much as 100% on exposure for 4 days.

2.4.8 Differences in Target and non-target mortality

The difference observed in percentage mortality of target and non-target insects suggest that susceptibility of one species may differ from the other (DiBartolomeis et al., 2019), which might

be linked to their differential metabolic system involved in detoxification of these xenobiotics. In line with these findings, Manjon et al. (2018) reported distinctive susceptibility of honey bees and bumble bees to various neonicotinoids like imidacloprid and thiacloprid, the foundation of which was the distinctive mechanism for detoxifying these neonicotinoids. However, intra-specific difference in toxicities of neonicotinoids have also been reported. For instance, Colgan et al. (2019) reported a stronger effect of imidacloprid and clothianidin on worker bees as compared to queens in colonies of bumble bees (*Bombus terrestris*). Nasreen et al. (2003) demonstrated the selectivity of some important commercial insecticides to *Chrysoperla carnea* that included abamectin, indoxacarb, chlorfenapyr, endosulfan, profenofos, spinosad and *Bacullis thuringiensis*. Of these, indoxacarb and profenofos were found extremely toxic, causing > 90% mortality while the rest were found safe as they caused less than 50% mortality in *Chrysoperla carnea*. Likewise, these differential toxicities may also be influenced by the degree of their penetration as well as persistence in the insect bodies (Nauen et al., 2003). Moreover, physiological and behavioural differences in larvae of the target and non-target insects with a differential mode of feeding may also be a likely explanation for the varied effect of the pesticides and their mixtures used in this study (Rodriguez-Saona et al., 2016).

Likewise, the lower mortality rate of fourth instars of *Plutella xylostella* may be because after fourth instar stage, the larvae enter the pre-pupal stage that lasts for 2 to 3 days. During this period the larvae stop further feeding on foliage and start preparing for pupal period (Golizadeh et al., 2007). Therefore, their chance of oral toxicity with pesticides may have reduced and hence, their mortality rate was relatively lower. Moreover, with increasing age, the build-in immunity of insects fortifies, which might provide them additional immunity against various xenobiotics (Mao et al., 2015; Vannette et al., 2015). In this study, however, this aspect of improved defence of fourth instars is of not much relevance because the insect colonies were cultured in laboratory and maintained in a pesticide-free environment. To achieve optimum control over the pests; therefore, timing the chemical application correctly can increase the effectiveness of pesticides to a greater extent (Wise et al., 2010). Similarly, Rodriguez-Saona et al. (2016) have reported greater susceptibility of younger larvae than older instars to acetamiprid and thiamethoxam in *Sparganothis sulfureana* and *Choristoneura parallela,* two important pests of cranberries. Likewise, Torres and Ruberson (2004) showed that the contact toxicity of imidacloprid and thiamethoxam to second instars of *Podisus nigrispinus* (the predatory stinkbug in cotton fields), was 3.8 and 8.2 times greater than their fifth instars. Toxicity of both neonicotinoids however, was similar in both instar stages of the stinkbug via oral route of exposure. In contrast, Rodriguez-Saona et al. (2016) reported lower efficacy of acetamiprid and thiamethoxam against younger larvae of *Sparganothis sulfureana* and *Choristoneura parallela,* two important pests of

cranberries. Acetamiprid however, showed a relatively pronounced adulticdal and ovicidal effect. Yue et al. (2003) demonstrated the differential toxicities of imidacloprid and thiamethoxam based on larval age of Indianmeal moth, *Plodia interpunctella.* They reported that higher concentrations and prolonged exposure periods to these neonicotinoids are required to avail maximum efficacy against the older instars. Likewise, Rodriguez-Saona et al. (2016) also reported the species-specific differential toxicity of indoxacarb as the insecticide was high in toxicity for *Sparganothis sulfureana* larvae in comparison to the larvae of *Choristoneura parallela*. Chapter 2 discussion

In the current study the mortality rate of Chrysoperla carnea was initially low which increased over increasing exposure time. This delayed mortality of *Chrysoperla carnea* at a higher rate over time may be linked to the combined effect of contact toxicity of the pesticides as well as consuming the contaminated green aphids. Aphids feed on plant juice by attacking their leaves, stems, buds and fruit. Therefore, in first 24 h of exposure, the mortality due to contact toxicity was low however, after 48 h both contact toxicity of treated leaf discs as well as consumption of contaminated prey grazing over the pesticides treated leaf discs may have given rise to increased mortality of *Chrysoperla carnea* larvae. This can be explained through Haber's rule that is C x T = constant, for detailed review see Witschi (1999). According to this law, the toxicity of a lower dose of a chemical over a longer period of exposure is equal to that of a higher dose for a shorter period (Tennekes and Sánchez-Bayo, 2019). Although the effect of pesticides was diluted via indirect exposure through contaminated prey as well as contact exposure, it increased over time. In concordance with the results of this study, Wanumen et al. (2016) reported an increase in the mortality rate of *Nesidiocoris tenuis*, a generalist predator used against tomato crop pests like *Trialeurodes vaporariorum*, *Bemisia tabaci* and *Tuta absoluta*. They tested the trophic level toxicity of six commercial insecticides to *Nesidiocoris tenuis* by feeding its adults on eggs of the mill moth *Ephestia kuehniella*, dipped in insecticide solutions. They observed a significant increase in the mortality rate of *Nesidiocoris tenuis* after 48 h and 72 h of consumption of the eggs contaminated with sulfoxafor (a competitive inhibitor of nAChR like neonicotinoids) and metaflumizone (Wanumen et al., 2016).

2.5 Conclusion

These results elicit concerns about the application of fungicides alone or in mixtures with insecticides in agricultural fields in terms of their toxicity to non-target insects. Previous studies have reported the synergistic relationship between DMI fungicides and neonicotinoids. The above results reveal that tebuconazole, a DMI fungicide, is toxic against insects (both target and non-target) to a degree that cannot be overlooked. Consequently, from the LC_{50} values that were below its authorised application rate, the notion that fungicides are safer to insects is

contradicted in this study. In current study however, the effect of all mixtures on *Chrysoperla carnea* was the same while its toxicity against *Plutella xylostella* increased with increase in the intensity of acetamiprid in mixtures as well as with time. Likewise, relatively lower mortality rate of advanced larval stages of *Plutella xylostella* on various pesticide treatments indicate the significance of life cycle stages of pests when effectiveness of a pesticide is under the question. To confirm and elaborate these findings further, there is a need to investigate these kinds of effects in insects by evaluating the effects of other combinations of fungicides and neonicotinoids having similar or different modes of action.

In addition, in current study the insect larvae showed an aversiveness from the leaf discs treated with the test pesticides and their mixtures. High mortality rates in larvae treated with acetamiprid, tebuconazole and their mixtures may be linked to their avoidance from the treated leaf surface accompanied with less feeding and drop in energy their content. However, these observations needed efficient evaluation and are therefore, were explored in next chapter.

Chapter 3 Behavioural alteration and sublethal effects of pesticides on *Plutella xylostella* and *Chrysoperla carnea*

3.1 Introduction

Sublethal behavioural responses induced by pesticides in insects has been an area that has received relatively less attention as most of the studies related to the effect of pesticides are based on the estimation of lethal toxicities. Pesticides sometimes do not show their overt acute lethal effects; rather they act slowly causing severe distress which can prove fatal for the organisms later on. Sublethal effects of pesticides are also an important part of suite of tests used in first tier risk assessments. Therefore, alongside the lethal assessment shown in chapter 2, it is important to carry out the pesticides risk assessment tests on the sublethal behavioural effects of insects (Tosi and Nieh, 2019). In general, the magnitudes of pesticides applied, is focused on prompt eradication of pest species. Nonetheless, residues gradually degrade in soil, water, plants and animals, resulting in subtle effects in the form of sublethal exposures (Solange et al., 2017). Moreover, studying sublethal effects of pesticides in both target and non-target insects is important not only because this can provide essential information about the overall success of a pesticide against target pest but also its selectivity for non-target species (Solange et al., 2017). In this way, not only the phenomenon underlying the gradual eradication of the pest can be understood, but prophylactic measures to safeguard non-target species can also be designed. It is, therefore, vital to evaluate the sublethal toxicities of these chemicals in insects, and this chapter has its main focus on sublethal effects of neonicotinoids and fungicides on target and non-target insects.

While the significance of the lethal effects of pesticides cannot be denied, underrating their potential sublethal impacts on organisms in the environment can have serious ecological consequences (Solange et al., 2017). Sublethal effects can be defined as the impact of a toxicant on behaviour, physiology, biology or demography of organisms or their populations that withstand its toxicity at lethal or lower than lethal concentrations. A sublethal concentration is that which induce no superficial mortality in a test population (Desneux et al., 2007). A wide variety of sublethal effects have been documented. Insects have evolved a range of reactions to overcome the effects of different toxins that they encounter in their environment. These

responses may be in the form of alteration in behaviour or physiology of the insects (Gómez-Guzmán et al., 2017; Nansen et al., 2016). Toxins sometimes, lead to many sublethal outcomes such as modifications in their activity, abnormal locomotion, feeding rate, metabolism and overall general physiological behaviour of the organism (Pisa et al., 2015). Behavorial modification like these over long term can lead to either increase their chances of survival or incur some fitness cost to the organisms. Different pesticides have been reported to produce different sublethal effects depending on their modes of action, methods of application, application rates, as well as the type of organisms (Pisa et al., 2015).

For instance, behavioural avoidance of insects to pesticides (Georghiou, 1972), which is referred to as the identification or detection of a hazard which lead the insects to restrain the toxin by simply stopping to feed on or escaping the area where the toxin is applied (Guedes et al., 2006). Modification of behaviour by insects to avoid the insecticide treated substrate can greatly increase their likelihood of survival. For example, between 1980s and 1990s, glucose based attracticide (chemical compounds containing phagostimulants such as glucose, fructose, sucrose and maltose to attract the organism and are capable of killing them when ingested) were largely in practice to control the German cockroaches (*Blatella germanica*) in eating places and food stores. These attracticides were usually used to lure the organism and feed on them, eventually exerting lethal and sublethal effects on the target organisms. Thus serving as an efficient mean of pest eradication. These controls however, are no more effective for German cockroches as they previously were. The reason is that cockroaches have developed a behavioural avoidance to feeding on these attracticides (Wang et al., 2004). This avoidance however, has some important fitness costs like reduction in size of oothecae and number of eggs in it (Shik et al., 2014).

Similarly, behavioural avoidance has also been reported in *Plutella xylostella*. Their behaviour on insecticides applied as foliar spray has been investigated and the findings suggest that DBM is capable of developing avoidance by selection of oviposition site (Sarfraz et al., 2005). Moreover, behavioural avoidance of *Plutella xylostella* to permethrin has also be reported (Jallow and Hoy, 2006; Jallow and Hoy, 2007). This avoidance may sometimes be due to irritation caused by pesticides. A study by Wiles and Jepson (1994), showed that the predatory *Coccinella septempunctata* (seven-spot ladybird) exhibited more frequent roaming behaviour accompanied with grooming and rubbing its body parts on exposure to deltamethrin sprayed plots. This was due to irritation produced by the pesticide that caused them to repel the treated areas. Moreover, the distress created by mobility can also make the insects more vulnerable to predation by their enemies. In addition, high doses of insecticide can also lower down the

mobility of insects. Suchail et al. (2001) assessed the impact of imidacloprid on *Apis mellifera* and found that mobility of bees was dependent on the insecticide dose. Highest motor activity was observed with the lowest dose of imidacloprid such that with increasing dose of imidacloprid the mobility of bees decreased. Furthermore, they suggested that the mobility could also change with time. Similarly, carabid beetle (*Harpalus pennsylvanicus*) when exposed to imidacloprid treated turf plots, exhibited a range of abnormalities in their behaviour such as impaired walking, excessive grooming and paralysis.

Likewise, changes in feeding rate is also one of the important sublethal products of pesticides. For example, Drobne et al. (2008) investigated the impact of imidacloprid on feeding rate, weight gain, digestive gland epithelial thickness and survival rate in adults and juveniles of the terrestrial isopod *Porcellio scaber* (woodlouse). After feeding on imidacloprid treated food for two weeks, they found the feeding rate and weight gain in juveniles, and feeding rate and digestive gland epithelial thickness in adults most affected. They also reported that imidacloprid can affect the isopods at similar concentrations to which insects are exposed. Similarly, poor foraging performance in bees has been reported due to prolonged exposure to imidacloprid (Gill and Raine, 2014; Lundin et al., 2015). Subsequently, the likelihood of insect's susceptibility to other environmental stressors increases (Alaux et al., 2010; Fauser-Misslin et al., 2014). In contrast, Rogers et al. (2007) conducted a study to determine the effect of soil-applied imidacloprid on survival and feeding behaviour of green lacewings. From their investigation, they found that adult lacewings fed on flowers from imidacloprid drenched soil plants, showed a lower survival rate. Feeding rate however, remained the same for all the treatments including control.

Furthermore, numerous pesticides groups have also been accounted for affecting the locomotion in insects. For example, clothianidin, thiamethoxam and imidacloprid have been recognised to instigate flight muscle paralysis in honey bees when these chemicals are ingested through contaminated guttation fluid by bees. This paralysis results in termination of wing movements (Girolami et al., 2009). Likewise, Medrzycki et al. (2012) reported that imidacloprid also effects the locomotion of bees by impairing their walking and running abilities. This in turn increase the stationary resting periods of insects on the treated surface and thus increase the exposure time to the contaminated substrate that can eventually lead to the death of bees. In contrast, Cresswell et al. (2012) in their study, found no effect on feeding rate and locomotion of honey bees when exposed to imidacloprid containing diet. In bumblebees, however, they found a progressive reduction in feeding rate.

While the toxic effects of insecticides are well-established in insects, information about the constraints laid by fungicides on insects is lacking. Though fungicides are generally considered benign for insects, they have been shown to disrupt development and cause impaired behaviours. Bernauer et al. (2015) investigated the effect of chlorothalonil fungicide exposure on bumble bees (*Bombus impatiens*) colony growth. Chlorothalonil was applied at field-relevant concentrations to flowers and colonies were exposed to them for one month. Total count and biomass at larval and pupal stages as well as adult bumble bees in colonies were assessed and used as an indicator of the colony success. Their results showed a reduction in the number of workers and total biomass as well as a fewer queen bees are seen in the colonies. They proposed that fungicides use during blooms has the capacity to cause severe disruption in the abundance of native bees of the agroecosystem.

Studies of pesticides on insects generally focus on their direct physiological impacts, while fairly little consideration is placed on the behavioural responses to their exposure (Guedes et al., 2006; Kongmee et al., 2004). Very less is known about the risk assessment practices which evaluate the sublethal effects of pesticides mixtures, particularly in the presence of fungicides, which is crucial, as in real world these might be the more important long term effects of pesticides application. Behavioural alteration of insects caused by fungicides individually or in the presence of insecticides is, therefore, an important area to explore. Similarly another important factor to influence the behaviour of insects is the exposure time to the pesticides. The current study was therefore, conducted to investigate and evaluate these factors.

3.1.1 Study objectives

The fundamental goal of the current study was to analyse a range of sublethal effects widely used for assessing the pesticidal impacts. To achieve this goal, the study was conducted with the following objectives:

1. To quantify behavioural avoidance of *Plutella xylostella* and *Chrysoperla carnea* as a measure of sublethal effect of acetamiprid and tebuconazole alone and their mixture.
2. To measure the degree to which feeding rate of the two insects was affected by acetamiprid and tebuconazole alone and in mixtures.
3. To determine the effect of acetamiprid and tebuconazole alone and in mixtures on locomotion of insects.

3.2 Materials and methods

3.2.1 The insects

Plutella xylostella, a deleterious pest of crucifers all over the world as a target model insect and *Chrysoperla carnea*, a common generalist predator in various agro systems, as a non-target model, were used in this study. For bioassays with *Plutella xylostella*, its larvae at second, third and fourth instar stages were selected because the damage caused to crucifers at these formative stages is massive and clear. In case of *Chrysoperla carnea*, larvae were picked randomly regardless of their developmental stages due to their predacious nature and importance at every instar stage. Another limiting factor to sort them according to their developmental stages was that larvae of *Chrysoperla carnea* were obtained from Agralan UK and they usually came in their early instar stages. These larvae on arrival to the insectary were allowed to acclimatize for 2 to 3 days in the rearing conditions used for *Plutella xylostella*, mentioned below. During acclimatising, they were allowed to graze on green aphids grown on Chinese cabbage plants for experimental purpose. Larvae of *Plutella xylostella* used in bioassays were obtained from the culture maintained for experimental purpose on Chinese cabbage plant under controlled conditions of temperature 25 ± 2ºC, relative humidity (RH) 60% and a photoperiod of 16:8 in ECR (Environmentally Controlled Room) of the invertebrate facility in Faculty of Environmental and Life Sciences, University of Southampton, UK.

3.2.2 Pesticides used

In this study, commercial formulations of tebuconazole (Folicur® 250 EW, Bayer CropSciences) and acetamiprid (Gazelle® SG 20%, CERTIS- Solution for Crop Protection) were used (Table 1). Three levels of acetamiprid and tebuconazole each were used as their individual treatments. These were 0.025 gL^{-1}, 0.05 gL^{-1}, 0.25 $gL^{-1}$for acetamiprid and 0.5 mlL^{-1}, 1 mlL^{-1} and 5 mlL^{-1} for tebuconazole (Table 2). A ten times lower quantity of the manufacturers recommended application rate of tebuconazole (details given in chapter 2 in section 2.2.4) was used in mixtures. Keeping this concentration constant, it was mixed with the above-mentioned three different concentrations of acetamiprid. Such that the three binary mixtures were in the combination of tebuconazole (mlL^{-1}) and acetamiprid (gL^{-1}) 0.5 + 0.025 (mix1), 0.5 + 0.05 (mix2) and 0.5 + 0.25 (mix3) respectively.

3.2.3 Bioassay

A leaf dip bioassay technique was used in the study for which solutions with the three concentrations were prepared in deionized water. The leaf discs for bioassay were prepared by cutting the leaves of 6 to 8 weeks old Chinese cabbage plant with the help of a sharp round shaped cutter. These leaf discs were then individually dipped in the solutions for 10 s, after which they were spread on a paper towel inside a fumehood with their abaxial surface facing upward and were left until air dry. The whole procedure was carried out in a fumehood to avoid the contamination of surrounding area. When dry, each leaf disc was transferred into a 100 mm Petri dish with a wet filter paper (Whatman no. 1, 70 mm) inside it.

3.2.4 Estimation of avoidance

Avoidance behaviour was defined as the escape of larvae from the pesticide treated leaf disc. The petri dishes with treated leaf disc served as substrate of contact as well as food for *Plutella xylostella* larvae. To measure the percentage avoidance, 10 larvae of the same instar stage, were picked from a *Plutella xylostella* cultured Chinese cabbage plant with a fine paint brush and were transferred to a Petri dish. Six Petri dishes each with 10 larvae of a single instar stage for each pesticide treatment and each instar stage were set up. In this way 6 replicates for each pesticide treatement as well as control were carried out for each instar stage, with the avoidance recorded as percentage based on the number of larvae avoiding the treated leaf surface.

Similarly, well-fed and active *Chrysoperla carnea* larvae were transferred in a group of 10 into each Petri dish where a leaf disc treated with one of the experimental pesticide. In this way, 60 *Chrysoprla carnea* instars divided in six Petri dishes, were exposed to each treatment. The pesticide treated leaf disc served as a substrate of contact for them while green aphids were supplied to them as food source.

Once ready all the Petri dishes were covered with a perforated plastic foil for ventilation. These were then retained in an environmentally controlled room for 24 h. After the interval, avoidance from the leaf discs was recorded such that each replicate was observed for the number of larvae resting away from the treated leaf surface. Same method was repeated after 48 h of exposure to the treated leaf discs for both insects' larvae.

3.2.5 Estimation of feeding rate

To determine the feeding rate of the *Plutella xylostella*, a photographic technique was utilised. For this purpose, Nikon camera was mounted on a tripod stand. The pesticide treated leaf discs were photographed from equal distance and same magnification each time, at three different intervals i.e., 0h (immediately after treatment, before the introduction of insect larvae), 24 h after the insects were introduced on the leaf disc surface followed by 48 h of the larval feeding on the leaf discs. These photographs were then analysed using the ImageJ software to determine the percentage of leaf disc area consumed after the respective intervals (Appendix A). Each leaf disc in a Petri dish served as a replicate. Six replicates for each treatment were carried out for each instar stage and each pesticide treatment as well as control. The difference in the percentage of leaf disc area left was used as an indicator of the feeding rate of the insect larvae. Percentage feeding was calculated as:

Percentage feeding = 100% leaf disc - % of leaf disc consumed

For *Chrysoperla carnea*, prior to the actual experiment a pilot experiment was conducted to determine the extent of aphids, a single *Chrysoperla carnea* larva can consume over 24 h. From that experiment it was established that a single lacewing larva in a pesticide free environment had an average consumption rate of five aphids per day. Therefore, keeping it as a reference, feeding bioassay for *Chrysoperla carnea* was conducted in such a manner that larvae still alive after avoidance bioassay were taken and placed singly in Petri dishes containing a leaf disc, over a wet filter paper. Each single larva in a petri dish was allowed to feed on 5 aphids supplied to them over the leaf surface. After 24 h the feeding rate was determined by counting the number of dead aphids inside the Petri dish. Likewise, the food source was renewed and feeding rate was determined after 48 h. Each single larva in a Petri dish served as a replicate and thirty replicates for each pesticide treatment as well as control were done. In a real field scinario as the *Chrysoperla carnea* were exposed to the pesticides via contact as well as feeding on the prey, (which was either contaminated by consuming the pesticide treated leaf juices or wandering over the pesticies spray surfaces). Therefore, both of these aspects were considered in this experimental design.

Percentage feeding for *Chrysoperla carnea* was calculated as

Percentage feeding = number of dead aphids in a petri dish / total number of aphids at the time of introduction in petri dish (5) x 100

3.2.6 Estimation of effect of pesticides on locomotion of insect larvae

Larval locomotion was assessed using video recording approach. For this purpose, 15 larvae of each insect from different concentrations of acetamiprid, tebuconazole and mixtures were picked randomly from different Petri dishes. Each larva was subjected to a video recording using Sony HDR-CX115 camera mounted on stand and fixed over a stable surface to ensure the videos were recorded at similar distance. The difference in locomotion of larvae at various treatments was determined. Each larva was subjected to move on a graph paper having 1 mm^2 gridlines. Two parameters were used to determine the efficiency of larval movement.

1. Speed v = distance covered by larva / time taken by larva in actual movement
   Where distance d = 20 mm
   Time t = total time (T) – time taken in Stationary periods (tSPn)

2. Number of stationary periods (SPn) = total count of resting periods taken by larva while travelling a distance of 20mm. One stationary period was defined as a resting period taken by a larva where it spent at least 5 seconds without moving in any direction.

3.2.7 Statistical analysis

The data were analysed using statistical software IBM SPSS version 24. Significant difference in avoidance rate, speed and stationary periods as well as feeding rate on various treatments of acetamiprid, tebuconazole and mixtures was determined using a general linear model univariate ANOVA (GLM) after log transformation of the data to meet the assumption of normality for parametric tests. Where the data was still non-normal after applying different types of log transformations, generalized linear model (GLZM) was used as it allows to work with non-normal distribution of data and is a parametric test which offers more power to the data than using a non-parametric alternative (Stroup, 2013). GLZM can also be used when data in various treatment groups have different forms of distributions i.e. normally distributed in one group and exponentially distributed in others. Due to these advantages, GLZM is more reliable abd valid testing technique when dealing with non normally distributed data (Kesleman et al., 2016). Where GLM was used for estimation of parameters, Tukeys HSD test was the post-hoc test used to estimate the effect of various concentrations of a pesticide on larvae. In case of GLZM Pairwise comparison offereing Bonferroni Significance, was followed.

3.3 Results

3.3.1 Effect of acetamiprid on avoidance behaviour of *Plutella xylostella*

3.3.1.1 Avoidance of *Plutella xylostella* at second instar stage

At second instar stage *Plutella xylostella* larvae exposed to different concentrations of acetamiprid demonstrated significant avoidance from treated leaf discs ($F_{(3, 40)} = 16.035$, $p = 0.000$) (Fig. 11a). The pattern of avoidance exhibited by larvae was such that, avoidance remained the same for control and 0.025 gL^{-1} acetamiprid, while it increased significantly with the two higher concentrations i. e. 0.025 gL^{-1} and 0.25 gL^{-1} ($p < 0.05$). In contrast, increasing the exposure time to the insecticide resulted in no significant change on avoidance of the larvae from the treated leaf discs ($F_{(1, 40)} = 2.884$, $p = 0.097$).

3.3.1.2 Avoidance of *Plutella xylostella* at third instar stage

Third instar larvae of *Plutella xylostella* showed a significant difference in their avoidance rate when came across the three concentrations of acetamiprid ($F_{(3, 40)} = 21.852$, $p = 0.000$) (Fig. 11b). Their pattern of avoidance was similar to that exhibited by second instar stage larvae. In other words, the rate of avoidance in larvae encountering 0.05 gL^{-1} and 0.25 gL^{-1} acetamiprid had no significant difference; however, was significantly higher than 0.025 gL^{-1} acetamiprid and control treatments. Moreover, avoidance rate also seemed to be dependent on exposure time to various treatments of acetamiprid such that it increased significantly over time from 24 h to 48 h ($F_{(1, 40)} = 28.622$, $p = 0.000$). Here the difference in avoidance rate of larvae exposed to 0.05 gL^{-1} and 0.25 gL^{-1} was much higher at 24 h and 48 h, as compared to control and 0.025 gL^{-1}.

3.3.1.3 Avoidance of *Plutella xylostella* at fourth instar stage

The difference in avoidance rate of fourth instar larvae was significant for acetamiprid concentrations ($F_{(3, 40)} = 96.366$, $p = 0.000$) (Fig. 11c). Here again, the degree of avoidance shown by fourth instar larvae to 0.05 gL^{-1} and 0.25 gL^{-1} acetamiprid was significantly higher than 0.025 gL^{-1} and control treatments. However, there existed no significant difference in avoidance of larvae exposed to 0.05 gL^{-1} and 0.25 gL^{-1} acetamiprid concentrations. Likewise, exposure time effect was significant ($F_{(1, 40)} = 10.903$, $p = 0.020$). In this case, the difference in avoidance of larvae exposed to 0.25 gL^{-1} was comparatively much greater over time than the difference between the remaining treatments.

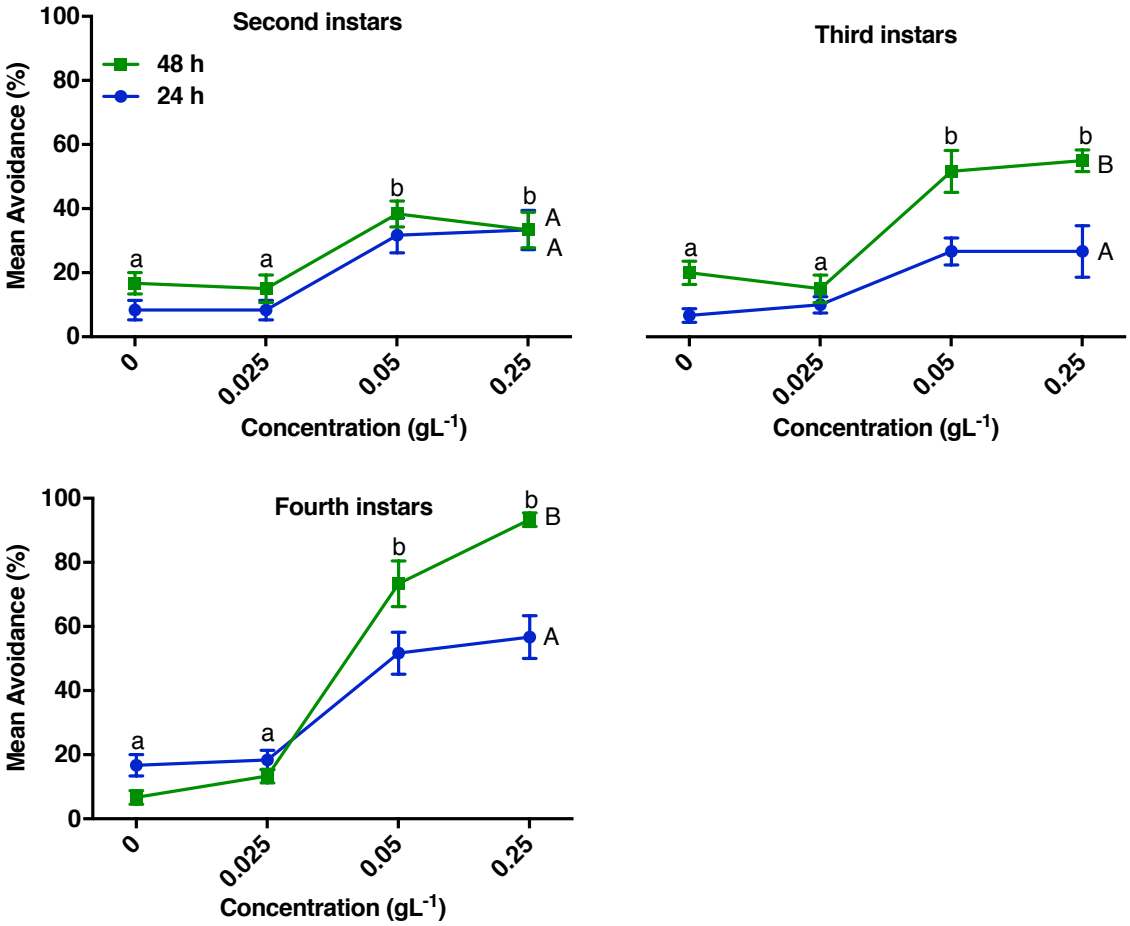

Figure 11 Mean percentage avoidance of three developmental stages of *Plutella xylostella* on various concentrations of acetamiprid. Error bars sharing the same lowercase letters show no significant difference in their behaviour ($p > 0.05$). Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.2 Effect of acetamiprid on avoidance behaviour of *Chrysoperla carnea*

For *Chrysoperla carnea* acetamiprid treatments used in the current study had a significant effect on avoidance of the larvae from the treated substrate ($F_{(3, 40)} = 10.394$, $p = 0.000$) (Fig. 12). Post-hoc test showed a significant rise in avoidance by larvae that encountered the leaf surface treated with 0.05 gL^{-1} and 0.25 gL^{-1} acetamiprid concentration. Avoidance shown by larval group exposed to control (deionized water only) was not significantly different than those exposed to 0.025 gL^{-1} acetamiprid; however, it was significantly lower than 0.05 gL^{-1} and 0.25 gL^{-1} acetamiprid. On the other hand, the rate of avoidance by larvae in 0.025 gL^{-1} treated group was significantly lower than 0.05 gL^{-1} group larvae but similar to 0.25 gL^{-1} group. In addition, the rate of avoidance

decreased slightly with increase in exposure time to the treatments, however, this reduction was not significant. In other words, no significant difference in avoidance of larvae existed between 24 h and 48 h exposure period ($F_{(1, 40)} = 2.647$, $p = 0.112$).

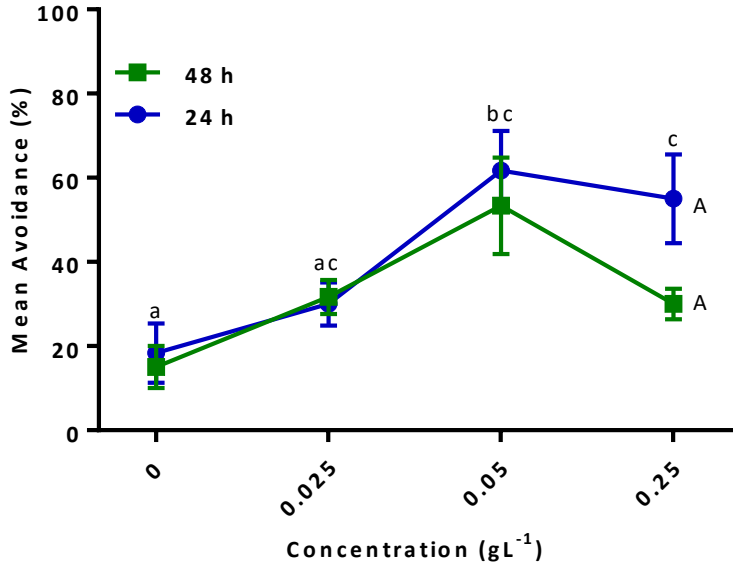

Figure 12 Mean percentage avoidance of *Chrysoperla carnea* larvae on various concentrations of acetamiprid. Error bars sharing the same lowercase letters show no significant difference in their avoidance behaviour ($p > 0.05$) with respect to concentrations. Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.3 Effect of tebuconazole on avoidance behaviour of *Plutella xylostella*

3.3.3.1 Avoidance of *Plutella xylostella* at second instar stage

The effect of different concentrations of tebuconazole showed a significant effect on avoidance of second instars of *Plutella xylostella* from the treated surface of leaf discs as acetamiprid. ($F_{(3, 40)} = 3.985$, $p = 0.014$) (Fig. 13a). The pattern of avoidance observed for tebuconazole treatments indicated a significant rise in avoidance with lowermost concentration (0.5 mlL^{-1}) following which the avoidance reduced with the two higher concentrations. This reduction in avoidance however, was not significant within the three tebuconazole concentrations ($p > 0.05$). This means, the larval group exposed to 0.5 mlL^{-1} tebuconazole showed a significantly higher avoidance than control while it remained similar to the rest of tebuconazole concentrations. Moreover, the larvae exposed to field recommended dose of tebuconazole showed no significant difference in avoidance from the rest of the treatments including control.

Likewise, avoidance of larvae was also significantly affected by increase in exposure time to the leaf disc surfaces treated with tebuconazole ($F_{(1, 40)} = 13.333$, $p = 0.001$).

3.3.3.2 Avoidance of *Plutella xylostella* at third instar stage

Difference in avoidance with different concentrations of tebuconazole for third instar larval group was also significant ($F_{(3, 40)} = 27.118$, $p = 0.000$) (Fig. 13b). Of the three concentrations of tebuconazole, rate of avoidance by larvae exposed to 1 mlL^{-1} was significantly higher than control as well as 5mlL^{-1} tebuconazole however, it was significantly lower than larvae that came across 1 mlL^{-1} tebuconazole concentration. On the other hand, the degree of avoidance shown by larvae when exposed to 5 mlL^{-1} tebuconazole treatment was not significantly different from that in control. Likewise, increase in exposure time also had a significant effect on avoidance rate of third instar larvae in case of tebuconazole ($F_{(1, 40)} = 9.159$, $p = 0.004$). It was such that the percentage of larvae avoiding the chemical treated surface went up with time.

3.3.3.3 Avoidance of *Plutella xylostella* at fourth instar stage

Fourth instar larvae also showed a significant difference in their rate of avoidance ($F_{(3, 40)} = 53.412$, $p = 0.000$) (Fig. 13c) from tebuconazole treated leaf surface. The rate of avoidance in control group larvae was significantly lower than all the three tebuconazole treatments. In addition, the rate of avoidance of larvae exposed to 1 mlL^{-1} and 5 mlL^{-1} was significantly higher than 0.5 mlL^{-1} tebuconazole, however, no significant difference existed between them. On the contrary, increase in exposure time had no significant effect on it ($F_{(1, 40)} = 2.805$ $p = 0.102$) though a slight rise in avoidance rate was observed over time.

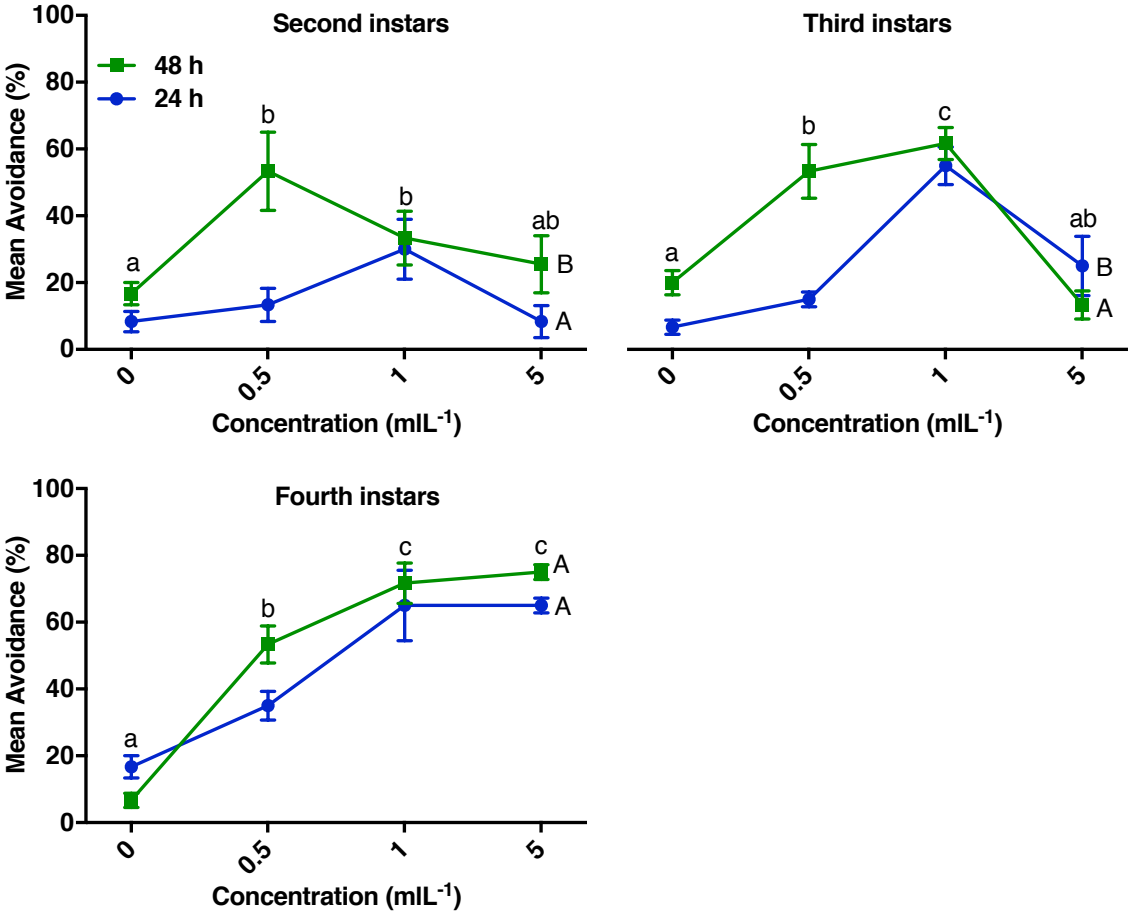

Figure 13 Mean percentage avoidance of three developmental stages of *Plutella xylostella* on various concentrations of tebuconazole. Error bars sharing the same lowercase letters show no significant difference in their avoidance behaviour with respect to concentrations of tebuconazole ($p > 0.05$). Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.4 Effect of tebuconazole on avoidance behaviour of *Chrysoperla carnea*

Tebuconazole treatments showed a significant effect on avoidance behaviour of *Chrysoperla carnea* larvae ($F_{(3, 40)} = 10.154$, $p = 0.017$) (Fig. 14). Relative to control, a significantly greater rate of avoidance was observed with the three tebuconazole treatments. No significant difference however, existed in rate of avoidance between 0.5 mlL^{-1}, 1 mlL^{-1} and 5 mlL^{-1} tebuconazole concentrations ($p > 0.05$). In the same manner, avoidance of larvae was not significantly affected by change in exposure time as it remained similar over the whole exposure period to tebuconazole and control treatments ($F_{(1, 40)} = 0.039$, $p = 0.844$).

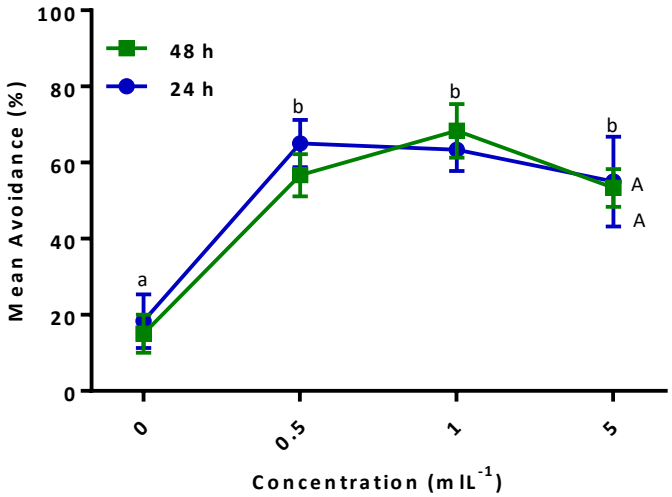

Figure 14 Mean percentage avoidance of *Chrysoperla carnea* larvae on various concentrations of tebuconazole. Error bars sharing the same lowercase letters show no significant difference in their avoidance behaviour ($p > 0.05$) with respect to concentrations of tebuconazole. Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.5 Effect of mixtures of tebuconazole and acetamiprid on avoidance behaviour of *Plutella xylostella*

3.3.5.1 Avoidance of *Plutella xylostella* at second instar stage

Mixtures of acetamiprid and tebuconazole used in this study had no significant effect on the avoidance behaviour of *Plutella xylostella* second instars ($F_{(3, 40)} = 1.116$, $p = 0.354$) (Fig. 15a). The percentage of avoidance remained similar between control as well as the three binary mixtures used in this study. In the same manner, exposure time effect on avoidance was not significant ($F_{(1, 40)} = 1.565$, $p = 0.218$).

3.3.5.2 Avoidance of *Plutella xylostella* at third instar stage

In case of third instars, rate of avoidance varied significantly with different mixtures ($F_{(3, 40)} = 6.607$, $p = 0.002$). Similarly, there was a significant difference in avoidance from the leaf disc in 24 h and 48 h when they were treated with various mixtures ($F_{(1, 40)} = 13.596$, $p = 0.001$) (Fig. 15b). The highest percentage avoidance was recorded with mix1 having 0.5 mlL^{-1} tebuconazole and 0.025 gL^{-1} acetamiprid followed by mix 2 (0.5 mlL^{-1} + 0.05 gL^{-1}) which gradually decreased with the following mixtures. Of the three mixtures, the difference remained non-significant between control, mix2 and mix3 (0.5 mlL^{-1} + 0.25 gL^{-1}) as well as between mix1 and mix2.

3.3.5.3 Avoidance of *Plutella xylostella* at fourth instar stage

With fourth instar larvae, avoidance was significantly different with various mixtures ($F_{(3, 40)}$ = 51.197, p = 0.000) (Fig. 15c). Moreover, avoidance was also highest relative to second and third instar larvae. Increase in exposure time also had a significant effect on avoidance of the fourth instar larvae ($F_{(1, 60)}$ = 8.154, p = 0.00). The difference in avoidance rate of fourth instar larvae was significant between control and all the three mixtures while there was no significant difference between mix1 and mix2. Moreover, rate of avoidance with mix1 and mix2 was significantly higher than mix3 and control. On the other hand, rate of avoidance in larvae facing mix3 was significantly higher than control only.

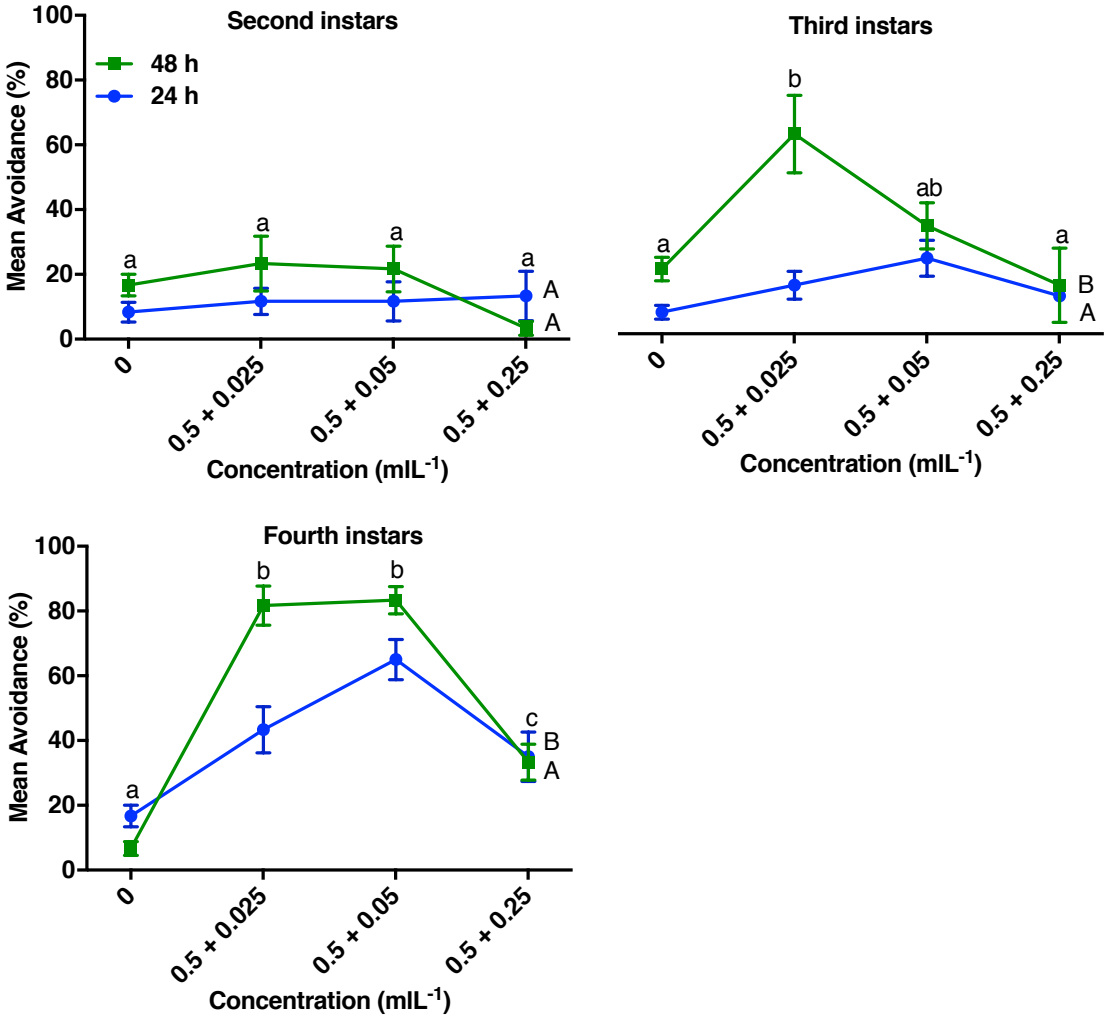

Figure 15 Mean percentage avoidance of three developmental stages of *Plutella xylostella* on various mixtures of tebuconazole and acetamiprid. Error bars sharing the same lowercase letters show no significant difference in their avoidance behaviour with respect to different treatments of mixtures (p > 0.05). Same uppercase letters on the

two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.6 Effect of mixtures of tebuconazole and acetamiprid on avoidance behaviour of *Chrysoperla carnea*

Mixtures of acetamiprid and tebuconazole indicated as least affecting the larvae in terms of avoidance from treated leaf discs. No significant difference in avoidance by larvae in control or those exposed to the three binary mixtures was found ($F_{(3, 40)} = 2.801$, $p = 0.423$) (Fig. 16). In the same way, rate of avoidance of larvae remained similar between 24 h and 48 h of contact with the treated substrate ($F_{(1, 40)} = 0.288$, $p = 0.592$). For simplicity of results, the graphs for sublethal parameters of *Chrysoperla carnea* presenting no significant difference are not shown any further in this chapter.

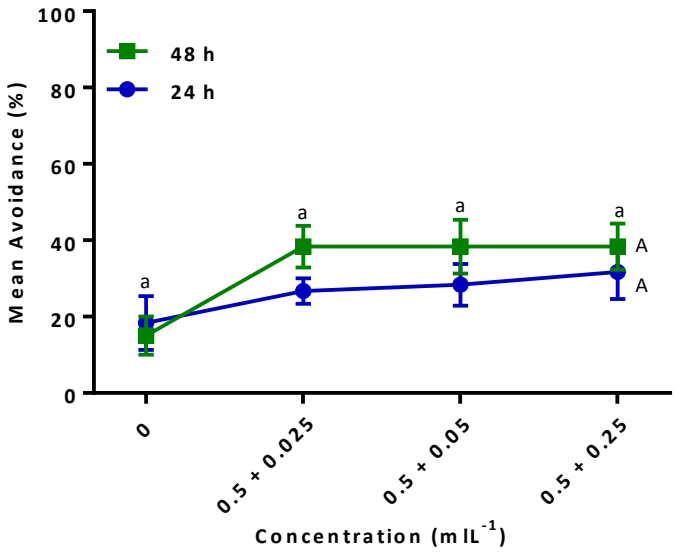

Figure 16 Mean percentage avoidance of *Chrysoperla carnea* larvae exposed to mixtures of tebuconazole and acetamiprid. Error bars sharing the same lowercase letters show no significant difference in their avoidance behaviour with respect to different treatments of mixtures ($p > 0.05$). Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.7 Effect of acetamiprid on speed of *Plutella xylostella*

3.3.7.1 Speed of *Plutella xylostella* at second instar stage

One important parameter of locomotion investigated in this study was the speed of larvae. Effect of acetamiprid on speed of second instars was significant ($F_{(3, 112)} = 9.610$, $p = 0.000$) (Fig. 17a). Of

the three concentrations of acetamiprid used in this study, the speed of larvae was significantly lower for 0.05 gL^{-1} and 0.25 gL^{-1} acetamiprid than control. No significant difference in speed of larvae with control and 0.025 gL^{-1} concentration of acetamiprid was found. The speed of the larvae within the three acetamiprid levels on the other hand, showed no significant difference between them. Moreover, the effect of change in exposure time also had a significant effect on it ($F_{(1, 112)} = 18.667$, $p = 0.000$). Interestingly, this effect was such that the speed of larvae was greater at 48 h than 24 h exposure to acetamiprid.

3.3.7.2 Speed of *Plutella xylostella* at third instar stage

Change in concentration of acetamiprid had no significant effect on the speed of third instars of *Plutella xylostella* ($F_{(3, 112)} = 0.242$, $p = 0.867$) (Fig. 17b). It remained below the range of 1.5 mm/s throughout the exposure of larvae to different acetamiprid treatments as well as control. Increase in exposure time however, resulted in a significant change in speed of larvae ($F_{(1, 112)} = 8.564$, $p = 0.004$), such that it increased significantly over time.

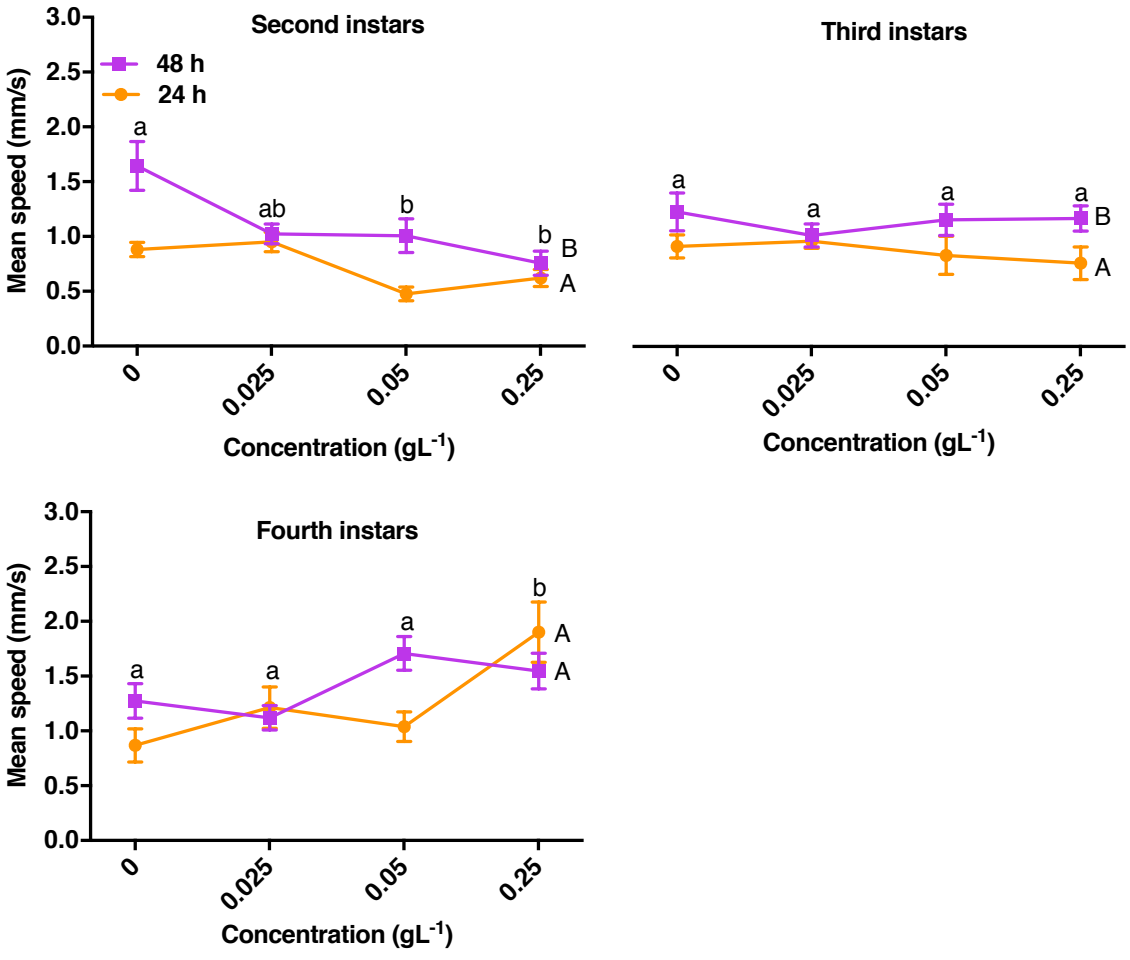

Figure 17 Mean speed of three developmental stages of *Plutella xylostella* larvae on various concentrations of acetamiprid. Error bars sharing the same lowercase letters show no significant difference in the speed of larvae. Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.7.3 Speed of *Plutella xylostella* at fourth instar stage

Different concentrations of acetamiprid showed a significant effect on the speed of larvae at fourth instar stage ($F_{(5, 127)} = 5.674$, $p = 0.001$) (Fig. 17c). Their speed tended to escalate significantly with 0.25 gL^{-1} while no significant difference in speed of larvae existed between the two lower concentrations of acetamiprid and that of control. Likewise, with increase in exposure time though the speed of larvae fluctuated with different concentrations, this change was not significant ($F_{(1, 127)} = 3.183$, $p = 0.077$).

3.3.8 Effect of acetamiprid on speed of *Chrysoperla carnea*

No significant difference was found in speed of *Chrysoperla carnea* larvae on control or acetamiprid concentrations ($F_{(3, 112)} = 0.290$, $p = 0.962$) (Fig. 18). Similarly, increasing the contact period to various acetamiprid treatments also had no significant effect on speed of larvae ($F_{(1, 112)} = 0.005$, $p = 0.943$).

3.3.9 Effect of tebuconazole on speed of *Plutella xylostella*

3.3.9.1 Speed of *Plutella xylostella* at second instar stage

A significant difference in speed of second instar larvae was observed on tebuconazole treatment ($F_{(3, 112)} = 9.839$, $p = 0.000$) (Fig. 18a). This significant difference in their speed only lied between control and the three tebuconazole concentrations, while no significant difference was observed on three concentrations of tebuconazole. Exposure time effect was also significant ($F_{(1, 112)} = 13.47$, $p = 0.000$) as the speed of the larvae increased significantly over the time course of 48 h.

3.3.9.2 Speed of *Plutella xylostella* at third instar stage

The third instar larvae also showed a significant difference in speed. In this case however, the difference was significant with various concentrations of tebuconazole ($F_{(3, 112)} = 7.226$, $p = 0.000$) as well as time of exposure ($F_{(1, 112)} = 6.056$, $p = 0.015$) (Fig. 18b). The speed of larvae dropped significantly with the lowest concentration of tebuconazole, then raised up with 1 mlL^{-1}. Afterwards another drop in speed was observed with the highest concentration that is 5 mlL^{-1}

tebuconazole however, it remained non-significant between control, 1 mlL^{-1} and 5 mlL^{-1} tebuconazole.

3.3.9.3 Speed of *Plutella xylostella* at fourth instar stage

The effect of different concentrations of tebuconazole on the speed of fourth instar larvae of *Plutella xylostella* was not significant ($F_{(3, 112)} = 1.012$, $p = 0.390$) (Fig. 18c). No significant difference existed between the larvae in control group as well as those coming across various tebuconazole concentrations. Exposure time again had no significant effect on speed of larvae ($F_{(1, 112)} = 0.475$, $p = 0.492$) therefore, the speed of larvae remained equal after 24 hr and 48 hr.

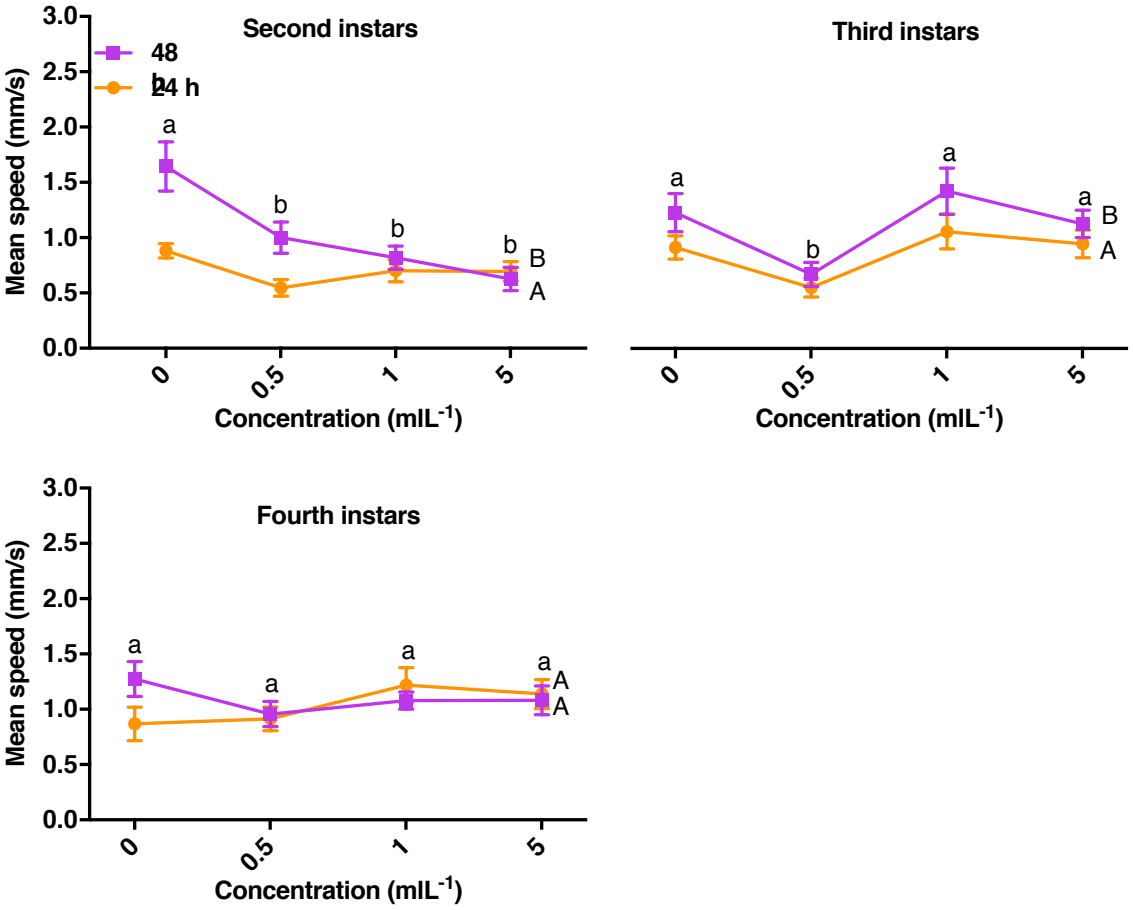

Figure 18 Mean speed of three developmental stages of *Plutella xylostella* on various concentrations of tebuconazole. Error bars sharing the same lowercase letters show no significant difference in the speed of larvae. Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.10 Effect of tebuconazole on speed of *Chrysoperla carnea*

Like acetamiprid, contact with tebuconazole of *Chrysoperla carnea* resulted in no significant rise or decline in their speed. The speed of larvae remained similar over 0 mlL^{-1}, 0.5 mlL^{-1}, 1 mlL^{-1} and 5 mlL^{-1} tebuconazole ($F_{(3, 112)} = 0.300$, $p = 0.960$). In a similar way, the larvae showed no significant change in their speed of locomotion over the entire contact period to the different treated substrates ($F_{(1, 112)} = 0.010$, $p = 0.919$).

3.3.11 Effect of mixtures of tebuconazole and acetamiprid on speed of *Plutella xylostella*

3.3.11.1 Speed of *Plutella xylostella* at second instar stage

Mixtures of the acetamiprid and tebuconazole under this study showed a significant effect on speed of *Plutella xylostella* larvae ($F_{(3, 112)} = 2.732$, $p = 0.047$) (Fig. 19a). This difference was such that the speed of larvae with mix3 (0.5 + 0.25) was significantly lower than control. Other than that, no statistically significant difference in speed of larvae was recorded with control or the two mixtures as well as between the three mixtures. On the contrary, the speed of larvae increased significantly with time of exposure ($F_{(1, 112)} = 41.105$, $p = 0.000$).

3.3.11.2 Speed of *Plutella xylostella* at third instar stage

No significant difference was indicated in speed of third instar larvae when exposed to mixtures having low or high intensity of acetamiprid ($F_{(3, 112)} = 2.079$, $p = 0.107$) (Fig. 19b). The speed remained the same for larvae encountering the control treatment or mixtures of tebuconazole and acetamiprid. Likewise, with increase in exposure time there was a rise in speed of larvae however, it was not significant ($F_{(1, 112)} = 3.964$, $p = 0.050$).

3.3.11.3 Speed of *Plutella xylostella* at fourth instar stage

Speed of fourth instar larvae also changed significantly with different mixtures $F_{(3, 112)} = 9.523$, $p = 0.000$) as well as with respect to exposure time ($F_{(1, 112)} = 32.483$, $p = 0.000$) (Fig. 19c). Speed was significantly high for mix1 (having lowest concentration of tebuconazole and acetamiprid) as compared to the two other mixtures (mix2 and mix3) and control. With increase in time of exposure, the speed increased significantly, however, it consistently decreased with the increasing intensity of pesticides in mixtures.

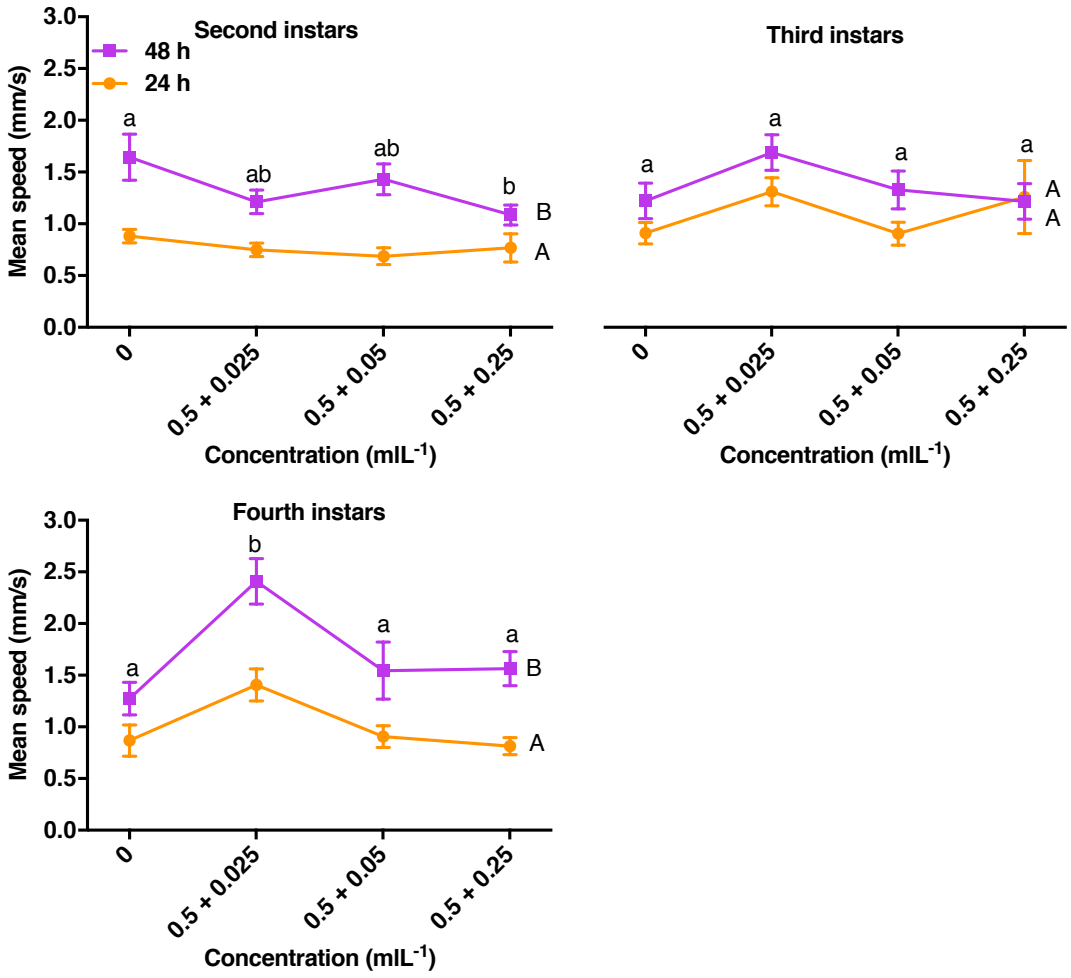

Figure 19 Mean speed of *Plutella xylostella* three developmental stages on various mixtures of tebuconazole and acetamiprid. Error bars sharing the same lowercase letters show no significant difference in the speed of larvae. Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.12 Effect of mixtures of tebuconazole and acetamiprid on speed of *Chrysoperla carnea*

Chrysoperla carnea larvae appeared to remain unaffected for their locomotion speed when they were exposed to mixtures of acetamiprid and tebuconazole. No significant difference existed between the speed of larvae in control group and those coming across the substrates treated with the three mixtures ($F_{(3, 112)} = 0.534$, $p = 0.911$). Same was the case for exposure time effect. Speed of larvae remained unchanged over the entire duration they were exposed to the mixtures or control treatments ($F_{(1, 112)} = 0.168$, $p = 0.682$).

3.3.13 Effect of acetamiprid on number of stationary periods (SPn) of *Plutella xylostella*

3.3.13.1 Number of stationary periods (SPn) of *Plutella xylostella* at second instar stage

Number of stationary periods (SPn) was another parameter studied to analyse the sublethal effects of pesticides on larvae. These kept on fluctuating with different concentrations however, the difference was not significant for second instar larvae ($F_{(3, 112)} = 1.970$, $p = 0.122$) (Fig 20a). The pattern was such that a reduced number of stationary periods with respect to that of control was recorded with 0.025 gL^{-1} concentration, followed by an increase with the two higher concentrations (0.05 gL^{-1} and 0.25 gL-1). This reduction or increase however, was not statistically significant ($p > 0.05$). Furthermore, number of stationary periods showed an inverse relationship with time such that it reduced with increase in exposure time to acetamiprid. The reduction however, was not significant ($F_{(1, 112)} = 3.364$, $p = 0.069$).

3.3.13.2 Number of stationary periods (SPn) of *Plutella xylostella* at third instar stage

At third instar stage different concentrations of acetamiprid used under this study had a significant effect on number of stationary periods ($F_{(3, 112)} = 2.898$, $p = 0.038$) (Fig. 20b). The number of stationary periods was significantly higher for larvae that encountered 0.25 gL^{-1} acetamiprid concentration from the rest of the two acetamiprid treatments as well as control. No significant difference was observed in number of stationary periods of larvae exposed to control 0.025 gL^{-1} and 0.05 gL^{-1}. Conversely, there was no significant variation in number of stationary periods with increase in exposure time ($F_{(1, 112)} = 3.802$, $p = 0.054$).

3.3.13.3 Number of stationary periods (SPn) of *Plutella xylostella* at fourth instar stage

For fourth instar larvae of *Plutella xylostella*, number of stationary periods was significantly different between various concentrations of acetamiprid ($F_{(3, 112)} = 4.833$, $p = 0.003$) as well as between 24 h and 48 h exposure time ($F_{(1, 112)} = 8.324$, $p = 0.005$) (Fig. 20c). In 24 h of exposure, the fourth instars exhibited the highest number of stationary periods when exposed to 0.05 gL^{-1} of acetamiprid concentration. This however, decreased significantly in 48 h of exposure.

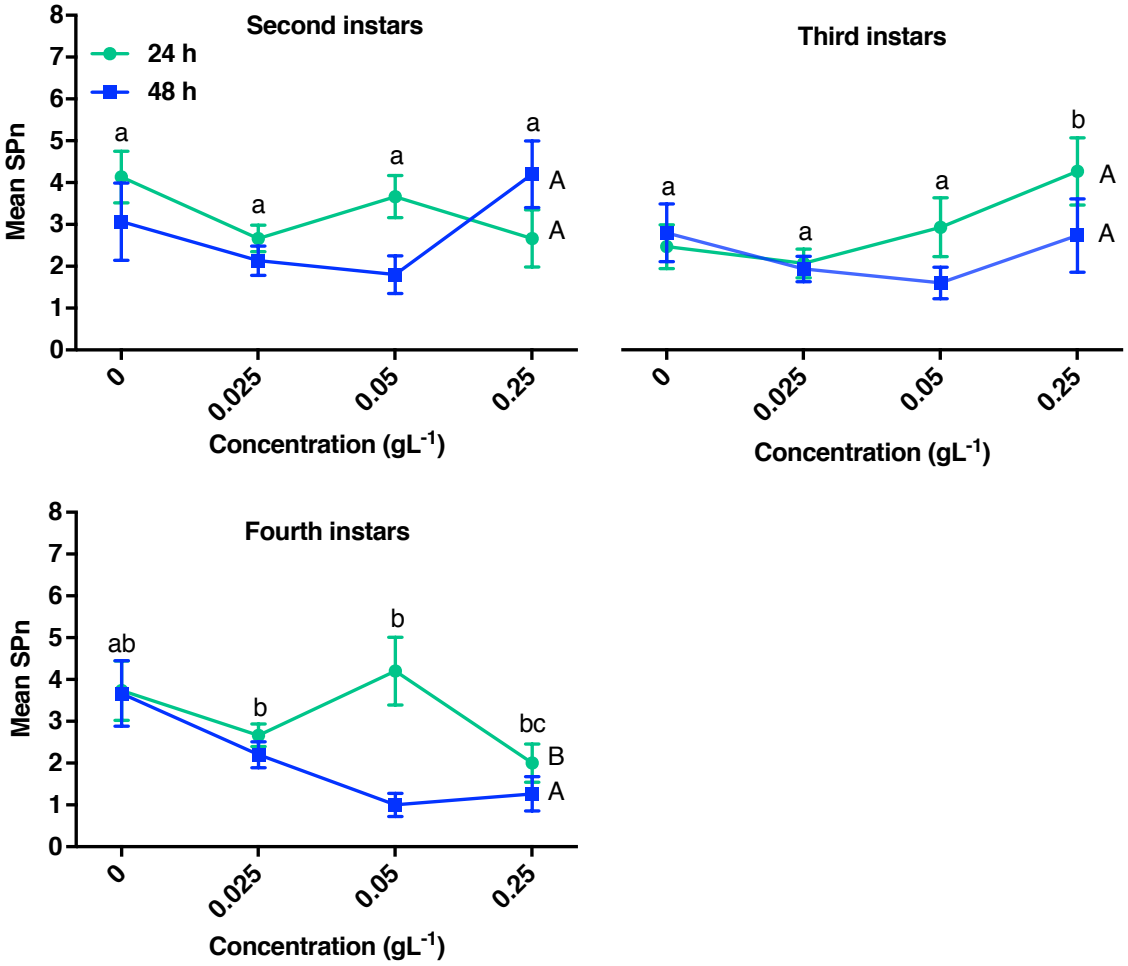

Figure 20 Mean number of stationary periods (SPn) of three developmental stages of *Plutella xylostella* on various concentrations of acetamiprid. Error bars sharing the same lowercase letters show no significant difference in SPn of larvae. Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.14 Effect of acetamiprid on number of stationary periods (SPn) of *Chrysoperla carnea*

Acetamiprid treatments did not proved to be a significant predictor of number of stationary periods of *Chrysoperla carnea* thus number of stationary periods during their locomotion was not significantly different with 0 gL^{-1}, 0.025 gL^{-1}, 0.05 gL^{-1} and 0.25 gL^{-1} acetamiprid concentrations ($F_{(3, 112)} = 1.773$, $p = 0.621$). In similar fashion, the larvae showed no significant change in their SPn with change in exposure time to the various treatments ($F_{(1, 112)} = 0.681$, $p = 0.409$).

3.3.15 Effect of tebuconazole on number of stationary periods of *Plutella xylostella*

3.3.15.1 Number of stationary periods (SPn) of *Plutella xylostella* at second instar stage

No statistically significant difference in SPn of *Plutella xylostella* second instar larvae was found between the concentrations of tebuconazole as well as control ($F_{(3, 112)} = 1.038$, $p = 0.379$) (Fig. 21a). Conversely, the exposure time effect of tebuconazole was significant ($F_{(1, 112)} = 8.358$, $p = 0.005$) and the number of stationary periods declined significantly after 48 h of exposure to tebuconazole. This means the larvae became hyperactive with less number of stationary periods and hence more mobile.

3.3.15.2 Number of stationary periods (SPn) of *Plutella xylostella* at third instar stage

For third instars, various concentrations of tebuconazole showed a significant effect ($F_{(3, 112)} = 3.340$, $p = 0.022$) (Fig. 21b). SPn was significantly high for 0.5 mlL^{-1} tebuconazole concentrations relative to 5 mlL^{-1}. On the other hand, exposure time effect was not significant in case of third instar larvae though the number slightly went down over time ($F_{(1, 112)} = 0.803$, $p = 0.372$).

3.3.15.3 Number of stationary periods (SPn) of *Plutella xylostella* at fourth instar stage

In case of fourth instar larvae of *Plutella xylostella*, the concentration effect of tebuconazole was not significant ($F_{(3, 112)} = 1.188$, $p = 0.318$) (Fig. 21c). Likewise, exposure time had no significant effect on number of stationary periods of larvae ($F_{(1, 112)} = 0.005$, $p = 946$). Here again, the number of stationary periods reduced for some concentrations and increased for others however, this fluctuation never resulted in a significant variation.

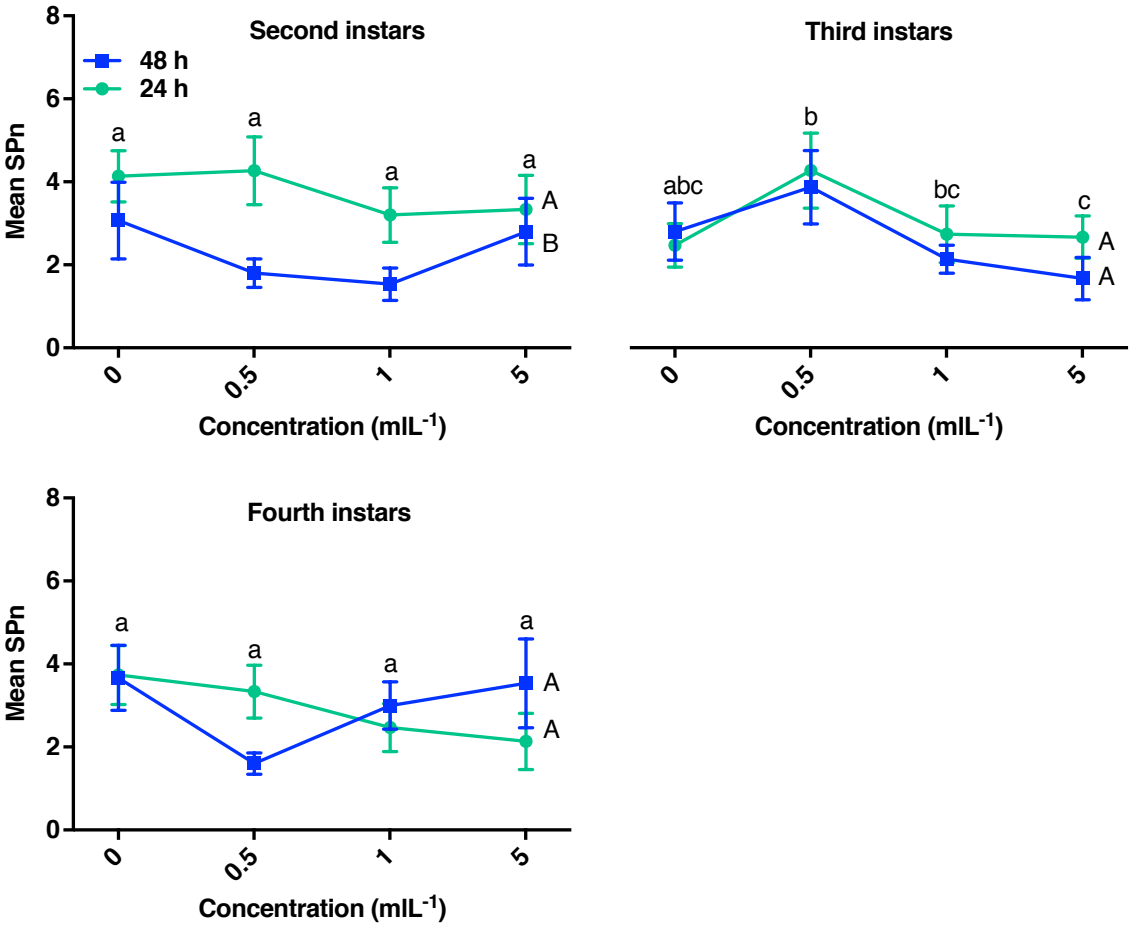

Figure 21 Mean number of stationary periods (SPn) of three developmental stages of *Plutella xylostella* on various concentrations of tebuconazole. Error bars sharing the same lowercase letters show no significant difference in SPn of larvae. Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.16 Effect of tebuconazole on number of stationary periods of *Chrysoperla carnea*

There was no significant difference in the frequency of stationary periods for *Chrysoperla carnea* larvae when exposed to three tebuconazole treatments ($F_{(3, 112)} = 2.463$, $p = 0.482$). Identically, any increase in contact duration of larvae with variously treated surfaces had no significant role in increasing or decreasing their number of resting periods while they moved in the provided space ($F_{(1, 112)} = 0.004$, $p = 0.950$).

3.3.17 Effect of mixtures of tebuconazole and acetamiprid on number of stationary periods of *Plutella xylostella*

3.3.17.1 Number of stationary periods (SPn) of *Plutella xylostella* at second instar stage

Mixtures of the acetamiprid and tebuconazole showed a significant effect on number of stationary periods of *Plutella xylostella* larvae ($F_{(3, 112)} = 3.469$, $p = 0.019$) (Fig. 22a). Similarly, the effect of time of exposure to mixture treatments was also significant ($F_{(1, 112)} = 7.631$, $p = 0.007$). Nevertheless, the difference was only between control and the mixtures. This means no significant difference existed between SPn of the three mixtures used in current study.

3.3.17.2 Number of stationary periods (SPn) of *Plutella xylostella* at third instar stage

The three mixtures under this study had no significant effect on the number of stationary periods (SPn) of third instar larvae ($F_{(3, 112)} = 1.488$, $p = 0.222$). Likewise, increase in exposure time to these mixtures produced no significant change in them ($F_{(1, 112)} = 2.708$, $p = 0.103$) (Fig. 22b). Stationary periods though showed a slight reduction with increased exposure time; however, this variation in SPn was not significant.

3.3.17.3 Number of stationary periods (SPn) of *Plutella xylostella* at fourth instar stage

At fourth instar stage, the larvae of *Plutella xylostella* showed no significant difference in SPn with different mixtures ($F_{(3, 112)} = 1.926$, $p = 0.129$) as well as with increase in exposure time ($F_{(1, 122)} = 1.177$, $p = 0.280$) to these mixtures (Fig. 22c). Here again the larvae tended to be faster after 48 h than 24 h but the difference was not significant.

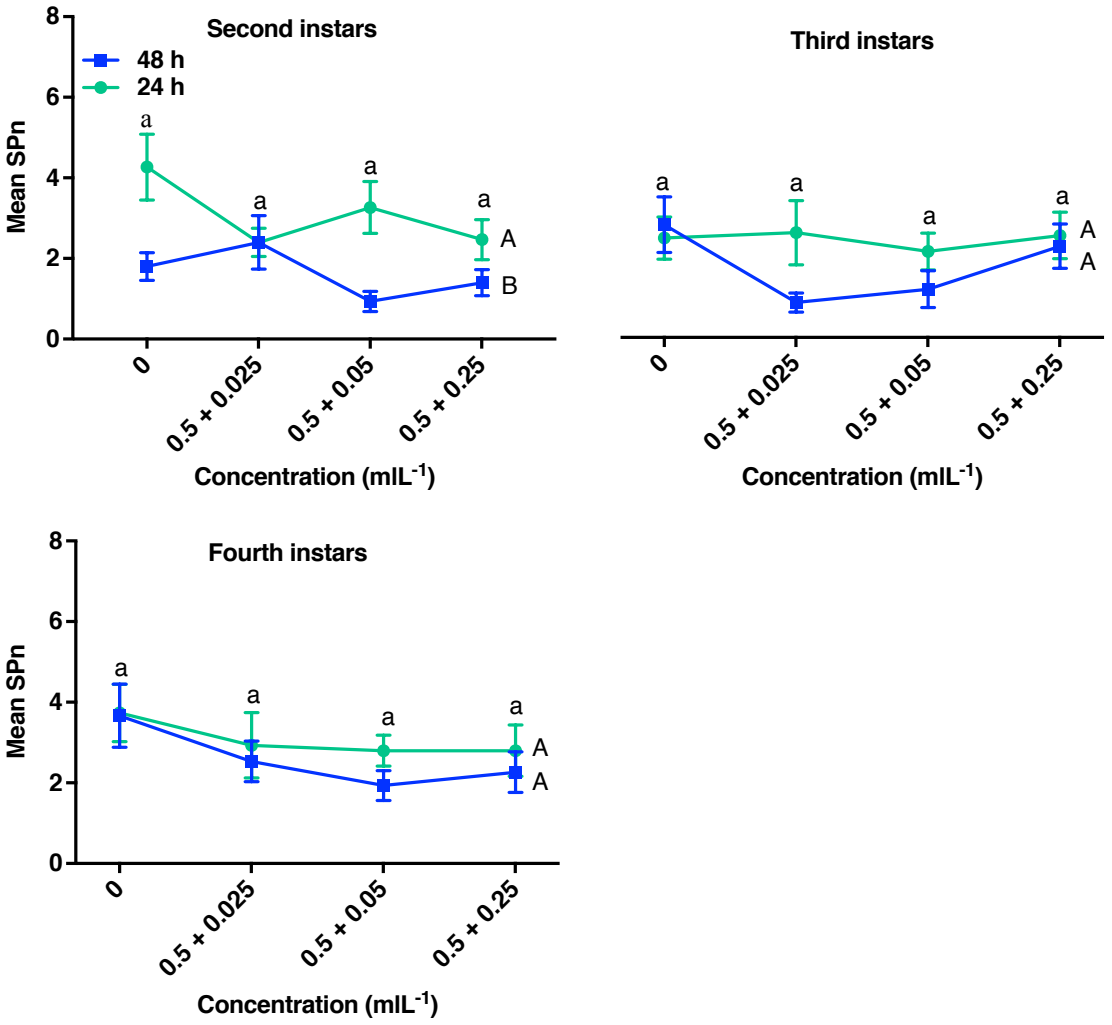

Figure 22 Mean number of stationary periods (SPn) of three developmental stages of *Plutella xylostella* on various mixtures of tebuconazole and acetamiprid. Error bars sharing the same lowercase letters show no significant difference in SPn of larvae. Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.18 Effect of mixtures of tebuconazole and acetamiprid on number of stationary periods of *Chrysoperla carnea*

In case of *Chrysoperla carnea*, the number of stationary periods of larvae treated with mixtures were not significantly different from control. These remained the same all over the entire set of treatments ($F_{(3, 112)} = 0.522$, $p = 0.668$). Similar was the case for exposure time effect. Throughout the whole contact period with the substrate, either treated with three mixtures individually or with no mixture control (deionized water only) no change in SPn was observed in the current study ($F_{(1, 112)} = 2.059$, $p = 0.110$).

3.3.19 Effect of acetamiprid on feeding rate of *Plutella xylostella*

3.3.19.1 Feeding rate of *Plutella xylostella* at second instar stage

The feeding rate of second instars of *Plutella xylostella* remained unaffected by acetamiprid treatments. No significant difference was found in the feeding rate of larvae in control and those treated with three concentrations of acetamiprid ($F_{(3, 40)} = 1.906$, $p = 0.144$) (Fig. 23a). With increase in exposure time however, percentage feeding of larvae increased significantly ($F_{(1, 40)} = 54.312$, $p = 0.000$).

3.3.19.2 Feeding rate of *Plutella xylostella* at third instar stage

Larvae of *Plutella xylostella* at third their instar stage too showed no significant change in their feeding rate with respect to varying concentrations of acetamiprid ($F_{(3, 40)} = 2.338$, $p = 0.088$) (Fig. 23b). On the other hand, the feeding rate increased significantly from 24 h to 48 h exposure to acetamiprid treatments ($F_{(1, 40)} = 30.615$, $p = 0.000$).

3.3.19.3 Feeding rate of *Plutella xylostella* at fourth instar stage

The feeding rate of fourth instar larvae of *Plutella xylostella* larvae was significantly affected by acetamiprid treatments ($F_{(3, 40)} = 23.719$, $p = 0.000$) (Fig. 23c). These were such that significantly higher feeding rate was observed with 0.25 gL^{-1} acetamiprid. Whereas, no significant difference existed between the feeding rate of the control group larvae as well as those treated with 0.025 gL^{-1} and 0.05 gL^{-1} acetamiprid. Moreover, the effect of exposure time on the feeding rate of larvae was also significant ($F_{(1, 40)} = 45.182$, $p = 0.000$) as their consumption rate was significantly greater in 48 h relative to 24 h.

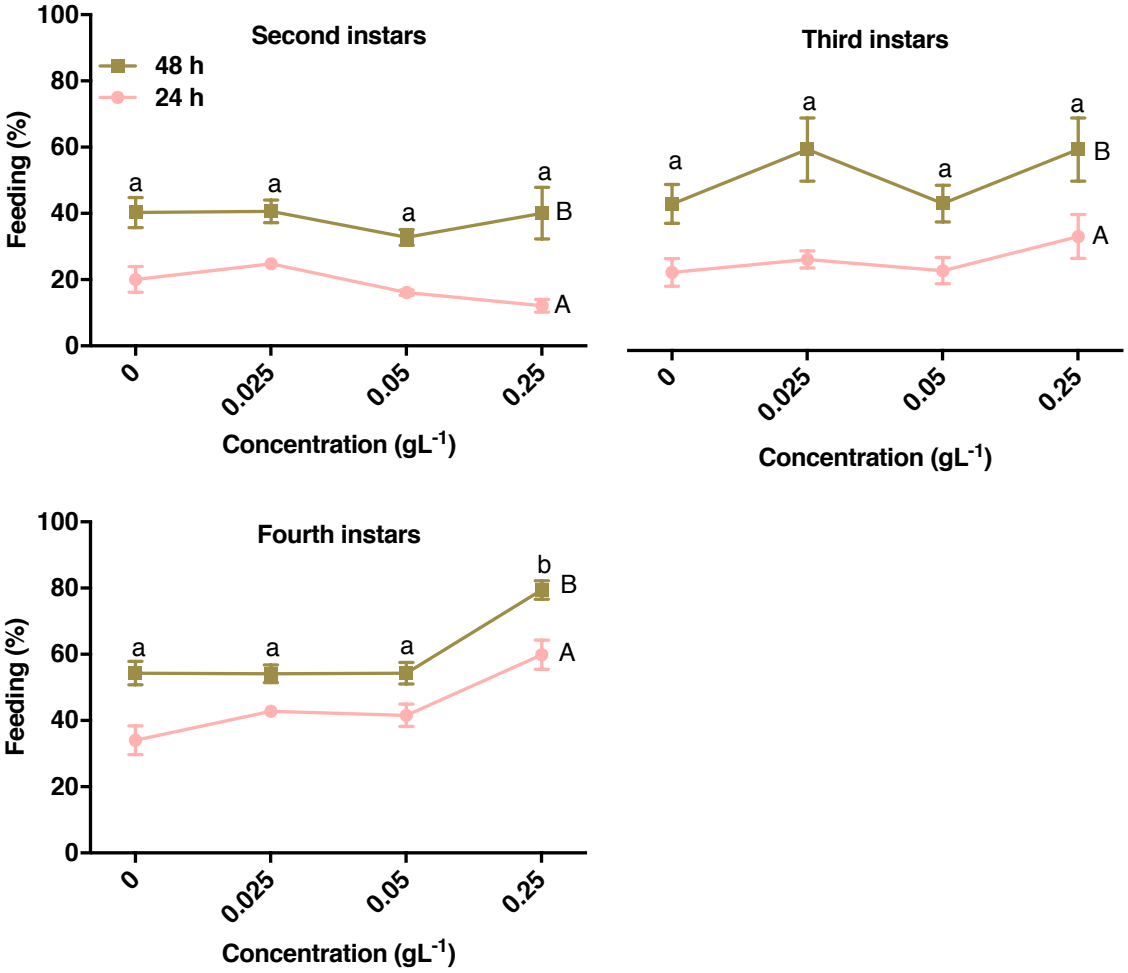

Figure 23 Mean percentage feeding of three developmental stages of *Plutella xylostella* on various concentrations of acetamiprid. Error bars sharing the same lowercase letters show no significant difference in feeding rate of larvae. Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.20 Effect of acetamiprid on feeding rate of *Chrysoperla carnea*

For *Chrysoperla carnea*, the three concentrations of acetamiprid and control showed no significant difference in larval feeding rate ($F_{(3, 196)} = 5.955$, $p = 0.114$). Throughout the treatments, however, the feeding rate remained below 50%. Furthermore, a slight decline was observed in the feeding rate of larvae over time, however, just as the effect of acetamiprid concentrations was non-significant, increasing the exposure time also had no significant role in changing the feeding rate of larvae ($F_{(1, 232)} = 1.592$, $p = 0.207$).

3.3.21 Effect of tebuconazole on feeding rate of *Plutella xylostella*

3.3.21.1 Feeding rate of *Plutella xylostella* at second instar stage

With tebuconazole, the feeding rate of larvae was significantly affected ($F_{(3, 40)} = 9.783$, $p = 0.000$) (Fig. 24a). Pairwise comparison showed a significant reduction in the feeding rate with the two highest levels of tebuconazole (1 mlL^{-1} and 5 mlL^{-1}) at $p < 0.05$. The percentage of feeding at 0.5 mlL^{-1} on the other hand, remained similar to that in control group larvae. Similarly, the effect of increase in exposure time was also significant on the feeding rate of the larvae and caused a significant rise in it ($F_{(1, 40)} = 17.659$, $p = 0.000$).

3.3.21.2 Feeding rate of *Plutella xylostella* at third instar stage

A significant difference in the feeding rate of third instar larvae was seen when treated with different concentrations of tebuconazole. ($F_{(3, 40)} = 39.062$, $p = 0.000$) (Fig. 24b). The feeding rate was significantly high for 0.5 mlL^{-1} tebuconazole concentration, than the larvae exposed to other tebuconazole treatments as well as control. Similarly, their feeding rate with the two higher concentrations of tebuconazole (1 mlL^{-1} and 5 mlL^{-1}), was significantly lower than control. The effect of increasing exposure time was also significant ($F_{(1, 40)} = 17.046$, $p = 0.000$). This means a significant rise in larval consumption rate was observed from 24 h to 48 h exposure.

3.3.21.3 Feeding rate of *Plutella xylostella* at fourth instar stage

The pattern of the feeding rate of fourth instar larvae was similar to that of third instar larvae. This means it was significantly affected by changing concentrations of tebuconazole ($F_{(3, 40)} = 96.504$, $p = 0.000$) (Fig. 24c). Furthermore, the degree of feed consumption was significantly high with 0.5 mlL^{-1} tebuconazole than control and the two stronger concentrations, 1 mlL^{-1} and 5 mlL^{-1} both of which limited the feeding of larvae significantly. Likewise, the effect of exposure time was also significant ($F_{(1, 40)} = 39.006$, $p = 0.000$), and the feeding rate increased with increase in exposure time from 24 h to 48 h.

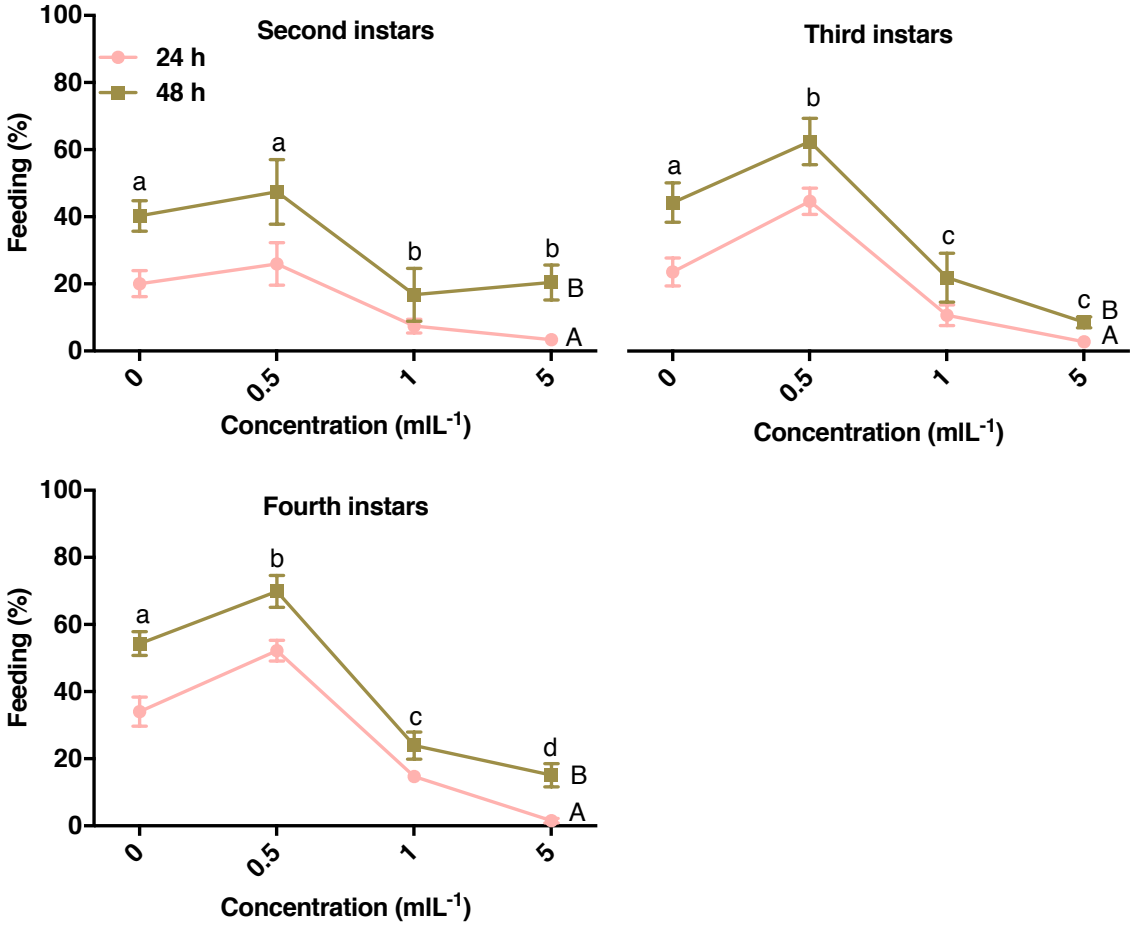

Figure 24 Mean percentage feeding of three developmental stages of *Plutella xylostella* on various concentrations of tebuconazole. Error bars sharing the same lowercase letters show no significant difference in feeding rate of larvae. Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.22 Effect of tebuconazole on feeding rate of *Chrysoperla carnea*

In case of *Chrysoperla carnea*, tebuconazole treatment caused a significant effect on their feeding rate ($F_{(3, 232)} = 72.344$, $p = 0.000$) (Fig. 25). Post-hoc test conducted showed a significant decline in feeding rate as compared to control in larval group encountering the surfaces treated with three tebuconazole doses ($p < 0.05$). In contrast, no significant difference existed in feeding rate of larvae exposed to 0.5 mlL^{-1} 1 mlL^{-1} and 5 mlL^{-1} tebuconazole ($p > 0.05$). On the other hand, feeding rate did not change significantly over time and remained closely similar at 24 h and 48 h contact period ($F_{(3, 232)} = 0.215$, $p = 0.643$).

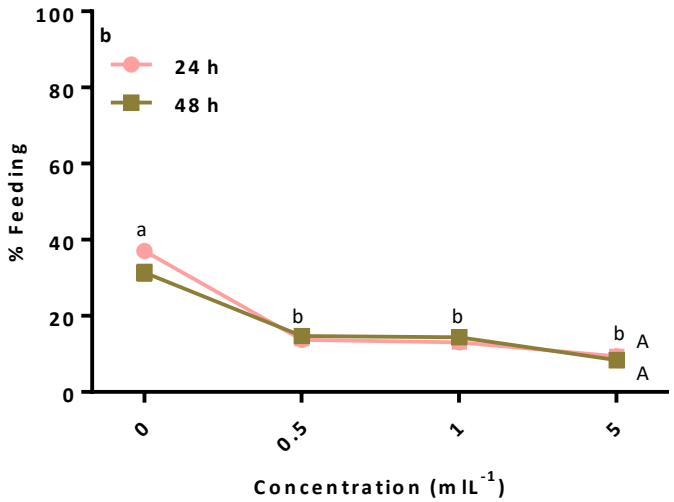

Figure 25 Mean percentage feeding of *Chrysoperla carnea* larvae on various concentrations of tebuconazole. Error bars sharing the same lowercase letters show no significant difference in feeding rate of larvae. Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.23 Effect of mixtures of tebuconazole and acetamiprid on feeding rate of *Plutella xylostella*

3.3.23.1 Feeding rate of *Plutella xylostella* at second instar stage

Mixtures of acetamiprid and tebuconazole affected the feeding rate of second instar larvae significantly ($F_{(3, 40)} = 14.781$, $p = 0.000$) (Fig. 26a). In this case, the difference in feeding rate was significant between control and the two mixtures i. e. mix2 and mix3 (having 0.05 gL^{-1} and 0.25 gL^{-1} acetamiprid respectively) mixed with 0.5 mlL^{-1} tebuconazole. Similarly, the percentage feeding with mix3, having the field relevant concentration of acetamiprid 0.25 gmL^{-1} mixed with 0.5 mlL^{-1} of tebuconazole, was significantly lower than mix1 (0.5 + 0.025). The larvae exposed to mix2 however, showed no significant difference in their feeding rate from mix1 and mix3. Likewise, with increase in exposure time, the feeding rate of larvae also increased between 24 h and 48 h ($F_{(1, 40)} = 34.444$, $p = 0.000$).

3.3.23.2 Feeding rate of *Plutella xylostella* at third instar stage

Larvae at third instar stage showed a significant change in feeding rate with respect to different mixtures ($F_{(3, 40)} = 19.074$, $p = 0.000$) (Fig. 26b) as well as exposure time ($F_{(1, 40)} = 56.616$, $p = 0.000$). This was such that the two mixtures with highest concentrations of acetamiprid mix2 and mix3 (0.5 + 0.05 and 0.5 + 0.25, tebuconazole + acetamiprid respectively) reduced the feeding rate of larvae significantly as compared to control and mix1 having the lowest concentration of

acetamiprid. No significant difference in feeding rate of larvae was observed when they were exposed to control and mix1. On the other hand, the feeding rate increased significantly between the intervals of 24 h and 48 h exposure to these mixtures.

3.3.23.3 Feeding rate of *Plutella xylostella* at fourth instar stage

The feeding rate of fourth instar larvae differed significantly with respect to varying mixtures ($F_{(3, 40)} = 19.851$, $p = 0.000$) as well as exposure time to the mixtures ($F_{(1, 40)} = 26.164$, $p = 0.000$) (Fig. 26c). Lowest consumption of leaf disc was recorded for mix3 having highest concentration of acetamiprid with tebuconazole. Whereas, with mix2, the rate of feeding was significantly lower than control and mix1 but higher than mix3.

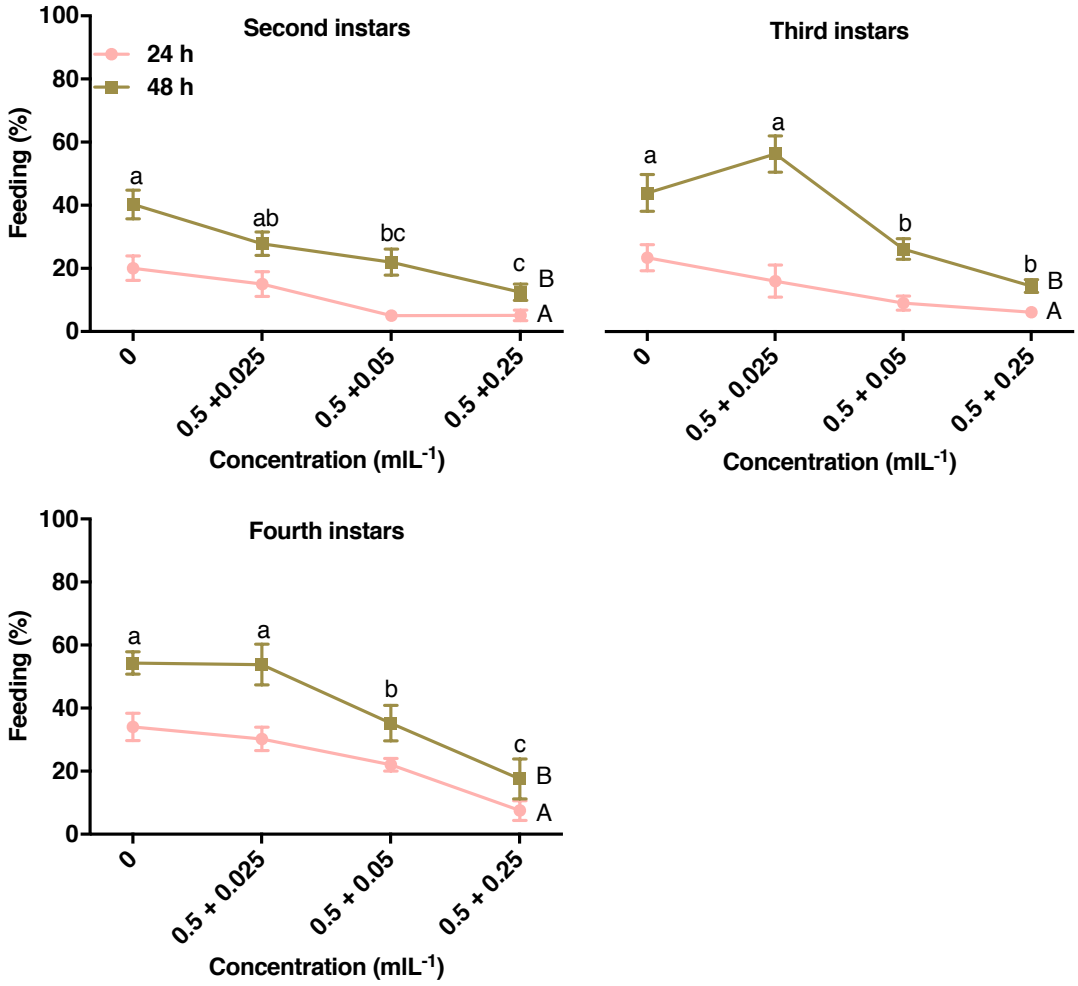

Figure 26 Mean percentage feeding of three developmental stages of *Plutella xylostella* larvae on various mixtures of tebuconazole and acetamiprid. Error bars sharing the same lowercase letters show no significant difference in feeding rate of larvae. Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

3.3.24 Effect of mixtures of tebuconazole and acetamiprid on feeding rate of *Chrysoperla carnea*

In case of *Chrysoperla carnea,* the mixtures of acetamiprid and tebuconazole demonstrated no significant effect on their feeding rate. It was such that no significant difference in feeding rate of control in relation to three mixture was found ($F_{(3, 232)} = 11.411$, $p = 0.101$). In the same pattern, exposure time had no significant effect on feeding rate of larvae in different groups ($F_{(3, 232)} = 1.674$, $p = 0.196$).

3.4 Discussion

The results of this study describes the sublethal effects on behaviour of *Plutella xylostella* and *Chrysoperla carnea* larvae. These sublethal effects included avoidance from treated leaf discs, alteration in the speed and the stationary periods demonstrated by the insects during their locomotion and their feeding rate as an indicator of the extent of the effect of pesticides on their behaviour. All these functions are important for the survival and propagation of an organism in its natural environment. For instance, the ability to efficiently forage or avoid the adverse situations, increase the chances of an organism's existence and adaptability to the prevailing environmental conditions.

3.4.1 Avoidance of *Plutella xylostella* larvae

In the present study, the avoidance of larvae from leaf disc treated with 0.05 gL^{-1} and 0.25 gL^{-1} acetamiprid was significantly high from 0 $gL^{-1}$and 0.025 gL^{-1}acetamiprid. A high rate of avoidance with the two upper concentrations indicate that the dose although was not enough to wipe out the insects completely, the neurotoxic effect was such that the larvae became hyperactive and hyperresponsive due to which they avoided the leaf discs. Acetamiprid is an N-cyanoguanidine neonicotinoid that acts as agonist of nicotinic acetylcholine receptors (nAChR) in central nervous system of insects and cause overstimulation and paralysis which may lead to death (Goulson, 2013b). Nansen et al. (2016) have reported their findings, which are in line with the present study. They found an increase in mobility and avoidance response in *Plutella xylostella* larvae from spinetoram and gamma-cyhalothrin insecticide treated leaves as compared to water treated leaves. Reduced rate of avoidance with lower doses could be related to minimal effect of the neonicotinoid, which was diluted to an extent where its efficacy was not strong enough to deter or kill the pest insects. Furthermore, Nansen et al. (2016) reported similar findings of the neurotoxic effect of imidacloprid and its metabolites on *Apis mellifera*. From their study, they reported that with imidacloprid and its metabolites, the early symptoms of neurotoxicity in worker bees were trembling, hyperactivity and hyperresponsiveness. With increased exposure time, the bees became hypoactive and hypo responsive and mortality started appearing after 4 h, which increased gradually over time. In current study, increase in exposure time increased the rate of avoidance of larvae except the larvae at second instar stage in which mortality rate was high. Thus, it can be deduced that the insects may became more hyperactive on exposure to

lower concentration over time and therefore, their degree of avoidance amplified. It may however, be different for different life stages of an insect as well as the concentration of the pesticides to which they are exposed. For instance, at higher concentration of the pesticdes in this study the mortality of second and third instar *Plutella xylostella* was higher than fourth instars. This may be under the influnce of greater toxicity of the pesticides for early instars stages than older instars. Similarly, Cordeiro et al. (2010) also reported a significant amount of avoidance of green lacewings from substrate treated with insecticides azadirachtin, permethrin and malathion.

In case of tebuconazole, the avoidance of second and third instar larvae with its lowest concentrations (0.5 mlL^{-1}) was highest than the two higher concentrations. This could be due to the lower intensity of 0.5 mlL^{-1} tebuconazole and hence low toxicity, which allow the insect to escape faster than being killed under the effect of stronger doses. The high mortality rate of larvae with stronger concentrations has been shown in chapter 2. Fourth instar on the other hand, showed greatest avoidance rate with the two highest concentrations of tebuconazole. This can be elucidated by their age and larger size and therefore, having a more developed response mechanism which may have enabled them to sense and respond more efficiently thus helping them to withstand the effect of stronger concentrations of tebuconazole and relatively killed less in number (as shown in chapter 2). In addition, lower rate of avoidance with low concentration suggests that its effect was not stronger enough to influence the behaviour of fourth instar larvae. Likewise, the rate of avoidance increased significantly over time of exposure to the concentrations of tebuconazole. These results suggest the residual toxicity of tebuconazole, which increased over time.

Results of current study demonstrates that avoidance of the *Plutella xylostella* second instar larvae was neither significantly different with type of mixture nor over exposure time. In contrast, rate of avoidance of third and fourth instar larvae was higher for mixtures with lower concentration of acetamiprid (mix1 (0.5 + 0.025) and mix2 (0.5 + 0.05)) than mix3 (0.5 + 0.025). Previous studies have reported the increased toxicity of mixtures of neonicotinoids and DMI fungicides (Tomé et al., 2017; Willow et al., 2019) which are in agreement with this study. This shows that combination of acetamiprid and tebuconazole has resulted in increasing the efficacy of these pesticides if compared to the individual concentrations of binary mixtures in terms of avoidance rate. Noosidum et al. (2014) compared the escape response of *Aedes aegypti* to essential oils applied singly and in mixtures. They found that mixture of oil extracted from *Litsea salicifolia* (LS) and *Litsea cubeba* (LC) at a concentration of 0.075% exhibited more potential for repellence (65.5%) than LS (20.3%) and LC (20%) oils at the same concentrations alone. Essential

oils however may not have the same effect on other insects at the same concentration or exposure time. Moreover, Fernandes et al. (2016) also reported the repellence of *Orius insidiosus, Cycloneda sanguinea* and *Chauliognathus flavipe* (important natural enemies of aphids, lepidopterus eggs, whitefly mites and thrips) from neonicotinoids treated filter papers. The findings support the phenomenon of avoidance in insects however; the degree of avoidance seems to be dependent on various factors such as the mode of action of a pesticide, type and strength of the chemical used and exposure time to the chemicals.

3.4.2 Avoidance of *Chrysoperla carnea* larvae

In *Chrysoperla carnea,* the avoidance response was highest with 0.05 gL^{-1} acetamiprid, which is, a five times lower than its manufacturer recommended rate of application. The avoidance at this concentration was identical to larval group exposed to 0.25 gL^{-1} but significantly higher than 0 control and 0.025 gL^{-1} acetamiprid. Easton and Goulson (2013) reported from their experiment on flying insects belonging to order Diptera and Coleoptera that insects from both of the taxonomic group exhibited a marked escape response from the traps treated with imidacloprid doses as low as 1 μgL^{-1} and 0.01 μgL^{-1}. Similarly, they reported a reduced number of spider catches in traps containing highest imidacloprid concentration as compared to traps treated with lower doses. These results are in line with the present study in which larvae of *Chrysoperla carnea* have shown a greater avoidance from higher concentrations of acetamiprid than lower. In tebuconazole treatment, the escape response from leaf discs treated with its three concentrations was significantly highest as compared to control. No significant difference however, was found in rate of avoidance by insect between the three-tebuconazole concentrations. These results suggest that tebuconazole is unacceptable for insect in a concentration as low as 10 time lower to manufacturer rate of application. The adverse effects of fungicides in beneficial insects have been established by some researchers in past. For instance, Pratissoli et al. (2010) identified negative impact of tebuconazole, thiophanate-methyl and chlorothalonil on parasitic capability of *Trichogramma atopovrilia* an important parasitoid species that parasitize *Diaphania hyalinata* eggs (a pest of Cucurbitaceous crops). Reports like these supports the findings from the current study of the adverse effects of tebuconazole in different forms. Similarly, the significant increase in avoidance of larvae over time suggest its toxicity that outstands and persist on the substrate thus enhancing its affect with time.

In case of mixtures of tebuconazole and acetamiprid, the degree of avoidance of insects from the treated leaves was identical for all the treatments including control. This means any rise or change

in concentration of the acetamiprid component of the mixtures had no effect on changing the escape response of *Chrysoperla carnea*. These finding suggest that for non-target insects the mixtures made by combining the lowest doses of individual components are equally repulsive than using and mixing the higher doses. This is in contrast to the studies that have reported the synergized effect of neonicotinoids in presence of tebuconazole (Willow et al., 2019).

3.4.3 Locomotion of *Plutella xylostella* larvae

Speed of *Plutella xylostella* larvae remained significantly low for the highest concentrations of acetamiprid with an exception of fourth instar larvae whose speed at 0.25 gL^{-1} was significantly high than control and the two lower concentrations. Speed of second instar larvae was significantly highest for control as compared to the two highest concentrations of acetamiprid (0.05 gL^{-1} and 0.25 gL^{-1}). In third instar larval set, however no significant difference in control and acetamiprid concentrations was seen. The relatively low speed of various developmental instars on acetamiprid treatments is in agreement with the findings of de Castro et al. (2013). They reported a reduced locomotor activity in *Supputius cincticeps* when exposed to spinosad, deltamethrin, chlorantraniliprole and methamidophos insecticides.

Tebuconazole concentrations showed a significant reduction in speed of larvae as compared to control except fourth instar larvae where tebuconazole had no significant effect on their speed. Change in concentration of acetamiprid in mixtures showed a significant effect on speed of *Plutella xylostella*. Relative to control speed of second instar larvae was lowest with mix3 that contained the highest concentration of acetamiprid. In third instar group, speed of larvae was not affected by any of the mixtures while speed of larvae at their fourth instar stage was significantly high with mix1 in comparison to control, mix2 and mix3. Behavioural irregularities of the similar kind such as hyperactivity and loss of motion coordination under the influence of combined exposure to flupyradifurone, an nAChR agonist insecticide, and propiconazole, an EBI fungicide has been reported in *Apis mellifera* by Tosi and Nieh (2019). Furthermore, the effect of duration of exposure to the chemicals was significant in case of second and fourth instars only. The reason for lower speed of instars with higher concentrations may be the greater toxicity of pesticides making the insects incapable of moving at their normal pace (Lambin et al., 2001).

The number of stationary periods recorded for second instar larvae was not affected by acetamiprid, tebuconazole and mixture treatments. In case of third instar larvae, SPn was significantly greater for 0.25 gL^{-1} acetamiprid. Tebuconazole on the other hand, caused a

significantly lowest number of stationary periods at its highest concentration in third instars whereas, showing no significant effect on stationary periods of fourth instar larvae. Likewise, various mixtures indicated no significant effect on larvae at their three developmental stages. Effect of increase in exposure time also varied with different chemical treatments as well as developmental stages however, at most places its effect remained non-significant on stationary periods. With some exceptions, number of stationary periods of *Plutella xylostella* significantly decreased with exposure time to acetamiprid, tebuconazole concentrations and mixtures treatments.

These findings are similar to those reported by Lambin et al. (2001). They found that bees treated with lowest dose of imidacloprid (1.25 ng/bee) resulted in increased motor activity while the higher doses (5–20 ng/bee) caused a reduction in their displacement. The results of present study are also in line with the findings of Suchail et al. (2001), who also reported a decline in motor activity of *Apis mellifera* when exposed to high doses of imidacloprid (2.5 to 20 ng per bee). Furthermore, Guedes et al. (2006) in their study also found a decline in flight taking off activity of pyrithroid susceptible population of maize weevil (*Sitophilus zeamais*) in response to increasing residue of deltamethrin. Likewise, Aliouane et al. (2009) investigated the motor activity of honey bees on topical and oral application of fipronil, acetamiprid and thiamethoxam and found that in all the treatments the bees demonstrated less activity in terms of their mobility as compared to control.

3.4.4 Locomotion of *Chrysoperla carnea* larvae

In *Chrysoperla carnea*, no significant effect on their speed and number of stationary periods was found with experimental pesticides, either alone or in mixtures. A study conducted by El Hassani et al. (2005) reported that regardless of route of exposure, no effect on locomotion activity of honey bees (*Apis mellifera*) was found when treated with fipronil. Fipronil is a broadspectrum neurotoxic insecticide similar to neonicotinoids. However, unlike neonicotionoids which targets the acetylcholine receptors in the central nervouse system, fipronil blocks the glutamate-gated chloride channels and GABA-gated chloride channels thus antagonize the calming-effect of GABA, which inturn leads to overexcitation of the affected nerves and muscles of the insects (Prullage et al. 2011). Similarly, Cordeiro et al. (2013) showed no significant difference in walking velocity of *Amblyseius herbicolus,* (a predator of red mite), between treated and non-treated substrates with deltamethrin. El Hassani et al. (2008) in another study, on the contrary, have also reported that thoracic administration of acetamiprid induced an increased motor activity of *Apis mellifera* at a

rate of 0.1 and 0.5 µg/bee. Thompson et al. (2014) identified increased stumbling and knockdown effect in honey bees after 4 h of exposure to clothianidin, thiamethoxam and imidacloprid treated cages. The fungicides used in their study however, showed no sublethal toxicity to bees whether alone or when combined with these neonicotinoids.

3.4.5 Feeding rate of *Plutella xylostella* larvae

Feeding rate of *Plutella xylostella* was identical for control and acetamiprid concentrations for second and third instar larvae. In fourth instar larvae, however, the feeding rate was significantly high for 0.25 gL^{-1} acetamiprid than control and the two lower concentrations of acetamiprid. On the other hand, feeding of larvae irrespective of their developmental stages, lowered down significantly when exposed to 1 mlL^{-1} and 5 mlL^{-1} as compared to 0 mlL^{-1} and 0.5 mlL^{-1} tebuconazole. This indicates an interesting result, which shows that the fungicide has both repelling property on insect pest as well as acting as antifeedant. This means the fungicide not only deter the insect away but also makes the foliage distasteful due to which the insect avoid to attack and feed on it. Likewise, the effect of mixtures was such that the larvae experienced a significantly higher reduction in their feeding when served on mix3 having the highest concentration of acetamiprid 0.25 gL^{-1} mixed with 0.5 mlL^{-1} tebuconazole. The reduction in feeding rate of *Plutella xylostella* larva on mix3 indicates the effect of acetamiprid in presence of tebuconazole as acetamiprid alone has no significant effect on feeding rate of the larvae. These results further strengthens the probability of antifeedant property of tebuconazole. A reduction in feeding rate of *Gammarus pulex* (an aquatic amphipod) fed on tebuconazole treated leaves has been reported by Dimitrov et al. (2014). Fernandes et al. (2016) reported a reduction in feeding rate of *Orius insidiosus* (insidious flower bug)*, Cycloneda sanguinea* (ladybird beetle) and *Chauliognathus flavipe* (soldier beetle) by 50%, 57% and 76% respectively when exposed to imidacloprid and 36%, 83% and 81% when exposed to thiamethoxam. Sancho et al. (2009) in their study found a reduction in energy content of *Daphnia magna* when exposed to concentrations of tebuconazole higher than 0.52 mgL^{-1}. Their findings suggested that tebuconazole has a moderate toxicity to *Daphnia magna* but at the same time, it cause serious impairment in metabolic functions as well as reduction in feeding rate after 5 hours of exposure to tebuconazole. These findings are in agreement with the present results as tebuconazole resulted a reduction in feeding of larvae relative to control.

3.4.6 Feeding rate of *Chrysoperla carnea* larvae

Feeding of *Chrysoperla carnea* larvae seemed to be least affected by acetamiprid as compared to control as no significant difference in feeding rate of larvae within the three acetamiprid treatments group and control was found. In contrast, Ail-Catzim et al. (2015), found imidacloprid highly toxic to *Chrysoperla carnea* because it reduced the average food intake of insect as compared to other insecticides abamectin, befenthrin and endosulfan. Similarly, larvae exposed to three mixtures showed no difference in their feeding from control. On the contrary, tebuconazole concentrations caused a significant reduction in feeding of larvae relative to control. These findings are interesting in terms of the effect of fungicide on the behavioural alteration of insects. At the same time, the results of current study suggest that tebuconazole was similar in its effect whether used at manufacturer recommended rate or five and ten times lower than that. Moreover, the effect of reduction in feeding rate was measured through contact toxicity as *Chrysoperla carnea* were not fed directly on tebuconazole treated prey. This is an indication towards a much potent toxic effect of tebuconazole towards this non-target insect. In concordance with current findings, Pratissoli et al. (2010) also reported that tebuconazole reduced fitness of *Trichogramma atopovirilia,* a natural enemy of *Diaphania hyalinata*, in terms of its parasitism as well as emergence on *Diaphania hyalinata* eggs.

3.5 Conclusion

Outcomes of the current study suggest that combining the pesticides even in the small concentrations, can be as effective against pest insect as they as they were in higher concentrations. Besides, the age of an insect plays a vital role in shaping its behaviour towards a chemical. Reduction in activity of early stage instars with the intensity of these chemicals propose that the pesticides attack the nervous system more efficiently. Eventually the insects became unable to avoid and escape the due to the high toxic effect of the chemicals. Hence increasing the chance of their eradication through high mortalities. At the same time, the damage caused by these high doses to non-target insects is alarming. For instance, reduction in feeding rate of *Chrysoperla carnea* upon exposure to tebuconazole treatments is of serious concern since these kinds of interference in the feeding of natural enemies might lead to long-lasting consequences that can scale-back the predatory efficacy of non-target insects. To avoid harming the beneficial non-target insects to an extent that is not restorable, there is a need to revise the levels at which pesticides are currently applied, especially when they are tank-mixed. Adopting such practices,

can not only be help conserve the populations of these non-target insects, whose role as biological control is crucial in agriculture, but also the process of pest eradication can be augmented. To support this argument, the risk assessment of combinations of other neonicotinoids and fungicides to analyse their effects is essential to be studied. In addition, the sublethal effects of tebuconazole and alike fungicides in insects are rare phenomena reported; therefore, it needs to be enlightened further to strengthen these facts about fungicides.

Chapter 4 Lethal and sublethal effects of thiacloprid alone and in mixtures with tebuconazole on *Chrysoperla carnea*

4.1 Introduction

Recent studies have shown an increased emphasis on investigation of the risks associated with co-application of fungicides and insecticides on various non-target organisms. This is because insecticides and fungicides are often tank-mixed to apply them simultaneously in agrarian fields (Thompson, 2012) and the existing risk assessment data about such practices is still very small and need further enrichment. In previous chapters the risk assessment of acetamiprid neonicotinoid and tebuconazole fungicide was carried out on target and non-target insects utilizing the first tier testing in the laboratory which allows manipulating the conditions as required. The results showed a significant effect of these pesticides mixtures on both target and non-target insects. These pesticides combinations were used to develop a methodology and look at what one would do in a classical tier testing. In higher tiered assessments, however, it is important to acquire as much field relevance as possible. Therefore, to take this study a step forward by assessing the impact of more field relevant combinations, the effect of mixtures of another neonicotinoid i. e. thiacloprid, having a similar mode of action as acetamiprid, with tebuconazole fungicide was evaluated in this chapter. Thiacloprid and tebuconazole are both known to come togather in tank-mixes for application on a wide range of crops such as cotton, orchards, wheat and oil seed rape, throughout Europe (Willow et al., 2019). According to a survey by Food and Environment Research Agency (fera), the total annual use of thiacloprid in UK was estimated as 6,091 kg by weight while Tebuconazole was used at the rate of 401,299 kg in the year 2016 only (https://secure.fera.defra.gov.uk/pusstats/myresults.cfm). Hence, looking ath their extensive use in the UK, the first tiered assessment was repeated using these two pesticides to see as to whether the effect of this neonicotinoid in combination with tebuconazole was similar to that investigated in earlier chapters.

Findings from recent studies suggest that toxicity of mixtures of insecticides and fungicides could be underestimated if they are only assessed based on their individual application, as is the current norm (Jansen et al., 2017). On the other hand, farmers would never hesitate to combine different products in a spray tank, which benefit them from saving time, as well as labour and energy. In

other words, such practices are performed to acquire an improved agronomic yield along with practical and economic advantage (Steven and Christopher, 2018). In addition, transportation of systemic pesticides along the plant body may result in accumulation of their residues in different parts thus increasing the potential exposure of beneficial insects to mixtures when other pesticides are sprayed (Thompson et al., 2014).

Currently, the systemic insecticides of fundamental concern are the neonicotinoids (Thompson et al., 2014). Thiacloprid is one of such widely used neonicotinoids in agriculture (Pesticide Action Network UK, 2016). It is among those neonicotinoids, which were patented for use as insecticides in April 2018, after imidacloprid, thiamethoxam and clothianidin were banned for outdoor use by all member countries of European Union, keeping in view a substantial body of evidence on their adverse effect on non-target insects (Giorio et al., 2017). Like all other neonicotinoids, its systemic nature allows it to travel through various parts of the plant (Johnson, 2015). It belongs to cyano group neonicotinoids (having cyanoguanidine pharmacophore). Cyanoguanidine neonicotinoids (acetamiprid and thiacloprid) have been reported as less toxic to insects as compared to the nitro-substituted neonicotinoids (imidacloprid, clothianidin, thiamethoxam) (Brunet et al., 2005). However, evidences of its high acute and chronic toxicities also exist in literature (Brandt et al., 2016; Tison et al., 2017). Moreover, previous experiments have shown a higher sensitivity of soil fauna and aquatic insects to this insecticide (Beketov and Liess, 2008; Mathieu et al., 2018). For instance, in a multigenerational study on springtail (*Folsomia candida*) response to thiacloprid, van Gestel et al. (2017) showed a reduction in its toxicity in second and third generation of the test species as compared to imidacloprid. This could be related to high persistence of imidacloprid for a longer period in soil than thiacloprid. On the other hand, the toxicity of thiacloprid was two folds on winter generation of mayflies as compared to thiamethoxam and imidacloprid (van den Brink et al., 2016).

Apart from acute toxic effects of pesticides, the chronic toxicity of these compounds to non-target organisms is also of utmost importance as this may lead to numerous behavioural sublethal effects. The most studied sublethal effects of agrochemicals on beneficial arthropod community include (a) knock down effect (a) tumbling and trembling, spinning and grooming behaviour that involves cleaning the abdomen and rubbing together the hind limbs (c) uncoordinated movements, (d) irritant and repellent effects of pesticides. Some endpoints used to determine the motor activity include the extent of time of their inactivity , the position of the arthropod in treated arena (Lambin et al., 2001), distance covered by insect and time spent in each level of arena (El Hassani et al., 2008). Imidacloprid applied at sublethal rate of 0.24 g/m^2 caused a

reduction in mass gain of wood cricket (*Nemobius sylvestris*) a herbivore, and nursery web spider (*Pisaura mirabilis*), a predator species found in wild strawberry (*Fragaria vesca*) food chain. Likewise, at a higher rate of 2.4 g/m^2 it caused impaired mobility, feeding, thorax growth as well as mass gain. Predatory capability of web spider was also adversely affected (Uhl et al., 2015; Uhl et al., 2016). Similarly, consumption of aphids in wheat field contaminated with thiamethoxam spray resulted in impairment in the predatory capability of ladybird (*Coleomegilla maculate*). The consequence of which was a reduction in its efficacy in eliminating the target pest effectively (Bredeson et al., 2015).

Most recent studies on the toxic impact of neonicotinoids are on pollinators specially bees, whereas, the volume of studies on other beneficial arthropods that have been published in last few years is not so big (Pisa et al., 2017). Moreover, majority of those studies were centred on the effect of imidacloprid. Only recently, studies have focused on other newly developed chemicals (Giorio et al., 2017). Both thiacloprid and tebuconazole are often applied as tank-mixex in a variety of agrian ecosystems which predominantly include wheat, cotton, orchards and oilseed rape. *Chrysoperla carnea* is an important natural enemy which is distributed worldwide and therefore, can be a part of many types of habitats (Willow et al., 2019). These types of natural predators offers theh first line of defence against pest in nature and hence, are a key to integrated pest management programs throughtout the world (Sabrey and El-Sayed 2011). Therefore, their role as a biological control species as well as their size and behavioural dissimilarities relative to bees, strongly suggests the inclusion of non-target beneficial insects like these in further additional ecotoxicological testing.

4.1.1 Objectives

The approach used in this study was to assess the toxicity of thiacloprid both alone and in combination with tebuconazole fungicide on *Chrysoperla carnea*. *Chrysoperla carnea* is an important biological control agent employed in Integrated Pest Management programmes and therefore, utilisation of selective insecticides against pests is essential for its survival. This entails the implementation of ecotoxicological investigations that can improve our understanding on the combined utilization of beneficial insects and insecticides (Medina et al., 2003). Moreover, due to the established synergism between DMI fungicides and neonicotinoids, it is also of vital importance to know the rates at which the fungicide and neonicotinoids become comparatively more toxic when applied together.

The study was carried out with the following objectives:

1. To determine the toxicity of thiacloprid alone and in mixtures on non-target insect model *Chrysoperla carnea*.
2. To determine whether there is a difference in toxicity of thiacloprid alone and its mixtures with tebuconazole in terms of LC_{50} of *Chrysoperla carnea* larvae.
3. To determine whether there is a difference in mortality rate and LC_{50} of *Chrysoperla carnea* larvae with respect to exposure time to thiacloprid and mixtures.
4. To determine the sublethal effects of thiacloprid and mixtures in the form of alteration in avoidance behaviour, feeding rate as well as mobility parameters (speed and number of stationary periods) of *Chrysoperla carnea* larvae.

4.2 Materials and methods

Two pesticides were used in the designed experiment. Details are given below in the Table 11.

Table 11 Pesticides used in bioassay on the non-target insect, *Chrysoperla carnea*.

Properties	**Chemical classification**	
	Insecticide	***Fungicide***
Commercial name	Biscaya	Folicur
Active ingredient	Thiacloprid	Tebuconazole
Mode of action	A neonicotinoid, disrupting the insects nervous system by stimulating nicotinic acetylcholine receptors	An EBI fungicide - inhibits P-450 enzyme, during sterol formation
Formulation	Oil dispersion concentrate 240 gL^{-1}	Oil Emulsion 250 gL^{-1}

4.2.1 Preparation of solutions

4.2.1.1 Individual application of thiacloprid

For bioassay, three solutions of thiacloprid were prepared. One of the three solutions was according to its label rate (0.4 Lha^{-1} in a water volume of 200 L) while the remaining two were 5 times and 10 times lower than label rate (Table. 12).

4.2.1.2 Mixtures of thiacloprid and tebuconazole

To determine the effect of mixtures in the non-target insect *Chrysoperla carnea*, binary mixtures of the two pesticides were prepared having different concentrations. Details of mixtures are given in Table 12.

Table 12 Concentrations of thiacloprid used alone and in its binary mixtures with tebuconazole.

Pesticide	Pesticides concentration per litre
Thiacloprid -1	10X lower than label rate (0.2 ml)
Thiacloprid -2	5X lower than label rate (0.4 ml)
Thiacloprid -3	label rate (2 ml)
Mix4	10X lower tebuconazole + 10X lower of thiacloprid (0.5 ml + 0.2 ml)
Mix5	10X lower tebuconazole + 5X lower thiacloprid (0.5 ml + 0.4 ml)
Mix6	10X lower tebuconazole + label rate of thiacloprid (0.5 ml + 2 ml)

4.2.2 Lethal toxicity on *Chrysoperla carnea*

To determine the toxic effect of thiacloprid and its binary mixtures with tebuconazole, *Chrysoperla carnea* larvae were obtained from Agralan limited UK. The larvae after arrival were subjected to freshly grown pesticide untreated Chinese cabbage plants infested by green aphids to acclimatize them for 2 to 3 days. After that, a lethal toxicity bioassay was carried out using thiacloprid and tebuconazole solutions alone as well as in combinations. These solutions were applied to the substrate (in this study, the leaf discs) by leaf dip bioassay technique mentioned in chapter 2 in detail. Once the leaf discs were ready, the acclimatized larvae were subjected to these treated leaf discs and green aphids were supplied to them as food source. Into each Petri dish, ten lacewing larvae were allowed to feed on aphids. Each treatment was replicated 12 times. After resting on pesticides treated leaf discs and feeding on aphids, first mortality counts were taken after 24 h. Following this the left over aphids were removed and the food source was replenished. A second mortality count was then recorded after 48 h of the initial exposure to the treated substrate.

4.2.3 Sublethal toxicity on *Chrysoperla carnea*

Sublethal changes in the behaviour of lacewings were recorded after exposure to the pesticide treated substrate. These sublethal parameters included avoidance of the larvae from the treated surface, alteration in feeding rate as well as pesticides effect on the mobility of insect larvae.

4.2.3.1 Avoidance from pesticide treated surface

To determine the avoidance behaviour of *Chrysoperla carnea* as a factor of sublethal effects of thiacloprid, the avoidance of 60 larvae spaced in six replicates (10 per each) on pesticide treated leaf discs was observed and recorded. This was done such that ten *Chrysoperla carnea* larvae were placed on a leaf disc treated with thiacloprid or mixtures or deionized water (serving as control) in a Petri dish. Into each Petri dish, green aphids were supplied to these larvae to feed on them. These Petri dishes were then covered with ventilated plastic foil. Number of larvae avoiding the treated leaf surface was recorded after 24 h of initial exposure. A second reading of avoidance was taken after 48 h of exposure to the pesticides.

4.2.3.2 Feeding rate

To determine the alteration in feeding rate of non-target insect *Chrysoperla carnea*, 30 lacewing larvae after contact toxicity test were randomly selected and transferred one each into a separate Petri dish. The larvae were provided with five aphids to feed on for the next 24 h. The aphids were allowed to rest on a leaf disc in petri dishes. After 24 h, the number of dead aphids were counted and consumption rate was recorded for each lacewing larvae. The same procedure was carried out after 48 h of exposure of larvae to the pesticides treated leaf discs to take the second count at 48 h. The percentage of feeding rate was recorded by counting the number of dead aphids in each replicate of each treatment.

4.2.3.3 Mobility parameters

Mobility of lacewings larvae was recorded after 24 h and 48 h of treatment to the chemical (individual or mixture) by subjecting a larva on a graph paper having 1 mm grid lines. Fifteen individual larvae were randomly selected to determine the mobility parameters. These included the speed of the larvae and the number of stationary periods demonstrated by the treated larvae while moving.

4.2.4 Statistical analysis

Before using parametric statistics, the data was tested for normality and the non-normal data was log transformed. Lethal and sublethal parameters for *Chrysoperla carnea* were determined by Generalized linear model (GLZM) as it is a parametric test applicable on non-normally distributed data and therefore, has an advantage over using a non-paramemeteric equivalent. Pairwise comparison offereing Bonferroni Significance, was followed. To estimate the effect of various

concentrations of a pesticide on larvae, Median lethal toxicity LC_{50} was determined using probit-analysis. All the statistical analysis was carried out using SPSS software.

4.3 Results

4.3.1 Lethal toxicity

4.3.1.1 Effect of thiacloprid on mortality of *Chrysoperla carnea*

Randomly selected *Chrysoperla carnea* larvae were subjected to the leaf discs treated with three different concentrations of thiacloprid and control in 12 replicates, 10 per each. Lethal toxicity of thiacloprid was determined in percentage mortality. The difference in percentage mortality was measured between three concentrations of tebuconazole and control as well as the time of exposure that was 24 h and 48 h. A significant difference in mortality was found between various concentrations of thiacloprid and control, which in this case was only deionized water ($F_{(3, 88)}$ = 73.930 p = 0.000) (Fig. 27a). Highest mortality was found with 2 mlL^{-1} concentration of thiacloprid. Pairwise comparison showed a significant difference between control and the three thiacloprid treatments as well as between 2 mlL^{-1} and 0.4 mlL^{-1} and 0.2 mlL^{-1} of thiacloprid concentrations used. Moreover, a slight increase in mortality was observed in mortality of larvae with increase in exposure time however, it was not significant ($F_{(1, 88)}$ = 2.629 p = 0.105).

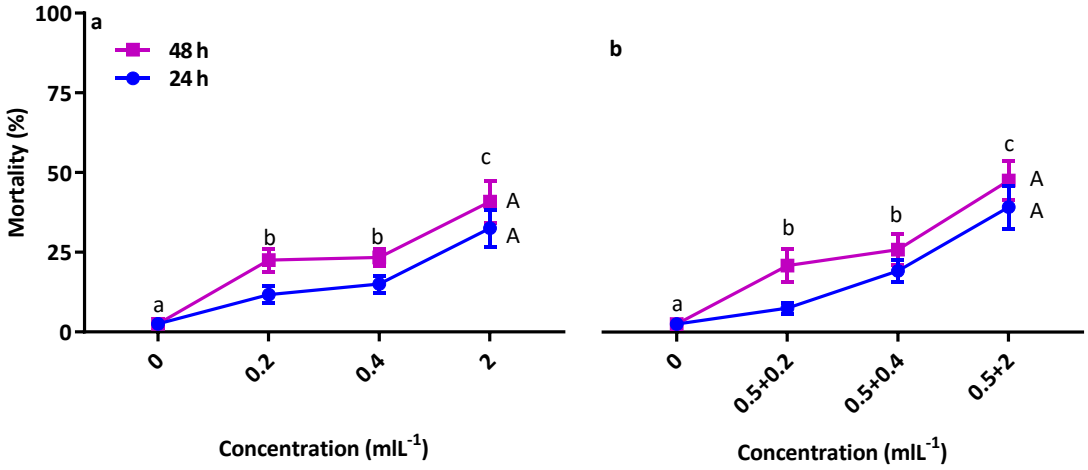

Figure 27 Mean percentage mortality of *Chrysoperla carnea* larvae on various treatments. a) Individual concentrations of thiacloprid b) Mixtures of tebuconazole and thiacloprid. Error bars sharing the same lowercase letters show no significant difference in mortality between different treatments (p > 0.05). Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure.

4.3.1.2 Effect of tebuconazole-thiacloprid mixtures on mortality of *Chrysoperla carnea*

In the current study, three different mixtures were used in which a 10X lower than field dose of tebuconazole was mixed with three concentrations of thiacloprid such that the three mixtures were (tebuconazole + thiacloprid) 0.5 + 0.2, 0.5 + 0.4 and 0.5 + 2 mlL^{-1}. Here the mortality rate was significantly affected by changing concentration of mixtures ($F_{(3, 88)} = 84.417$ $p = 0.000$). Pairwise comparison however, showed a significantly high mortality rate by mixtures than control. Likewise, mix6 (0.5 mlL^{-1} tebuconazole + 2 mlL^{-1} thiacloprid) resulted in a significantly higher mortality rate of the larvae as compared to the two other mixtures that contained lower levels of thiacloprid in them (Fig. 27b). Increase in the time of exposure to the mixtures showed no significant effect on mortality ($F_{(1, 88)} = 3.214$ $p = 0.073$).

4.3.1.3 Effect of thiacloprid and tebuconazole-thiacloprid mixtures on LC_{50} of *Chrysoperla carnea*

In the current study, the toxicity of thiacloprid as well as mixtures for *Chrysoperla carnea* larvae were similar as shown by LC_{50} values (Table. 6). Mixtures although had a slightly lower LC_{50} values than thiacloprid, this difference was not statistically significant ($p > 0.01$; overlapping 95% FL). In addition, the toxicity of thiacloprid as well as its mixtures with tebuconazole on *Chrysoperla carnea* although increased with time as shown by a drop in LC_{50} values. This decline in LC_{50} values however, was not significantly different for individual thiacloprid treatments or its mixtures ($p > 0.01$; overlapping 95% FL). Likewise, the LC_{50} values were always above the concentrations of thiacloprid applied alone or in mixtures.

Table 13 Toxicity (LC_{50}) of thiacloprid and its mixtures with tebuconazole on *Chrysoperla carnea* after 24 h and 48 h exposure.

Pesticide	**24 h LC_{50} (95% FL)**	**48 h LC_{50} (95% FL)**
Thiacloprid	4.075[a] (3.486 – 5.148)	3.466[a] (3.009 – 4.227)
Mixtures	3.500[a] (3.120 – 4.093)	3.094[a] (2.668 – 3.816)

Values within column sharing the same lowercase letter have overlapping 95% FL and are not significantly different at $p > 0.01$.

4.3.2 Sublethal toxicity

4.3.2.1 Effect of thiacloprid on avoidance behaviour of *Chrysoperla carnea*

To determine the avoidance behaviour of *Chrysoperla carnea* as a factor of sublethal effects of thiacloprid, the avoidance of 60 larvae spaced in six replicates (10 per each) on pesticide treated leaf discs was observed and recorded. The mean percentage avoidance of larvae was significantly different among the various treatments used ($F_{(3, 40)} = 22.20$ $p = 0.000$). Post-hoc test of multiple comparison between treatments showed a significantly higher avoidance by larvae subjected to the three individual treatments of thiacloprid 0.2 mlL^{-1}, 0.4 mlL^{-1} and 2 mlL^{-1} than control. Conversely, avoidance of larvae with the three thiacloprid concentrations, remained similar ($p > 0.05$) (Fig. 28a). Furthermore, the effect of change in exposure time had no significant effect on avoidance behaviour of the larvae ($F_{(1, 40)} = 0.076$ $p = 0.784$).

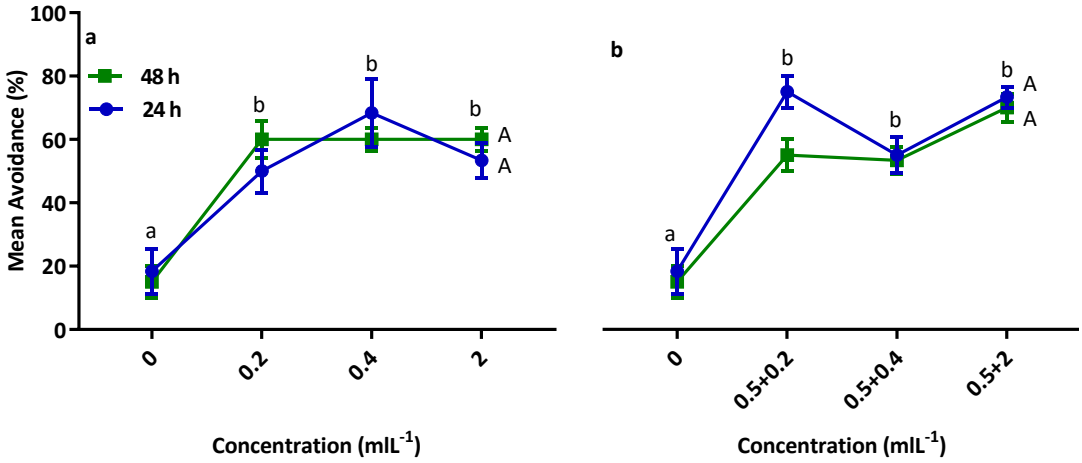

Figure 28 Mean percentage avoidance of *Chrysoperla carnea* larvae on various treatments. a) Individual concentrations of thiacloprid b) Mixtures of tebuconazole and thiacloprid. Error bars sharing the same lowercase letters show no significant difference in avoidance between different treatments ($p > 0.05$). Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

4.3.2.2 Effect of tebuconazole-thiacloprid mixtures on avoidance behaviour of *Chrysoperla carnea*

Various mixtures significantly affected the avoidance of larvae from the leaf discs ($F_{(3, 40)} = 11.18$ $p = 0.011$). Exposure time effect on the other hand, was not significant in case of mixtures ($F_{(1, 40)} = 0.197$ $p = 0.657$). The post-hoc test for multiple comparison between treatments showed a

significant increase in avoidance of larvae of the three mixture when compared to control. This increase in avoidance among the mixtures however, remained non-significant (Fig. 28b).

4.3.2.3 Effect of thiacloprid on feeding rate of *Chrysoperla carnea*

Feeding rate of *Chrysoperla carnea* larvae was determined by measuring the number of aphids each larva consumed during 24 h and 48 h after exposure to the leaf discs treated with pesticides. Consumption of 30 larvae was observed for each concentration of pesticides used under this study.

For thiacloprid alone treatments, no significant change was found in percentage feeding of larvae within its three concentrations as well as control ($F_{(3, 232)} = 2.546$ $p = 0.467$) (Fig. 29a). In contrast, the feeding rate decreased with increase in time ($F_{(1, 232)} = 4.548$ $p = 0.033$). This decrease may be related to the prolonged exposure of larvae to pesticides and residual toxicity of insecticide, which has adversely affected the feeding capability of *Chrysoperla carnea* over time.

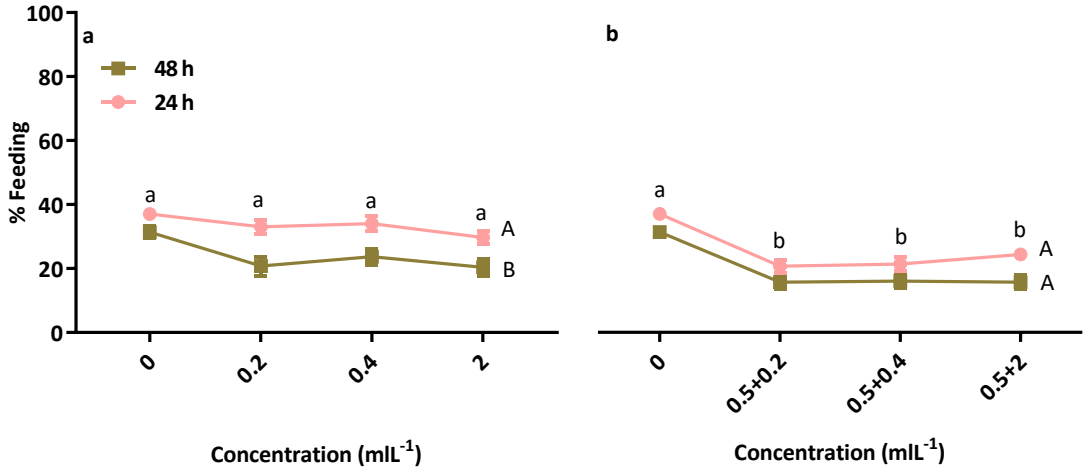

Figure 29 Mean percentage feeding of *Chrysoperla carnea* larvae on various treatments. a) Individual concentrations of thiacloprid b) Mixtures of tebuconazole and thiacloprid. Error bars sharing the same lowercase letters show no significant difference in percentage feeding between different treatments ($p > 0.05$). Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

4.3.2.4 Effect of tebuconazole-thiacloprid mixtures on feeding rate of *Chrysoperla carnea*

The three binary mixtures of tebuconazole plus thiacloprid when compared to control, had a significant effect on percentage feeding of larvae under this study ($F_{(3, 232)} = 11.922$ $p = 0.008$).

This difference however, was due to control where the feeding rate was significantly higher than the three mixtures ($p < 0.05$). In contrast, exposure time had no significant effect on feeding rate of larvae ($F_{(1, 232)} = 3.458$ $p = 0.063$) (Fig. 29b).

4.3.2.5 Effect of thiacloprid on speed of *Chrysoperla carnea*

For various concentrations of thiacloprid although the larvae showed some fluctuation in their speed, this change in speed was not statistically significant ($F_{(3, 112)} = 0.221$ $p = 0.974$). In addition, the speed of larvae also showed no significant change with respect to increase in exposure time ($F_{(1, 112)} = 0.012$ $p = 0.912$) (Fig. 30a).

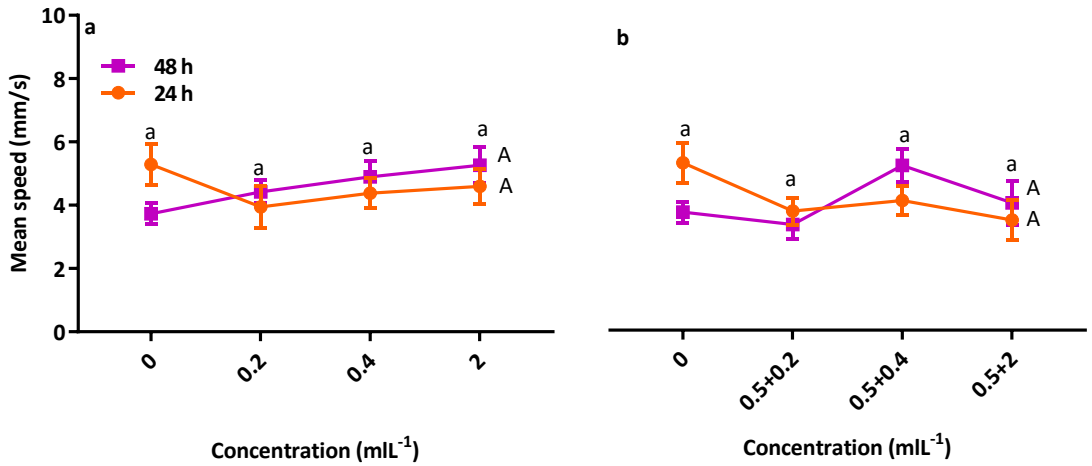

Figure 30 Mean speed of *Chrysoperla carnea* larvae on various treatments. a) Individual concentrations of thiacloprid b) Mixtures of tebuconazole and thiacloprid. Error bars sharing the same lowercase letters show no significant difference in mean speed between different treatments ($p > 0.05$). Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

4.3.2.6 Effect of tebuconazole-thiacloprid mixtures on speed of *Chrysoperla carnea*

The larvae of *Chrysoperla carnea* showed some alteration in their speed when exposed to mixtures however, this change in speed was not significant ($F_{(3, 112)} = 0.538$ $p = 0.910$). Similarly, the exposure time effect was not a significant predictor of larval speed ($F_{(1, 112)} = 0.00$ $p > 0.991$) (Fig. 30b).

4.3.2.7 Effect of thiacloprid on number of stationary periods (SPn) of *Chrysoperla carnea*

The number of stationary periods of *Chrysoperla carnea* wasn't affected significantly by thiacloprid treatments ($F_{(3, 112)} = 0.484$ $p = 0.785$). This means there was no statistically significant

difference between numbers of stationary periods observed with control treatment as well as with the various concentrations of thiacloprid used. Similarly, no significant change in SPn was found with respect to change in exposure time ($F_{(1, 112)} = 0.898$ $p = 0.343$) (Fig. 31a).

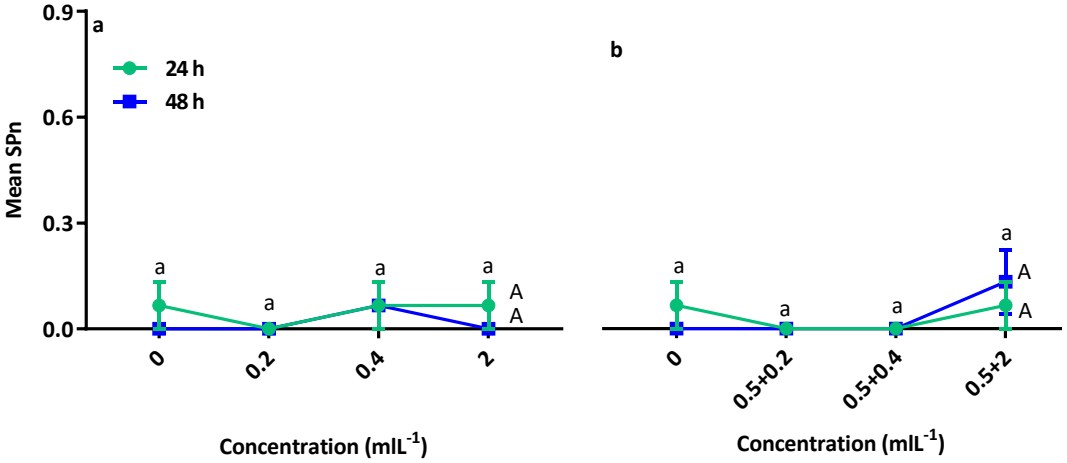

Figure 31 Mean number of stationary periods (SPn) of *Chrysoperla carnea* larvae on various treatments. a) Individual concentrations of thiacloprid b) Mixtures of tebuconazole and thiacloprid. Error bars sharing the same lowercase letters show no significant difference in mean speed between different treatments ($p > 0.05$). Same uppercase letters on the two lines indicate no significant difference with respect to time of exposure ($p > 0.05$).

4.3.2.8 Effect of tebuconazole-thiacloprid mixtures on number of stationary periods (SPn) of *Chrysoperla carnea*

Mixtures of thiacloprid and tebuconazole had no significant effect on the number of stationary periods of *Chrysoperla carnea* ($F_{(3, 112)} = 0.863$ $p = 0.353$). In addition, the SPn of larvae also showed no significant change with respect to increase in exposure time ($F_{(1, 112)} = 0.001$ $p = 0.976$) (Fig. 31b).

4.4 Discussion

4.4.1 Lethal toxicity of thiacloprid on *Chrysoperla carnea*

The results demonstrate an increase in the mortality rate of *Chrysoperla carnea* as the concentration of thiacloprid increased. The mortality rate although raised over time of exposure to thiacloprid, it was not significant. Similarly, the lethal median concentration (LC_{50}) although declined from 24 h to 48 h, still remained non-significant. Earlier studies (Tennekes and Sánchez-Bayo, 2011; Tennekes, 2010a) have pointed out the rise in mortality rate over time on exposure to neonicotinoids. The underlying mechanism for these delayed mortalities involves the binding of these neonicotinoids to nicotinergic acetylcholine receptors (nAChR) that are rooted in synaptic membranes of nerve cells. The continuous stimulation of these receptors produce electric impulses, which eventually lead to the death of nerve cells. Binding of more neonicotinoid molecules to nAChR receptors cause more neurons to die which accumulates with time until the insect is incapable to cope with the damage and thus ultimately die. This is termed as reinforced or time cumulative toxicity (Tennekes and Sánchez-Bayo, 2013).

van den Brink et al. (2016)looked at seasonal variability in acute and chronic toxicity of thiamethoxam, imidacloprid and thiacloprid on mayfly (*Cloeon dipterum*) reported a 6 – fold decline in their LC_{50} from 24 h to 96 h of exposure regardless of season. This study demonstrates the increase in toxicity of thiacloprid over time. Here however, the change in mortality rate was not significantly greater between 24 h and 48 h, may be due to the difference in exposure here compared to van den Brink et al. (2016). The experiment described in this chapter also demonstrated that although the reduction in LC_{50} was not as large, it revealed the residual effect of thiacloprid in terms of increase in mortality rate and decline in LC_{50} over exposure period. Similarly, Luna-Cruz et al. (2015) reported a mortality rate of 38% in a parasitoid, *Tamarixia triozae* on exposure to tomato leaves treated with 1115 ppm imidacloprid concentration after 24 h. Likewise, Krischik et al. (2015) reported the negative impact of imidacloprid in terms of reduction in survival rate of two butterfly species, *Danaus plexippus* and *Vanessa cardui*. In another study by Fogel et al. (2016), they found a mortality rate of 90% in ladybird (*Eriopis connexa*) when they were treated with 200 mgL^{-1} of acetamiprid for 15 days. On the other hand, only 15% of mortality rate was observed when the application rate of acetamiprid was reduced to half (100 mgL^{-1}) of its initial application. Similarly, Gontijo et al. (2015) in their study on sunflower crop grown from thiamethoxam treated seeds, reported a mortality rate of 48% in *Chrysoperla carnea* after 8 days. In the current study, however, mortality was above 40% after 48 h only.

Although thiacloprid and acetamiprid belong to the same cyano group neonicotinoids and studies have shown their small toxicities in comparison to thiamethoxam and other neonicotinoids, here it was found that mortality rate of *Chrysoperla carnea* was close to 50% in 48 h and could even exceed it, if the insect was exposed to it for a longer period. Moreover, the difference in findings could also be due to the method of exposure as in the current study, the non-target *Chrysoperla carnea* was tested for contact toxicity following the leaf dip bioassay while Gontijo et al. (2015) adopted the seed treatment procedure. Seed treatment or seed dressing is a procedure in which seeds are treated with the desired pesticides prior to planting them. This can be environmentally friendly as the amount of chemical used in this procedure can be lowered as compared to foliar spray. Malaker and Mian (2009) investigated the effect of two seed treating fungicides and a foliar spray fungicide on the black point disease in wheat. From their study they reported that relative to seed treatment, the foliar spray fungicide suppressed the severity of wheat black point disease significantly. This might be because the effect of a pesticide would get diluted as it travels to different parts of the plant from the seed while the foliar spray offers an instant and more stronger control for the disease.

4.4.2 Lethal toxicity of tebuconazole-thiacloprid mixtures on *Chrysoperla carnea*

Mortality rate of the non-target insect *Chrysoperla carnea* larvae also increased significantly with different mixtures. The changing concentration of thiacloprid in mixtures showed its significant effect at its highest concentration, that was in mix6 having 0.5 mlL^{-1} + 2 mlL^{-1} of tebuconazole and thiacloprid respectively. Whereas, the rest of the two mixtures with lower concentrations of thiacloprid showed no significant difference between them in affecting the mortality rate of *Chrysoperla carnea*. Likewise, no significant increase in mortality rate was observed over time of exposure from 24 h to 48 h. Moreover, the LC_{50} values for mixtures were above the range of all mixtures used over the entire exposure period. In addition, it also did not decrease significantly between 24 h and 48 h as well as there was no significant difference in LC_{50} of mixtures and that of thiacloprid alone. Jansen et al. (2017) investigated the effects of binary mixtures of fungicides and insecticides on larvae of the ladybird *Adalia bipunctata*, parasitic wasp *Aphidius rhopalosiphi* and the predatory mite *Typhlodromus pyri*. Their results revealed that mixture of thiacloprid and epoxiconazole (a triazole EBI fungicide) had a very strong synergistic effect on all species tested as their LD50 values were up to 11 times lower than thiacloprid alone. On the other hand, epoxiconazole showed no toxic effect on either of the insects when tested alone. By contrast in

present study the LC_{50} value of mixtures of tebuconazole and thiacloprid was not significantly different than thiacloprid alone. Likewise, tebuconazole was also found toxic to both target insect and non-target insect under this study as shown in previous chapters.

These differences in findings could either be due to the variation in exposure method or sometimes due to species specificity, as the response of one species of insect to a chemical may differ from the other. In another study conducted by Lanteigne et al. (2015), the detrivorous amphipod of organic matter *Hyalella azteca* showed that mixture of imidacloprid and cyfluthrin caused a 1.7 and 2.7 folds of higher mortality rate than the two insecticides used alone. However, findings so far shows that insects are comparatively more susceptible to neonicotinoids than amphipods (Morrissey et al., 2015). At the same time, these effects are also species specific. For instance, Englert et al. (2017) have reported that susceptibility of *Gammarus fossarum* an (amphipod crustacean) to thiacloprid, acetamiprid and imidacloprid was greater than caddisfly *Chaetopteryx villosa* (a hexapod insect) both in mixtures and in individual exposures.

4.4.3 Sublethal toxicity of thiacloprid and tebuconazole-thiacloprid mixtures on *Chrysoperla carnea*

Rate of avoidance of *Chrysoperla carnea* was significantly higher in thiacloprid treated leaf discs as than control. However, the three concentrations of thiacloprid indicated no significant difference in repellence of the insect form leaf surfaces treated with them. Likewise, there existed no significant difference in avoidance rate of the insect larvae between the three mixtures of thiacloprid and tebuconazole but it was significantly greater than control. Moreover, feeding rate of *Chrysoperla carnea* larvae exposed to three thiacloprid concentrations was similar to that in control. However, it decreased significantly with time. This decrease in feeding rate may be related to the prolonged exposure of larvae to pesticides and residual toxicity of insecticide, which has adversely affected the feeding capability of *Chrysoperla carnea* over time. On the other hand, feeding rate of larvae with the three binary mixtures was significantly less than control and decreased only slightly over time which was not significant. Similarly, the speed and number of stationary periods of *Chrysoperla carnea* remained similar for control and thiacloprid as well as for mixtures and control groups. In the same way, exposure time had no significant effect in either case. The findings of existing study are in line with those of Fernandes et al. (2016). They reported the repellence of predatory bug *Orius insidiosus,* as well as the predatory beetles *Chauliognathus flavipes* and *Cycloneda sanguinea* from filter paper surfaces treated with thiamethoxam and imidacloprid. Likewise, Krischik et al. (2015) have reported feeding disruptions and modification in

oviposition behaviour in two butterfly species (*Vanessa cardui and Danaus plexippus*) under the effect of various insecticides like naled, imidacloprid, resmethrin, permethrin, dichlorvos, and malathion. In another field trial conducted by Gontijo et al. (2015), sunflower plants grown from thiamethoxam treated seeds although had no significant effect on mortality of *Orius insidiosus* a predatory bug, yet it caused a decline in fertility of female bugs and their egg viability which resulted in decreasing the survival of nymphs by 40%. In line with the findings of the existing study, Uhl et al. (2015) also demonstrated that exposure to 2.4 g/m^2 of imidacloprid caused a decline in herbivory of the ground crickets (*Nemobius sylvestris*) on strawberry leaves as well as its mobility in the experimental set up. On the other hand, web spiders (*Pisaura mirabilis*) tended to be more agile and its rate of predation on crickets increased within the arena after exposure to the same dose of imidacloprid.

4.5 Conclusion

Findings from this study demonstrate no big difference in toxicity of mixtures of tebuconazole and thiacloprid relative to thiacloprid alone treatments used on *Chrysoperla carnea* under this study. This could possibly be due to the lower concentration of tebuconazole used here. This study suggests that the synergistic effect of neonicotinoids and fungicides that have been previously reported can be mitigated if the concentration of one of the component of the mixtures can be reduced. Further investigation in the same line of mixtures risk assessment however, is required for elaborate understanding. Moreover, it is also important to compare the effects of individual neonicotinoid treatments and mixtures on target and non-target insects in order to gain some information on how to manage and make the best use of pesticides when they are mixed.

Chapter 5 General discussion

The aim of this study was to analyse the effects of two neonicotinoids and a fungicide, both individually and in combinations, on target and non-target insects and from these results suggest ways how manage those effects. In this way, this innovative technique of mixture risk assessment can provide some risk management options for the use of pesticides mixtures which can also assist to improve the decision-making process already in place. This is significant because most conventional risk assessment procedures evaluate the effects of a single chemical, or more precisely the single 'active ingredient' on organisms and the environment at a given time. This does not however, match with the real world scenario, where variable chemical cocktails are formed by the interaction of different pesticides (David et al., 2016; Rizzati et al., 2016; Hernandez et al., 2017). These cocktails of plant protection products are the outcome of either tank-mixing two or more chemicals before their field application or by applying them sequentially during a cropping season (Solecki et al., 2014).

The study was, therefore, carried out within the context of ecological risk analysis; that is a combination of two separate elements, the risk assessment and risk management process. Although based in the laboratory, this study was conducted in such a way that could replicate the real field scenario. Therefore, the environmental factors such as temperature, relative humidity and photoperiod which has a vital role in the survival and propagation of organisms in their environment were kept optimum for the test insects. When developing new chemicals and introducing them on the market, a critical step required for their approval and acceptance is to test them on their targets and associated non-target organisms. Hence, in this study, the effects of two types of pesticides, the neonicotinoids (acetamiprid and thiacloprid) and a fungicide (tebuconazole) both alone and in mixtures, were quantified on *Plutella xylostella*, a model target and *Chrysoperla carnea*, a model non-target insect.

To achieve the primary goal of the study, the main objectives were divided into experimental objectives on the basis of risk assessment and risk management framework. These were:

1. To determine the lethal effects of acetamiprid, thiacloprid, tebuconazole both individually and in mixtures on *Plutella xylostella* and *Chrysoperla carnea***,** *Chapter 2 and Chapter 4***.**
2. To determine the sublethal effects of acetamiprid, thiacloprid, tebuconazole both individually and in mixtures on *Plutella xylostella* and *Chrysoperla carnea***,** *Chapter 3 and Chapter 4***.**
3. To determine the difference in the effects of these pesticides on target and non-target insects, *Chapter 2*, thereby making some possible risk management options at conclusion.

5.1 Summary of the key findings

5.1.1 Lethal effects of acetamiprid, thiacloprid, tebuconazole and their mixtures on target and non-target insects

- The mortality rate of all developmental stages of *Plutella xylostella* significantly increased with the increasing acetamiprid and tebuconazole concentrations, either alone or in mixtures. In addition, mortality was also found age-dependent in *Plutella xylostella*, such that fourth instar larvae indicated significantly lower mortality rate than second and third instar larvae on various concentrations of acetamiprid, tebuconazole and their mixtures.

- The mortality rate of *Chrysoperla carnea* increased significantly with increasing concentrations of acetamiprid and was greatest for the highest dose of acetamiprid. By contrast, three concentrations of tebuconazole, though significantly higher than control, had a similar effect on *Chrysoperla carnea*. This was also the case for mixtures of tebuconazole and acetamiprid.

- The mortality rate of *Chrysoperla carnea* increased with increasing concentration of thiacloprid both alone and in its mixtures with tebuconazole. This was significantly high for 2 mlL^{-1} thiacloprid and mix6 (0.5 mlL^{-1} tebuconazole + 2 mlL^{-1} thiacloprid).

- Exposure time effect of acetamiprid, tebuconazole or its binary mixtures varied among the three developmental stages of *Plutella xylostella*, giving a mixed picture of significant and non-significant change in mortality rate over time.

- Increasing the exposure time of *Chrysoperla carnea* larvae to acetamiprid alone and acetamiprid - tebuconazole mixtures caused a significant increase in larval mortality. But rise in exposure time to tebuconazole alone, thiacloprid alone and thiacloprid – tebuconazole mixtures had no significant effect on their mortality rate.

- LC_{50} values of acetamiprid, tebuconazole and their mixtures dropped over time. In the case of acetamiprid, LC_{50} value for *Plutella xylostella* was much lower than *Chrysoperla carnea* suggesting its high toxicity against *Plutella xylostella*.

- Tebuconazole, on the other hand, proved more toxic to *Chrysoperla carnea* and all developmental stages of *Plutella xylostella*, indicated by the LC_{50} values that were below its recommended application rate after 48 h.

- Toxicity of three binary mixtures in terms of LC_{50} against *Chrysoperla carnea* and fourth instar larvae of *Plutella xylostella* was low (due to high LC_{50} values) as compared to second and third instar larvae of *Plutella xylostella* indicating the higher efficacy against second and third instar larvae of *Plutella xylostella*.

- No significant difference in LC_{50} values of thiacloprid and thiacloprid - tebuconazole mixtures existed for *Chrysoperla carnea* larvae over the whole exposure period.

5.1.2 Sublethal effects of acetamiprid, thiacloprid, tebuconazole and their mixtures on target and non–target insects

- Avoidance in *Plutella xylostella* increased with increase in the concentration of acetamiprid. In contrast, on tebuconazole treatments, it seemed to be age dependent as second and third instars tended to avoid lower concentrations more than higher ones, whereas fourth instar larvae showed a tendency of avoiding all the three concentrations of tebuconazole. Likewise, mix1 and mix2 caused a greater repulsion in third and fourth instars but had no significant effect on second instar larvae. With mix3, third and fourth instars experienced a significant reduction in avoidance due to high mortality rate.

- In *Chrysoperla carnea* rate of avoidance increased with increase in the concentration of acetamiprid; however, tebuconazole concentrations remained equally and highly repulsive for *Chrysoperla carnea* larvae relative to control. On the contrary, no significant effect of acetamiprid – tebuconazole mixtures was observed on their avoidance rate. In addition, exposure to three thiacloprid concentrations as well as three thiacloprid – tebuconazole mixtures increased the rate of avoidance in *Chrysoperla carnea* significantly as compared to control.

- Speed of larvae of *Plutella xylostella* declined and number of stationary periods increased with the intensity of acetamiprid, tebuconazole alone or in mixtures. Feeding rate of second and third instars was not significantly affected by acetamiprid concentrations however, fourth instars feeding rate was significantly higher at the highest concentration of acetamiprid. In contrast

feeding rate of all developmental stages declined with increase in the concentration of tebuconazole as well as with intensity of acetamiprid - tebuconazole mixtures.

- Mobility parameters (speed and stationary periods) of *Chrysoperla carnea* remained unaffected with individual concentrations of acetamiprid, thiacloprid and tebuconazole as well as their binary mixtures with tebuconazole. Likewise, the feeding rate of *Chrysoperla carnea* remained unaffected with acetamiprid or thiacloprid individual concentrations as well as acetamiprid – tebuconazole mixtures. However, tebuconazole alone concentrations and thiacloprid – tebuconazole mixtures reduced the feeding rate of larvae significantly relative to control.

- Exposure time to these neonicotinoids and fungicide alone and their mixtures mostly affected the sublethal parameters of *Plutella xylostella.* In contrast, they remained unaffected for *Chrysoperla carnea* except with individual concentrations of thiacloprid which caused a significant reduction in its feeding rate over exposure time.

5.1.3 Mean difference in mortality rate of target and non-target insects

- A maximum difference of 53.3% and 45% in mortality rate was found between *Chrysoperla carnea* and second instars of *Plutella xylostella* with mix3 after 24 h and 48 h respectively. This was followed by the difference between *Chrysoperla carnea* larvae and third instars of *Plutella xylostella* with mix3 which was 43.3% and 36.7% after 24 h and 48 h respectively.

- Besides mortality rate of fourth instar larvae was always greater than *Chrysoperla carnea* larvae irrespective of the chemical treatments which indicates the significance of time of application of insecticides and mixtures since the efficacy of mixtures and individual insecticides is gradually lost as the target insect ages.

5.1.4 Maximum differences in mortality rate

- The maximum difference in mortality rate existed between *Chrysoperla carnea* and second instar larvae of *Plutella xylostella* within 24 h of exposure to mix3 (0.25 gL^{-1}acetamiprid – 0.5 mlL^{-1} tebuconazole).
- This difference declined over 48 h due to relatively greater mortality of *Chrysoperla carnea* larvae.

- The mortality rate of *Chrysoperla carnea* larvae was always lower than fourth instar larvae of *Plutella xylostella* regardless of the pesticide treatment indicating the significance of the time of application of pesticides for its optimal efficacy.
- The risk management options and decisions made will depend on the primary objective of pesticides use. For instance, the primary goal could be to achieve optimal control over pest population or it could be to conserve the non-target community.

5.2 Discussion

This study adopted the first tier testing approach in risk assessment of pesticides to analyse the effects of neonicotinoids (acetamiprid and thiacloprid) both individually and in the presence of a fungicide (tebuconazole) on target and non-target insects. This makes it possible to identify a dose or range of doses at which optimal control over the target organism can be achieved whilst remaining relatively less harmful to non-target species. The study chose *Chrysoperla carnea* as a non-target species and *Plutella xylostella* as a target insect. The same approach can be applied to a more realistic scenario containing amendable target and non-targets as well as the pesticides.

Furthermore, tiered testing is the key to risk assessment. This tiered arrangement extends the standard, relatively simple toxicity assessment (first or lower tiered test) to a more intricate and multiplex situation (second and third or higher tiered evaluation) involving the integration of processes and functions taking place in a naturally existing ecological unit. For instance, semi-field and real field multispecies testing (Schafer et al., 2019).

Lower tier testing, is the most popular and relatively more adopted tool in scientific research. This is because it is more profitable such that it can be used to determine whether higher tier investigation is required at all (Rohr et al., 2016). It is more simplistic and less resource-intensive level of screening. Thus saving time and money. Higher tier experiments are more realistic and relevant as they can further refine the estimatmation of risk by adding more informed data through extended laboratory and extrapolation models (Homman et al., 2010). it is however, not always easy and possible to carry out such experimental procedures due to certain constraints such as environmental and seasonal variations, life stages of the organisms under consideration and economic and budgetary limitations. Lower tier testing are therefore, employed at first place in ecological risk assessment and further higher tier tests are decided based on its outcomes.

5.2.1 Risk assessment of pesticides mixtures, conceptual framework

An environmental risk assessment examines the adverse impact of chemicals on various target and non-target organisms, and the data obtained then functions as a tool to assist the regulatory decision-making process (Hill and Sendashonga, 2003; Lee et al., 2015). A risk assessment should therefore be efficient enough to extract important information from experimental trials to make an informed decision to a regulatory framework. Therefore, the structural clarity of the risk assessment framework is vital to reach an informed, effective decision (Raybould and Wilkinson, 2005). Similarly, when designing an experiment, realism should be considered as the most crucial entity which signifies its relevance to the system for which it is meant to be implemented. This is

particularly the case in laboratory studies where the selected indicator species represent the actual species of the functional group (Sutherland and Poppy, 2005; Weber, 2014).
In traditional risk assessments of pesticides, organisms are exposed to a single compound. Nonetheless, from the management perspective, it is also vital to compare the effects of these pesticides and find the difference between them on the target and non-target insects taken into account in this study. This is an important step a risk manager would adopt for the safe use of pesticides to investigate whether and how their effects vary at a certain period when used alone or in mixtures. Mixture risk assessments on the other hand, is a relatively newly emerging science in its very early stage. This means that the framework is in a continuous process of development and requires meaningful, operative planning to facilitate effective decision-making (Reffstrup et al., 2010).

Ideally, when evaluating pesticides mixtures for their toxicological attributes, detailed information about the constituent parts of mixtures accompanied by their mechanism of action is significant to acquire. Furthermore, to execute an effective risk assessment, appropriate information on exposure to these mixtures is required, which generally is not always available. On the other hand, the residues of pesticides mixtures that an individual encounter in its environment via different routes of exposure may also change in intensity and quality over time. Likewise, another limitation is the occurrence of enormously vast numbers of chemical toxins in the environment which makes it realistically impossible to run toxicity checks for each potentially existing mixture (Kienzler et al., 2016). It also makes it impossible to know the extent of the interaction between these toxins, and whether those interactions constitute antagonism, additivity or synergism (Reffstrup et al., 2010; Beyer et al., 2014). Thus a robust, scientifically rigorous plan of action for assessment of chemical mixtures is required, which is capable of evaluating the ecological risks in diverse sectors (Kienzler et al., 2016).

Over the last decade therefore, chemical mixtures risk assessment has gained significant attention, as evident from several reports published by American and European organisations (OECD, 2011; EFSA, 2013c; EC, 2012; USEPA, 2016) (OECD 2011, EFSA 2014a, OECD 2011, USEPA 2016). Bopp et al. (2016) reported that whilst evaluation of individual chemicals is central to regulate their use, assessment of mixtures across these chemical groups is also of legislative significance. Moreover, the combined effect of individual compounds found in their Predicted No-effect Concentration has been acknowledged by ecotoxicologists (Pery et al., 2013). Likewise, when evaluating mixtures for authorisation, the interaction between their components such as the active ingredients, adjuvants and co-formulants as well as synergists should be taken into

account (EC, 2009). In this regard, the two most popular and widely applied models of mixtures risk assessment are the 'Independent Action model' (IA) and the 'Concentration Addition' (CA) approach (Panizzi et al., 2017; Cedergreen, 2014). The latter implies that the toxicity of a mixture is an outcome of the respective potency and concentrations of its individual constituents. This model usually assumes that the constituent elements of a mixture possess similar target sites and mode of action in an individual (Kortenkamp et al., 2009) whereas, the IA approach is generally suggested for mixes that contain components having dissimilar modes of action, each of which act strictly independently. These models, however, are criticised for as not being appropriate for every situation (Cedergreen et al., 2008; Borgert, 2004; Schindler, 2017) because they have their limitations when mixtures with non-CA effects need to be addressed. The non-CA concept applies to mixtures whose components have interactions exhibiting synergism or antagonism. Therefore, mixture risk assessments are still carried out in a non-harmonized fashion. For this reason, more elaborative research is required to obtain and integrate the experimentally collected information on the effects of pesticides mixtures on overall animal diversity (Panizzi et al., 2017).

In this study, one such non-CA cumulative exposure approach to chemicals has been explored with the main aim of outlining a possible risk assessment procedure for pesticides mixtures and subsequently making some possible risk management recommendations. The layout of the framework involves first tier testing, which compares the effects of individual neonicotinoids (acetamiprid and thiacloprid) on target and non-target insects both with and without a fungicide (tebuconazole) by combining them in different concentrations and evaluating the lethal and sublethal effects of every single and combined doses.

To fetch the productive information, problem formulation and the consequential planning to address the problem are the two fundamental phases in risk assessment framework. During problem formulation, the stressors, receptors, their exposure routes and potential adverse ecological effects are identified, whereas planning involves the conceptual plan of work that carries out the technical analysis needed to efficiently address the quantified problem (National Research Council, 2015). In this context, the first important decision is the selection of stressors to evaluate an appropriate model organism (Juntti, 2019). A model organism is a species that is studied to recognise the basis of biological processes taking place not only in that species but are more widespread and general. Hence using a representative model animal, we can understand the principles that operate similarly in many, if not all of the organisms (Segner and Baumann, 2016). Consequently, the choice of a model species is influenced by many factors; however, ideally it should be the species that best corresponds to the needs of the research question. Likewise, another major factor is to know whether the organism of choice for an experiment can be studied in a certain set of laboratory conditions. The significance of this aspect is that some

animal species may exhibit fascinating performances in the wild, but in captivity may not display such ideal behaviour due to the absence of natural stimuli and the stress caused by the altered conditions in the enclosure. Similarly, the reproductive rate of the organism should be considered when a maximized sample size is required in a short time (Juntti, 2019). In this context, organisms with low ecological demand, high reproductive rate and shorter generation time are preferred as laboratory models (Jaspers, 2015). A model species is valuable as it can serve to understand the ecotoxicological impact of chemical exposure, which can then be extrapolated to a more general population of species. Nevertheless, the domain of application has to be kept in mind while using a model animal, which means ensuring homogeneity of biological characteristics between the actual and model species (Segner and Baumann, 2016).

Plutella xylostella, a pest of cruciferous crops and *Chrysoperla carnea*, a generalist predator were the two model insects in this study. These insects have long been used as suitable representative species of pests and natural enemies in diverse laboratory settings due to their cosmopolitan distribution, high reproductive rate, multivoltinity and year round availability (Maia et al., 2016; Machekano et al., 2017; Chen et al., 2017). Larvae of these two insects were chosen because the caterpillar is the destructive stage of *Plutella xylostella.* Similarly, as a natural enemy, instars of *Chrysoperla carnea* are efficient predators of several aphid species, whiteflies, thrips, eggs and caterpillars of moths, leafhoppers, larvae of beetles and spider mites and therefore are very important biocontrol species (Lavagnini et al., 2015). Hence, evaluvating the impact of plant protection products like neonicotioids on such biocontrol insects is of fundamental importance. The information obtained through such investigations can inform the growers to select the chemicals that are effective against pest but least toxic to such biocontrol fauna. Secondly, the cultures of these target and non-target insects can be well maintained under laboratory conditions.

Likewise, the test pesticides were selected based on their frequency of application as well as their occurrence in agricultural fields reported by previous studies. For instance, thiacloprid a cyano group neonicotinoid and tebuconazole, a DMI fungicide used in this study, are reportedly among the most frequently recovered pesticides from pollens of oilseed rape and wildflowers growing in the peripheries of cultivable lands. In addition, tebuconazole was reported as one of the most frequently applied fungicides in the experimental oilseed rape fields (David et al., 2015; David et al., 2016; Botias et al., 2016). Synergistic interaction of tebuconazole and its compatibility with neonicotinoids in tank-mixes has also been reported, and as such has been used as mixtures in some recent studies (Willow et al., 2019). Acetamiprid, the second neonicotinoid of choice, has

been reportedly found less toxic to non-target pollinators, however, in the presence of triazole fungicides its toxicity increases as much as 105 fold (Iwasa et al., 2004).

Due to the large scale application of tebuconazole in different crops, it is also one of the most frequently detected pesticides from pollens collected by honey bees, bumblebees and wild bees (Johnson, 2015; Thompson et al., 2014; David et al., 2015; David et al., 2016; Botias et al., 2017). In addition, this fungicide is compatible with cyano group neonicotinoids (acetamiprid and thiacloprid, having cyano (-C=N) functional group instead of nitro ($-NO_2$) functional group) in tank mixes and has been used in mixtures in a few recent studies (Ostyn, 2017; Vanderhaegen, 2017). Moreover, tebuconazole has been categorised as toxic towards aquatic life and can cause substantial and long-established negative effects in the aquatic domain (Bayer CropScience Limited, 2016).

To achieve the primary goal of the study, pesticides were observed both individually and in mixtures. In terms of individual application, the approach adopted was to use these three pesticides individually at concentrations similar to manufacturer-recommended application (MRA) rates as well as the two lower concentrations that were 5X and 10X lower than MRAs (details given in chapter 2 and 4). In mixtures, however, combining and then analysing the effects of each concentration was not realistic due to time constraints, therefore, the method used was the binary combination of each single neonicotinoid concentration tested in this study, with a fixed single dose of tebuconazole.

5.2.2 Outcomes of the study

Insect pests are the primary element that limits the commercial crop production. Customers and consumers always demand for clean and spotless harvest. Therefore, it is crucial for the growers to identify pest problem and promptly apply the control measures to avoid a buildup that can lead to an uncontrollable pest infestation. Thus preventing the pest to have a devastating effect of the crop yield. Immature stages of insects are comparatively more susceptible to pesticides. Susceptibility, in most cases, declines as the insect progresses through its developmental stages (Galvan et al., 2005; Cutler et al., 2006). In this study, young larvae of *Plutella xylostella* were more likely affected in terms of their lethal and sublethal endpoints. Overall, however, larval mortality increased with increase in the concentration of acetamiprid, tebuconazole and their mixtures. Likewise, the mortality rate of *Chrysoperla carnea* increased with increase in the strength of acetamiprid, thiacloprid and thiacloprid- tebuconazole mixtures. Although, tebuconazole was toxic to *Chrysoperla carnea*, the toxicity of the three concentrations was similar in terms of a non-significant difference between their mortality rates. These findings suggest the

relatively high sensitivity of newly emerged larvae to the pesticides. Their protective barriers are not as substantial as those of an egg which has a waterproof and impermeable chorionic membrane or of the pupa with silken cocoon (Medina et al., 2003; Schneider et al., 2004). These findings corroborate Fogel et al. (2013) who reported a higher susceptibility of second instars of *Eriopis connexa* (a predator of whiteflies and aphids in Neotropics) to acetamiprid than the fourth instar larvae. Moreover, the susceptibility of these larvae was positively correlated with the strength of acetamiprid concentrations. In addition, the detoxification mechanism of older larvae may be more efficient than that of younger instars, which have fewer functional enzymes to detoxify the same dose of single or multiple chemicals they encounter (Stark et al., 2004). The sensitivity of *Chrysoperla carnea* on the other hand, varied between two types of pesticides mixtures, as the three acetamiprid – tebuconazole mixtures were equal in their effect, whereas with thiacloprid – tebuconazole mixtures, the mortality rate increased as the mixtures got the strength of combining pesticides. This indicates the difference in strength and potency of the two types of neonicotinoids being used in mixtures for this non-target insect.

In the present study, although larvae of *Chrysoperla carnea* were obtained from an external source, most of them were in their first and second instar stages. The mortality rate of these larvae was comparatively lower than second instar larvae and to some extent third instar larvae of *Plutella xylostella* with various pesticide treatments but higher than fourth instars of *Plutella xylostella*. These findings indicate the species-specific toxicity of the tested neonicotinoids or their mixtures. This is in line with the results from Li et al. (2017), who reported that relative to *Apis mellifera*, *A. cerana* exhibited greater sensitivity to clothianidin and imidacloprid. This was indicated by low LC_{50} values of 0.5 ng and 2.7 ng per bee for clothianidin and imidacloprid respectively, while for *A. mellifera* these were 2.0 ng and 8.6 ng per bee. Furthermore, at the prepupal stage, the fourth instars stop feeding, whereas younger instars are gluttonous consumers of their food. Therefore, this may be a reasonable explanation for the increased death rate of second and third instar larvae caused by the joint effect of oral route of exposure via feeding as well as through direct contact with these pesticides. Cutler et al. (2006) also found that susceptibility of second instars of *Podisus maculiventris* (predatory bug, natural enemy of potato beetle, *Leptinotarsa decemlineata*) to novaluron treated potato leaves via direct contact exposure augmented by ingestion of the treated foliage.

In addition, the difference in sensitivity of the two insects may also be due to their differential responses to co-formulants or adjuvants such as surfactants, dyes and foaming agents that are routinely used in chemical formulations to improve their performance. These ingredients are

typically a trademark of manufacturing companies and are not publically disclosed (Mesnage and Antoniou, 2018). According to the review by Mullin et al. (2016), the rapid decline of honey bees may be partly the outcome of unmonitored use of these so-called 'inert ingredients'. In their study, they reported the toxicity to bees of organosilicon surfactants that are tank-mixed with various fungicides including the ergosterol biosynthesis inhibitors and insecticides of different classes. Therefore, along with the dose, it may also be these supplementary components which augment the negative effects of a pesticide.

One of the fundamental mechanisms that the insects have evolved to escape the adverse effects of chemical toxins is their metabolic detoxification of xenobiotics. This involves the chief enzyme families of carboxylesterases, cytochrome P450 monooxygenases and glutathione transferases (Li et al., 2007). In the current study, acetamiprid alone and acetamiprid – tebuconazole mixtures were comparatively less toxic to *Chrysoperla carnea*, as evident by their higher LC_{50} values for larvae as compared to *Plutella xylostella* larvae. Thiacloprid and thiacloprid–tebuconazole mixtures revealed no significant difference in their toxicity to *Chrysoperla carnea*. These outcomes indicate the relatively high tolerance of *Chrysoperla carnea* to both, individual as well as combined pesticides. In contrast, Hardstone and Scott (2010) reported that while honey bees' vulnerability to pesticides may not be greater than other insects, their higher sensitivity to multiple contaminants may be due to the lower number of genes involved in detoxification of these toxins. This makes them more vulnerable and susceptible to synergistic actions of multiple pesticides. Fewer than 50% of the genes in the honey bee genome are involved in synthesizing the detoxifying enzymes low, compared to other insects (Claudianos et al., 2006). Given this, the tolerance of *Chrysoperla carnea* larvae to the test pesticides in the current study may be linked to more efficiently evolved genomics which may produce a greater number of genes encoding for insecticide detoxification enzymes, thus offering the insect more resistance against the insecticides. For instance, pipronyl butoxide (PBO) inhibits the esterase and microsomal oxidase enzymes in *Chrysoperla carnea*, therefore resistance of this insect to nitenpyram neonicotinoid declined when exposed to it in the presence of PBO (Mansoor et al., 2017). Similarly, the resistance of *Chrysoperla carnea* to a variety of insecticides has been linked to the activity of esterase and monooxygenases (Abbas et al., 2014; Mansoor et al., 2013).

The common practice of applying multiple pesticides in a tank-mix usually involves combining them at their manufacturers recommended application rates (MRAs). Therefore, their simultaneous application leads to the synergistic lethal and sublethal effects that have been reported for many chemical classes previously (Iverson et al., 2019; Raimets et al., 2018). However, the co-application of neonicotinoids like thiacloprid and the fungicide tebuconazole can have a synergizing effect on insects, for instance, a parasitoid, *Aphelinus abdominalis* at as low as

on tenth of their MRAs (Willow et al., 2019). In the current study however, no such effect was observed when tebuconazole was combined with either acetamiprid or thiacloprid at one tenth of their MRAs on *Chrysoperla carnea* or *Plutella xylostella*. This might be due to the inter-specific physiological and structural differences, which might also be the reason for their modified behavioural response to deal with different toxins in their environment. Likewise, Willow et al. (2019) reported a significant effect on loss of motion control of *A. abdominalis* attributed to increasing concentration of tebuconazole in five mixtures of tebuconazole and thiacloprid used in their study. Thiacloprid concentration was kept constant at one-tenth of its MRA, while tebuconazole concentration progressively increased between one-hundredth of its MRA and its MRA. In the current study, a decline in speed and increase in stationary periods of *Plutella xylostella* was observed as tebuconazole and acetamiprid concentrations increased either individually or in their mixtures. However, in *Chrysoperla carnea* no such sublethal effect on its locomotion was observed. These results suggest that *Chrysoperla carnea* has evolved a proficient mechanism for coping with various pesticides and their mixtures. Its molecular basis, however, still needs further investigation to enable a clear insight about inter-specific variations involved in the process.

Pesticide induced selection pressure in an agricultural regime is a fundamental aspect influencing the distribution of insects spatially and temporally as well as shaping their life history and behavioural attributes (Guedes et al., 2016; Desneux et al., 2007). Behavioural aversiveness or avoidance is one of the vital behavioural adaptations that insects have evolved to ensure survival and success in their environment. This behavioural aspect relies on the insect's ability to identify the hazard (in this study, pesticide sprayed leaves) and, where possible, respond either by escaping to an area where no spray has occurred or sometimes to stop feeding on the sprayed leaves or crop (Nansen et al., 2016). This kind of behavioural avoidance was observed in current study in both *Plutella xylostella* as well as *Chrysoperla carnea*. In young instars of *Plutella xylostella*, avoidance was high in lower doses of test pesticides due to low mortality rate, while in older instars avoidance was also high in greater concentrations. In agreement with these findings, Nansen et al. (2016) reported that different mechanisms might be involved in behavioural avoidance in the various life stages of an insect. Likewise, the repulsion rate of *Chrysoperla carnea* increased with increasing concentrations of acetamiprid, thiacloprid, tebuconazole and thiacloprid – tebuconazole; however, the effect of three acetamiprid - tebuconazole mixtures was equal on their avoidance rate. This further strengthens the argument that *Chrysoperla carnea*'s behavioural avoidance has evolved and that their relatively low mortality rate may also be linked to this aspect of their behaviour.

Pesticides usually kill the pest population via direct contact exposure and ingestion. Therefore, these two modes of exposure and action are significant for the effectiveness of a pesticide (Nansen et al., 2016). Hence the feeding rate can be a critical sublethal endpoint measured to analyse the effect of pesticides on insect fauna exposed to it. In this study, feeding rate of *Plutella xylostella* declined significantly with the increasing concentrations of tebuconazole and acetamiprid – tebuconazole mixtures. With acetamiprid, the feeding rate of larvae was like that of the control, except in fourth instars which consumed more leaf mass at the highest concentration of acetamiprid. Likewise, the feeding rate of *Chrysoperla carnea* declined with tebuconazole and thiacloprid-tebuconazole mixtures. These findings are in agreement with those of Sgolastra et al. (2018) who reported a higher feeding rate of *Osmia bicornis* (solitary bee) on clothianidin treated sugar solution than in those exposed to sugar solution containing a mixture of propiconazole and clothianidin. In addition, the antifeedant property of tebuconazole has been established in different taxonomic groups. For instance, Dimitrov et al. (2014) demonstrated a significant decline in the feeding rate of *Gammarus pulex* (an amphipod crustacean) when fed tebuconazole sprayed leaves. The decline of feeding rate of *Chrysoperla carnea* with thiacloprid – tebuconazole mixtures in comparison to acetamiprid – tebuconazole mixtures suggest the relative potency of the former mixture against this non-target insect. Secondly, the effect of tebuconazole treatments in terms of declining the feeding rate of both insects further signifies the indiscriminative impact of this fungicide on insect taxa. These findings suggest need for more comprehensive and rigorous investigation of the effect of this and other frequently used fungicides on the non-target insects' populations.

The impact of pesticide exposure time in terms of its efficacy has great relevance to the process of environmental risk assessment. With a decreased exposure level, the time required for a pesticide to produce a specified effect increases. Furthermore, if the effect caused by binding the pesticide molecules to its receptor is irreversible, it is reinforced by the time of exposure (Tennekes, 2010b). For instance, in the current study, although it differed between the two insects and also the developmental stages of the insects, median lethal concentration (LC_{50}) of individual pesticides as well as their mixtures decreased over time. This time-to-effect approach is therefore, essential in order to gain information about the toxic effects of a pesticide for an organism based on concentration and exposure time (Tennekes and Sánchez-Bayo, 2019). The toxicity of neonicotinoids can be explained in light of the above argument. Neonicotinoids are nicotine agonists and have a high and irreversible binding affinity for nicotinic acetylcholine receptors (nAChRs) (Godfray et al., 2014). After binding, these receptors are stimulated by acetylcholine neurotransmitters, causing nerve stimulation. The irreversible binding of neonicotinoid molecules to the receptors results in their blockage and thus hyperexcitation of the nervous system leads to

paralysis and death of the insects (Belzunces et al., 2012). Furthermore, as the binding is irreversible, even low doses of neonicotinoids over a prolonged exposure period can be substantially toxic to insects (Tsvetkov et al., 2017) and further synergised in the presence of some fungicides (Thompson et al., 2014; Raimets et al., 2018).

5.2.3 Management options and recommendations

Risk assessment is an integral part of the broader risk analysis framework, and the information obtained via this process communicates the associated risks with the use of a certain chemical. An equally important aspect however, is to know the extent of the risks and whether any possible adoptable management options are available to mitigate those risks. This is where the risk managers play their vital role in the regulation of these chemicals. Researchers in the past have shown a higher degree of toxicity of pesticides mixtures to insects, specifically to the non-target community. Moreover, only a few studies have compared the efficacy of individual insecticides and combinations on various life stages of pests relative to their natural enemies. In this study, a higher degree of control was demonstrated with mixtures for target insect larvae than their toxicity to non-target, which means specificity of pesticides and their mixtures against the target and non-target insects. Development of target larvae through their life stages, however, gave rise to a negative impact of both individual pesticides and their mixtures on non-target larvae. The mortality rate of the non-target insect larvae was higher relative to the advanced stages larvae of the target insect.

In 2013, a partial restriction on the use of three neonicotinoids i. e. imidacloprid, thiamethoxam and clothianidin, driven by the plummeting number of natural pollinators, especially bees, was put by European Commission, such that they were only allowed for indoor use in greenhouses, on winter cereals and on some plants post-flowering, but completely restricted on plants attractive to bees (Woodcock et al., 2018; McGrath, 2014). However, EFSA evaluated the effects of these neonicotinoids through the data collected till 2018. The conclusion drawn from that information was presented to the European Commission who examined them thoroughly and decided to place a total ban on the outdoor use of these three neonicotinoids (Stokstid, 2018). The decision was made by incorporating both scientific as well as public and political interests.

Likewise, due to the increasing concern over the cocktail toxicity of plant protection products on human health and other non-target organisms in the environment, the European and United States regulatory bodies such as EFSA, EC and EPA are thriving to provide technical guidance on mixtures risk assessment and management (Solecki et al., 2014). There is, however, a lack of joint

agreement on adequately specific guidelines, which is nurturing the discrepancy in the practice of mixtures risk assessment and decision-making. These differences in mutual agreement may arise because a regulatory decision typically involves many other non-scientific aspects such as political, economic, public and statutory. Therefore, the regulatory bodies always encourage recommendations that could be in the interest of all the aspects mentioned earlier. In this study, keeping various factors in view, a few such recommendations are provided which can either help focus on higher tier tests or these recommendations from tier 1 assessment on approved chemicals could be enough whilst continuing to monitor the situation.

1. From risk management viewpoint, if the decision has to be focused on optimal control of pest organism while still allowing some harm to non-target insect population, the following recommendations can be taken under consideration. For instance, in the present study, based on maximum control of target insect, the mixtures with ideal efficacy were found to be mix2 and mix3 over the entire exposure period (Table 8). This efficacy however, was restricted as well as dependent on the developmental stage of the target larvae. Such that the optimal effect can be achieved when instars at their early developmental stages are targeted.
2. Likewise, the maximum difference between mortality rates of the non-target insect existed with the early life stages of target insect as well as with mix3 and mix2 (Table 9). However, the difference declined over time of exposure essentially due to an increased mortality of non-target insect. The enhancing effect of time however, is a factor that cannot be mitigated therefore, whilst analysing the maximum difference in the effect of a pesticides on target and non-target insect this factor has to be kept in mind.
3. On the other hand, if the emphasis of a risk management decision is to conserve the population of non-target insect to a maximum level, the subsequent effect of pesticides on target insect has to be carefully analysed because exclusively focusing on non-target insect population health can sometimes be of little benefit in terms of controlling the target insect to a lower than optimal level. In this study, for example, if the mixtures are chosen based on the maximum protection they offer to the non-target insect (Table 10), their resultant effect on target insect is quite low which is not ideal from a farmer's perspective in a real field.

The present study is the first to use the effective-mixture-dose-based approach for mixture risk assessment on target and non-target organisms. This method can be used as a tool to withdraw meaningful information via risk assessment procedures taking various classes of target and non-target organisms into consideration. Such studies can be helpful in providing useful information for regulatory risk management decisions.

By Taking the two neonictionds and a fungicides as a test case, this study has explored the ongoing debate about improving the mixture risk assessment procedures. Thus by adopting a newer approach of investigating the toxicity of pesticides mixtures, it represents a positive and progressive step towards mixture risk assessment. The current study, within this framework, gives some risk management recommendations for safe and effective use of Non-CA pesticides mixtures against target and non-target insects. This work also highlights the significance of the effect of exposure time as a reinforcing factor towards the toxicity of pesticides. All these results suggest that mixture risk assessment approaches need to be updated in order to make them more realistic and field relevant. Nonetheless, this is a process still at its foundational stage, and there is much more to uncover.

5.3 Future work

The present study has highlighted some questions that are truly important to be answered in order for future exploration to gain more insight in the ecological risk assessment of pesticides mixtures. Further research and development operating at different scales and with different approaches is necessary in order to move this field of investigation forward in the following two ways 1) To continue analysing the details of the work explored in the current study in a more focused and micro-detailed way. Section 1 to 5 below indicates this type of future work. 2) To look through the gaps that exists and where some of the really big overarching questions lies that could form the basis of a completely new work. Section 6 to 7 below identifies the future investigations of this nature.

1) To analyse the effects of thiacloprid and tebuconazole mixtures on the same target model so as to evaluate the difference in their effects between these target and non-target insects. Likewise, sensitivity for a single pesticide or a mixture may vary amongst various species (Beketov and Liess, 2008; Tomé et al., 2017). Therefore, studies like these can be performed to see if the same kind of effects of pesticides used in the current study also occur in other target and non-target insects belonging to same taxonomic group, or if they can be extended to other taxa.

2) Similarly, exploring the potential impacts of other neonicotinoids and alike fungicides via different routes of exposure in similar as well as other target and non-target organisms can enhance this line of research. In this way, a better evaluation of the effectiveness or the toxicity of a pesticides/mixtures can be done.

3) When assessing the behaviour of a pesticide alone or in mixtures, the developmental stages of an insect as well as the exposure time are important aspects to consider. An insecticide may have different effects at different stages of an insect life cycle (Bhojani et al., 2018). Therefore, it would be interesting to investigate the effects of pesticides mixtures at different exposure time and developmental stages in other model species to understand maximum efficacy of pesticides mixtures. This can be achieved by targeting the most vulnerable stage of the target organism while at the same time carrying out effective measures to ensure the safety or to comparatively minimise the harm induced to non-target animal communities.

4) Evaluation of the toxic effects of sterol biosynthesis inhibitor (SBI) fungicides on insects has been an interesting line of research that has recently gathered momentum due to the reports on their synergistic toxicities in the presence of certain insecticides, especially the neonicotinoids. In the present study however, the individual toxicity to both target and non-target insects, of

tebuconazole fungicide was high. Similarly, the combinatorial toxic effects of some fungicides has been reported as being hundreds of times higher than in a single insecticide (Tomé et al., 2017). Therefore, more studies to create an increased database on the toxicity of fungicides as well as other pesticides with insects not being their primary target would be of considerable scientific interest and could serve as a tool for the revision of currently used risk assessment procedures.

5) The effects of Ergosterol Biosynthesis inhibitor (EBI) fungicides has been explored to a considerable extent. However, the nature of interactions that exist between non-EBI fungicides and insecticides has been investigated to that extent. Although the current investigation on EBI fungicides and neonicotinoids interface occupies a paramount position in the mixture risk assessments, the use of pesticides is not confined to these two classes. A number of other novel combinations (non-neonicotinoid insecticides and non-EBI fungicides) may also contribute to the cocktail effects of pesticides which usually receive little attention. An important contribution to fill this gap would therefore, be to include these kind of interactions in the mixtures risk assessment.

6) Equally as interesting would be the investigation of individual components of agrochemical formulations and their toxicity on various target and non-target organisms as there exist enough corroborations about the augmentative effects of active ingredients in the presence of other constituents of commercial agrochemical formulations (Mullin et al., 2016; Mullin et al., 2015; Zhu et al., 2014; Mesnage and Antoniou, 2018). This would enable the selection of those supplementary elements that are effective and selective for target and non-target insects.

7) To date the majority of the research on mixtures risk assessments has been conducted on different species of bees due to the status they have acquired in agriculture as being the key-pollinators. The role and contribution of bees to ecosystem services is undeniably significant; however, the ecological health of other non-bee pollinators as well as natural enemies of pests, is just as important. Therefore, studies considering the ecotoxicological impacts of pesticides mixtures on non-bee beneficial species such as spiders, molluscs etc would bring more productive data and would be an interesting part of work to understand the inter-specific response to chemical pesticides present in their surroundings. This can paint a clearer picture on associated risks and their management in a vast array of beneficial insects that could boost agricultural productivity in the presence of natural pollinators.

Appendix A Method used for analysing the images for feeding bioassay of *Plutella xylostella* using ImageJ

=> Drag and drop the image from saved image file in ImageJ to open.

=> Select image from the tool bar menu => colour => split channels

Keep the blue channel and close the others.

Scale the image:

Using line tool, draw a known distance on ruler as 10mm

=> Analyze => set scale => in known distance enter 10 and in units enter mm => tick Global

Remove unwanted material => use free hand tool with control X

Threshold:

=> Image => adjust => threshold

If white or black => edit => invert

Selection:

=> Edit => Selection => Create selection

Measure:

=> Analyze => set measurements => CTRL + M to measure

Bibliography

Abbas N., Mansoor, M. M., Shad, S. A., Pathan, A. K., Waheed, A., Ejaz, M., Razaq, M. & Zulfiqar, M. A. 2014. Fitness cost and realized heritability of resistance to spinosad in *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Bulletin of Entomological Research,* 104**,** 707-715.

Abt E., Rodricks, J. V., Levy, J. I., Zeise, L. & Burke, T. A. 2010. Science and decisions: advancing risk assessment. *Risk Analysis,* 30**,** 1028 -1036.

Ahmad M., Saleem, M. A. & Sayyed, A. H. 2009. Efficacy of insecticide mixtures against pyrethroid- and organophosphate-resistant populations of *Spodoptera litura* (Lepidoptera: Noctuidae). *Pest Management Science,* 65**,** 266-274.

Ahmed S., Nisar, M., Shakir, M., Imran, M. & Iqbal, K. 2014. Comparative efficacy of some neonicotinoids and traditional insecticides on sucking insect pests and their natural enemies on Bt-121 cotton crop. *Journal of Animal and Plant Sciences,* 24**,** 660-663.

Ail-Catzim C. E., Cerna-Chavez, E., Landeros-Flores, J., Ochoa-Fuentes, Y., Garcia-Lopez, A. M. & Gonzalez, R. E. R. 2015. Effect of insecticides on the mortality and predation of *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Southwestern Entomologist,* 40**,** 565-573.

Akhtar N., Shoaib, A. & Nafisa, A. A. 2014. Identification and in vitro control of canola spot disease pathogen. *Pakistan Journal of Phytopathology,* 26**,** 103-108.

Aktar W., Sengupta, D. & Chowdhury, A. 2009. Impact of pesticides use in agriculture: their benefits and hazards. *Interdisciplinary Toxicology,* 2**,** 1-12.

Alaux C., Brunet, J. L., Dussaubat, C., Mondet, F., Tchamitchan, S., Cousin, M., Brillard, J., Baldy, A., Belzunces, L. P. & Le Conte, Y. 2010. Interactions between N*osema microspores* and a neonicotinoid weaken honeybees (*Apis mellifera*). *Environmental Microbiology,* 12**,** 774-782.

Aliouane Y., El Hassani, A. K., Gary, V., Armengaud, C., Lambin, M. & Gauthier, M. 2009. Subchronic exposure of honeybees to sublethal doses of pesticides: effects on behavior. *Environmental Toxicology and Chemistry,* 28**,** 113-122.

Altieri M. A. 2004. Linking ecologists and traditional farmers in the search for sustainable agriculture. *Frontiers in Ecology and the Environment,* 2**,** 35-42.

Amalin D. M., Peña, J. E., Duncan, R., Leavengood, J. & Koptur, S. 2009. Effects of pesticides on the arthropod community in the agricultural areas near the Everglades National Park. *Proceedings of Florida State Horticultural Society,* 122**,** 429-437.

Anjum F. & Wright, D. 2016. Relative toxicity of insecticides to the crucifer pests *Plutella xylostella* and *Myzus persicae* and their natural enemies. *Crop Protection,* 88**,** 131-136.

Austin R. 1999. Yield of wheat in the United Kingdom: recent advances and prospects. *Crop Science,* 39**,** 1604-1610.

Backhaus T., Blanck, H. & Faust, M. 2010. *Hazard and risk assessment of chemical mixtures under REACH: state of the art, gaps and options for improvement*, Swedish chemicals agency.

Backhaus T. & Faust, M. 2012. Predictive Environmental Risk Assessment of Chemical Mixtures: A Conceptual Framework. *Environmental Science & Technology,* 46**,** 2564-2573.

Barrett G. E., Alexander P. D., Robinson J. S. & Bragg N. C. 2016. Achieving environmentally sustainable growing media for soilless plant cultivation system - a review. *Scientia Horticulturae*, 212, 220 - 234.

Basallote-Ureba M. J., Prados-Ligero, A. M. & Melero-Vara, J. 1998. Effectiveness of tebuconazole and procymidone in the control of *Stemphylium* leaf spots in garlic. *Crop Protection,* 17**,** 491-495.

Bayer Cropscience Limited 2016. Environmental Information Sheet Folicur MAPP 16731.

Beketov M. A. & Liess, M. 2008. Acute and delayed effects of the neonicotinoid insecticide thiacloprid on seven freshwater arthropods. *Environmental Toxicology and Chemistry,* 27**,** 461-470.

Beloti V. H., Alves, G. R., Araujo, D. F. D., Picoli, M. M., Moral, R. D., Demetrio, C. G. B. & Yamamoto, P. T. 2015. Lethal and sublethal effects of insecticides used on citrus, on the ectoparasitoid *Tamarixia radiata*. *PLoS ONE,* 10.

Belzunces L. P., Tchamitchian, S. & Brunet, J. L. 2012. Neural effects of insecticides in the honey bee. *Apidologie,* 43**,** 348-370.

Bernauer O. M., Hannah, R. G.-D. & Shawn, A. S. 2015. Colonies of bumble bees (*Bombus impatiens*) produce fewer workers, less bee biomass, and have smaller mother queens following fungicide exposure. *Insects,* 6**,** 478 - 488.

Beyer J., Petersen, K., Song, Y., Ruus, A., Grung, M., Bakke, T. & Tollefsen, K. E. 2014. Environmental risk assessment of combined effects in aquatic ecotoxicology: A discussion paper. *Marine Environmental Research,* 96**,** 81-91.

Bhojani D. V., Desai, H. R., Shinde, C. U. & Bhanderi, G. R. 2018. Relative toxicity of commonly used insecticides and combination products in cotton ecosystem to *Chrysoperla zastrowi sillemi* (Esben–Peterson) under laboratory condition. *International Journal of Current Microbiology and Applied Sciences,* 7**,** 1523-1533.

Blacquiere T., Smagghe, G., Van Gestel, C. A. & Mommaerts, V. 2012. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology,* 21**,** 973-992.

Bonmatin J.-M., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D., Krupke, C., Liess, M., Long, E., Marzaro, M. & Mitchell, E. 2015. Environmental fate and exposure; neonicotinoids and fipronil. *Environmental Science and Pollution Research,* 22**,** 35-67.

Bopp S. K., Kienzler, A., Van Der Linden, S., Lamon, L., Paini, A., Parissis, N., Richarz, A. N., Triebe, J. & A., W. 2016. Review of case studies on the human and environmental risk assessment of chemical mixtures. Luxembourg: European Commission.

Borgert C. J. 2004. Chemical mixtures: An unsolvable riddle? *Human and Ecological Risk Assessment,* 10**,** 619-629.

Botias C., David, A., Hill, E. M. & Goulson, D. 2016. Contamination of wild plants near neonicotinoid seed-treated crops, and implications for non-target insects. *Science of the Total Environment,* 566**,** 269-278.

Botias C., David, A., Hill, E. M. & Goulson, D. 2017. Quantifying exposure of wild bumblebees to mixtures of agrochemicals in agricultural and urban landscapes. *Environmental Pollution,* 222**,** 73 - 82.

Bourguet D. & Guillemaud, T. 2016. The hidden and external costs of pesticide use. *In:* LICHTFOUSE, E. (ed.) *Sustainable Agriculture Reviews.* Springer, Cham.

Brandon K. 2015. *Bees feeding on fungicide-dosed flowers develop health issues, studies say* [Online]. Available: https://www.theguardian.com/environment/2015/jun/18/bees-fungicide-flowers-farm-insecticide [Accessed].

Brandt A., Gorenflo, A., Siede, R., Meixner, M. & Buchler, R. 2016. The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (*Apis mellifera* L.). *Journal of Insect Physiology,* 86**,** 40 - 47.

Bredeson M. M., Reese, R. N. & Lundgren, J. G. 2015. The effects of insecticide dose and herbivore density on tri-trophic effects of thiamethoxam in a system involving wheat, aphids, and ladybeetles. *Crop Protection,* 69**,** 70 - 76.

Brittain C. A., Vighi, M., Bommarco, R., Settele, J. & Potts, S. G. 2010. Impacts of a pesticide on pollinator species richness at different spatial scales. *Basic and Applied Ecology,* 11**,** 106 - 115.

Brown M. J. & Paxton, R. J. 2009. The conservation of bees: a global perspective. *Apidologie,* 40**,** 410-416.

Brunet J.-L., Badiou, A. & Belzunces, L. P. 2005. In vivo metabolic fate of [14C]-acetamiprid in six biological compartments of the honeybee, *Apis mellifera* L. *Pest Management Science,* 61**,** 742 - 748.

Brush S. B. 2008. Locating Crop Diversity in the Contemporary World. *In:* SCOTT, J. C. (ed.) *Farmers' Bounty.* London: Yale University Press.

Brussaard L. 1997. Biodiversity and ecosystem functioning in soil. *Ambio***,** 563-570.

Bundschuh M., Goedkoop, W. & Kreuger, J. 2014. Evaluation of pesticide monitoring strategies in agricultural streams based on the toxic-unit concept - Experiences from long-term measurements. *Science of the Total Environment,* 484**,** 84-91.

Burkle L. A., Marlin, J. C. & Knight, T. M. 2013. Plant-pollinator interactions over 120 years: loss of species, co-occurrence, and function. *Science,* 339**,** 1611-1615.

Canico A., Santos, L. & Massing, R. 2013. Development and adult longevity of diamondback moth and its parasitoids *Cotesia plutellae* and *Diadegma semiclausum* in uncontrolled conditions. *African Crop Science conference proceedings.,* 11**,** 257 - 262.

Carpenter D., Arcaro, K. & Spink, D. 2002. Understanding the human health effects of chemical mixtures. *Environmental Health Perspect,* 110**,** 25 - 42.

Carreck N. L. & Ratnieks, F. L. 2014. The dose makes the poison: have “field realistic” rates of exposure of bees to neonicotinoid insecticides been overestimated in laboratory studies? *Journal of Apicultural Research,* 53**,** 607-614.

Carriger J. F., Rand, G. M., Gardinali, P. R., Perry, W. B., Tompkins, M. S. & Fernandez, A. M. 2006. Pesticides of potential ecological concern in sediment from south Florida canals: an

ecological risk prioritization for aquatic arthropods. *Soil & Sediment Contamination,* 15**,** 21-45.

Cartea M., Lema, M., Francisco, M. & Velasco, P. 2011. Basic information on vegetables Brassica crops. *In:* SADOWSKI, J. & KOLE, C. (eds.) *Genetics, Genomics and Breeding of Vegetable Brassicas.* Enfield, New Hampshire: Science Publishers.

Cartea M. E., Francisco, M., Soengas, P. & Velasco, P. 2010. Phenolic compounds in Brassica vegetables. *Molecules,* 16**,** 251-280.

Carvalho F. P. 2017. Pesticides, environment, and food safety. *Food and Energy Security,* 6**,** 48 - 60.

Carvalho G. A., Godoy, M. S., Parreira, D. S., Lasmar, O., Souza, J. R. & Moscardini, V. F. 2010. Selectivity of growth regulators and neonicotinoids for adults of *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae). *Revista Colombiana de Entomología,* 36**,** 195-201.

Casida J. E. & Durkin, K. A. 2013. Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. *Annual Review of Entomology,* 58**,** 99-117.

Cassman K. G. 1999. Ecological intensification of cereal production systems: yield potential, soil quality, and precision agriculture. *Proceedings of the National Academy of Sciences,* 96**,** 5952-5959.

Cedergreen N. 2014. Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. *PLoS ONE,* 9**,** e96580.

Cedergreen N., Christensen, A. M., Kamper, A., Kudsk, P., Mathiassen, S. K., Streibig, J. C. & Sorensen, H. 2008. A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. *Environmental Toxicology and Chemistry,* 27**,** 1621-1632.

Chandler D. & Davidson, G. 2005. Evaluation of entomopathogenic fungus *Metarhizium anisopliae* against soil-dwelling stages of cabbage maggot (Diptera: Anthomyiidae) in glasshouse and field experiments and effect of fungicides on fungal activity. *Journal of Economic Entomology,* 98**,** 1856-1862.

Chandran N. N., Fojtova, D., Blahova, L., Rozmankova, E. & Blaha, L. 2018. Acute and (sub)chronic toxicity of the neonicotinoid imidacloprid on *Chironomus riparius*. *Chemosphere,* 209**,** 568-577.

Chauzat M.-P., Faucon, J.-P., Martel, A.-C., Lachaize, J., Cougoule, N. & Aubert, M. 2006. A survey of pesticide residues in pollen loads collected by honey bees in France. *Journal of Economic Entomology,* 99**,** 253-262.

Chen J. Q., Saleem, M., Wang, C. X., Liang, W. X. & Zhang, Q. M. 2018. Individual and combined effects of herbicide tribenuron-methyl and fungicide tebuconazole on soil earthworm *Eisenia fetida*. *Scientific Reports,* 8.

Chen Z. Z., Liu, L. Y., Liu, S. Y., Cheng, L. Y., Wang, X. H. & Xu, Y. Y. 2017. Response of *Chrysoperla nipponensis* (Okamoto) (Neuroptera: Chrysopidae) under long and short photoperiods. *Journal of Insect Science,* 17.

Chiu W. A. & Rusyn, I. 2018. Advancing chemical risk assessment decision-making with population variability data: challenges and opportunities. *Mammalian Genome,* 29**,** 182-189.

Ciach M. & Peksa, L. 2019. Human-induced environmental changes influence habitat use by an ungulate over the long term. *Current Zoology,* 65**,** 129-137.

Claudianos C., Ranson, H., Johnson, R. M., Biswas, S., Schuler, M. A., Berenbaum, M. R., Feyereisen, R. & Oakeshott, J. G. 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. *Insect Molecular Biology,* 15**,** 615-636.

Cloyd R. A. 2009. Getting mixed-up: Are greenhouse producers adopting appropriate pesticide mixtures to manage arthropod pests? *HortTechnology,* 19**,** 638 - 646.

Cloyd R. A. & Dickinson, A. 2006. Effect of insecticides on mealybug destroyer (Coleoptera: Coccinellidae) and parasitoid *Leptomastix dactylopii* (Hymenoptera: Encyrtidae), natural enemies of citrus mealybug (Homoptera: Pseudococcidae). *Journal of Economic Entomology,* 99**,** 1596-1604.

Cloyd R. A. & Raudenbush, A. L. 2014. Efficacy of binary pesticide mixtures against western flower thrips. *HortTechnology,* 24.

Colgan T. J., Fletcher, I. K., Arce, A. N., Gill, R. J., Rodrigues, A. R., Stolle, E., Chittka, L. & Wurm, Y. 2019. Caste- and pesticide-specific effects of neonicotinoid pesticide exposure on gene expression in bumblebees. *Molecular Ecology,* 28**,** 1964-1974.

Cook N., Green, J., Shuker, D. M. & Whitehorn, P. R. 2016. Exposure to the neonicotinoid imidacloprid disrupts sex allocation cue use during superparasitism in the parasitoid wasp *Nasonia vitripennis*. *Ecological Entomology,* 41**,** 693-697.

Cooper J. & Dobson, H. 2007. The benefits of pesticides to mankind and the environment. *Crop Protection,* 26**,** 1337-1348.

Cordeiro E. M. G., Correa, A. S., Venzon, M. & Guedes, R. N. C. 2010. Insecticide survival and behavioral avoidance in the lacewings *Chrysoperla externa* and *Ceraeochrysa cubana*. *Chemosphere,* 81**,** 1352-1357.

Cordeiro E. M. G., De Moura, I. L. T., Fadini, M. a. M. & Guedes, R. N. C. 2013. Beyond selectivity: Are behavioral avoidance and hormesis likely causes of pyrethroid-induced outbreaks of the southern red mite *Oligonychus ilicis*? *Chemosphere,* 93**,** 1111-1116.

Coules A. E., Lunn, G. D. & Rossall, S. Year. Disease and canopy control in oilseed rape using triazole fungicides. The BCPC Conference: Pests and diseases, 2002 Brighton Hilton Metropole Hotel, Brighton, UK. British Crop Protection Council, 617-622.

Crawley M. J. 2007. *The R Book,* Chichester,West Sussex, UK, John Wiley and Sons Ltd.

Cresswell J. E. 2011. A meta-analysis of experiments testing the effects of a neonicotinoid insecticide (imidacloprid) on honey bees. *Ecotoxicology,* 20**,** 149-157.

Cresswell J. E., Page, C. J., Uygun, M. B., Holmbergh, M., Li, Y. R., Wheeler, J. G., Laycock, I., Pook, C. J., De Ibarra, N. H., Smirnoff, N. & Tyler, C. R. 2012. Differential sensitivity of honey bees and bumble bees to a dietary insecticide (imidacloprid). *Zoology,* 115**,** 365-371.

Curtis C., Jana-Kara, B. & Maxwell, C. 2003. Insecticide treated nets: impact on vector populations and relevance of initial intensity of transmission and pyrethroid resistance. *Journal of Vector Borne Diseases,* 40**,** 1-8.

Cutler G. C., Scott-Dupree, C. D., Tolman, J. H. & Harris, C. R. 2006. Toxicity of the insect growth regulator novaluron to the non-target predatory bug *Podisus maculiventris* (Heteroptera : Pentatomidae). *Biological Control,* 38**,** 196-204.

Das S. K. 2014. Scope and Relevance of using Pesticide Mixtures in Crop Protection: A Critical Review. *International Journal of Environmental Science and Toxicology Research,* 2**,** 119 - 123.

David A., Botías, C., Abdul-Sada, A., Goulson, D. & Hill, E. M. 2015. Sensitive determination of mixtures of neonicotinoid and fungicide residues in pollen and single bumblebees using a scaled down QuEChERS method for exposure assessment. *Analytical and Bioanalytical Chemistry,* 407**,** 8151-8162.

David A., Botías, C., Abdul-Sada, A., Nicholls, E., Rotheray, E. L., Hill, E. M. & Goulson, D. 2016. Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. *Environment International,* 88**,** 169-178.

De Castro A. A., Correa, A. S., Legaspi, J. C., Guedes, R. N. C., Serrao, J. E. & Zanuncio, J. C. 2013. Survival and behavior of the insecticide-exposed predators *Podisus nigrispinus* and *Supputius cincticeps* (Heteroptera: Pentatomidae). *Chemosphere,* 93**,** 1043-1050.

Delpuech J.-M. & Allemand, R. 2011. Side effects of fungicides on the abundance and the species diversity of the natural populations of *Drosophila* and their hymenopterous parasitoids in orchards. *Phytoparasitica,* 39**,** 429-435.

Desneux N., Decourtye, A. & Delpuech, J.-M. 2007. The sublethal effects of pesticides on beneficial arthropods. *Annual Review of Entomology,* 52**,** 81-106.

Dibartolomeis M., Kegley, S., Mineau, P., Radford, R. & Klein, K. 2019. An assessment of acute insecticide toxicity loading (AITL) of chemical pesticides used on agricultural land in the United States. *PLoS ONE,* 14.

Dimitrov M. R., Kosol, S., Smidt, H., Buijse, L., Van Den Brink, P. J., Van Wijngaarden, R. P. A., Brock, T. C. M. & Maltby, L. 2014. Assessing effects of the fungicide tebuconazole to heterotrophic microbes in aquatic microcosms. *Science of the Total Environment,* 490**,** 1002-1011.

Drobne D., Blazic, M., Van Gestel, C. a. M., Leser, V., Zidar, P., Jemec, A. & Trebse, P. 2008. Toxicity of imidacloprid to the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea). *Chemosphere,* 71**,** 1326-1334.

Dubey N., Shukla, R., Kumar, A., Singh, P. & Prakash, B. 2010. Prospects of botanical pesticides in sustainable agriculture. *Current Science,* 98**,** 479-480.

Easton A. H. & Goulson, D. 2013. The neonicotinoid insecticide imidacloprid repels pollinating flies and beetles at field-realistic concentrations. *PLoS ONE,* 8.

Ec 2009. REGULATION (EC) No 1107/2009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. *Official Journal of the European Union,* 309**,** 1 - 50.

Ec 2012. Communication from the commission to the council - the combination effects of chemicals. Chemical mixtures. Brussels: COM(2012), 10.

Efsa 2013a. EFSA Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees).

Efsa 2013b. Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera, Bombus* spp. and solitary bees). *EFSA Journal.*

Efsa 2013c. Scientific Opinion on the identification of pesticides to be included in cumulative assessment groups on the basis of their toxicological profile. *EFSA Journal,* 11**,** 3293.

Efsa 2014. Risk assessment vs risk management: What's the difference?

El Hassani A. K., Dacher, M., Gary, V., Lambin, M., Gauthier, M. & Armengaud, C. 2008. Effects of sublethal doses of acetamiprid and thiamethoxam on the behavior of the honeybee (*Apis mellifera*). *Archives of Environmental Contamination and Toxicology,* 54**,** 653-661.

El Hassani A. K., Dacher, M., Gauthier, M. & Armengaud, C. 2005. Effects of sublethal doses of fipronil on the behavior of the honeybee (*Apis mellifera*). *Pharmacology Biochemistry and Behavior,* 82**,** 30-39.

Elbert A., Haas, M., Springer, B., Thielert, W. & Nauen, R. 2008. Applied aspects of neonicotinoid uses in crop protection. *Pest Management Science,* 64**,** 1099-1105.

Englert D., Zubrod, J. P., Link, M., Mertins, S., Schulz, R. & Bundschuh, M. 2017. Does Waterborne Exposure Explain Effects Caused by Neonicotinoid-Contaminated Plant Material in Aquatic Systems? *Environmental Science & Technology,* 51**,** 5793-5802.

Epa 1992. Framework for ecological risk assessment. Environmental Protection Agency, Washington, DC (United States). Risk Assessment Forum.

Epa 1998. Guidelines for Ecological Risk Assessment. *In:* AGENCY, U. E. P. (ed.). Washington DC: Risk Assessment Forum.

Epa 2003. Pesticides factsheet clothianidin. Washington, DC.

Evans S. C., Shaw, E. M. & Rypstra, A. L. 2010. Exposure to a glyphosate-based herbicide affects agrobiont predatory arthropod behaviour and long-term survival. *Ecotoxicology,* 19**,** 1249-1257.

Faucon J. P., Aurières, C., Drajnudel, P., Mathieu, L., Ribière, M., Martel, A. C., Zeggane, S., Chauzat, M. P. & Aubert, M. F. 2005. Experimental study on the toxicity of imidacloprid given in syrup to honey bee (*Apis mellifera*) colonies. *Pest Management Science,* 61**,** 111-125.

Fauser-Misslin A., Sadd, B. M., Neumann, P. & Sandrock, C. 2014. Influence of combined pesticide and parasite exposure on bumblebee colony traits in the laboratory. *Journal of Applied Ecology,* 51**,** 450-459.

Fernandes M. E., Alves, F. M., Pereira, R. C., Aquino, L. A., Fernandes, F. L. & Zanuncio, J. C. 2016. Lethal and sublethal effects of seven insecticides on three beneficial insects in laboratory assays and field trials. *Chemosphere,* 156**,** 45-55.

Finizio A. & Villa, S. 2002. Environmental risk assessment for pesticides A tool for decision making. *Environmental Impact Assessment Review,* 22**,** 235 - 248.

Fogel M. N., Schneider, M. I., Desneux, N., Gonzalez, B. & Ronco, A. E. 2013. Impact of the neonicotinoid acetamiprid on immature stages of the predator *Eriopis connexa* (Coleoptera: Coccinellidae). *Ecotoxicology,* 22**,** 1063-1071.

Fogel M. N., Schneider, M. I., Rimoldi, F., Ladux, L. S., Desneux, N. & Ronco, A. E. 2016. Toxicity assessment of four insecticides with different modes of action on pupae and adults of *Eriopis connexa* (Coleoptera: Coccinellidae), a relevant predator of the Neotropical Region. *Environmental Science and Pollution Research,* 23**,** 14918-14926.

Fountain M. T., Brown, V. K., Gange, A. C., Symondson, W. O. & Murray, P. J. 2007. The effects of the insecticide chlorpyrifos on spider and Collembola communities. *Pedobiologia,* 51**,** 147-158.

Francesena N., Desneux, N., De Campos, M. R. & I., S. M. 2017. Side effects of spirotetramat on pupae and adults of a neotropical strain of *Eretmocerus mundus* (Hymenoptera: Aphelinidae): Effects on the life parameters and demography. *Environmental Science and Pollution Research,* 24**,** 17719 – 17730.

Furlan L. & Kreutzweiser, D. 2015. Alternatives to neonicotinoid insecticides for pest control: case studies in agriculture and forestry. *Environmental Science and Pollution Research,* 22**,** 135-147.

Furlong M. J., Wright, D. J. & Dosdall, L. M. 2013. Diamondback moth ecology and management: problems, progress, and prospects. *Annual Review of Entomology,* 58**,** 517-541.

Galvan T. L., Koch, R. L. & Hutchison, W. D. 2005. Toxicity of commonly used insecticides in sweet corn and soybean to multicolored Asian lady beetle (Coleoptera : Coccinellidae). *Journal of Economic Entomology,* 98**,** 780-789.

Garnett T. & Godfray, C. 2012. Sustainable intensification in agriculture. Navigating a course through competing food system priorities. *Food Climate Research Network and the Oxford Martin Programme on the Future of Food, University of Oxford, UK***,** 51.

Gavin W. 2015. *Pesticides and sustainable agricultural productivity* [Online]. Adhacent Government. Available: www.adjacentgovernment.co.uk/.../pesticides-sustainable-agricultural-productivity [Accessed].

Gazziero D. L. P. 2015. Misturas de agrotóxicos em tanque nas propriedades agrícolas do Brasil. *Planta Daninha,* 33**,** 83-92.

Georghiou G. P. 1972. The evolution of resistance to pesticides. *Annual Review of Ecology and Systematics***,** 133-168.

Ghananand T., Prasad, C. & Nath, L. 2011. Effect of insecticides, bio-pesticides and botanicals on the population of natural enemies in brinjal ecosystem. *Vegetos-An International Journal of Plant Research,* 24**,** 40-44.

Gibbons D., Morrissey, C. & Mineau, P. 2015. A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. *Environmental Science and Pollution Research,* 22**,** 103-118.

Giglio A., Giulianini, P. G., Zetto, T. & Talarico, F. 2011. Effects of the pesticide dimethoate on a non-target generalist carabid, *Pterostichus melas italicus* (Dejean, 1828)(Coleoptera: Carabidae). *Italian Journal of Zoology,* 78**,** 471-477.

Gill H. K. & Garg, H. 2014. Pesticide: environmental impacts and management strategies. *In:* MARCELO, L. L. & SONIA, S. (eds.) *Pesticides-Toxic Effects.* Rijeka, Croatia: Intech.

Gill R. J. & Raine, N. E. 2014. Chronic impairment of bumblebee natural foraging behaviour induced by sublethal pesticide exposure. *Functional Ecology,* 28**,** 1459-1471.

Gill R. J., Ramos-Rodriguez, O. & Raine, N. E. 2012. Combined pesticide exposure severely affects individual-and colony-level traits in bees. *Nature,* 491**,** 105-108.

Giorio C., Safer, A., Sánchez-Bayo, F., Tapparo, A., Lentola, A., Girolami, V., Van Lexmond, M. B. & Bonmatin, J. M. 2017. An update of the Worldwide Integrated Assessment (WIA) on systemic insecticides Part 1: new molecules, metabolism, fate, and transport. *Environmental Science and Pollution Research*.

Girolami V., Mazzon, L., Squartini, A., Mori, N., Marzaro, M., Di Bernardo, A., Greatti, M., Giorio, C. & Tapparo, A. 2009. Translocation of neonicotinoid insecticides from coated seeds to seedling guttation drops: A novel way of intoxication for bees. *Journal of Economic Entomology,* 102**,** 1808-1815.

Godfray H. C. J., Blacquiere, T., Field, L. M., Hails, R. S., Petrokofsky, G., Potts, S. G., Raine, N. E., Vanbergen, A. J. & Mclean, A. R. Year. A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. Proc. R. Soc. B, 2014. The Royal Society, 20140558.

Golizadeh A., Kamali, K., Fathipour, Y. & Abbasipour, H. 2007. Temperature-dependent development of diamondbackmoth, *Plutella xylostella* (Lepidoptera: Plutellidae) on two brassicaceous host plants. *Insect Science,* 14**,** 306 - 316.

Gómez-Guzmán J. A., García-Marín, F. J., Sáinz-Pérez, M. & González-Ruiz, R. 2017. Behavioural resistance in insects: Its potential use as bio indicator of organic agriculture. *IOP Conference Series: Earth and Environmental Science.* IOP Publishing Ltd.

Gontijo P. C., Moscardini, V. F., Michaud, J. P. & Carvalho, G. A. 2015. Non-target effects of two sunflower seed treatments on *Onus insidiosus* (Hemiptera: Anthocoridae). *Pest Management Science,* 71**,** 515-522.

Goulson D. 2013a. Neonicotinoids and bees: What's all the buzz? *Significance,* 10**,** 6-11.

Goulson D. 2013b. Review: An overview of the environmental risks posed by neonicotinoid insecticides. *Journal of Applied Ecology,* 50**,** 977-987.

Goulson D. 2015. Neonicotinoids impact bumblebee colony fitness in the field; a reanalysis of the UK's Food & Environment Research Agency 2012 experiment. *PeerJ,* 3**,** e854.

Goulson D., Nicholls, E., Botías, C. & Rotheray, E. L. 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science,* 347**,** 1255957.

Grzywacz D., Rossbach, A., Rauf, A., Russell, D., Srinivasan, R. & Shelton, A. 2010. Current control methods for diamondback moth and other brassica insect pests and the prospects for improved management with lepidopteran-resistant Bt vegetable brassicas in Asia and Africa. *Crop Protection,* 29**,** 68-79.

Guedes N., Silva, L. & Guedes, R. Year. Deltamethrin-induced behavioral responses of pyrethroid-resistant and-susceptible populations of the maize weevil (*Sitophilus zeamais*). Proceedings of the 9th International Working Conference on Stored-Product Protection, ABRAPOS, Passo Fundo, RS, Brazil, 15-18 October 2006., 2006. Brazilian Post-Harvest Association (ABRAPOS), 299-305.

Guedes R. N. C., Smagghe, G., Stark, J. D. & Desneux, N. 2016. Pesticide-induced stress in arthropod pests for optimized integrated pest management programs. *Annual Review of Entomology, Vol 61,* 61**,** 43-62.

Hardstone M. C. & Scott, J. G. 2010. Is *Apis mellifera* more sensitive to insecticides than other insects? *Pest Management Science,* 66**,** 1171-1180.

Heath A. G. 1995. *Water pollution and fish physiology,* New York Lewis Publishers, CRC press

Hernandez A. F., Gil, F. & Lacasana, M. 2017. Toxicological interactions of pesticide mixtures: an update. *Archives of Toxicology,* 91**,** 3211-3223.

Hill R. A. & Sendashonga, C. 2003. General principles for risk assessment of living modified organisms: lessons from chemical risk assessment. *Environmental Biosafety Research,* 2**,** 81 - 88.

Hill T. A. & Foster, R. E. 2000. Effect of insecticides on the diamondback moth (Lepidoptera: Plutellidae) and its parasitoid *Diadegma insulare* (Hymenoptera: Ichneumonidae). *Journal of Economic Entomology,* 93**,** 763-768.

Hommen U., Baveco J. M., Galic N., van den Brink P.J. 2010. Potential application of ecological models in the European environmental risk assessment of chemicals I: review of protection goals in EU dirctives and regulations. Integrated Environmental Assessment and Management. 6, 325–337.

Hopwood J., Vaughan, M., Shepherd, M., Biddinger, D., Mader, E., Black, S. H. & Mazzacano, C. 2012. Are neonicotinoids killing bees? A review of research into the effects of neonicotinoid insecticides on bees, with recommendations for action. Xerces Society for Invertebrate Conservation, USA. *The Xerces Society for Invertebrate Conservation***,** 33.

Imran M. 2017. Economic Insect Pests of Brassica. *In:* EL-ESAWI, M. A. (ed.) *Brassica germplassm characterization, breeding and utilization.* Intechopen.

Iverson A., Casey, H., Leeah, R., Olivia, M. & Scott, M. 2019. Synergistic effects of three sterol biosynthesis inhibiting fungicides on the toxicity of a pyrethroid and neonicotinoid insecticide to bumble bees. *Apidologie***,** 1 -12.

Iwasa T., Motoyama, N., Ambrose, J. T. & Roe, R. M. 2004. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Protection,* 23**,** 371-378.

Jackson L. E., Pascual, U. & Hodgkin, T. 2007. Utilizing and conserving agrobiodiversity in agricultural landscapes. *Agriculture, Ecosystems & Environment,* 121**,** 196-210.

Jallow M. F. & Hoy, C. W. 2006. Quantitative genetics of adult behavioral response and larval physiological tolerance to permethrin in diamondback moth (Lepidoptera: Plutellidae). *Journal of Economic Entomology,* 99**,** 1388-1395.

Jallow M. F. & Hoy, C. W. 2007. Indirect selection for increased susceptibility to permethrin in diamondback moth (Lepidoptera: Plutellidae). *Journal of Economic Entomology,* 100**,** 526-533.

Jankowska B. & Wiech, K. 2006. The composition and role of parasitoids in reducing population densities of diamondback moth *Plutella xylostella* L. on different cabbage vegetables. *Journal of Plant Protection Research,* 46**,** 275-284.

Jansen J. P., Stephane, L., Justine, G. & Jessica, D. 2017. Possible synergistic effects of fungicide-insecticide mixtures on beneficial arthropods. *Pesticides and Beneficial Organism IOBC-WPRS Bulletin,* 125**,** 28 - 35.

Jaspers V. L. B. 2015. Selecting the right bird model in experimental studies on endocrine disrupting chemicals. *Frontiers in Environmental Science,* 3.

Jeschke P., Nauen, R., Schindler, M. & Elbert, A. 2011. Overview of the status and global strategy for neonicotinoids. *Journal of Agricultural and Food Chemistry,* 59**,** 2897-2908.

Johnson R. M. 2015. Honey bee toxicology. *Annual Review of Entomology,* 60**,** 415.

Johnson R. M., Mao, W., Pollock, H. S., Niu, G., Schuler, M. A. & Berenbaum, M. R. 2012. Ecologically appropriate xenobiotics induce cytochrome P450s in *Apis mellifera*. *PLoS ONE,* 7**,** e31051.

Juntti S. 2019. The Future of Gene-Guided Neuroscience Research in Non-Traditional Model Organisms. *Brain Behavior and Evolution,* 93**,** 108-121.

Kamuanga M., Sigué, H., Swallow, B., Bauer, B. & D'ieteren, G. 2001. Farmers' perceptions of the impacts of tsetse and trypanosomosis control on livestock production: evidence from southern Burkina Faso. *Tropical Animal Health and Production,* 33**,** 141-153.

Kapusta-Duch J., Kopeć, A., Piatkowska, E., Borczak, B. & Leszczyńska, T. 2012. The beneficial effects of *Brassica* vegetables on human health. *Roczniki Państwowego Zakładu Higieny,* 63**,** 389-95.

Kesleman H. J., Othman A.R. & Wilcox R.R. 2016. Generalised linear model analysis fot treatment group equality when data is non normal. *Journal of Modern Statistical Methods,* 15, 32 - 61.

Khalid M. T., Sarwar, M. F., Sarwar, M. H. & Sarwar, M. 2015. Current role of physiotherapy in response to changing healthcare needs of the society. *International Journal of Education and Information Technology,* 1**,** 105 - 110.

Khan M. A., Khan, H. & Ruberson, J. R. 2015. Lethal and behavioral effects of selected novel pesticides on adults of *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae). *Pest Management Science,* 71**,** 1640-1648.

Kienzler A., Bopp, S. K., Van Der Linden, S., Berggren, E. & Worth, A. 2016. Regulatory assessment of chemical mixtures: Requirements, current approaches and future perspectives. *Regulatory Toxicology and Pharmacology,* 80**,** 321-334.

Kimber D. S. & Mcgregor, D. I. 1995. The species and their origin, cultivation and world production. *In:* KIMBER, D. S. & MCGREGOR, D. I. (eds.) *Brassica Oilseed; Production and Utilization.* Cambridge: Centre for Agriculture and Biosciences International, University Press.

Kongmee M., Prabaripai, A., Akratanakul, P., Bangs, M. J. & Chareonviriyaphap, T. 2004. Behavioral responses of *Aedes aegypti* (Diptera : Culicidae) exposed to deltamethrin and possible implications for disease control. *Journal of Medical Entomology,* 41**,** 1055-1063.

Kortenkamp A., Backhaus, T. & Faust, M. 2009. State of the art report on mixture toxicity. Final report.

Kremen C., Williams, N. M. & Thorp, R. W. 2002. Crop pollination from native bees at risk from agricultural intensification. *Proceedings of the National Academy of Sciences,* 99**,** 16812-16816.

Krischik V., Rogers, M., Gupta, G. & Varshney, A. 2015. Soil-applied imidacloprid translocates to ornamental flowers and reduces survival of adult *Coleomegilla maculata*, *Harmonia axyridis*, and H*ippodamia convergens* ladybeetles, and larval *Danaus plexippus* and *Vanessa cardui* butterflies. *PLoS ONE,* 10**,** 1 - 22.

Krupke C. H., Hunt, G. J., Eitzer, B. D., Andino, G. & Given, K. 2012. Multiple routes of pesticide exposure for honey bees living near agricultural fields. *PLoS ONE,* 7**,** e29268.

Kucharik C. J. & Ramankutty, N. 2005. Trends and variability in US corn yields over the twentieth century. *Earth Interactions,* 9**,** 1-29.

Kumar R., Kranthi, S., Nitharwal, M., Jat, S. & Monga, D. 2012. Influence of pesticides and application methods on pest and predatory arthropods associated with cotton. *Phytoparasitica,* 40**,** 417-424.

Kusznierewicz B., Bartoszek, A., Wolska, L., Drzewiecki, J., Gorinstein, S. & Namieśnik, J. 2008. Partial characterization of white cabbages (*Brassica oleracea* var. *capitata f. alba*) from different regions by glucosinolates, bioactive compounds, total antioxidant activities and proteins. *LWT-Food Science and Technology,* 41**,** 1-9.

Ladurner E., Bosch, J., Kemp, W. P. & Maini, S. 2005. Assessing delayed and acute toxicity of five formulated fungicides to *Osmia lignaria* and *Apis mellifera*. *Apidologie,* 36**,** 449-460.

Lambert O., Piroux, M., Puyo, S., Thorin, C., L'hostis, M., Wiest, L., Buleté, A., Delbac, F. & Pouliquen, H. 2013. Widespread occurrence of chemical residues in beehive matrices from apiaries located in different landscapes of western France. *PLoS ONE,* 8**,** e67007.

Lambin M., Armengaud, C., Raymond, S. & Gauthier, M. 2001. Imidacloprid-induced facilitation of the proboscis extension reflex habituation in the honeybee. *Archives of Insect Biochemistry and Physiology,* 48**,** 129-134.

Lanteigne M., Whiting, S. A. & Lydy, M. J. 2015. Mixture Toxicity of imidacloprid and cyfluthrin to two non-target Species, the fathead minnow *Pimephales promelas* and the amphipod *Hyalella azteca*. *Archives of Environmental Contamination and Toxicology,* 68**,** 354-361.

Lash H. E., Warnock, D. F. & Cloyd, R. A. 2007. Effect of pesticide mixtures on the survival of the predatory mite *Neoseiulus cucumeris* (Acarina : Phytoseiidae). *Journal of Entomological Science,* 42**,** 311-319.

Lavagnini T. C., Morales, A. C. & Freitas, S. 2015. Population genetics of *Chrysoperla externa* (Neuroptera: Chrysopidae) and implications for biological control. *Brazilian Journal of Biology,* 75**,** 878-885.

Laycock I., Lenthall, K. M., Barratt, A. T. & Cresswell, J. E. 2012. Effects of imidacloprid, a neonicotinoid pesticide, on reproduction in worker bumble bees (*Bombus terrestris*). *Ecotoxicology,* 21**,** 1937-1945.

Lee J. W., Won, E. J., Raisuddin, S. & Lee, J. S. 2015. Significance of adverse outcome pathways in biomarker-based environmental risk assessment in aquatic organisms. *Journal of Environmental Sciences-China,* 35**,** 115-127.

Levine S. L., Giddings, J., Valenti, T., Cobb, G. P., Carley, D. S. & Mcconnell, L. L. 2019. Overcoming challenges of incorporating higher tier data in ecological risk assessments and risk management of pesticides in the United States: Findings and recommendations from the 2017 workshop on regulation and innovation in Agriculture. *Integrated Environmental Assessment and Management,* 15**,** 714-725.

Li X. C., Schuler, M. A. & Berenbaum, M. R. 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annual Review of Entomology,* 52**,** 231-253.

Li Z. G., Li, M., He, J. F., Zhao, X. M., Chaimanee, V., Huang, W. F., Nie, H. Y., Zhao, Y. Z. & Su, S. K. 2017. Differential physiological effects of neonicotinoid insecticides on honey bees: A comparison between *Apis mellifera* and *Apis cerana*. *Pesticide Biochemistry and Physiology,* 140**,** 1-8.

Li Y., Liu Z., Wang Y., Yang N., Xin X., Yang S. & feng H. 2012. identification of quantitative trait loci for yellow inner leaves in Chinese cabbage (Brassica rapa L. ssp. pekinensis) bsed on SSR and SRAP markers. *Scientia Horticulturae*, 133, 10 - 17.

Lindblade K. A., Eisele, T. P., Gimnig, J. E., Alaii, J. A., Odhiambo, F., Ter Kuile, F. O., Hawley, W. A., Wannemuehler, K. A., Phillips-Howard, P. A. & Rosen, D. H. 2004. Sustainability of reductions in malaria transmission and infant mortality in western Kenya with use of insecticide-treated bednets: 4 to 6 years of follow-up. *Jama,* 291**,** 2571-2580.

Linthurst R., Bourdeau, P. & Tardif, R. 1995. *Methods to assess the effects on chemical ecosystems,* New York, Wiley.

Loru L., Fois, X., Ramasani, S. V., Fadda, L. M. & Pantaleoni, R. A. 2014. An innovative, low-cost, small-scale rearing method for green lacewings (Neuroptera Chrysopidae). *Biodiversity Journal,* 5**,** 221-224.

Luna-Cruz A., Rodriguez-Leyva, E., Lomeli-Flores, J. R., Ortega-Arenas, L. D., Bautista-Martinez, N. & Pineda, S. 2015. Toxicity and residual activity of insecticides against *Tamarixia triozae* (Hymenoptera: Eulophidae), a parasitoid of *Bactericera cockerelli* (Hemiptera: Triozidae). *Journal of Economic Entomology,* 108**,** 2289-2295.

Lundin O., Rundlöf, M., Smith, H. G., Fries, I. & Bommarco, R. 2015. Neonicotinoid insecticides and their impacts on bees: a systematic review of research approaches and identification of knowledge gaps. *PLoS ONE,* 10**,** e0136928.

Machekano H., Mvumi, B. M. & Nyamukondiwa, C. 2017. Diamondback Moth, *Plutella xylostella* (L.) in southern africa: Research trends, challenges and insights on sustainable management options. *Sustainability,* 9.

Maia J. B., Carvalho, G. A., Medina, P., Garzon, A., Gontijo, P. D. & Vinuela, E. 2016. Lethal and sublethal effects of pesticides on *Chrysoperla carnea* larvae (Neuroptera: Chrysopidae) and the influence of rainfastness in their degradation pattern over time. *Ecotoxicology,* 25**,** 845-855.

Manjon C., Bartlomiej, J. T., Marion, Z., Katherine, B., Emma, R., Gillian, H., Kumar, S. S., Christoph, T. Z., Rafael, A. H., Bettina, L., Rebecca, R., Laura, K., Maxie, K., Jurgen, B., Martin, S. W., Davies, T. E., Linda, M. F., Chris, B. & Ralf, N. 2018. Unravelling the molecular determinants of bee sensitivity to neonicotinoid insecticides. *Current Biology,* 28**,** 1 - 7.

Manning P., Ramanaidu, K. & Cutler, G. C. 2017. Honey bee survival is affected by interactions between field-relevant rates of fungicides and insecticides used in apple and blueberry production. *Facets,* 2**,** 910-918.

Mansoor M. M., Abbas, N., Shad, S. A., Pathan, A. K. & Razaq, M. 2013. Increased fitness and realized heritability in emamectin benzoate-resistant *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Ecotoxicology,* 22**,** 1232-1240.

Mansoor M. M., Raza, A. M., Abbas, N., Aqueel, M. A. & Afzal, M. 2017. Resistance of green lacewing, *Chrysoperla carnea* Stephens to nitenpyram: cross-resistance patterns, mechanism, stability, and realized heritability. *Pesticide Biochemistry and Physiology,* 135**,** 59-63.

Mao W., Schuler, M. A. & Berenbaum, M. R. 2015. Task-related differential expression of four cytochrome P450 genes in honeybee appendages. *Insect Molecular Biology,* 24**,** 582-588.

Mao W. F., Schuler, M. A. & Berenbaum, M. R. 2017. Disruption of quercetin metabolism by fungicide affects energy production in honey bees (Apis mellifera). *Proceedings of the National Academy of Sciences of the United States of America,* 114**,** 2538-2543.

Martinez R. S., Di Marzio, W. D. & Sáenz, M. E. 2015. Genotoxic effects of commercial formulations of chlorpyrifos and tebuconazole on green algae. *Ecotoxicology,* 24**,** 45-54.

Mathieu R., A., T., Natal-Da-Luza, T., Sara, L., Joao, R., Fernando, R., Jose, P. S., Henrique, M. V. S. & Azevedo-Pereira 2018. Effects of the neonicotinoids acetamiprid and thiacloprid in their commercial formulations on soil fauna. *Chemosphere,* 194**,** 85 - 93.

McArt S.H., Urbanowicz C., McCoshum S., Irwin R.E., Adler L.S. 2017. Landscape predictors of pathogen prevalence and range contractions in US bumblebees. Proceedings of Royal Society B. 284, 20172181.

Mcewen P. K., New, T. R. & Whittington, A. E. 2007. *Lacewings in the crop environment*, Cambridge University Press.

Mcgrath P. F. 2014. Politics meets science: The case of neonicotinoid insecticides in Europe. *S.A.P.I.E.N.S,* 7**,** 1 - 10.

Mcneely J. A. & Scherr, S. J. 2003. *Ecoagriculture: strategies to feed the world and save wild biodiversity*, Island Press.

Medina P., Flor, B., Pedro, D. E., Angeles, A. & Elisa, V. 2003. Side effects of six insecticides on different developmental stages of *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Pesticides and Beneficial Organisms,* 26**,** 33 - 40.

Medrzycki P., Sgolastra, F., Bogo, G., Tosi, S. & Venturi, S. 2012. Influence of some experimental conditions on the results of laboratory toxicological tests on honeybees. *Hazards of Pesticides to Bees: 11th International Symposium of the Icp-Pr Bee Protection Group,* 437**,** 104-104.

Meek M. E., Boobis, A. R., Crofton, K. M., Heinemeyer, G., Van Raaij, M. & Vickers, C. 2011. Risk assessment of combined exposure to multiple chemicals: A WHO/IPCS framework. *Regulatory Toxicology and Pharmacology,* 60**,** S1-S14.

Mesnage R. & Antoniou, M. N. 2018. Ignoring Adjuvant Toxicity Falsifies the Safety Profile of Commercial Pesticides. *Frontiers in Public Health,* 5.

Messelink G. J., Bennison, J., Alomar, O., Ingegno, B. L., Tavella, L., Shipp, L., Palevsky, E. & Wäckers, F. L. 2014. Approaches to conserving natural enemy populations in greenhouse crops: current methods and future prospects. *BioControl,* 59**,** 377-393.

Millar N. S. & Denholm, I. 2007. Nicotinic acetylcholine receptors: targets for commercially important insecticides. *Invertebrate Neuroscience,* 7**,** 53 - 66.

Millennium Ecosystem Assessment 2005. *Ecosystems and human well-being: Health synthesis,* Washington D. C. (USA), World Resources Institute.

Moffat C., Buckland, S. T., Samson, A. J., Mcarthur, R., Pino, V. C., Bollan, K. A., Huang, J. T.-J. & Connolly, C. N. 2016. Neonicotinoids target distinct nicotinic acetylcholine receptors and neurons, leading to differential risks to bumblebees. *Scientific Reports,* 6.

Mommaerts V., Reynders, S., Boulet, J., Besard, L., Sterk, G. & Smagghe, G. 2010. Risk assessment for side-effects of neonicotinoids against bumblebees with and without impairing foraging behavior. *Ecotoxicology,* 19**,** 207-215.

Money C. 2018. Improving the relevance and efficiency of human exposure assessments within the process of regulatory risk assessment. *Environmental Science-Processes & Impacts,* 20**,** 12-19.

Moniski S. 1998. Pesticide Mixtures. *In:* VALKENBURG, W. V., SUGAVANAM, B. & KHAITAN, S. K. (eds.) *Pesticide Formulation-Recent Development and their Applications in Developing Countries.* United Nations Industries Development Organization, New Delhi: New Age International (P) Ltd. Publishers.

Monosson E. 2005. Chemical mixtures: Considering the evolution of toxicology and chemical assessment. *Environmental Health Perspectives,* 113**,** 383-390.

Moretto A., Bachman, A., Boobis, A., Solomon, K. R., Pastoor, T. P., Wilks, M. F. & Embry, M. R. 2017. A framework for cumulative risk assessment in the 21st century. *Critical Reviews in Toxicology,* 47**,** 85-97.

Morrissey C. A., Mineau, P., Devries, J. H., Sanchez-Bayo, F., Liess, M., Cavallaro, M. C. & Liber, K. 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: A review. *Environment International,* 74**,** 291-303.

Mostafalou S. & Abdollahi, M. 2012. Concerns of environmental persistence of pesticides and human chronic diseases. *Clinical and Experimental Pharmacology,* S5**,** e002.

Mullin C. A., Chen, J., Fine, J. D., Frazier, M. T. & Frazier, J. L. 2015. The formulation makes the honey bee poison. *Pesticide Biochemistry and Physiology,* 120**,** 27-35.

Mullin C. A., Fine, J. D., Reynolds, R. D. & Frazier, M. T. 2016. Toxicological risks of agrochemical spray adjuvants: organosilicone surfactants may not be safe. *Frontiers in public health,* 4.

Mullin C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R. & Pettis, J. S. 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PLoS ONE,* 5**,** e9754.

Nansen C., Baissac, O., Nansen, M., Powis, K. & Baker, G. 2016. Behavioral avoidance - will physiological insecticide resistance level of insect strains affect their oviposition and movement responses? *PLoS ONE,* 11**,** 1 - 12.

Nasreen A., Mustafa, G. & Ashfaq, M. 2003. Selectivity of some insecticides to *Chrysoperla carnea* (stephen) (neuroptera: Chrysopidae) in laboratory. *Pakistan Journal of Biological Sciences,* 6**,** 536 - 538.

National Research Council 2009. Science and Decisions: Advancing Risk Assessment. Washington DC: National Academies Press.

National Research Council 2015. Review of California's Risk-Assessment Process for Pesticides. *Risk-Assessment Practices for Pesticides.* Washngton, DC: National Academies Press.

Nauen R., Ebbinghaus-Kintscher, U., Salgado, V. L. & Kaussmann, M. 2003. Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. *Pesticide Biochemistry and Physiology,* 76**,** 55-69.

Nguyen C., Bahar M. H., Baker G. & Andrew N. R. 2014. Therma tolerence limits of diamondback moth in ramping and plunging assays. *PLoS ONE*. 0087535.

Ninsin K. D., Mo, J. & Miyata, T. 2000. Decreased susceptibilities of four field populations of the diamondback moth, *Plutella xylostella* (L.)(Lepidoptera: Yponomeutidae), to acetamiprid. *Applied Entomology and Zoology,* 35**,** 591-595.

Noosidum A., Chareonviriyaphap, T. & Chandrapatya, A. 2014. Synergistic repellent and irritant effect of combined essential oils on *Aedes aegypti* (L.) mosquitoes. *Journal of Vector Ecology,* 39**,** 298-305.

Norgaard K. B. & Cedergreen, N. 2010. Pesticide cocktails can interact synergistically on aquatic crustaceans. *Environmental Science and Pollution Research,* 17**,** 957 - 967.

OECD 2011. Series on Testing and Assessment, No 140, WHO OECD ILSI/HESI International Workshop on risk assessment of combined exposures to multiple chemicals. *ENV/JM/MONO(2011)10.* Paris, France.

Oerke E.-C. & Dehne, H.-W. 2004. Safeguarding production—losses in major crops and the role of crop protection. *Crop Protection,* 23**,** 275-285.

Onyilagha J., Bala, A., Hallett, R., Gruber, M., Soroka, J. & Westcott, N. 2003. Leaf flavonoids of the cruciferous species, *Camelina sativa*, *Crambe* spp., *Thlaspi arvense* and several other genera of the family Brassicaceae. *Biochemical Systematics and Ecology,* 31**,** 1309-1322.

Ostyn S. 2017. *Risk assessment of thiacloprid in combination with tebuconazole on Bombus terrestris.* Master, Ghent University.

Pallab G. 2016. *'Biblical' moth influx threatens to devastate crops* [Online]. Science and Environment, BBC News. Available: http://www.bbc.co.uk/news/science-environment-36516619 [Accessed 29/07/ 2016].

Panizzi S., Suciu, N. A. & Trevisan, M. 2017. Combined ecotoxicological risk assessment in the frame of European authorization of pesticides. *Science of the Total Environment,* 580**,** 136-146.

Paoletti M. G. 1999. Using bioindicators based on biodiversity to assess landscape sustainability. *Agriculture, Ecosystems & Environment,* 74**,** 1-18.

Papachristos D. P. & Milonas, P. G. 2008. Adverse effects of soil applied insecticides on the predatory coccinellid *Hippodamia undecimnotata* (Coleoptera: Coccinellidae). *Biological Control,* 47**,** 77-81.

Pappas M. L., Broufas, G. D. & Koveos, D. S. 2011. Chrysopids predators and their role in biological control. *Journal of Economic Entomology,* 8**,** 301 - 326.

Paradis D., Bérail, G., Bonmatin, J.-M. & Belzunces, L. P. 2014. Sensitive analytical methods for 22 relevant insecticides of 3 chemical families in honey by GC-MS/MS and LC-MS/MS. *Analytical and Bioanalytical Chemistry,* 406**,** 621-633.

Paramasivam M. & Selvi, C. 2017. Laboratory bioassay methods to assess the insecticide toxicity against insect pests-A review. *Journal of Entomology and Zoology Studies,* 5**,** 1441-1445.

Parmar B. S. & Tomar, S. S. 2004. Pesticide formulation theory and practice. New Delhi: CBS Publishers and Distributors.

Pasini R. A., Grutzmacher, A. D., Pazini, J. D., De Armas, F. S., Bueno, F. A. & Pires, S. N. 2018. Side effects of insecticides used in wheat crop on eggs and pupae of *Chrysoperla externa* and *Eriopis connexa*. *Phytoparasitica,* 46**,** 115-125.

Paterson A. H., Lan, T.-H., Amasino, R., Osborn, T. C. & Quiros, C. 2001. *Brassica* genomics: a complement to, and early beneficiary of, the *Arabidopsis* sequence. *Genome Biology,* 2**,** 1339-1347.

Pelosi C., Barot, S., Capowiez, Y., Hedde, M. & Vandenbulcke, F. 2014. Pesticides and earthworms. *Agronomy for Sustainable Development,* 34**,** 199-228.

Pery A. R. R., Schuurmann, G., Ciffroy, P., Faust, M., Backhaus, T., Aicher, L., Mombelli, E., Tebby, C., Cronin, M. T. D., Tissot, S., Andres, S., Brignon, J. M., Frewer, L., Georgiou, S., Mattas, K., Vergnaud, J. C., Peijnenburg, W., Capri, E., Marchis, A. & Wilks, M. F. 2013. Perspectives for integrating human and environmental risk assessment and synergies with socio-economic analysis. *Science of the Total Environment,* 456**,** 307-316.

Pesticide Action Network Uk 2016. List of Home and Garden Pesticides Containing Neonicotinoids. http://www.pan-uk.org/home-garden/list-of-home-and-gardenpesticides-containing-neonicotinoids.

Petter F., Seate, D., Pacheco, L. A., Antonio De Almeida, F. & De Alcântara Neto, F. 2012. Incompatibilidade fisica de misturas entre herbicidas e fungicidas. *Planta Daninha,* 30**,** 449-457.

Philips C. R., Fu Z., Kuhar T. P., Shelton M. & Cordero R. J. 2014. Natural history, ecology and management of diamondback moth (Lepidoptera: Plutellidae) with emphasis on the United States. *Journal of Integrated Pest Management*, 5. DOI: http://dx.doi.org/10.1603/IPM14012.

Pilling E. D. & Jepson, P. C. 1993. Synergism between EBI fungicides and a pyrethroid insecticide in the honeybee (*Apis mellifera*). *Pesticide Science,* 39**,** 293-297.

Pisa L., Goulson, D., Yan, E. C., Gibbons, D., Sánchez-Bayo, F., Mitchell, E., Van Der Sluijs, J., Macquarrie, C., Giorio, C., Long, E. Y., Mcfield, M., Bijleveld Van Lexmond, M. & Bonmatin, J. M. 2017. An update of the world integrated assessment (WIA) on systemic insecticides. Part 2: impacts on organisms and ecosystems. *Environmental Science and Pollution Research*.

Pisa L. W., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J.-M., Downs, C. A., Goulson, D., Kreutzweiser, D. P., Krupke, C., Liess, M. & Mcfield, M. 2015. Effects of neonicotinoids and fipronil on non-target invertebrates. *Environmental Science and Pollution Research,* 22**,** 68-102.

Potts S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O. & Kunin, W. E. 2010. Global pollinator declines: trends, impacts and drivers. *Trends in Ecology & Evolution,* 25**,** 345-353.

Powlson D., Gregory, P. J., Whalley, W., Quinton, J., Hopkins, D., Whitmore, A., Hirsch, P. & Goulding, K. 2011. Soil management in relation to sustainable agriculture and ecosystem services. *Food Policy,* 36**,** S72-S87.

Pratissoli D., Milanez, A. M., Barbosa, W. F., Celestino, F. N., Andrade, G. S. & Polanczyk, R. A. 2010. Side effects of fungicides used in cucurbitaceous crop on *Trichogramma atopovirilia*

oatman & platner (Hymenoptera: Trichogramatidae). *Chilean Journal of Agricultural Research***,** 323-327.

Pretty J. 2008. Agricultural sustainability: concepts, principles and evidence. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* 363**,** 447-465.

Prullage J. B, Trans H. V., Timmon P., Harriman J., Chester S. T.& Powell K. 2011. The combined mode of action of fipronil and amitraz on the motility of *Rhipicephalus sanguineus*.*Veterinary Parasitology*, 179, 302 - 310.

Quarles W. 2014. Neonicotinoids, Bees, Birds and Beneficial Insects. *Common Sense Pest Control Quarterly*.

Ragsdale N. N. 2006. The role of pesticides in agricultural crop protection. *Annals of the New York Academy of Sciences,* 894**,** 199-205.

Raimets R., Karise, R., Mand, M., Kaart, T., Ponting, S., Song, J. M. & Cresswell, J. E. 2018. Synergistic interactions between a variety of insecticides and an ergosterol biosynthesis inhibitor fungicide in dietary exposures of bumble bees (*Bombus terrestris* L.). *Pest Management Science,* 74**,** 541-546.

Raj J., Chandra M., Dogra T. D., Pahuja M., & Raina A. 2013. Determination of median lethal dose of combination of endosulfan and cypermethrin in wistar rat. *Toxicology international*, 20(1), 1–5.

Rakow G. 2004. Species origin and economic importance of Brassica. *Brassica.* Springer.

Ratnieks F. L. & Carreck, N. L. 2010. Clarity on honey bee collapse? *Science,* 327**,** 152-153.

Raybould A. & Wilkinson, M. J. 2005. Assessing the environmental risks of gene flow from GM crops to wild relatives. *In:* POPPY, G. M. & WILKINSON, M. J. (eds.) *Gene Flow from GM plants – A manual for assessing, measuring and managing the risks.*: Blackwell Publishing.

Reffstrup T. K., Larsen, J. C. & Meyer, O. 2010. Risk assessment of mixtures of pesticides. Current approaches and future strategies. *Regulatory Toxicology and Pharmacology,* 56**,** 174-192.

Reynard B. W. 2012. The producer-pollinator dilemma: Neonicotinoids and honeybee colony collapse. 53-65.

Richmond D. & Patton, A. 2014. Neonicotinoid insecticides and pollinators: What's all the buzz about? *Golf Course Manangement: Research.* US.

Rizzati V., Briand, O., Guillou, H. & Gamet-Payrastre, L. 2016. Effects of pesticide mixtures in human and animal models: An update of the recent literature. *Chemico-Biological Interactions,* 254**,** 231-246.

Rodriguez-Saona C., Wanumen, A. C., Salamanca, J., Holdcraft, R. & Kyryczenko-Roth, V. 2016. Toxicity of insecticides on various life stages of two tortricid pests of cranberries and on a non-target predator. *Insects,* 7.

Rogers M. A., Krischik, V. A. & Martin, L. A. 2007. Effect of soil application of imidacloprid on survival of adult green lacewing, *Chrysoperla carnea* (Neuroptera : Chrysopidae), used for biological control in greenhouse. *Biological Control,* 42**,** 172-177.

Rohr J. R., Salice, C. J. & Nisbet, R. M. 2016. The pros and cons of ecological risk assessment based on data from different levels of biological organization. *Critical Reviews in Toxicology,* 46**,** 756-784.

Romeis J., Bartsch, D., Bigler, F., Candolfi, M. P., Gielkens, M. M., Hartley, S. E., Hellmich, R. L., Huesing, J. E., Jepson, P. C. & Layton, R. 2008. Assessment of risk of insect-resistant transgenic crops to nontarget arthropods. *Nature Biotechnology,* 26**,** 203-208.

Rondeau G., Sánchez-Bayo, F., Tennekes, H. A., Decourtye, A., Ramírez-Romero, R. & Desneux, N. 2014. Delayed and time-cumulative toxicity of imidacloprid in bees, ants and termites. *Scientific Reports,* 4.

Rosell G., Quero, C., Coll, J. & Guerrero, A. 2008. Biorational insecticides in pest management. *Journal of Pesticide Science,* 33**,** 103-121.

Rother H. A. 2008. South African farm workers' interpretation of risk assessment data expressed as pictograms on pesticide labels. *Environmental Research,* 108**,** 419-427.

Rotteveel A. 2013. Directive 2009/128/EC on the sustainable use of pesticides. *Julius-Kühn-Archiv***,** 21.

Roubos C. R., C Rodriguez-Saona C., Holdcraft R., Mason K. S. & Isaacs R. (2014). Relative toxicity and residual activity of insecticides used in blueberry pest management: mortality of natural enemies. *Journal of Economic Entomology*, 107, 277-285.

Royal Society 2009. Reaping the benefits: science and the sustainable intensification of global agriculture. The Royal Society London.

Rugno G. R., Zanardi, O. Z. & Yamamoto, P. T. 2015. Are the pupae and eggs of the lacewing *Ceraeochrysa cubana* (Neuroptera: Chrysopidae) tolerant to insecticides? *Journal of Economic Entomology,* 108**,** 2630-2639.

Sabrey K. H. & El-Sayed A. A. 2011. Biosafety of a biopesticide and some pesticides used on cotton crop against green lacewings, *Chrysoperla carnea* (Stehens) (Neuroptera: Chrysopidae). Journal of Biopesticides, 4, 214 - 218.

Saljoqi A., Asad N., Khan J., Haq E., Rehman S., Huma Z., Saeed H. G., Nadeem M., salim M., Ahmad B and Zada H. 2015. The impact of temperature on biological and life table parameters of *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) fed on cabbage aphid, *Brevicoryne brassicae* (Linneaus). *Journal of Entomology and Zoology Studies*, 3, 238 - 242.

Sanchez-Bayo F. & Goka, K. 2014. Pesticide residues and bees–a risk assessment. *PLoS ONE,* 9**,** e94482.

Sancho E., Villarroel, M., Andreu, E. & Ferrando, M. 2009. Disturbances in energy metabolism of *Daphnia magna* after exposure to tebuconazole. *Chemosphere,* 74**,** 1171-1178.

Sancho E., Villarroel, M., Fernández, C., Andreu, E. & Ferrando, M. 2010. Short-term exposure to sublethal tebuconazole induces physiological impairment in male zebrafish (*Danio rerio*). *Ecotoxicology and Environmental Safety,* 73**,** 370-376.

Sanlier N. & Guler, S. M. 2018. The benefits of *Brassica* vegetables on human health. *Journal of Health Research,* 1**,** 1-13.

Sarfraz M., Dosdall, L. & Keddie, B. 2005. Evidence for behavioural resistance by the diamondback moth, *Plutella xylostella* (L.). *Journal of Applied Entomology,* 129**,** 340-341.

Sarfaraz M., Keddie A. B. & Dosdall L. M. 2007. Biological control of the diamondback moth, *Plutella xylostella*, a review. *Biocontrol Science and Technology*, 15, 763-789

Sarwar M. & Salman, M. 2015. The paramount benefits of using insecticides and their worldwide importance in food production. *International Journal of BIoinformatics and Biomedical Engineering,* 1**,** 359 - 365.

Sasaki K. & Takahashi, T. 2002. A flavonoid from *Brassica* rapa flower as the UV-absorbing nectar guide. *Phytochemistry,* 61**,** 339-343.

Schafer R. B., Liess, M., Altenburger, R., Filser, J., Hollert, H., Ross-Nickoll, M., Schaffer, A. & Scheringer, M. 2019. Future pesticide risk assessment: narrowing the gap between intention and reality. *Environmental Sciences Europe,* 31.

Schindler M. 2017. Theory of synergistic effects: Hill-type response surfaces as 'null-interaction' models for mixtures. *Theoretical Biology and Medical Modelling,* 14.

Schmuck R., Schöning, R., Stork, A. & Schramel, O. 2001. Risk posed to honeybees (*Apis mellifera* L, Hymenoptera) by an imidacloprid seed dressing of sunflowers. *Pest Management Science,* 57**,** 225-238.

Schmuck R., Stadler, T. & Schmidt, H. W. 2003. Field relevance of a synergistic effect observed in the laboratory between an EBI fungicide and a chloronicotinyl insecticide in the honeybee (*Apis mellifera* L, Hymenoptera). *Pest Management Science,* 59**,** 279-286.

Schneider M. I., Smagghe, G. & Vinuela, E. 2004. Comparative effects of several insect growth regulators and spinosad on the different developmental stages of the endoparasitoid *Hyposoter didymator* (Thunberg). *Pesticides and Beneficial Organism IOBC/wprs Bulletin,* 27**,** 13 - 19.

Segner H. & Baumann, L. 2016. What constitutes a model organism in ecotoxicology? *Integrated Environmental Assessment and Management,* 12**,** 199-200.

Sgolastra F., Arnan, X., Cabbri, R., Isani, G., Medrzycki, P., Teper, D. & Bosch, J. 2018. Combined exposure to sublethal concentrations of an insecticide and a fungicide affect feeding, ovary development and longevity in a solitary bee. *Proceedings of the Royal Society B-Biological Sciences,* 285.

Shankarganesh K., Naveen, N. & Bishwajeet, P. 2016. Effect of Insecticides on Different Stages of Predatory Green Lacewing, *Chrysoperla zastrowi sillemi* (Esben.-Petersen). *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences***,** 1-8.

Shentu X. P., Wang, X. L., Xiao, Y. & Yu, X. P. 2019. Effects of Fungicide Propiconazole on the Yeast-Like Symbiotes in Brown Planthopper (BPH, *Nilaparvata lugens* Stal) and Its Role in Controlling BPH Infestation. *Frontiers in Physiology,* 10.

Shik J. Z., Schal, C. & Silverman, J. 2014. Diet specialization in an extreme omnivore: nutritional regulation in glucose-averse German cockroaches. *Journal of Evolutionary Biology,* 27**,** 2096-2105.

Simon-Delso N., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J.-M., Chagnon, M., Downs, C., Furlan, L., Gibbons, D. W., Giorio, C. & Girolami, V. 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. *Environmental Science and Pollution Research,* 22**,** 5-34.

Solange M. F., Breda, M. O., Barbosa, D. R. S., Araujo, A. M. N. & Guedes, C. A. 2017. The sublethal effects of insecticides in insects. *In:* VONNIE, D. (ed.) *Biological Control of Pest and Vector Insects.* IntechOpen.

Solecki R., Stein, B., Frische, T., Matezki, S., Wogram, J. & Streloke, M. 2014. Paradigm shift in the risk assessment of cumulative effects of pesticide mixtures and multiple residues to humans and wildlife: German proposal for a new approach. *Journal Fur Verbraucherschutz Und Lebensmittelsicherheit-Journal of Consumer Protection and Food Safety,* 9**,** 329-331.

Solomon K. R., Wilks, M. F., Bachman, A., Boobis, A., Moretto, A., Pastoor, T. P., Phillips, R. & Embry, M. R. 2016. Problem formulation for risk assessment of combined exposures to chemicals and other stressors in humans. *Critical Reviews in Toxicology,* 46**,** 835-844.

Stark J. D., Banks, J. E. & Acheampong, S. 2004. Estimating susceptibility of biological control agents to pesticides: influence of life history strategies and population structure. *Biological Control,* 29**,** 392-398.

Steven L. L. & Christopher, J. B. 2018. Review and recommendations on criteria to evaluate the relevance of pesticide interaction data for ecological risk assessments. *Chemosphere,* 209**,** 124-136.

Stevens S. & Jenkins, P. 2014. Heavy costs: Weighing the value of neonicotinoid insecticides in agriculture. *Center for Food Safety Available from http://www.centerforfoodsafety. org/files/neonic-efficacy_digital_29226. pdf (accessed August 20, 2014)*.

Stokstid E. 2018. European agency concludes controversial 'neonic' pesticides threaten bees. *Science.* New York, Washington, DC: American Association for Advancement in Science.

Stroup, W. W. (2013). Generalized linear mixed models: Modern concepts, methods andapplications. Boca Raton, FL: CRC Press.

Suchail S., Guez, D. & Belzunces, L. P. 2000. Characteristics of imidacloprid toxicity in two *Apis mellifera* subspecies. *Environmental Toxicology and Chemistry,* 19**,** 1901-1905.

Suchail S., Guez, D. & Belzunces, L. P. 2001. Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in *Apis mellifera*. *Environmental Toxicology and Chemistry,* 20**,** 2482-2486.

Survilienė E. & Dambrauskienė, E. 2006. Effect of different active ingredients of fungicides on Alternaria spp. growth in vitro. *Pest Management Science,* 4**,** 403-406.

Sutherland J. P. & Poppy, G. M. 2005. Quantifying exposure. *In:* POPPY, G. M. & WILKINSON, M. J. (eds.) *Gene Flow from GM Plants.* Blackwell.

Tabashnik B. E., Van Rensburg, J. & Carrière, Y. 2009. Field-evolved insect resistance to Bt crops: definition, theory, and data. *Journal of Economic Entomology,* 102**,** 2011-2025.

Taillebois E., Cartereau, A., Jones, A. K. & Thany, S. H. 2018. Neonicotinoid insecticides mode of action on insect nicotinic acetylcholine receptors using binding studies. *Pesticide Biochemistry and Physiology,* 151**,** 59-66.

Taillebois E. & Thany, S. H. 2016. The Differential Effect of Low-Dose Mixtures of Four Pesticides on the Pea Aphid *Acyrthosiphon pisum*. *Insects,* 7**,** 53.

Takahashi H., Mitsui, J., Yano, M., Take, T. & Yamada, T. 1999. Efficacy of acetamiprid 2% granule against diamondback moth on cabbage by various application methods. *Nippon Noyaku Gakkaishi,* 24**,** 23-27.

Talekar N. & Shelton, A. 1993. Biology, ecology, and management of the diamondback moth. *Annual Review of Eentomology,* 38**,** 275-301.

Tappert L., Pokorny, T., Hofferberth, J. & Ruther, J. 2017. Sublethal doses of imidacloprid disrupt sexual communication and host finding in a parasitoid wasp. *Scientific Reports,* 7.

Tennekes H. A. 2010a. The significance of the Druckrey-Küpfmüller equation for risk assessment--the toxicity of neonicotinoid insecticides to arthropods is reinforced by exposure time. *Toxicology,* 276**,** 1 - 4.

Tennekes H. A. 2010b. The significance of the Druckrey-Kupfmuller equation for risk assessment-The toxicity of neonicotinoid insecticides to arthropods is reinforced by exposure time. *Toxicology,* 276**,** 1-4.

Tennekes H. A. & Sánchez-Bayo, F. 2011. Time-dependent toxicity of neonicotinoids and other toxicants: Implications for a new approach to risk assessment. *Journal of Environmental and Analytical Toxicology,* 4**,** 1-8.

Tennekes H. A. & Sánchez-Bayo, F. 2013. The molecular basis of simple relationships between exposure concentration and toxic effects with time. *Toxicology,* 309**,** 39 - 51.

Tennekes H. A. & Sánchez-Bayo, F. 2019. Time-dependent toxicity of neonicotinoids and other toxicants:Implications for a new approach to risk assessment. *Journal of Environmental and Analytical Toxicology,* 4**,** 1 - 8.

Thiyam U., Kuhlmann, A., Stöckmann, H. & Schwarz, K. 2004. Prospects of rapeseed oil by-products with respect to antioxidative potential. *Comptes Rendus Chimie,* 7**,** 611-616.

Thompson H. M. 2012. Interaction between pesticides and other factors in effects on bees. EFSA Supporting Publication EN-340.

Thompson H. M., Fryday, S. L., Harkin, S. & Milner, S. 2014. Potential impacts of synergism in honeybees (*Apis mellifera*) of exposure to neonicotinoids and sprayed fungicides in crops. *Apidologie,* 45**,** 545-553.

Tian D. Y., Mao, H. C., Lv, H. C., Zheng, Y., Peng, C. H. & Hou, S. G. 2018. Novel two-tiered approach of ecological risk assessment for pesticide mixtures based on joint effects. *Chemosphere,* 192**,** 362-371.

Tilman D., Cassman, K. G., Matson, P. A., Naylor, R. & Polasky, S. 2002. Agricultural sustainability and intensive production practices. *Nature,* 418**,** 671-677.

Tison L., Holtz, S., Adeoye, A., Kalkan, O., Irmisch, N. S., Lehmann, N. & Menzel, R. 2017. Effects of sublethal doses of thiacloprid and its formulation Calypso (R) on the learning and memory performance of honey bees. *Journal of Experimental Biology,* 220**,** 3695-3705.

Tomé H. V., Ramos, G. S., Araújo, M. F., Santana, W. C., Santos, G. R., Guedes, R. N. C., Maciel, C. D., Newland, P. L. & Oliveira, E. E. 2017. Agrochemical synergism imposes higher risk to Neotropical bees than to honeybees. *Royal Society Open Science,* 4**,** 160866.

Tomizawa M. & Casida, J. E. 2005. Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu. Rev. Pharmacol. Toxicol.,* 45**,** 247-268.

Torres J. & Ruberson, J. N. E. 2004. Toxicity of thiamethoxam and imidacloprid to *Podisus nigrispinus* (Dallas) (Heteroptera:Pentatomidae) nymphs associated to aphid and whitefly control in cotton. *Neotropical Entomology,* 33**,** 99–106.

Tosi S. & Nieh, J. C. 2019. Lethal and sublethal synergistic effects of a new systemic pesticide, flupyradifurone (Sivanto (R)), on honeybees. *Proceedings of the Royal Society B-Biological Sciences,* 286.

Townson H., Nathan, M., Zaim, M., Guillet, P., Manga, L., Bos, R. & Kindhauser, M. 2005. Exploiting the potential of vector control for disease prevention. *Bulletin of the World Health Organization,* 83**,** 942-947.

Tsvetkov N., Samson-Robert, O., Sood, K., Patel, H. S., Malena, D. A., Gajiwala, P. H., Maciukiewicz, P., Fournier, V. & Zayed, A. 2017. Chronic exposure to neonicotinoids reduces honey bee health near corn crops. *Science,* 356**,** 1395-+.

Udeigwe T. K., Teboh, J. M., Eze, P. N., Stietiya, M. H., Kumar, V., Hendrix, J., Mascagni, H. J., Ying, T. & Kandakji, T. 2015. Implications of leading crop production practices on environmental quality and human health. *Journal of Environmental Management,* 151**,** 267-279.

Uhl P., Bucher, R., Schäfer, R. B. & Entling, M. H. 2015. Sublethal effects of imidacloprid on interactions in a tritrophic systemof non-target species. *Chemosphere,* 132**,** 152 – 158.

Uhl P., Franke, L. A., Rehberg, C., Wollmann, C., Stahlschmidt, P., Jeker, L. & Brühl, C. A. 2016. Interspecific sensitivity of bees towards dimethoate and implications for environmental risk assessment. *Scientific Reports,* 6.

Usepa 2016. Pesticide Cumulative Risk Assessment: Framework for Screening Analysis Purpose, EPA-HQ-OPP-2015-0422, Office of Chemical Safety and Pollution Prevention. Washington, DC: U.S. Environmental Protection Agency.

Van Den Brink P. J., Smeden, J. M. V., Bekele, R. S., Dierick, W., Gelder, D. D., Noteboom, M. & Roessink, I. 2016. Acute and chronic toxicity of neonicotinoids to nymphs of a mayfly species and some notes on seasonal differences. *Environmental Toxicology Chemistry,* 35**,** 128 – 133.

Van Gestel C. a. M., Cdle, S., Lam, T., Koekkoek, J. C., Lamoree, M. H. & Verweij, R. A. 2017. Multigeneration toxicity of imidacloprid and thiacloprid to *Folsomia candida*. *Ecotoxicology,* 26**,** 320 – 328.

Van Leeuwen C. J. & Vermeire, T. G. 2007. *Risk assessment of chemicals: An Introduction 2nd edition,* Dordrecht, The Netherlands, Springer Science and Buisness media.

Vanbergen A. J. 2013. Threats to an ecosystem service: pressures on pollinators. *Frontiers in Ecology and the Environment,* 11**,** 251-259.

Vanderhaegen R. 2017. *Effects of thiacloprid in combination with tebuconazole on different developmental stages of Osmia cornuta.* Master of Science, Ghent University.

Vannette R. L., Mohamed, A. & Johnson, B. R. 2015. Forager bees (*Apis mellifera*) highly express immune and detoxification genes in tissues associated with nectar processing. *Scientific Reports,* 5.

Verkerk R., Dekker, M. & Jongen, W. M. F. 2001. Post-harvest increase of indolyl glucosinolates in response to chopping and storage of *Brassica* vegetables. *Journal of the Science of Food and Agriculture,* 81**,** 953-958.

Vidau C., Diogon, M., Aufauvre, J., Fontbonne, R., Viguès, B., Brunet, J.-L., Texier, C., Biron, D. G., Blot, N. & El Alaoui, H. 2011. Exposure to sublethal doses of fipronil and thiacloprid highly increases mortality of honeybees previously infected by *Nosema ceranae*. *PLoS ONE,* 6**,** e21550.

Wallingford A. K., Kuhar, T. P. & Schultz, P. B. 2012. Toxicity and field efficacy of four neonicotinoids on harlequin bug (Hemiptera: Pentatomidae). *Florida Entomologist,* 95**,** 1123-1126.

Wang W., Wan Q., Li Y. & Yu X. 2019. Uptake, translocation and subcellular distribution of pesticides in Chinese cabbage (*Brassica rapa* var. *chinensis*). *Ecotoxicology and Environmental Safety*, 183, 109488.

Wang C., Scharf, M. E. & Bennett, G. W. 2004. Behavioral and physiological resistance of the German cockroach to gel baits (Blattodea: Blattellidae). *Journal of Economic Entomology,* 97**,** 2067-2072.

Wang M. L., Aai X. Z. & Zheng N. 2008. Study on pigment content and light requiring characteristics of different leaf position leaves in purple cabbage. *Acta Agriculturae Borealli-occidentalis Sinica*, 17, 221–225.

Wanumen A., Sanchez-Ramos, I., Vinuela, E., Medina, P. & Adan, A. 2016. Impact of feeding on contaminated prey on the life parameters of *Nesidiocoris tenuis* (Hemiptera: Miridae) adults. *Journal of Insect Science,* 16**,** 1-7.

Weber M. 2014. Experiments in Biology. *In:* EDWARD, N. Z. (ed.) *The Stanford Encyclopedia of Philosophy* Summer ed.

Wei S-J, Shi B-C, Gong Y-J, Jin G-H, Chen X-X, Meng X-F. 2013. Genetic structure and demographic history reveal migration of the diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae) from the southern to northern regions of China. *PLoS ONE* 8(4): e59654

Whitehorn P. R., O'connor, S., Wackers, F. L. & Goulson, D. 2012. Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science,* 336**,** 351-352.

Wiles J. A. & Jepson, P. C. 1994. Sub-lethal effects of deltamethrin residues on the within-crop behaviour and distribution of *Coccinella septempunctata*. *Entomologia Experimentalis et Applicata,* 72**,** 33-45.

Williamson S. M. & Wright, G. A. 2013. Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. *The Journal of Experimental Biology,* 216**,** 1799-1807.

Willow J., Silva, A., Veromann, E. & Amagghe, G. 2019. Acute effect of low-dose thiacloprid exposure synergised by tebuconazole in a parasitoid wasp. *PLoS ONE,* 14**,** 1 -13.

Winfree R., Williams, N. M., Gaines, H., Ascher, J. S. & Kremen, C. 2008. Wild bee pollinators provide the majority of crop visitation across land-use gradients in New Jersey and Pennsylvania, USA. *Journal of Applied Ecology,* 45**,** 793-802.

Wise J. C., Jenkins, P. E., Vander Poppen, R. & Isaacs, R. 2010. Activity of Broad-Spectrum and Reduced-Risk Insecticides on Various Life Stages of Cranberry Fruitworm (Lepidoptera: Pyralidae) in Highbush Blueberry. *Journal of Economic Entomology,* 103**,** 1720-1728.

Witschi H. 1999. Some notes on the history of Haber's law. *Toxicological Sciences,* 50**,** 164-168.

Wood T. J. & Goulson, D. 2017. The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. *Environmental Science and Pollution Research,* 24**,** 17285-17325.

Woodcock B., Ridding, L., Freeman, S. N., Pereira, M. G., Sleep, D., Redhead, J., Aston, D., Carreck, N. L., Shore, R. F., Bullock, J. M., Heard, M. S. & Pywell, R. F. 2018. Neonicotinoid residues in UK honey despite European Union moratorium. *PLoS ONE,* 13**,** 1-15.

Wright D. 2004. Biological control of DBM: a global perspective. *In:* BORDAT, D. & KIRK, A. A. (eds.) *Improving Biocontrol of Plutella xylostella, .* Montpellier, France: CIRAD.

Yadav R. S., Sampath, R. & Sharma, V. 2001. Deltamethrin treated bednets for control of malaria transmitted by *Anopheles culicifacies* (Diptera: Culicidae) in India. *Journal of Medical Entomology,* 38**,** 613-622.

Yang E., Chuang, Y., Chen, Y. & Chang, L. 2008. Abnormal foraging behavior induced by sublethal dosage of imidacloprid in the honey bee (Hymenoptera: Apidae). *Journal of Economic Entomology,* 101**,** 1743-1748.

Yi F., Zou, C., Hu, Q. & Hu, M. 2012. The joint action of destruxins and botanical insecticides (rotenone, azadirachtin and paeonolum) against the cotton aphid, *Aphis gossypii* Glover. *Molecules,* 17**,** 7533-7542.

Youn Y.-N., Seo, M., Shin, J., Jang, C. & Yu, Y. 2003. Toxicity of greenhouse pesticides to multicolored Asian lady beetles, *Harmonia axyridis* (Coleoptera: Coccinellidae). *Biological Control,* 28**,** 164-170.

Yu C. H., Lin, R. H., Fu, M. R., Zhou, Y. M., Zong, F. L., Jiang, H., Lv, N., Piao, X. Y., Zhang, J., Liu, Y. Q. & Brock, T. C. M. 2014. Impact of imidacloprid on life-cycle development of *Coccinella septempunctata* in laboratory microcosms. *Ecotoxicology and Environmental Safety,* 110**,** 168-173.

Yue B. S., Wilde, G. E. & Arthur, F. 2003. Evaluation of thiamethoxam and imidacloprid as seed treatments to control European corn borer and Indianmeal moth (Lepidoptera : Pyralidae) larvae. *Journal of Economic Entomology,* 96**,** 503-509.

Zalucki M. P., Shabbir, A., Silva, R., Adamson, D., Shu-Sheng, L. & Furlong, M. J. 2012. Estimating the economic cost of one of the world's major insect pests, *Plutella xylostella* (Lepidoptera: Plutellidae): just how long is a piece of string? *Journal of Economic Entomology,* 105**,** 1115-1129.

Zhang L., Yan, C. X., Guo, Q., Zhang, J. B. & Ruiz-Menjivar, J. 2018. The impact of agricultural chemical inputs on environment: global evidence from informetrics analysis and visualization. *International Journal of Low-Carbon Technologies,* 13**,** 338-352.

Zhu W., Schmehl, D. R., Mullin, C. A. & Frazier, J. L. 2014. Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. *PLoS ONE,* 9**,** e77547.

Zhu Y. C., Yao, J. X., Adamczyk, J. & Luttrell, R. 2017. Synergistic toxicity and physiological impact of imidacloprid alone and binary mixtures with seven representative pesticides on honey bee (*Apis mellifera*). *PLoS ONE,* 12.