DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Assessment of the Effects of the Neonicotinoids Thiacloprid and Acetamiprid on Soil Fauna

Tolutope Oluseyi Akeju

2014

DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Assessment of the Effects of the Neonicotinoids Thiacloprid and Acetamiprid on Soil Fauna

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica do Professor Doutor José Paulo Sousa, Professor Auxiliar do Departamento de Ciências da Vida da Universidade de Coimbra e do Doutor Henrique Azevedo Pereira, do IMAR-CMA, Universidade de Coimbra

Tolutope Oluseyi Akeju

2014

ACKNOWLEDGEMENTS

Special thanks to Almighty God and to my Savior and Lord Jesus Christ for bringing me to this particular juncture in life.

My sincere appreciation goes to Prof. Dr. José Paulo Sousa for the opportunity to carry out this research project. I am equally grateful to Dr. Henrique Azevedo Pereira for the support and supervision that he gave me during the laboratory work and the write-up process. Also to Dr. Joana Alves who has been extremely helpful to me in resolving personal administrative issues during the master's program. I will not forget in a hurry the support of Tiago Natal da Luz, Sónia Chelinho, Jean Mathieu Renaud, Paulo Roger, Carla Pereira, Filipa Reis and all other members of the Soil Ecology and Ecotoxicology Laboratory. Thank you for all the assistance rendered especially during the experimental set-ups and manuscript writing to make this thesis a reality.

I wish to thank my family; i.e. my Dad and Mum, Bro Olumide, my elder brother and sisters for their concern particularly at a distressing period of my life. For your encouragement and prayers, I am able to complete this thesis.

Finally, I like to thank the European Commission and the EMAE consortium for the 2-year opportunity that was given to me to study in Europe. And the privilege of studying with colleagues from different countries around the world is an experience that will remain indelible in my mind. I wish all EMAE students (2012/2014) success in their future endeavors.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

RESUMO

Actualmente, os inseticidas neonicotinóides são considerados como o grupo mais importante de inseticidas no mundo. Têm sido comercializados em mais de 120 países para o controlo de pragas agrícolas, devido ao seu amplo espectro de actividade e versatilidade na sua aplicação. De uma forma geral, os neonicotinóides são agonistas selectivos dos receptores nicotínicos de acetilcolina dos insetos (nAChRs). Tiaclopride e acetamipride são neonicotinóides que pertencem ao mesmo grupo químico (cyanoamidines), são altamente solúveis em água e não-persistentes no meio ambiente. Estes compostos são normalmente aplicados em pomares ou plantas ornamentais através de pulverização foliar dos respectivos produtos formulados (por exemplo Calypso® e Epik®). Apesar de os organismos não-alvo do solo serem muito susceptíveis à exposição durante estas aplicações, há escassez de informação na literatura científica sobre a toxicidade do acetamipride e tiaclopride, especialmente para invertebrados não-alvo do solo. Este estudo tenta preencher esta lacuna através da avaliação da toxicidade de tiaclopride e acetamiprida em quatro invertebrados do solo: *Folsomia candida*, *Eisenia andrei*, *Enchytraeus crypticus* e *Hypoaspis aculeifer*, utilizando solo artificial. De uma forma geral, os resultados obtidos indicam que a sensibilidade relativa dos organismos de teste para tiaclopride e acetamiprida, usando tanto a reprodução e sobrevivência como parâmetros, é similar e pode ser expressa como: *F. candida* > *E. andrei* > *E. crypticus* > *H. aculeifer*, com *F. candida* como organismo de teste mais sensível. Extrapolando os resultados dos testes laboratoriais válidos com todos os invertebrados (excepto *E. andrei*) para as condições de campo, as condições ambientais previstas (PECs) e as condições ambientais previstas que não demonstram efeitos ($PNEC_{tiaclopride}$ = 0,024 mg kg^{-1}; $PNEC_{acetamiprida}$ = 0,004 mg kg^{-1}) foram derivadas para tiaclopride e acetamiprida. Os rácios de exposição de toxicidade calculados (ETRs; PEC/EC_{10}) e os quocientes de risco (PEC/PNEC) demonstraram que o risco de tiaclopride para os invertebrados de teste em particular, e para o compartimento do solo, em geral, é insignificante. Embora o risco de acetamiprida às populações de campo dos invertebrados teste tenha sido caracterizado como baixo, o quociente de risco calculado (PEC/PNEC) foi maior que 1, que é o valor de risco predefinido pela Comissão Europeia. Portanto, pode concluir-se que o efeito do neonicotinóide acetamiprida no compartimento do solo apresenta um risco significativo e indesejável.

ABSTRACT

Presently, neonicotinoid insecticides are the most prominent group of insecticides in the world. They have been commercialized in over 120 countries for the control of agricultural pests due to their broad spectrum activity and versatility in application. Generally, all neonicotinoids are selective agonists of insect nicotinic acetylcholine receptors (nAChRs). Thiacloprid and acetamiprid belong to the same chemical group (cyanoamidines), and like other neonicotinoids, they are highly soluble in water and non-persistent in the environment. Acetamiprid and thiacloprid are usually applied to orchard or ornamental crops through foliar spraying of respective formulated products (e.g. Calypso® and Epik®). Though non target soil organisms are very likely to be exposed during these applications, there is paucity of information in scientific literature regarding the toxicity of acetamiprid and thiacloprid especially to non-target soil invertebrates. This study attempts to fill this gap by evaluating the toxicity of thiacloprid and acetamiprid in artificial OECD soil to four soil invertebrates namely: *Folsomia candida*, *Eisenia andrei*, *Enchytraeus crypticus* and *Hypoaspis aculeifer*. Results obtained indicate that generally, relative sensitivity of the test organisms to thiacloprid and acetamiprid using both reproduction and survival endpoint parameters are the same and can be expressed as: *F. candida* > *E. andrei* > *E. crypticus* > *H. aculeifer* with *F. candida* as the most sensitive test organism. To extrapolate from valid laboratory test results for all invertebrates (except *E. andrei*) to field conditions, predicted environmental concentrations (PECs) and predicted no-effect concentrations ($PNEC_{thiacloprid}$ = 0.024 mg kg^{-1}; $PNEC_{acetamiprid}$ = 0.004 mg kg^{-1}) were derived for thiacloprid and acetamiprid. Calculated exposure-toxicity ratios (ETRs; PEC/EC10) and hazard quotients (PEC/PNEC) showed that the risk of thiacloprid to the test invertebrates in particular and to the soil compartment in general is negligible. Although the risk of acetamiprid to field populations of the test invertebrates was characterized as low, calculated risk quotient (PEC/PNEC) was greater than 1, the trigger value preset by the European Commission. Therefore, it can be concluded that the risk of acetamiprid to the soil compartment is significant and undesirable.

Chapter 1

1. GENERAL INTRODUCTION

1.1 Soil biodiversity as an integral aspect of global biodiversity

The soil is arguably the most diverse habitat within terrestrial ecosystems. In fact, survey data from several studies revealed that a quarter of invertebrate and vertebrate species inhabit the soil (Decaëns et al. 2006) and it has recently been estimated that the biodiversity of soil animals comprises 23% of the biodiversity of all described species (Lavelle et al. 2006). Therefore, soil biodiversity is an essential component of the general biodiversity concept and nowadays it is recognized that soil organisms are responsible for the provision of many ecosystem services necessary for human wellbeing. Some of these services include soil organic matter decomposition, soil fertility, regulation of nutrient cycles (carbon, nitrogen, phosphorus and sulphur), water flow regulation and detoxification or bioremediation of pollutants (Lavelle et al. 2006; Turbe et al. 2010).

Soil biodiversity is especially needed for agricultural sustainability which aims to improve food production while conserving the fertility and productivity of soils (Beare et al., 1997). As a result, the contributions of decomposer soil organisms in maintaining the structural and functional properties of agro-ecosystems have been sufficiently described in scientific literature (Fragoso et al., 1997; Beare et al., 1997). There is evidence that the biodiversity of soil organisms in agro-ecosystems confers resistance to stress and disturbances such as fires, pathogenic disease and pest outbreaks (Altieri 1999). For instance, N-fixing leguminous plants introduced into Nitrogen deficient African soils mitigated the invasion of maize fields with the parasitic weed named *Striga sp* (Barrios 2007).

The conservation of soil biodiversity is of immense economic importance (Figure 1) as ecosystem services provided by soil organisms have been valued to exceed US$ 1.5 trillion (Brussaard et al. 2007). Among these ecosystem services, global recycling or decomposition of organic wastes was estimated to be most essential, with a monetary value estimated to be greater than US$ 760 billion (Brussaard et al., 2007). In addition, soil microorganisms in agricultural and natural ecosystems are known to fix an estimated minimum amount of 140 million tons of Nitrogen (US$ 90 billion) per year (Brussaard et al., 2007).

Despite the high level of biodiversity present in the soil, there are still many knowledge gaps. Therefore, the soil has been described as one of the last great frontiers of scientific investigation and research efforts are being made to increase understanding of the effects of soil biodiversity on ecosystem functioning and the exact roles played by functional groups of soil biota in

regulating biogeochemical cycles. This is essential as reduced soil biodiversity will negatively affect the composition and stability of terrestrial communities and ecosystems (Jones & Bradford 2001; Fitter et al. 2005).

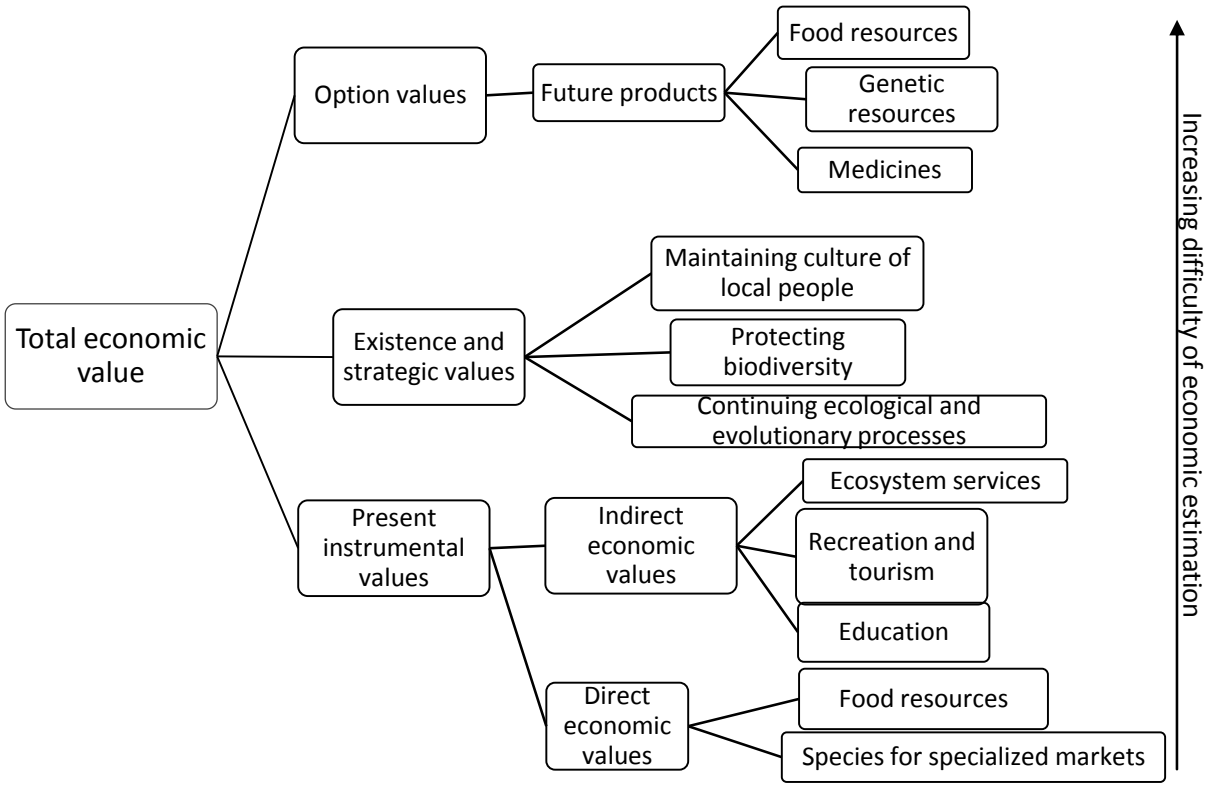


Figure 1: The global value of soil biodiversity in an ecosystem: A conceptual diagram. Adapted from Decaëns et al. (2006)

The 21st century has seen increasing anthropogenic activities that have significantly altered natural ecosystems. The Millennium Ecosystem Assessment noted that climate change and pollution are impacting natural ecosystems at a very rapid rate on a global scale (Reid 2005). Soil biota is highly affected by climate change and pollution, and sampling evidences studies have shown that drivers (e.g. habitat loss, agricultural intensification, etc) causing extinctions and reduction of biodiversity in aboveground organisms also affects belowground biota. For example, sampling records have reported the decline of mushroom species in the Netherlands over a 20-year period by 65% and the rareness of over 50% of earthworm species found across Europe (Gardi et al. 2013).

Despite the unclear relative impacts of these drivers at a regional scale, over-exploitation of land has been estimated to be a main threat to soil biodiversity in the European Union (EU) (Gardi et

al. 2013). Because soil protection is crucial to the long-term preservation of biological diversity as required by the Rio-Convention (Hagvar 1998), the European Commission took legislative steps in 2006 to safeguard the soil by developing and adopting the Soil Thematic Strategy (COM(2006) 231) (EC 2006b) which includes the proposal for a Soil Framework Directive (COM(2006) 232) (EC 2006a).

1.2 Ecology of soil fauna

1.2.1 Classification, distribution, species abundance and richness

One can consider soil fauna as all species of micro and macroscopic animals dwelling in a particular soil and they can be classified into 4 groups based on their relative sizes. This definition excludes microorganisms such as bacteria and fungi (Fortuna 2012). Microfauna are microscopic soil organisms with body sizes of 20 μm - 200 μm (e.g. nematodes and protozoa). Mesofauna have body sizes between 200 μm – 2 mm and are mainly represented by mites and collembolans which make approximately 95% of soil micro-arthropods, and also enchytraeids. The macrofauna (2 mm – 20 mm) include earthworms, millipedes and other macroarthropods. Larger organisms like some species of earthworms (e.g. *Lumbricus terrestris*), snails, reptiles and amphibians with body sizes > 20 mm are classified as megafauna (Cole et al. 2006; Menta 2012; EC 2010; Neher & Barbercheck 1998).

Body size may exert a strong influence on distribution, species abundance, richness, and the ecosystem function of soil organisms. Generally, smaller soil organisms tend to possess higher abundance (see Table 1) and a wider geographic distribution (Anderson 1977).

Table 1: Species richness and abundance of some soil fauna taxa in Mediterranean soils

Organism group	Abundance (ind m^{-2})	Biomass (mg DW m^{-2})	Species number
Nematoda*	3000 – 13000	~ 440	17 – 20**
Acari (mites)	< 1000 – 5000	~ 120	3 – 10
Collembola	1500 – 33000	~ 120	17 – 38
Enchytraeidae	2000 – 3000	110 – 640	3 – 22
Lumbricidae	0 – 200	100 – 12100	1 – 7

*numbers given per kg soil; DW (dry weight); ** families, not species; ~ numbers deduced from grassland sites
Source: European Atlas of Soil Biodiversity (EC 2010)

The ubiquitous distribution of microfauna and mesofauna is attributed to their inhabitation of small water films and pore spaces (micro-sites; diameter ≤ 100μm) present in moist soils with decomposing organic matter (Anderson 1977; Neher 1999; Neher & Barbercheck 1998). Species richness in soil fauna have been empirically observed to increase 100 times for a 10 fold decrease in length (Erwin 1994). However, exceptions to this empirical observation exist at body

sizes below 1cm. For example, studies have revealed that mites (Acari) with sizes below the modal range (0.3 – 1 mm) are less diverse, probably due to a corresponding decrease in host specificity and microhabitat diversity (Walter & Behan-Pelletier 1999; Erwin 1994).

There are several other factors affecting soil fauna biodiversity apart from body size. For example, habitat heterogeneity was found to significantly improve the diversity of collembolan and earthworm species at local and landscape scales (Vanbergen et al. 2007). In addition, the distribution and diversity of soil organisms is determined by physical and chemical soil properties such as the pore size of soil particles, soil moisture, soil pH, etc. Vegetation composition and physiology, particularly plant litter chemistry, has been an intensively researched factor influencing the distribution of soil invertebrates (Sylvain & Wall 2011). Finally, biotic interactions among soil fauna within the same trophic group (e.g. competition) and across different trophic levels (e.g. predation) are important regulators of soil biodiversity (Wardle 2006). Role of soil fauna in ecosystem functioning

Charles Darwin in his 1881 book, "The formation of vegetable mould through the action of worms", described the decomposition of organic matter by earthworms through their feeding and burrowing activities in the soil. Subsequently, ecological experiments have established the importance and role of soil fauna communities on the two major ecosystem processes namely production and decomposition, in addition to their effects on biochemical and physical soil properties (Huhta, 2007; Brussaard et al., 2007).

Rivet, redundant and keystone hypotheses are among several hypotheses proposed to explain species diversity and ecosystem function linkage. While the rivet hypothesis suggests that each species has a unique role to play in ecosystem functioning (Lawton 1994), the redundant hypothesis point to the existence of functional redundancy among species (Setala et al. 2005; Naeem 2008; Wolters 2001). The realization that several species perform similar function has led to the functional classification of soil fauna based on life-history, eco-physiology, food preferences, feeding mode and microhabitat criteria (Brussaard et al., 1997; Brussaard 1998). Five functional classes of soil fauna together with their ecosystem functions are described below:

Collembola: also called springtails, they are very abundant and widely distributed across most terrestrial ecosystems and their ecology could be strongly affected by human activities (Rusek 1998). Together with mites, they constitute 95% of total microarthropod numbers (Neher & Barbercheck 1998; Bardgett & Cook 1998). When compared to collembolans found in litter layers and the 0 – 2 cm upper soil horizon e.g. *Isotoma viridis*, those found at deeper soil layers lack eyes and pigmentation and possess soft bodies with short appendages (e.g. *Protaphorura fimata*) (Bardgett & Cook 1998; EC 2010). They feed mainly on soil microbiota (fungi, bacteria, actinomycetes, algae) and organic matter. Hence, they are usually called 'litter transformers' and are important in the formation of soil microstructure in several terrestrial ecosystems (Lavelle 1997; Rusek 1998; Heneghan & Bolger 1998).

Earthworms: are considered the most significant soil invertebrates in most terrestrial ecosystems worldwide (Rombke et al. 2005). As a result of their size, they are called 'ecosystem engineers' because of their ability to create or modify the soil habitat through various activities such as burrowing, casting, breakdown of organic matter, seed dispersal, ingestion of soil particles and symbiotic interactions with soil microbes (Rombke et al. 2005; Jouquet et al. 2006; Cole et al. 2006). Earthworms are also known to improve primary production by stimulating plant growth (Lavelle 1997).They can be classified into three ecological groups namely: epigeics (they dwell in upper litter layers without creating burrows in the soil e.g. *Eisenia fetida*), anecics (vertical burrowers (> 2 m deep) and have darkly colored bodies) and endogeics (soil-feeding, whitish earthworms living in the mineral layers of the soil) (Rombke et al. 2005; Fragoso et al. 1997).

Enchytraeidae: also called potworms, they are grouped within the phylum Annelida (like the earthworms), are generally whitish in appearance and highly abundant in coniferous forests and grasslands (EC 2010; van Vliet et al. 1995; Brussaard 1997). *Cognettia sphagnetorum* is usually the most abundant species, constituting up to 95% of total enchytraeid biomass (Briones et al. 2004). Enchytraeids are known to mostly inhabit the upper layer (0-5 cm) of the soil and their feeding activities have been found to increase the respiration of soil organisms (Didden & de Fluiter 1998; Cole et al. 2000). Apart from their role in organic matter breakdown and nutrient turn-over, they also influence soil structure through their burrowing capacities, deposition of fecal pellets and ingestion of mineral particles (van Vliet et al. 1995).

Mites (Acari): Soil mites are arguably the most diverse and abundant arthropods in agroecosystems worldwide. In the EU, soil mite species richness is known to be highest in the Mediterranean and Balkan regions (EC 2010). There are three main suborders: Mesostigmata, Prostigmata, and Oribatida (Behan-Pelletier 2003). Orbatid mites usually participate in the decomposition and nutrient cycling process by feeding on plant litters, fungi and algae (Franklin et al. 2004). Prostigmatans are fluid feeders and they could be predators, fungivores or parasites. Mesostigmatic mites occupy a central position in soil food web as nonspecific top predators that feed on nematodes, collembola and other small insects. Thus, they influence ecosystem processes by regulating populations of other soil organisms. Gasmasid mites in particular are very sensitive to environmental disturbances within a short time scale, making them suitable bioindicators (Beaulieu & Weeks 2007; Bedano & Ruf 2007; Behan-Pelletier 2003). Most mites are found in the 0 – 5 cm soil layer probably due to the occurrence of higher moisture and microbial activities in the top layers of soil (Perdue & Crossley Jr 1990).

1.3 Soil Ecotoxicology: Pesticides and their effects on living organisms

1.3.1 Background

Ecotoxicology aims to protect ecosystems by studying the effects of chemicals on populations and communities of organisms. In most cases, the general approach is to extrapolate from effects in single-species organisms to effects at the ecosystem level (van Gestel 2012). Ecotoxicology as

a scientific discipline started 52 years ago with a publication by Rachel Carson called "Silent Spring" which described the disastrous effects of several pesticides such as dichlordiphenyltrichlorethane (DDT) and other chlorinated pesticides used during and after the Second World War (Werner & Hitzfeld 2012).

In the period immediately after WW II, most synthetic insecticides could be classified into two groups based on their structural properties and mode of toxicity: organochlorines and organophosphates (Werner & Hitzfeld 2012). Organochlorines such as DDT and Polychlorinated biphenyls (PCBs) are carcinogenic and persistent in the environment. They bioaccumulate and biomagnify in ecosystems while exhibiting reproductive toxicity such as thinning of egg shells and endocrine disruption in vertebrates (Fry 1995). Consequently, their use has been regulated internationally. Organophosphates (e.g. parathion), often known to be neurotoxic and less persistent, are more toxic especially to humans and many organophosphates have been banned in Europe and the US. They have been gradually replaced by neonicotinoids and pyrethroids over the decades for reasons that include among others their high persistence in the environment, chronic toxicity to humans and the development of resistant pest strains (Werner & Hitzfeld 2012; Salvi et al. 2003).

1.3.2 Neonicotinoids

Since the hugely successful commercialization of imidacloprid in 1991, neonicotinoids have gone on to become the most important group of insecticides in the world due to their effective insecticidal properties (Elbert et al. 2008; Legocki & Połeć 2008). In 2006, neonicotinoids accounted for 17% of the global insecticide market (Jeschke & Nauen 2008). Neonicotinoids are systemic, water soluble and broad spectrum insecticides applied to plants through foliar sprays, seed coatings or via the soil. They are absorbed through the leaves or roots and distributed throughout the tissues of a plant (Goulson 2013; Miao et al. 2013). The high systemicity and prophylactic use of neonicotinoids makes them efficient in the control of sap sucking and boring insect pest groups, especially Hemiptera (e.g. aphids, whiteflies, planthoppers), and Coleoptera (e.g. beetles) (Elbert et al. 2008; Nauen & Denholm 2005; Miao et al. 2013).

All neonicotinoids are nicotine derivatives and they can be classified into three chemical groups: N-nitro-guanidines (imidacloprid, thiamethoxam, clothianidin and dinotefuran); N-cyanoamidines (acetamiprid and thiacloprid); nitromethylenes (nithiazine and nitenpyram) (Jeschke & Nauen 2008; Goulson 2013). They are agonists of the nicotinic acetylcholinesterase receptors (nAChRs) in the central nervous system of insects and mammals (Tomizawa & Casida 2005; Sánchez-bayo et al. 2013). The high selective toxicity of neonicotinoids to insects compared to vertebrates is assumed to be due to their binding to specific cationic sub-sites in insect nAChRs although they bind to an anionic sub-site in mammalian nAChRs (Tomizawa et al. 2000; Tomizawa & Casida 2003). Research has however shown that acetamiprid and imidacloprid stimulate mammalian nAChRs at low concentrations (1-100 μM) and thus may affect the developing human brain (Kimura-Kuroda et al. 2012).

Recent independent scientific studies have established the considerable lethal and sub-lethal toxicity of neonicotinoids to target insect pests (Vojoudi & Saber 2013; Miao et al. 2013). There have also been significant research efforts to assess the effects of neonicotinoids on bees and other non-target invertebrates in aquatic and terrestrial ecosystems. Neonicotinoids have been implicated in the pollinator crisis (colony collapse disorder), currently a major environmental concern (Werner & Hitzfeld 2012). Field studies have revealed that trace amounts of neonicotinoids can reduce the foraging ability of bees. Also, field-realistic concentrations of imidacloprid was shown to cause an 85% decline in queen reproduction and a significant reduction of growth rate in the bumble bee *Bombus terrestris* (Whitehorn et al. 2012). Furthermore, nitro-group neonicotinoids (clothianidin, dinotefuran, imidacloprid, thiamethoxam, nitenpyram) was found to show high contact toxicity to honeybees compared to cyano-group neonicotinoids (Blacquière et al. 2012; Decourtye & Devillers 2010).

In aquatic ecosystems, imidacloprid (IMI) as a pesticide active ingredient was shown to reduce the growth of *Chironomus riparius* larvae during a constant 10-day exposure (Azevedo-Pereira et al. 2010). The decline of macro-invertebrates in surface water polluted with IMI also highlights the negative impact of neonicotinoids on non-target aquatic invertebrates (Van Dijk et al. 2013). Though it has been estimated that more than 90% of neonicotinoid pesticide active ingredient used as seed coatings enters the soil, information on the toxicity of neonicotinoids on soil fauna in scientific literature have been scant and where available, have mostly focused on toxicity assessment of imidacloprid to earthworms (Kreutzweiser et al. 2008; Goulson 2013; Capowiez et al. 2005).

1.4 Ecological Risk Assessment (ERA) of Pesticides

Frequent release of chemicals such as hydrocarbons, heavy metals and agricultural pesticides usually lead to accumulated concentrations harmful to the survival and growth of soil organisms (Cardoso & Alves 2012). Particularly, an estimated 2.5 million metric tons of pesticides are applied each year, making pesticide release a major environmental concern (van der Werf 1996; Finizio & Villa 2002). Consequently, the sensitivity of soil organisms to chemicals, their ability to accumulate pollutants (e.g. earthworms) and change spatial patterns (e.g. arthropods) have led to their use as suitable indicators of soil pollution (Eijsackers 1983; Santorufo et al., 2012). Ecological risk assessment can be prognostic (prospective) or diagnostic (retrospective) (Calow & Forbes 2003). While prospective risk assessment is usually deployed in pesticide registration and review, retrospective assessment is used in site-specific risk assessment of contaminated lands (van Gestel 2012).

1.4.1 Assessing the effects of pesticides on soil fauna

Pesticide ERA normally involves characterizing effects and exposure to non-target organisms through the development of a predicted no-effect concentration (PNEC) and predicted effect concentration (PEC) values respectively to generate a toxicity exposure ratio (PNEC/PEC) which is used to estimate ecological risk (van der Werf 1996; van Straalen & van Rijn 1998). In

Europe, standard laboratory toxicity tests aimed at assessing the effects of pesticides to soil organisms have been developed for *Eisenia fetida*/*andrei* (earthworm: Lumbricidae), *Folsomia candida* (springtail: Collembola), *Enchytraeus albidus or E. crypticus* (potworm: Enchytraeidae) and *Hypoaspis aculeifer* (Acari: Laelapidae) (Jänsch et al. 2006). These toxicity tests can either be acute (endpoint: mortality) or chronic (endpoint: reproduction, growth) and are measured by appropriate parameters such as LC_{50} (concentration causing 50% mortality for an exposed organism group); NOEC and LOEC (no-observable and lowest-observable effect concentration); EC_{10} and EC_{50} (effect concentrations causing 10% and 50% reductions respectively in a measured endpoint) (Cortet et al. 1999; Stark & Banks 2003). It is important to note that whenever comparisons of toxicity parameters obtained from laboratory tests with the predicted environmental concentration (PEC) indicate unacceptable risk, higher-tier testing with an increase in ecological realism should be carried out with semi-field, terrestrial model ecosystems (TME) or field tests (Jänsch et al. 2006; Frampton et al. 2006).

1.4.2 Problem formulation

Significant concentrations of pesticide active ingredients are usually deposited on the soil through direct application and also through drift or foliar wash-offs during pest control programs in agricultural fields (Racke 2003). In assessing the effects of neonicotinoids on nontarget soil organisms, studies have mainly focused on assessing the effects of the neonicotinoid imidacloprid on earthworms. This study aims to assess the toxic effects that neonicotinoid pesticides potentially constitute to earthworms and other nontarget soil invertebrates.

1.4.3 Selection of test organisms

Four ecologically relevant soil invertebrate species were selected: earthworms (*Eisenia andrei*), collembola (*Folsomia candida*), enchytraeids (*Enchytraeus crypticus*) and predatory mites (*Hypoaspis aculeifer*). Earthworms are suitable bioindicator species in soil eco-toxicity testing (Yasmin & D'Souza 2010) and despite the fact that they represent over 80% of terrestrial invertebrates by biomass (Yasmin & D'Souza 2010), studies have confirmed the lower sensitivity of *Eisenia fetida* to broad-spectrum insecticides compared to soil arthropods e.g. *Folsomia candida*. Therefore, first-tier risk assessment of chemicals should include laboratory toxicity tests of different soil organisms representative of sensitive non-target taxa in the ecosystem (Daam et al. 2011). *F. candida* is known to be very sensitive to organic chemicals and researchers have found it to be one of the most sensitive among soil invertebrates (Fountain & Hopkin 2005).

In general, enchytraeids are not less sensitive to chemical stressors than earthworms or collembolans (Rombke 2003), and they have been described as suitable indicator organisms for chemical risk assessment in terrestrial ecosystems (Didden & Römbke 2001). *E. crypticus* was specifically chosen due to its ease of culture in the laboratory, shorter reproductive cycle and high tolerance range for varying soil properties (Castro-Ferreira et al. 2012). Selection of the gasmid predatory mite *H. aculeifer* was premised on its widespread distribution and ecosystem

role in various habitats; it usually occur in agricultural lands and represents an additional trophic level to the other standard model species available for terrestrial ecotoxicity testing (Smit et al. 2012).

1.4.4 Neonicotinoid insecticides tested

Thiacloprid and acetamiprid were the tested active constituents in the respective commercial formulations Calypso® and Epik®, which are mainly employed as spray applications. These two insecticides are chemically different but similar in their insecticidal toxic mechanism to clothianidin, imidacloprid and thiametoxam, which are mainly applied as seed treatments and are the only group of neonicotinoids to have their use restricted for two years by the EC since 2013 due to their toxicity to bees (EC 2013). It is expected that nontarget soil organisms are more exposed to thiacloprid and acetamiprid as the amount of active ingredient (g of a.i ha^{-1}) used during spray applications is much greater than that for granular or seed treatments (Jeschke et al. 2011).

1.4.5 Experiments conducted

Acute (range-finding) and chronic (definitive) soil ecotoxicity tests were conducted. Range-finding tests were carried out to obtain the acute lethal toxicity and results obtained were then used to refine concentration ranges for subsequent chronic toxicity tests using both survival and reproduction as endpoints. In most ecotoxicological assessments, reproduction has been shown to be a more sensitive and reliable endpoint compared to survival and growth for collembola, enchytraeids (Didden & Römbke 2001) and earthworms (Robidoux et al. 2004), although test duration is longer. In comparison with other sublethal parameters, avoidance response has been shown to be equally or perhaps more sensitive than reproduction (Schaefer 2003) and despite its usefulness as a rapid toxicity screening tool (Natal da Luz et al. 2004), it is unsuitable for assessing the chronic toxicities of persistent chemical stressors due to its shorter test duration.

1.5 Research objectives

The aim of this study was to assess the effects of thiacloprid and acetamiprid on soil fauna (earthworms, springtails, enchytraeids and predatory mites) in order to provide first-tier ecotoxicity data that would be useful in the estimation of a predicted no-effect concentration (PNEC) for a more accurate ecological risk assessment (ERA) of thiacloprid and acetamiprid. To achieve this goal, the following objectives were defined:

1. To determine the acute and chronic toxicities of thiacloprid and acetamiprid on soil invertebrates using mortality and reproduction endpoints by calculating appropriate lethal and effect concentration (LC & EC) toxicity parameters.
2. To evaluate the relative sensitivities of the soil invertebrates to thiacloprid and acetamiprid.
3. To derive an initial predicted no-effect concentration (PNEC) using an assessment factor and predicted environmental concentrations (PECs) in order to characterize the risk posed by thiacloprid and acetamiprid to the soil invertebrates.

1.6 References

Altieri, M.A. (1999). The ecological role of biodiversity in agroecosystems. *Agriculture, Ecosystems & Environment*, 74: 19–31.

Anderson, J.M. (1977). The organization of soil animal communities. *Ecological Bulletin*, 25: 15–23.

Azevedo-Pereira, H.M.V.S., Lemos, M.F.L. & Soares, A.M.V.M. (2010). Behaviour and growth of *Chironomus riparius* Meigen (Diptera: Chironomidae) under imidacloprid pulse and constant exposure scenarios. *Water, Air, & Soil Pollution*, 219: 215–224.

Bardgett, R.D. & Cook, R., (1998). Functional aspects of soil animal diversity in agricultural grasslands. *Applied Soil Ecology*, 10: 263–276.

Barrios, E. (2007). Soil biota, ecosystem services and land productivity. *Ecological Economics*, 64(2): 269–285

Beare, M.H., Reddy, M.V., Tian, G. and Srivastava, S.C. (1997). Agricultural intensification, soil biodiversity and agroecosystem function in the tropics : the role of decomposer biota, 6: 87–108.

Beaulieu, F. & Weeks, A.R. (2007). Free-living mesostigmatic mites in Australia: their roles in biological control and bioindication. *Australian Journal of Experimental Agriculture*, 47(4): 460–478. Available at: http://www.publish.csiro.au/?paper=EA05341.

Bedano, J.C. & Ruf, A. (2007). Soil predatory mite communities (Acari: Gamasina) in agroecosystems of Central Argentina. *Applied Soil Ecology*, 36(1): 22–31.

Behan-Pelletier, V.M. (2003). Acari and Collembola biodiversity in Canadian agricultural soils. *Canadian Journal of Soil Science*, 83: 279 – 288.

Blacquière, T., Smagghe, G., van Gestel, C.A.M., Mommaerts, V. (2012). Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology*, 21: 973–992.

Briones, M.J.I., Poskitt, J. & Ostle, N. (2004). Influence of warming and enchytraeid activities on soil CO_2 and CH_4 fluxes. *Soil Biology and Biochemistry*, 36(11): 1851–1859.

Brussaard, L. (1997). Biodiversity and ecosystem functioning in soil. *Ambio*, 26(8): 563–570.

Brussaard, L. (1998). Soil fauna, guilds, functional groups and ecosystem processes. *Applied Soil Ecology*, 9: 123-135

Brussaard, L., Pulleman, M.M., Ouedraogo, E., Mando, A., Six, J. (2007). Soil fauna and soil function in the fabric of the food web. *Pedobiologia*, 50(6): 447–462.

Brussaard, L., De Ruiter, P.C. & Brown, G.G., (2007). Soil biodiversity for agricultural sustainability. *Agriculture, Ecosystems & Environment*, 121(3): 233–244. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0167880906004476 [Accessed January 10, 2014].

Capowiez, Y., Rault, M., Costagliola, G. & Mazzia, C. (2005). Lethal and sublethal effects of imidacloprid on two earthworm species (*Aporrectodea nocturna* and *Allolobophora icterica*). *Biology and Fertility of Soils*, 41(3): 135–143.

Cardoso, E.J.B.N. & Alves, P.R.L. (2012). Soil Ecotoxicology. *In* G. Begum, (Ed.) *Ecotoxicology* pp. 47-51. InTech, ISBN: 978-953-51-0027-0, Available at: http://www.intechopen.com/books/ecotoxicology/soil-ecotoxicology.

Castro-Ferreira, M.P., Roelofs, D., van Gestel, C.A.M., Verweij, R.A., Soares, A.M.V.M., Amorim, M.J.B. (2012). *Enchytraeus crypticus* as a model species in soil ecotoxicology. *Chemosphere*, 87(11): 1222–1227. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22365279

Cole, L., Bradford, M.A., Shaw, P.J.A., Bardgett, R.D. (2006). The abundance, richness and functional role of soil meso- and macrofauna in temperate grassland—A case study. *Applied Soil Ecology*, 33(2): 186–198.

Cole, L., Bardgett, R. & Ineson, P. (2000). Enchytraeid worms (Oligochaeta) enhance mineralization of carbon in organic upland soils. *European Journal of Soil Science*, 51: 185–192.

Crouau, Y. & Moïa, C. (2006). The relative sensitivity of growth and reproduction in the springtail, *Folsomia candida*, exposed to xenobiotics in the laboratory: an indicator of soil toxicity. *Ecotoxicology and environmental safety*, 64(2): 115–121.

Daam, M.A., Leitao, S., Cerejeira, M.J., & Sousa, J.P. (2011). Comparing the sensitivity of soil invertebrates to pesticides with that of *Eisenia fetida*. *Chemosphere*, 85(6): 1040–1047.

Decaëns, T., Jimenez, J.J., Gioia, C., Measey, G.J. & Lavelle, P. (2006). The values of soil animals for conservation biology. *European Journal of Soil Biology*, 42: S23–S38.

Decourtye, A. & Devillers, J. (2010). Ecotoxicity of neonicotinoid insecticides to bees. *In* Thany, S.H (Ed.), Insect Nicotinic Acetylcholine Receptors (pp 85-95). Landes Bioscience and Springer Science+Business Media.

Didden, W.A.M. & de Fluiter, R. (1998). Dynamics and stratification of Enchytraeidae in the organic layer of a Scots pine forest. *Biology and Fertility of Soils*, 26(4): 305–312. Available at: http://link.springer.com/10.1007/s003740050381.

Didden, W. & Römbke, J., (2001). Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. *Ecotoxicology and environmental safety*, 50(1): 25–43.

Domene, X., Alcañiz, J.M. & Andrés, P. (2007). Ecotoxicological assessment of organic wastes using the soil collembolan *Folsomia candida*. *Applied Soil Ecology*, 35(3): 461–472.

EC European Commission (2006a). *Communication COM(2006) 232 final - "Establishing a framework for the protection of soil and amending Directive 2004/35/EC,"* Brussels. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2006:0232:FIN:EN:PDF.

EC European Commission (2006b). *Communication COM(2006)231 final - "Thematic Strategy for Soil Protection,"* Brussels. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2006:0231:FIN:EN:PDF.

EC European Commission (2010). European Atlas of Soil Biodiversity. S. Jeffery, C. Gardi, A. Jones, L. Montanarella, L. Marmo, L. Miko, K. Ritz, G. Peres, J. Römbke and W. H. van der Putten et al., (Eds.), Publications Office of the European Union, Luxembourg.

Eijsackers, H. (1983). Soil fauna and soil microflora as possible indicators of soil pollution. *Environmental Monitoring and Assessment*, 3: 307–316.

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.

Andre, H.M., Noti, M.I. & Lebrun, P. (1994). The soil fauna: the other last biotic frontier. *Biodiversity and Conservation*, 56: 45–56.

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

Fitter, A.H., Gilligan, C.A., Hollingworth, K., Kleczkowski, A., Twyman, R.M. & Pitchford, J.W. (2005). Biodiversity and ecosystem functioning in soil. *Functional Ecology*, 19: 369–377.

Fortuna, A. (2012). The soil biota. *Nature Education Knowledge*, 3(10): 1. Available at: http://www.nature.com/scitable/knowledge/library/the-soil-biota-84078125 [Accessed February 9, 2014].

Fragoso, C., Brown, G.G., Patron, J.C., Blanchart, E., Pashanasi, B., Senapati, B. & Kumar, T. (1997). Agricultural intensification, soil biodiversity and agroecosystem function in the tropics: the role of earthworms. *Applied Soil Ecology*, 6: 17–35.

Frampton, G.K. Jansch, S., Scott-Fordsmand, Janeck J., Rombke, J. & Van Den Brink, P.J. (2006). Effects of pesticides on soil invertebrates in laboratory studies: A review and analysis using species sensitivity distributions. *Environmental Toxicology and Chemistry*, 25(9): 2480–2489.

Franklin, E., Hayek, T., Fagundes, E.P. & Silva, L.L. (2004). Oribatid mite (Acari: Oribatida) contribution to decomposition dynamic of leaf litter in primary forest, second growth, and polyculture in the Central Amazon. *Brazilian Journal of Biology*, 64(1): 59–72.

Fry, D.M. (1995). Reproductive effects in birds exposed to pesticides and industrial chemicals. *Environmental health perspectives*, 103(19): 165–171.

Gardi, C., Jeffery, S. & Saltelli, A. (2013). An estimate of potential threats levels to soil biodiversity in EU. *Global change biology*, 19(5): 1538–1548. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23505125 [Accessed January 5, 2014].

Cortet, J., Vauflery, G.A., Pointsot-Balaguer, N., Gomot, L., Texier, C. & Cluzeau, D.N. (2000). The use of invertebrate soil fauna in monitoring pollutant effects. *European Journal of Soil Biology*, 35(3): 115–134.

Goulson, D. (2013). An overview of the environmental risks posed by neonicotinoid insecticides. *Journal of Applied Ecology*, 50(4): 977–987.

Hagvar, S. (1998). The relevance of the Rio-Convention on biodiversity to conserving the biodiversity of soils. *Applied Soil Ecology*, 9: 1–7.

Heneghan, L. & Bolger, T. (1998). Soil microarthropod contribution to forest ecosystem processes : the importance of observational scale. *Plant and Soil*, 205: 113–124.

Huhta, V. (2007). The role of soil fauna in ecosystems: A historical review. *Pedobiologia*, 50(6): 489–495.

Jänsch, S., Frampton, G.K., Römbke, J., Van den Brink, P.J. & Scott-Fordsmand, J.J. (2006). Effects of pesticides on soil invertebrates in model ecosystem and field studies: a review and comparison with laboratory toxicity data. *Environmental Toxicology and Chemistry*, 25(9): 2490–2501. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16986805.

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(7): 2897–908.

Jeschke, P. & Nauen, R. (2008). Neonicotinoids – from zero to hero in insecticide chemistry. *Pest Management Science*, 64: 1084–1098.

Jones, T.H. & Bradford, M.A. (2001). Assessing the functional implications of soil biodiversity in ecosystems. *Ecological Research*, 16(5): 845–858.

Jouquet, P., Dauber, J., Lagerlöf, J., Lavelle, P. & Lepage, M. (2006). Soil invertebrates as ecosystem engineers: Intended and accidental effects on soil and feedback loops. *Applied Soil Ecology*, 32(2): 153–164.

Kimura-Kuroda, J., Komuta, Y., Kuroda, Y., Hayashi, M. & Kawano, H. (2012). Nicotine-like effects of the neonicotinoid insecticides acetamiprid and imidacloprid on cerebellar neurons from neonatal rats. *PloS One*, 7(2): e32432.

Kreutzweiser, D.P., Good, K.P., Chartrand, D.T., Scarr, T.A., Holmes, S.B. & Thompson, D.G. (2008). Effects on litter-dwelling earthworms and microbial decomposition of soil-applied imidacloprid for control of wood-boring insects. *Pest Management Science*, 64: 112–118.

Lavelle, P. (1997). Faunal activities and soil processes: Adaptive strategies that determine ecosystem function. *Advances in Ecological Research*, 27: 93–112.

Lavelle, P. Decaëns, T. Aubert, M. Barot, S. Blouin, M. Bureau, F. Margerie, P. Mora, P. Rossi, J.-P. (2006). Soil invertebrates and ecosystem services. *European Journal of Soil Biology*, 42: S3–S15.

Lawton, J.H., 1994. What do species do in ecosystems. *Oikos*, 71: 367–364.

Legocki, J. & Połeć, I. (2008). Contemporary trends in development of active substances possessing the pesticidal properties: neonicotinoid insecticides. Pestycydy, (1-2): 143–159.

Da Luz, T.N., Ribeiro, R. & Sousa, J.P. (2004). Avoidance tests with collembola and earthworms as early screening tools for site-specific assessment of polluted soils. *Environmental toxicology and chemistry / SETAC*, 23(9): 2188–2193.

Menta, C. (2012). Soil fauna diversity – Function, soil degradation, biological indices, soil restoration. *In* Gbolagade Akeem Lameed (Ed.), *Biodiversity Conservation and Utilization in a Diverse World (*pp. 49–94). ISBN: 978-953-51-0719-4, InTech, DOI: 10.5772/51091.

Miao, J., Du, Z., Wu, Y., Gong, Z., Jiang, Y., Duan, Y., Li, T. & Lei, C. (2013). Sub-lethal effects of four neonicotinoid seed treatments on the demography and feeding behaviour of the wheat aphid *Sitobion avenae*. *Pest Management Science*, 70(1): 55–59.

Naeem, S. (1998). Species redundancy and ecosystem reliability. *Conservation Biology*, 12(1): 39–45. Available at: http://doi.wiley.com/10.1111/j.1523-1739.1998.96379.x.

Nauen, R. & Denholm, I. (2005). Resistance of insect pests to neonicotinoid insecticides: current status and future prospects. *Archives of Insect Biochemistry and Physiology*, 58(4): 200–215.

Neher, D.A. (1999). Soil community composition and ecosystem processes: Comparing agricultural ecosystems with natural ecosystems. *Agroforestry Systems*, 45: 159–185.

Neher, D.A. & Barbercheck, M.E. (1998). Diversity and function of soil mesofauna. *In* Collins, W.W. & Qualset, C.O. (Eds), *Biodiversity in Agroecosystems* (pp. 27–47). CRC Press

Perdue, J.C. & Crossley Jr, D.A. (1990). Vertical distribution of soil mites (Acari) in conventional and no-tillage agricultural systems. *Biology and Fertility of Soils*, 9: 135 – 138.

Reid, W.V. et al., (2005). Ecosystems and human well-being: biodiversity synthesis. *Millenium Ecosystem Assessment*, World Resources Institute, Washington, DC. Available at: http://www.maweb.org/documents/document.354.aspx.pdf.

Robidoux, P.Y., Sunahara, G.I., Savard, K., Berthelot, Y., Dodard, S., Martel, M., Gong, P. & Hawari, J. (2004). Acute and chronic toxicity of the new explosive CL-20 to the earthworm (*Eisenia andrei*) exposed to amended natural soils. *Environmental Toxicology and Chemistry / SETAC*, 23(4): 1026 – 1034.

Rombke, J. (2003). Ecotoxicological laboratory tests with enchytraeids: A review. *Pedobiologia*, 47: 607–616.

Rombke, J., Jansch, S. & Didden, W. (2005). The use of earthworms in ecological soil classification and assessment concepts. *Ecotoxicology and Environmental Safety*, 62: 249–265.

Rusek, J. (1998). Biodiversity of Collembola and their functional role in the ecosystem. *Biodiversity and Conservation*, 7: 1207–1219.

Salvi, R.M., Lara, D.R., Ghisolfi, E.S., Portela, L.V., Dias, R.D. & Souza, D.O. (2003). Neuropsychiatric evaluation in subjects chronically exposed to organophosphate pesticides. Toxicological Sciences, 72(2): 267–271.

Sánchez-Bayo, F., Tennekes, H.A. & Goka, K. (2013). Impact of Systemic Insecticides on Organisms and Ecosystems. *In* Trdan, S. (Ed.), *Insecticides - Development of Safer and More Effective Technologies* (pp. 367-416), ISBN: 978-953-51-0958-7, InTech, DOI: 10.5772/52831

Santorufo, L., Van Gestel, C.A.M., Rocco, A. & Maisto, G. (2012). Soil invertebrates as bioindicators of urban soil quality. *Environmental Pollution*, 161: 57–63.

Schaefer, M. (2003). Behavioural endpoints in earthworm ecotoxicology. *Journal of Soils and Sediments*, 3(2): 79–84.

Setala, H., Berg, M.P. & Jones, H.T. (2005). Trophic structure and functional redundancy in soil communities. *In* Bardgett, R.D., Usher, M.B. & Hopkins, D.W. (Eds), *Biological Diversity and Function in Soils* (pp. 236–249). Cambridge University Press.

Smit, C.E., Moser, T. & Römbke, J. (2012). A new OECD test guideline for the predatory soil mite *Hypoaspis aculeifer*: results of an international ring test. *Ecotoxicology and Environmental Safety*, 82: 56–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22698879.

Stark, J.D. & Banks, J.E. (2003). Population-level effects of pesticides and other toxicants on arthropods. *Annual Review of Entomology*, 48: 505–19.

Van Straalen, N.M. & Van Rijn, J.P. (1998). Ecotoxicological risk assessment of soil fauna recovery from pesticide application. *Review of Environmental Contamination and Toxicology*, 154: 83 – 141.

Sylvain, Z.A. & Wall, D.H. (2011). Linking soil biodiversity and vegetation: implications for a changing planet. *American Journal of Botany*, 98(3): 517–527. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21613143 [Accessed March 20, 2014].

Tomizawa, M. & Casida, J.E. (2005). Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annual Review of Pharmacology and Toxicology*, 45: 247–68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15822177 [Accessed March 31, 2014].

Tomizawa, M. & Casida, J.E. (2003). Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annual Review of Entomology*, 48: 339–364.

Tomizawa, M., Lee, D.L. & Casida, J.E. (2000). Neonicotinoid insecticides: molecular features conferring selectivity for insect versus mammalian nicotinic receptors. *Journal of agricultural and food chemistry*, 48(12): 6016–6024.

Turbe, A., De Toni, A., Benito, P., Lavelle, P., Lavelle, P., Ruiz, N., Van der Putten, W.H. Labouze, E. & Mudgal, S. (2010). Soil biodiversity: functions, threats and tools for policy makers. Bio-intelligence Service, IRD, and NIOO, *Report for European Commission (DG Environment)*, Paris.

Vanbergen, A.J. et al., (2007). Scale-specific correlations between habitat heterogeneity and soil fauna diversity along a landscape structure gradient. *Oecologia*, 153(3): 713–725.

Van Dijk, T.C., Van Staalduinen, M.A. & Van der Sluijs, J.P. (2013). Macro-invertebrate decline in surface water polluted with imidacloprid. *PloS one*, 8(5), p.e62374.

Van Gestel, C.A.M. (2012). Soil ecotoxicology: state of the art and future directions. In: Štrus J, Taiti S, Sf- enthourakis S (Eds), *Advances in Terrestrial Isopod Biology*. *ZooKeys*, 176: 275–296

Van Vliet, P.C., Beare, M. & Coleman, D. (1995). Population dynamics and functional roles of Enchytraeidae (Oligochaeta) in hardwood forest and agricultural ecosystems. *Plant and Soil*, 170: 199–207.

Vojoudi, S. & Saber, M. (2013). Lethal and sublethal effects of thiacloprid on survival, growth and reproduction of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Archives of Phytopathology and Plant Protection*, 46(15): 1769–1774.

Walter, D.E. & Behan-Pelletier, V. (1999). Mites in forest canopies: filling the size distribution shortfall? *Annual Review of Entomology*, 44: 1–19.

Wardle, D.A. (2006). The influence of biotic interactions on soil biodiversity. *Ecology letters*, 9(7): 870–886. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16796577.

Van der Werf, H.M. (1996). Assessing the impact of pesticides on the environment. *Agriculture, Ecosystems & Environment*, 60: 81–96.

Werner, I. & Hitzfeld, B. (2012). 50 Years of Ecotoxicology since Silent Spring – A Review. *GAIA: Ecological Perspectives for Science and Society*, 21(3): 217–224.

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.

Wolters, V. (2001). Biodiversity of soil animals and its function. *European Journal of Soil Biology*, 37: 221–227.

Yasmin, S. & D'Souza, D. (2010). Effects of pesticides on the growth and reproduction of earthworms: A review. *Applied and Environmental Soil Science*, 2010: 1–9.

Chapter 2

2 Ecotoxicity of the neonicotinoid thiacloprid to Folsomia candida, Eisenia andrei, Enchytraeus crypticus and Hypoaspis aculeifer

2.1 ABSTRACT

Neonicotinoid insecticides are the most important group of insecticides in the world, widely adopted in pest management programs due to their broad spectrum activity and versatility in application. Thiacloprid is a member of the neonicotinoid insecticide class and like others, it act as selective agonist of insect nicotinic acetylcholine receptors (nAChRs). Though non-target soil organisms are likely to be exposed to thiacloprid during spray applications of formulated product Calypso®, there is virtually no existing information in published scientific literature on its toxicity to soil organisms. This study evaluated the chronic effects of thiacloprid on four terrestrial invertebrates (*Folsomia candida*, *Eisenia andrei*, *Enchytraeus crypticus* and *Hypoaspis aculeifer*) in artificial OECD soil. From the results obtained, relative sensitivity of the test species can be expressed as *F. candida* > *E. andrei* > *E. crypticus* > *H. aculeifer* (survival) and *E. andrei* > *F. candida* > *E. crypticus* > *H. aculeifer* (reproduction). Among other probable reasons, relative sensitivity could be due to inter-specific differences in the exposure routes of these invertebrates to thiacloprid in soil pore water. Predicted environmental concentration (PEC = 0.03 mg kg^{-1}) and predicted no-effect concentration (PNEC = 0.024 mg kg^{-1}) were derived for thiacloprid based on the valid test results of *F. candida*, *E. crypticus* and *H. aculeifer*. Calculated exposure-toxicity ratios (ETRs; PEC/EC_{10}) and risk quotient (PEC/PNEC) were lesser and approximately equal to trigger values 0.2 and 1 respectively as defined by the European Commission. Therefore, it can be concluded that the risk of thiacloprid to the above-mentioned soil invertebrates and consequently to the soil compartment is acceptable. Notwithstanding the low risk of thiacloprid to soil invertebrates as indicated by this study, additional first-tier eco-toxicity tests that may lead to the refinement of the PEC and/or PNEC are recommended.

2.2 INTRODUCTION

Neonicotinoids are now the most prominent class of insecticides in the world. They are registered in at least 120 countries globally with annual sales estimated at $1.5 billion (Jeschke *et al*. 2011). As systemic pesticides, they are selective agonists of insect nicotinic acetylcholine receptors (nAChRs) (Jeschke et al. 2011; Szczepaniec et al. 2013). Although 60% of neonicotinoid insecticides are used as seed coatings, some are applied as foliar sprays, granular treatments or by chemigation. Non-agricultural uses of neonicotinoids include professional usage for controlling cockroaches, ants and termites in households, and also in veterinary medicine for the topical control of ectoparasites in pets (Jeschke et al. 2011; Goulson 2013).

Thiacloprid is the second member of Bayer's chloronicotinyl insecticide family launched in 2000 under the formulation Calypso® and exclusively utilized for foliar application (Yu et al. 2007; Elbert et al. 2008). Apart from its selective toxicity to insect pests, thiacloprid is known to be ecologically benign and its bee safety profile has encouraged its use on flowering plants (Buchholz & Nauen 2002). Like other pesticides, inefficient foliar application of sprayed Calypso® formulation causes an unintended exposure of non-target terrestrial and aquatic organisms to thiacloprid. This dissipation could occur through spray drift, soil deposition, foliar wash-off, leaching via rainfall, surface water run-offs, etc (van der Werf 1996; Linders et al. 2000; Palumbo et al. 2001; Racke 2003).

Independent research has documented the high toxicity of thiacloprid in aquatic ecosystems. For example, thiacloprid was shown to impact the sediment-dwelling nontarget insect *Chironomus riparius* at concentrations ≥ 0.5 µg L^{-1} (Langer-Jaesrich et al. 2010). In a different study, the 5% hazardous concentration (HC5) of thiacloprid (0.72 µg L^{-1}) for freshwater arthropods based on acute exposure and chronic post-exposure observations was found to be lower than predicted environmental concentrations ($PEC_{orchard}$: 1.99 µg L^{-1}; $PEC_{ornamental}$: 17.52 µg L^{-1}) for surface water under a worst-case scenario (Beketov & Liess 2008). These research findings thereby highlight the need to reduce the potential toxicity of thiacloprid to nontarget freshwater organisms.

Field dissipation studies have revealed that thiacloprid is easily biodegraded in the terrestrial compartment with DT_{50} and DT_{90} of 9-27 days and 31-91 days respectively in the top soil layer (Barden 2001; EC 2004; EC 2008). However, its metabolites can be classified as persistent with DT_{90} > 100 days (EPPO 2003b; EC 2004). Beneficial soil organisms are expected to be repeatedly exposed to significant amounts of thiacloprid as the formulated product Calypso® is usually applied at several intervals to orchard and ornamental plants for the control of sucking and boring pests before harvesting. It is therefore disconcerting that there is little or no information in scientific literature on the toxicity of thiacloprid to soil invertebrates.

This chapter aims to assess the effects of thiacloprid to the soil invertebrates *Folsomia candida*, *Eisenia andrei*, *Enchytraeus crypticus* and *Hypoaspis aculeifer* by determining its acute and chronic toxicity to these organisms using survival and reproduction endpoints. In addition, the

relative sensitivities of the invertebrates to thiacloprid are evaluated and the implications of the toxicity effects for terrestrial ecological risk assessment are discussed.

2.3 MATERIALS AND METHODS

2.3.1 Culture conditions and test substance

Springtails were cultured in plastic containers lined with an 11:1 mixture of plaster of Paris and activated charcoal. Food was added twice a week in small amounts to avoid fungal spoilage. Earthworms were cultured in aerated plastic containers with horse manure and peat mixture as substrate and fed twice a month with oat porridge. Enchytraeids were cultured in agar and fed *ad-libitum* with oatmeal twice a week. Predatory mites were cultured in plastic containers lined with an 8:1 ratio of plaster of Paris and activated charcoal. Feeding with cheese mites (*Tyrophagus putrescentiae*) took place 2-3 times a week. All specimens were cultured at 20 ^{o}C ± 2 ^{o}C, 16h: 8h light-dark cycle and an illumination of 400 - 800 Lux.

2.3.2 Test soil

Tests were conducted using artificial soil prepared from the constituent mixture (75% sand, 5% peat and 20% kaolin clay) according to OECD (1984) guideline. Though the tested invertebrates possess a world-wide distribution, the OECD artificial soil was chosen to standardize soil properties and to facilitate easy interpretation and comparison of results obtained. According to EPPO (2003), a typical agricultural soil has a maximum organic matter content of 5%. Therefore, peat content of the artificial soil was decreased to 5% and sand content increased to 75% accordingly. Also, sphagnum peat was sieved through 5-mm mesh for *E. andrei, F. candida* and *E. crypticus*, and through 2-mm mesh for *H. aculeifer*. $CaCO_3$ was added to adjust the pH of the artificial soil to 6.0±0.5, and soil water holding capacity (WHC) was determined to ensure that soil water content was around 40% to 60% of the maximum WHC.

2.3.3 Test Substance

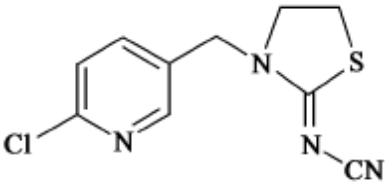
The tested chemical was Calypso® 480 SC produced by Bayer CropScience. Active constituent: Thiacloprid (480 g L^{-1}). IUPAC name: (Z)-3-(6-chloro-3-pyridylmethyl)-1, 3-thiazolidin-2-ylidenecyanamide. Physicochemical characteristics are shown in Table 2. Toxicity assessments were based on active ingredient concentrations.

2.3.4 Toxicity assessments

All tests were performed according to OECD and ISO guidelines. The water content of the test soils was determined to ensure the required moisture content which is around 40 to 60% of the

maximum water-holding capacity (WHCmax). For both range-finding and definitive tests, the soil pH and moisture content for each treatment concentration including the controls were determined at the beginning and end of each experiment. Test vessels were incubated under controlled laboratory conditions: temperature of 20 ± 2ºC, light-dark cycle of 16h: 8h and an illumination of 400 - 800 Lux.

Table 2: Physicochemical characteristics of the neonicotinoid thiacloprid

 Chemical structure	Empirical formula	$C_{10}H_9ClN_4S$
	Molecular mass	252.73 g mol^{-1}
	Relative density	1.46 g cm^{-3}
	Solubility (pH7, 20°C)	184 mg L^{-1}
	Log K_{ow} (20°C)	1.26
	Henry's Law constant	5 x 10^{-10} Pa m^{-3} mol^{-1}

2.3.5 Treatment concentrations

Each test concentration was prepared from a stock solution, diluted with distilled water and mixed into artificial soil at a volume equivalent to the corresponding 50% WHC for that amount of soil. Range-finding tests with mortality as the only endpoint were conducted for each of the test species except *Eisenia andrei* to refine the range of concentrations chosen for the definitive reproduction tests. The concentrations used for the *E. andrei* reproduction test were based on the 14-day acute toxicity (LC_{50}) value obtained from the European Commission review report on thiacloprid (EC 2004). For each range-finding test, artificial soils were spiked with nominal thiacloprid concentrations of 0.1, 1, 10, 100, 1000 mg kg^{-1}. Eight test concentrations (including the control) were used in definitive testing with five replicates (Collembola, enchytraeid and predatory mite) or four replicates (earthworm). Nominal concentrations of thiacloprid for each of the test species include: 0, 1, 2.1, 4.4, 9.2, 19.3, 40.5 and 85.1 mg kg^{-1} (*F. candida*); 0, 0.52, 1.26, 3.01, 7.2, 17.36, 41.7 and 100.0 mg kg^{-1} (*E. andrei*); 0, 0.5, 1.4, 3.9, 11.0, 30.7, 86.1 and 240.9 mg kg^{-1} (*E. crypticus*); 0, 16.38, 40.96, 102.4, 256, 640, 1600 and 4000 mg kg^{-1} (*H. aculeifer*).

2.3.6 Initial development of Predicted Environmental Concentrations (PECs)

Based on the use pattern of Calypso® 480 SC through foliar spray for apples and pears during their inflorescence emergence, flowering and fruit development growth stages (BBCH code = 54-75), an initial and time-weighted average PEC_{intial} & PEC_{twa} (mg kg^{-1}) of the active substance thiacloprid in the top soil was calculated (see Table 3). This estimation was done by assuming a uniform distribution in the upper soil layer of 5 cm depth and a dry soil bulk density of 1.5 g/cm^3. Given that maximum dissipation time (DT_{50lab}) of thiacloprid is 5 d, the maximum application rate was taken to be 180 g a.i ha^{-1} (EC 2004). The interception fraction (F = 0.4) for apples during the spring growing phase was obtained from standard interception factors of fruit trees in the Netherlands (Linders et al. 2000).

Table 3: Worst-case predicted environmental concentrations (PECs) estimated for thiacloprid

Initial (mg kg^{-1})	Time-weighted (mg kg^{-1})	
	14 days	28 days
0.14	0.06	0.03

2.3.7 Definitive reproduction test

Folsomia candida: The collembolan reproduction test (ISO, 1999) was chosen to assess adult survival and juvenile production by the springtail *Folsomia candida*. Test duration was 28 days. Each glass test vessel was filled with 30 g of artificial soil (wet mass). At the start of the experiment, 10 synchronized juveniles (10 – 12 days old) were added to each vessel and fed with 2 – 10 mg of granulated dry yeast. Thereafter, test vessels were aerated and dry yeast was added once every week, whilst dry yeast was added and moisture loss replenished after two weeks. After 4 weeks, surviving adults and juvenile numbers were photographed and counted using the Image Tool software (Wilcox et al. 2002) after flotation.

Enchytraeus crypticus: Though the ERT (*Enchytraeus* reproduction test) guidelines ISO 16387 (ISO, 2003) recommended the use of *Enchytraeus albidus, E. crypticus,* a suggested alternative testing species was used due to practical advantages especially its shorter reproductive cycle. 20 g of artificial soil (dry weight) were introduced into each glass test vessels. 10 adults of *E. crypticus* with well-developed clitella and up to 50 mg of finely ground dry oats were supplied into each test vessel. Aeration and feeding were done each week and after 14 days, moisture loss exceeding 2% was replenished. The ERT was terminated after 28 days and organisms were fixed and stained with alcohol and Bengal red. Surviving adults and juveniles were counted after a minimum period of 12 hours under illuminated lenses.

Hypoaspis (Geolaelaps) aculeifer*:* Test was conducted according to the guideline OECD 226 (OECD, 2008). Ten adult females of 28 – 35 days old were introduced into test vessels containing 20 g of artificial soil (dry mass) and then fed with cheese mites. Test vessels were weighed to serve as reference for soil moisture loss check-up. The mites were fed twice a week and soil moisture content in the test vessels was monitored. After 14 days, test was terminated and *H. aculeifer* adults and juveniles were separated from the soil substrate using the heat extraction method described in the guideline OECD 226 (OECD, 2008). Finally, surviving adult and juvenile mites were then counted under a dissecting microscope.

Eisenia andrei: The epigeic earthworm *Eisenia andrei* was selected for the earthworm reproduction test and test procedures followed the international guideline ISO 11268-2 (ISO, 1998). Before testing, synchronized adult worms (2 to 12 months old) with clitella were selected and acclimatized for 24 hours. 500 – 600 g (dry mass) of artificial soil was measured in each test container and 10 adult worms (250 – 600 mg) were added to each test container. Moistened cow

manure (15 g) was added and the test containers were weighed for periodic (after 14 days) monitoring of soil moisture content. On day 28, surviving adults in each vessel were counted. Test was terminated after 56 days with the counting of the juveniles hatched from the cocoons.

2.3.8 Data analyses

For the definitive reproduction tests, mortality (LC_{50}) and reproduction (EC_{10} & EC_{50}) endpoint values were calculated by fitting various nonlinear regression models in the R package 'drc' used for the analysis of dose-response curve data (Ritz & Streibig 2005). Generally, the logistic model (two or three parameter) provided the best fit for most toxicity data as given below:

$$Y = f(x) = c + \frac{d - c}{1 + \exp(b(\log(x) - \log(e)))} \quad (1)$$

Where Y is the number of juveniles or adult survival (fraction), x is the nominal test concentration (mg a.i/kg dry soil), b is the maximal slope of the logistic function, d is the maximum response in the controls (upper limit), c is the minimum response (lower limit) at $x \sim \infty$ and e is the LC_{50} or EC_{50} value. *ED.drc* function was used to estimate 10%, 20% and 50% lethal and effective concentrations (LC/EC) respectively. Equation 2 gives the initial soil exposure concentration (*Ci*) of thiacloprid (mg kg^{-1}) for the assessment of acute effects (EPPO 2003a).

$$Ci = \frac{A \times (1 - F)}{L \times 10^2 \times D} \quad (2)$$

The time-weighted average concentration (*TWAC*) of thiacloprid (mg kg^{-1}) for chronic toxicity assessment was calculated using the following equation (EPPO 2003a):

$$TWAC = Ci \times \left(\frac{DT50}{t.In2}\right) \times \left[1 - \exp\left(-t \times \frac{In2}{DT50}\right)\right] \quad (3)$$

Where A = application rate (g a.i ha^{-1}); F = interception factor; L = soil layer depth (cm); D = soil bulk density (g cm^{-3}); t = duration of toxicity test and DT_{50} = 50% laboratory dissipation time. To determine NOEC and LOEC values, the normality and homogeneity of reproduction, survival and earthworm weight loss data were tested using Shapiro-Wilk and Levene's test before statistical analysis. Where normality and homoscedascity assumptions are satisfied, significant differences between treatment response means were tested using one-way ANOVA analysis followed by post-hoc trend (Tukey HSD) or pairwise comparison (Dunnett) tests (*p-value* = 0.05). Otherwise, appropriate functions in the R package 'pgirmess' (Giraudoux 2013) were applied to determine NOECs/LOECs using the non-parametric Kruskal-Wallis test followed by post-hoc multiple comparisons (*p-value* = 0.05) as described by Siegel & Castellan (1988).

For acute range-finding tests, the exponential decay model as obtained in the 'drc' R package was chosen as it provided the best LC_{50} estimates with lowest-bound confidence intervals. The model equation is given below:

$$Y = f(x) = c + (d - c)(\exp(-x/e)) \quad (4)$$

Where d is the estimated survival rate at $x = 0$, c is the estimated survival rate at $x \sim \infty$ and $e > 0$ determines the steepness of the decay curve. All statistical analyses were performed according to OECD standard guidelines (OECD 2006) using R (R Core Team 2014) and Microsoft Excel.

2.4 RESULTS

2.4.1 Test validation

As prescribed by the test protocols, validity requirements were satisfied in the controls of the definitive reproduction tests for all test organisms except *E. andrei*. Mean adult mortality for *F. candida*, *E. crypticus* and *H. aculeifer* was < 20%. The average reproduction rate for *F. candida* was 273 juveniles. In addition, *H. aculeifer* had 250 juveniles per replicate while *E. crypticus* produced an average of 885 juveniles. Coefficient of variation (CV) for all test organisms was below 30%. Although adult mortality after 4 weeks for *E. andrei* was << 10%, unfortunately, there were less than 30 juveniles in the control replicates. Therefore, *E. andrei* reproduction test was invalid. For the range-finding tests, adult mortality in the controls was well below 20% (*E. crypticus* & *H. aculeifer*) or approximately 20% (*F. candida*).

2.4.2 Acute (range-finding) toxicity

Results below (Table 4) for the range-finding toxicity tests showed that thiacloprid was lethal to both *F. candida* and *E. crypticus* according to the estimated 50% lethal concentrations (LC_{50}) values, though *F. candida* was more sensitive as expected. In *H. aculeifer*, adult mortality was extremely low and insufficient to allow for LC_{50} estimation.

Table 4: Estimated lethal concentrations (acute) for *F. candida* and *E. crypticus*

Organism	Parameter (mg/kg)	Estimate	95% CI
F. candida	LC_{10}	6.46	2.6 - 10.29
	LC_{50}	42.47	17.22 - 67.71
E. crypticus	LC_{10}	48.78	35.82 - 61.73
	LC_{50}	320.88	235.67 - 406.10

2.4.3 Definitive Tests

The chronic sub-lethal toxicity of thiacloprid to *Folsomia candida* is shown below (Figure 2, Table 5) by both concentration-response modelling and hypothesis-testing methods. The EC_{10} and EC_{50} values for *F. candida* reproduction were 1.0 and 2.1 mg kg^{-1} respectively, which is approximately one-half adult survival LC_{10} & LC_{50} values of 1.92 and 4.38 mg kg^{-1}. In the same vein, adult survival NOEC & LOEC values ($p < 0.05$) of 2.1 and 4.4 mg kg^{-1} respectively were higher than those for reproduction (1.0 and 2.1 mg kg^{-1}) by a factor of 2. It can also be noted that for both reproduction and adult survival in *F. candida*, bounded NOEC & LOEC values were very similar to estimated EC_{10} & EC_{50} values respectively.

Again, for *Enchytraeus crypticus*, both hypothesis testing and dose-response modelling proved reproduction to be the more sensitive endpoint. EC_{10} and EC_{50} values of 1.27 and 5.60 mg kg^{-1} respectively were lower than LC_{10} (15.91 mg kg^{-1}) and LC_{50} (25.58 mg kg^{-1}) for adult survival. Similarly, adult survival NOEC and LOEC values (11.0 & 30.7 mg kg^{-1}) were considerably higher that EC_{10} & EC_{50} values respectively. It is impressive to also note that estimated LC and EC values for *E. crypticus* were similar to NOEC & LOEC values ($p < 0.05$) for both adult survival and juvenile production taking into consideration the 95% CIs (see Figure 2, Table 5).

Effect concentrations for *Eisenia andrei* were: EC_{10} = 0.002; EC_{50} = 0.048. NOEC and LOEC values for reproduction could not be determined for *E. andrei* probably due to the invalidation of the toxicity test as described above. Therefore, results concerning reproduction endpoints are de-emphasized due to its unreliability. It is however noteworthy to report that the NOEC (7.2 mg kg^{-1}) and LOEC (17.36 mg kg^{-1}) values for adult survival in *E. andrei* ($p < 0.05$) did not significantly deviate from estimated LC_{10} and LC_{50} values which are 12.25 (6.53 – 17.97) mg kg^{-1} and 18.21 (16.87 – 19.54) mg kg^{-1} respectively. As presented in Figure 2, weight change as a sublethal endpoint in *E. andrei* (measured as 28-day percentage weight loss) was sensitive and significant differences from the controls were found in treatment concentrations starting from 7.2 mg kg^{-1} and above. Just as described in the range-finding test, mortality in *Hypoaspis aculeifer* adults was too low to allow for LC_{50} estimation either by interpolation or extrapolation. Reproductive toxicity was very low, when compared to other test organisms, with EC_{10} & EC_{50} values of 967.69 mg kg^{-1} and 3673.87 mg kg^{-1} respectively. NOEC and LOEC values determined ($p < 0.05$) are also similar to the estimated EC values (Table 5).

As laid out by EU technical guidance document on the risk assessment of chemicals (EC 2003), $PNEC^{1}_{soil}$ (0.024 mg kg^{-1}) for thiacloprid was derived by dividing the lowest EC_{10} value by an assessment factor of 50. This procedure was based on the EC_{10} values of *F. candida, E. crypticus* and *H. aculeifer* only. EC_{10} for *F. candida* was used as it was the lowest and an assessment factor of 50 was selected because the test organisms fall into two different trophic levels.

[1] Predicted no effect concentration for the soil compartment

Table 5: Ecotoxicity parameters based on adult survival and reproduction endpoints for four soil invertebrate species exposed to thiacloprid in artificial OECD soil.

Organism	Endpoint	Parameter[a] (mg kg^{-1})	Estimate with 95% CI[b]	SSI[d]
F. candida[f]	Adult survival	LC_{10}	1.92 (1.31 – 2.53)	3.65
		LC_{50}	4.38 (3.34 – 5.41)	
		NOEC	2.1	
		LOEC	4.4	
	Reproduction	EC_{10}	1.20 (0.77 – 1.63)	
		EC_{50}	2.13 (1.87 – 2.38)	
		NOEC	1.0	
		LOEC	2.1	
E. andrei[g]	Adult survival	LC_{10}	12.25 (6.53 – 17.97)	9105
		LC_{50}	18.21 (16.87 – 19.54)	
		NOEC	7.2	
		LOEC	17.36	
	Reproduction	EC_{10}	0.002 (-0.010 – 0.015)	
		EC_{50}	0.048 (-0.090 – 0.19)	
		NOEC	NA[c]	
		LOEC	NA[c]	
E. crypticus[h]	Adult survival	LC_{10}	15.91 (9.88 – 21.92)	20.14
		LC_{50}	25.58 (22.64 – 28.51)	
		NOEC	11	
		LOEC	30.7	
	Reproduction	EC_{10}	1.27 (0.26 – 2.29)	
		EC_{50}	5.60 (3.81 – 7.38)	
		NOEC	1.4	
		LOEC	3.9	
H. aculeifer[i]	Reproduction	EC_{10}	967.69 (424.2 – 1511.2)	NA[e]
		EC_{50}	3673.87 (2646.21 – 4701.5)	
		NOEC	1600	
		LOEC	4000	

[a]Parameter = 10% lethal concentration (LC_{10}); median lethal concentration (LC_{50}), 10% effective concentration (EC10); median effective concentration (EC_{50}); lowest-observed-effect concentration (LOEC); no-observed-effect concentration (NOEC). [b]CI = Confidence intervals. [c]NA = NOEC/LOEC could not be determined probably due to test invalidity. [d]SSI = Sublethal sensitivity index (LC_{50}/EC_{10}). [e]NA = SSI could not be calculated due to inability to estimate LC_{50}. NOEC/LOEC for reproduction: [f]determined by One-way ANOVA followed by Tukey's HSD; [g]determined by Kruskal's test, followed by Siegel-Castellan multiple comparisons; [h]determined in the same way as for *E. andrei*; [i]determined by One-way ANOVA, followed by Dunnett pairwise comparison.

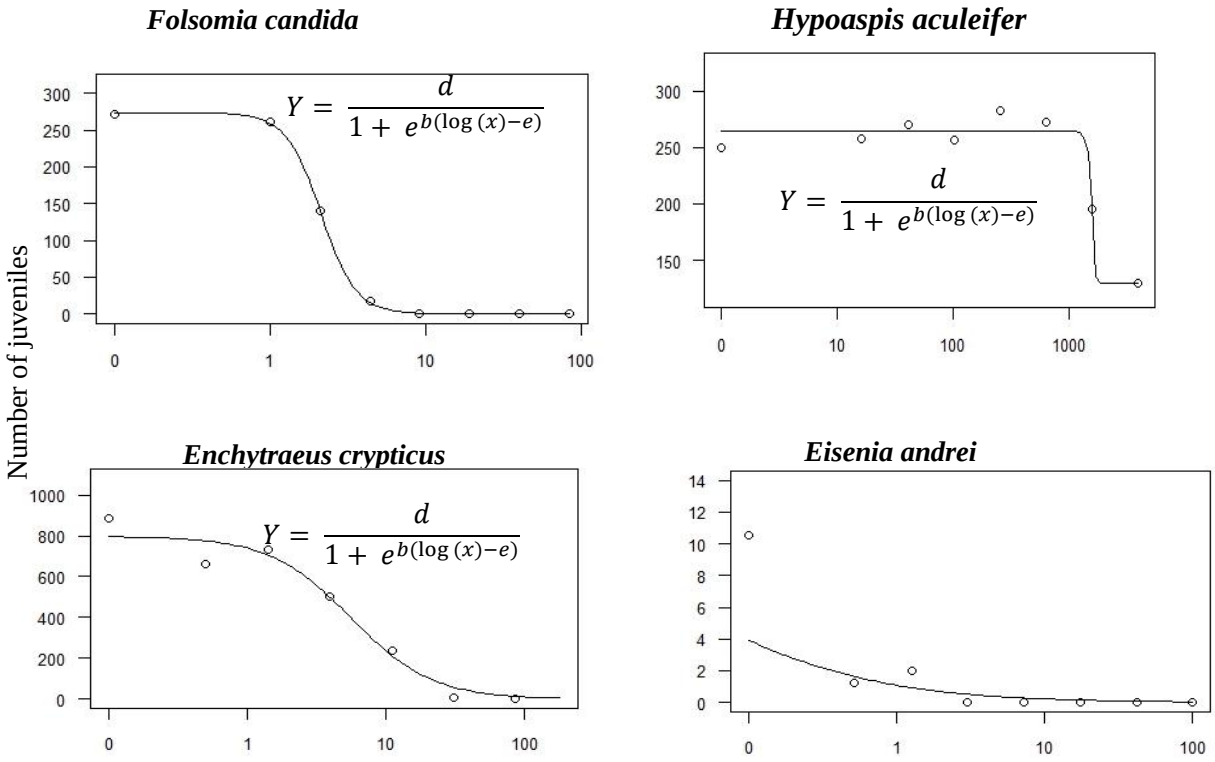


Figure 2: Sublethal effects of thiacloprid on *F. candida*, *E. crypticus*, *E. andrei* and *H. aculeifer* using reproduction as endpoint. Graphs show concentration-response relationships as predicted by the model.

2.5 DISCUSSION

2.5.1 Relative sensitivity of the four invertebrate species

From the result obtained, the relative sensitivity of the tested invertebrates to thiacloprid based on median lethal concentrations (LC_{50}) can be expressed as: *F. candida* > *E. andrei* > *E. crypticus* > *H. aculeifer*. When sublethal sensitivity comparisons are made on the basis of median effect concentrations (EC_{50}), we have: *E. andrei* > *F. candida* > *E. crypticus* > *H. aculeifer*. It is important to state here that 'ceteris paribus', *F. candida* is expected to be the most sensitive organism in both cases and several eco-toxicity studies even in tropical ecosystems have confirmed the relative higher sensitivity of *F. candida* to insecticides (Frampton et al. 2006; Daam et al. 2011; Chelinho et al. 2012). Also, due to the invalidity of the *E. andrei* reproduction test, sensitivity comparisons using the EC_{50} for *E. andrei* may not be reproducible.

H. aculeifer is unarguably the least sensitive invertebrate tested for thiacloprid. For all that is known, *H. aculeifer* seems to exhibit lower or intermediate relative sensitivity to certain chemicals when compared to other soil invertebrates (Owojori et al. 2014) but generalizations cannot be made as information for many other chemicals are scarce in scientific literature. Though *H. aculeifer* is thought to be potentially sensitive to chemicals (EC_{50} ranges for

dimethoate = 3.0 – 7.0 mg kg^{-1}; boric acid = 71 – 402 mg kg^{-1}) (Smit et al. 2012), it has also been found to exhibit low sensitivity to natural and synthetic naphthoquinones (Whitaker et al. 2009), chemicals that possess certain similar physico-chemical properties with neonicotinoids. The extremely low sensitivity or insensitivity of *H. aculeifer* obtained in this study could be attributed to fact that exposure to thiacloprid through cheese mites (prey) is likely to be negligible due to the low Log K_{ow} (1.26) of thiacloprid which suggest a low potential for bioconcentration.

On the contrary, the high sensitivity of *F. candida* could be attributed to the highly selective neurotoxic mode of action as thiacloprid, like other neonicotinoids, binds strongly and irreversibly to insect nicotinic acetylcholine receptors (nAChRs) (Jeschke et al. 2011). Also, the thin-walled ventral tube of *F. candida* serves as a major exposure route to dissolved thiacloprid in pore soil water (Fountain & Hopkin 2005). Soft-bodied oligochaetes i.e. *E. crypticus* and *E. andrei* are likely to be exposed to thiacloprid mainly through pore soil water. Exposure through food or ingestion of soil particles is likely to be negligible especially for thiacloprid with Log K_{ow} < 5 (Bezchlebová et al. 2007). As a result, an explanation for the intermediate relative sensitivities of *E. crypticus* and *E. andrei* may be that thiacloprid is less selective for the nAChRs of oligochaetes compared to that of arthropods.

2.5.2 Relating Lethal and Sublethal effects

Generally for the four invertebrates tested, reproduction appears to be more sensitive than survival for all estimated endpoint parameters as presented in Figure 3 and Table 5 which is the consensus for most ecotoxicity studies given the relevance of reproductive endpoints for assessing population-level effects of pesticides (Stark & Banks 2003; van Gestel 2012). When chronic LC_{50}/EC_{10} ratios, also called sublethal sensitivity index (SSI), are compared among these organisms, *F. candida* has the lowest value followed by *E. crypticus.* No explanation could be given at the moment for the differences in the sublethal sensitivity indices, because even though *E. andrei* has the highest SSI value, conclusions cannot be drawn given the invalidity of the *E. andrei* reproduction test.

2.5.3 Comparing concentration-response modeling with hypothesis testing

The use of hypothesis testing in generating ecotoxicity data for regulatory testing have been criticized and the replacement of the NOEC with point estimates have been proposed due to problems in the testing and statistical procedures used in determining its values (Fox 2008; Warne & Dam 2008). For instance, NOECs/LOECs are highly variable and inaccurate; NOECs normally correspond to 10 – 30% effect concentrations, while LOECs correspond to > 30% effect concentrations (Warne & Dam 2008). However, this study found NOEC and LOEC values to correspond to EC_{10} & EC_{50} values respectively (see Table 4) though estimated EC values were

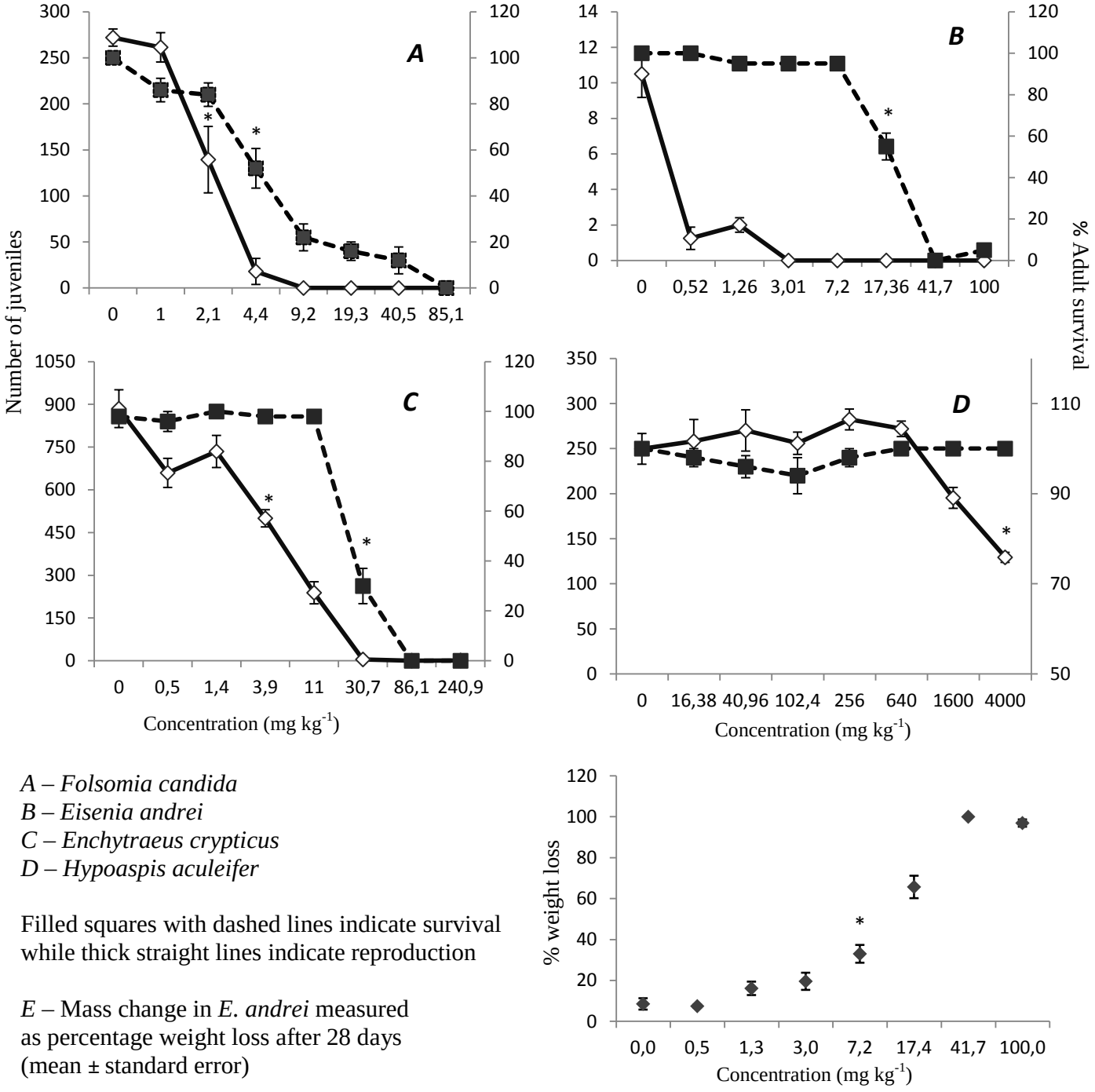
more conservative and hence more accurate. These results show that NOEC/LOEC values can still be generated as an alternative to regression-based EC_X method provided eco-toxicity tests are properly designed so as to improve the precision and accuracy of results. This could be achieved through increased replication, choosing an appropriate lowest treatment concentration, choosing a spacing factor ≤ 3.2, etc.

2.5.4 Initial considerations for Environmental Risk Assessment

Following the risk assessment scheme defined by the European and Mediterranean Plant Protection Organization (EPPO 2003b) in order to assess the chronic toxicity of thiacloprid to soil fauna, predicted environmental concentrations, calculated as 14 & 28-day TWACs, was compared to EC_{10} values (PEC/EC_{10}) to obtain exposure-toxicity ratios (ETRs) for all test invertebrates except *E. andrei*. ETRs obtained were found to be less than the trigger value of 0.2 preset by the EU (EC 1991). It can therefore be maintained with some measure of confidence that thiacloprid in the formulation Calypso® poses low risk to field populations of *F. candida*, *E. crypticus* and *H. aculeifer* following a single maximum application rate of 180 g a.i ha^{-1} by foliar spraying to apple plants. Subsequently, the risk of thiacloprid to the soil ecosystems was characterized since *F. candida* may not be the most sensitive species in the soil ecosystem. Calculated hazard quotient i.e. PEC/PNEC ratio = 1.25. Since this value is approximately equal to 1 which is the trigger value, we come to a definite conclusion that based on the first-tier eco-toxicity results generated by this study, the risk posed by thiacloprid to the soil compartment is acceptable and there is no need for additional risk mitigation measures. It should be noted that though a hazard quotient ~ 1 indicate there may be no need for additional eco-toxicity testing, the invalidation of the *E. andrei* reproduction test necessitates additional first-tier toxicity data that will be useful in refining the PNEC and PEC.

2.6 CONCLUSION

Among the tested invertebrates, *F. candida* is arguably the most sensitive to thiacloprid while *H. aculeifer* is least sensitive. In addition, survival endpoint parameters proved to be less sensitive compared to reproduction as expected while NOEC/LOEC values corresponded with estimated LC/EC values. This study supports the widely held notion that thiacloprid is ecological benign particularly to soil organisms as calculated ETRs and hazard quotient did not exceed preset trigger values at a recommended maximum field dose of 180 g a.i ha^{-1} to apple plants. Finally, the toxicity of thiacloprid to other soil invertebrates should be assessed to enable PNEC adjustment through species sensitivity distribution. Thiacloprid toxicity to soil invertebrates may be investigated in natural soils for improved ecological realism. Also, the influence of varying soil properties on thiacloprid bioavailability should be an important consideration for future research.



A – Folsomia candida
B – Eisenia andrei
C – Enchytraeus crypticus
D – Hypoaspis aculeifer

Filled squares with dashed lines indicate survival while thick straight lines indicate reproduction

E – Mass change in *E. andrei* measured as percentage weight loss after 28 days (mean ± standard error)

Figure 3: Number of juveniles (reproduction) and adult survival (percentage) for the four (A – D) test invertebrate species (mean ± standard error) as a response to different treatment concentrations of thiacloprid in OECD artificial soil. In addition, growth as sub-lethal endpoint was measured in *E. andrei* during the reproduction test as shown (E). *Asterisk denotes that response means from that treatment concentration and above are significantly different from respective controls ($P < 0.05$) (see Table 5)

.

2.7 REFERENCES

Barden, G. (2001). Evaluation of the new active Thiacloprid in the new product Calypso 480 SC Insecticide. National Registration Authority for Agricultural and Veterinary Chemicals, Canberra, Australia

Beketov, M.A. & Liess, M. (2008). Acute and delayed effects of the neonicotinoid insecticide thiacloprid on seven freshwater arthropods. *Environmental toxicology and chemistry / SETAC*, 27(2): 461–70. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18348641.

Bezchlebová, J., Cernohlakova, J., Lana, J., Sochova, I., Kobeticova, K. & Hofman, J. (2007). Effects of toxaphene on soil organisms. *Ecotoxicology and Environmental Safety*, 68(3): 326–334.

Buchholz, A. & Nauen, R. (2002). Translocation and translaminar bioavailability of two neonicotinoid insecticides after foliar application to cabbage and cotton. *Pest management science*, 58(1): 10–16.

Chelinho, S., Lopes, I., Natal-da-Luz, T., Domene, X. & Nunes, M.E.T., Espindola, E.L.G., Ribeiro, R. & Sousa, J.P. (2012). Integrated ecological risk assessment of pesticides in tropical ecosystems: a case study with carbofuran in Brazil. *Environmental Toxicology and Chemistry / SETAC*, 31(2): 437–445.

Daam, M.A., Leitao, S., Cerejeira, M.J. & Sousa, J.P. (2011). Comparing the sensitivity of soil invertebrates to pesticides with that of *Eisenia fetida*. *Chemosphere*, 85(6): 1040–1047.

Drozdzyński, D. & Kowalska, J. (2009). Rapid analysis of organic farming insecticides in soil and produce using ultra-performance liquid chromatography/tandem mass spectrometry. *Analytical and bioanalytical chemistry*, 394(8): 2241–2247.

EC (1991). European Commission council directive of July 1991 concerning the placing of plant protection products on the market (91/414/EEC). *Official Journal of the European Communities*, No L 230/1.

EC (2008). Directive 98/8/EC of the European Commission concerning the placing of biocidal products on the market. Thiacloprid (Product-type 8). *Assessment Report, Annex I - UK.*

EC (2004). European Commission review report for the active substance thiacloprid SANCO/4347/2000 - Final. *Commission Working Document.*

EC (2003). Technical Guidance Document on Risk Assessment Part II. *In support of* Commission Regulation (EC) No 1488/94 on Risk Assessment for existing substances, Institute for Health and Consumer Protection, European Chemicals Bureau.

Elbert, A., Matthias, H., Bernd, S., Wolfgang, T. & Ralf, N. (2008). Applied aspects of neonicotinoid uses in crop protection. *Pest Management Science*, 64: 1099–1105.

EPPO (2003a). European and Mediterranean Plant Protection Organization (EPPO) Standards. Environmental risk assessment scheme for plant protection products. Chapter 4: Soil. *OEPP/EPP0 Bulletin*, 33: 147–149.

EPPO (2003b). EPPO Standards. Environmental Risk Assessment Scheme for Plant Protection Products. Chapter 8: Soil Organisms and Functions. *Bulletin OEPP/EPPO*, 33: 147–149.

Fountain, M.T. & Hopkin, S.P. (2005). *Folsomia candida* (Collembola): A "standard" soil arthropod. *Annual review of entomology*, 50: 201–222.

Fox, D.R. (2008). NECS , NOECS AND THE ECx. *Australasian Journal of Ecotoxicology*, 14: 7–9.

Frampton, G.K., Jansch, S., Scott-Fordsmand, J.J., Rombke, J. & Van Den Brink, P.J. (2006). Effects of pesticides on soil invertebrates in laboratory studies: A review and analysis using species sensitivity distributions. *Environmental Toxicology and Chemistry*, 25(9): 2480–2489.

Van Gestel, C. A. M. (2012). Soil ecotoxicology: state of the art and future directions. *ZooKeys*, 296(176): 275–96.

Giraudoux, P. (2013). Pgirmess: Data analysis in Ecology. R package version 1.5.8. Available at: http://cran.r-project.org/package=pgirmess.

Goulson, D. (2013). Review: An overview of the environmental risks posed by neonicotinoid insecticides. *Journal of Applied Ecology*, 50(4): 977–987.

ISO (1998). Soil quality - Effects of pollutants on earthworms (*Eisenia fetida*). Part 2: Determination of Effects on Reproduction, No 11268-2. ISO, Geneve.

ISO (2003). Soil quality - Effects of pollutants on Enchytraeidae (*Enchytraeus sp*) - Determination of effects on reproduction and survival. International Standard ISO 16387. Geneva, Switzerland.

ISO (1999). Soil quality - Inhibition of reproduction of Collembola (*Folsomia candida*) by soil pollutants, No. 11267. ISO, Geneve.

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(7): 2897–2908. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20565065.

Langer-Jaesrich, M., Köhler, H.-R. & Gerhardt, A. (2010). Assessing toxicity of the insecticide thiacloprid on *Chironomus riparius* (Insecta: Diptera) using multiple end points. *Archives of Environmental Contamination and Toxicology*, 58(4): 963–972.

Linders, J., Mensink, H., Stephenson, G., Wauchope, D. & Racke, K. (2000a). Foliar interception and retention values after pesticide application. A proposal for standardized values for environmental risk assessment. *Pure and Applied Chemistry*, 72(11): 2199–2218.

OECD (1984). Earthworm, Acute Toxicity Tests. OECD guideline for testing of chemicals (207), Paris.

OECD (2006). Current approaches in the statistical analysis of ecotoxicity data: a guidance to application. OECD Environmental Health and Safety Publications Series on Testing and Assessment No 54, ENV/JM/MONO(2006), Paris.

OECD (2008). Predatory mite (*Hypoaspis* (*Geolaelaps*) *aculeifer*) reproduction test in soil. OECD guidelines for testing chemicals 226, Paris.

Owojori, O.J., Waszak, K. & Roembke, J. (2014). Avoidance and reproduction tests with the predatory mite *Hypoaspis aculeifer*: effects of different chemical substances. *Environmental toxicology and chemistry / SETAC*, 33(1): 230–237.

Palumbo, J., Horowitz, A. & Prabhaker, N. (2001). Insecticidal control and resistance management for Bemisia tabaci. *Crop Protection*, 20(9): 739–765. Available at: http://linkinghub.elsevier.com/retrieve/pii/S026121940100117X.

R Core Team, (2014). R a language and environment for statistical computing. Available at: http://www.r-project.org/.

Racke, K.D. (2003). Release of pesticides into the environment and initial concentrations in soil, water, and plants. *Pure and Applied Chemistry*, 75(11-12): 1905–1916.

Ritz, C. & Streibig, J.C. (2005). Bioassay Analysis using R. *Journal of Statistical Software*, 12(5): 1-22

Siegel, S. & Castellan, J.N. (1988). Non-parametric statistics for the behavioural sciences, New York: MacGraw Hill International.

Smit, C.E., Moser, T. & Römbke, J. (2012). A new OECD test guideline for the predatory soil mite *Hypoaspis aculeifer*: results of an international ring test. *Ecotoxicology and Environmental Safety*, 82: 56–62.

Stark, J.D. & Banks, J.E. (2003). Population-level effects of pesticides and other toxicants on arthropods. *Annual Review of Entomology*, 48: 505–519.

Szczepaniec, A., Raupp, M.J., Parker, R.D., Kerns, D. & Eubanks, M.D. (2013). Neonicotinoid insecticides alter induced defenses and increase susceptibility to spider mites in distantly related crop plants. *PloS one*, e62620, 8(5): 1-11.

Warne, M.S.J. & Dam, R.V. (2008). NOEC and LOEC data should no longer be generated or used. *Australasian Journal of Ecotoxicology*, 14: 1–5.

Van der Werf, H.M.G. (1996). Assessing the impact of pesticides on the environment. *Agriculture, Ecosystems & Environment*, 60: 81–96.

Whitaker, J., Chaplow, J.S., Potter, E., Scott, W.A., Hopkin, S., Harman, M., Sims, I. & Sorokin, N. (2009). The comparative toxicity to soil invertebrates of natural chemicals and their synthetic analogues. *Chemosphere*, 76(3): 345–52.

Wilcox, D.C., Dove, B.C., McDavid, D.W. & Greer, D.B. (2002). UTHSCSA Image Tool version 3.00. *The University of Texas Health Science Center, San Antonio, Texas*. Available at: http://compdent.uthscsa.edu/dig/itdesc.html#What.

Yu, Y., Wu, J., Stahler, M. & Pestemer, W. (2007). Residual dynamics of thiacloprid in medical herbs marjoram, thyme, and camomile in soil. *Journal of Environmental Sciences*, 19: 205–209.

Chapter 3

3 Toxicity of the neonicotinoid acetamiprid to Folsomia candida, Eisenia andrei, Enchytraeus crypticus and Hypoaspis aculeifer

3.1 ABSTRACT

Neonicotinoid insecticides, commercialized in over 120 countries worldwide, have proved to be very effective for controlling agricultural pests. They act as agonist of insect nicotinic acetylcholine receptors (nAChRs). Acetamiprid is a second generation neonicotinoid that is mainly applied as foliar spray to orchard and ornamental plants. Relevant scientific information concerning the toxicity of acetamiprid particularly to soil invertebrates is very limited though non target terrestrial and aquatic organisms are usually exposed to through spray applications of formulated acetamiprid. In this paper, reproduction tests were conducted according to standard guidelines to assess the chronic toxicity of acetamiprid to four terrestrial invertebrates in artificial OECD soil. Results obtained indicated that for survival and reproduction, relative sensitivity of the test species can be expressed as *Folsomia candida* > *Eisenia andrei* > *Enchytraeus crypticus* > *Hypoaspis aculeifer*. Acetamiprid was extremely toxic to *F. candida* (EC_{50} = 0.29 mg/kg) while *H. aculeifer* was least sensitive (EC_{50} = 650.60 mg/kg). Differences in exposure routes and higher selectivity of acetamiprid for nAChRs of arthropods may be responsible for the relative sensitivities observed. To extrapolate from these valid tests for *F. candida*, *E. crypticus* and *H. aculeifer*, predicted environmental concentrations (PECs) and predicted no-effect concentration (PNEC = 0.004 mg kg^{-1}) were derived for acetamiprid. Risk of acetamiprid to field populations of these three invertebrates was characterized as low since calculated exposure-toxicity ratios (ETRs; PEC/EC10) for the three invertebrates were lesser than 0.2. However, calculated risk quotient (PEC/PNEC) was greater than 1, the preset trigger value. This means that the risk of formulated acetamiprid to the soil compartment following a single maximum application rate of 100 g a.i ha^{-1} to young citrus plants is unacceptable

3.2 INTRODUCTION

Neonicotinoids are now the most effective class of insecticides in the world since the commercialization of pyrethroids, registered in at least 120 countries globally with annual sales estimated at $1.5 billion (Jeschke et al. 2011). As systemic pesticides, they are selective agonists of insect nicotinic acetylcholine receptors (*n*AChRs) (Jeschke et al. 2011; Szczepaniec et al. 2013). 60% of neonicotinoids are used as prophylactic seed dressings worldwide, but they are also applied as foliar sprays on orchard and ornamental crops for the control of lepidopteran, coleopteran and hemipteran pest species e.g. aphids, plant-hoppers, moths, etc (Jeschke & Nauen 2010; Goulson 2013). Therefore, their broad-spectrum insecticidal properties, low mammalian toxicity, high flexibility of use and lower application rates (g of active ingredient per hectare) compared to organophosphate and carbamate insecticides have led to their widespread acceptance (Zalom et al. 2005; Elbert et al. 2008).

Acetamiprid (Figure 4), a second-generation neonicotinoid was initially commercialized in Japan in 1995 by Nippon Soda mainly for foliar applications while direct soil uses are restricted (Elbert et al. 2008). Today, acetamiprid is marketed under several brands such as Mosipilan®, Epik®, Assail® and Chipco™ with different formulations (e.g. 20% acetamiprid SP, 3% acetamiprid EC, etc). In addition, acetamiprid has been proved to be more effective against pests (e.g. *Bemisia tabaci*) when used as foliar sprays than when applied directly to the soil (Palumbo et al. 2001). The ecological risks posed by acetamiprid to nontarget aquatic and terrestrial organisms are still relatively unclear. Acetamiprid is highly soluble (4250 mg L^{-1} at 25°C) and stable in water (Jeschke & Nauen 2010). Like other pesticides, nontarget aquatic organisms are usually exposed through off-target spray drift, surface water runoffs, etc (Racke 2003). However, there is paucity of independent published scientific literature regarding the toxicity of acetamiprid to aquatic nontarget invertebrates.

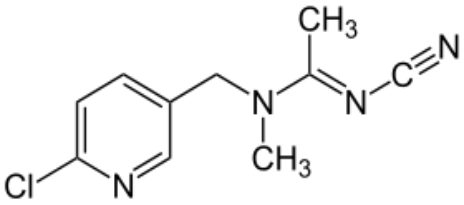


Figure 4: Chemical structure of acetamiprid

Concerning the ecotoxicity of acetamiprid to terrestrial nontarget invertebrates, one study found acetamiprid to negatively impact the behavior of the honeybee (*Apis mellifera*) at sublethal doses (El Hassani et al. 2008). Acetamiprid is easily biodegraded in soils with half-life (DT_{50}) of 2.9 days from field dissipation studies (EC 2004). However, spray applications of commercial formulations to orchard crops at several intervals during pest control programs might imply that populations of beneficial soil organisms are repeatedly exposed to significant amounts of acetamiprid. It is therefore remarkable that there is no published information in scientific literature on the effects of acetamiprid to soil invertebrates.

This study aims to assess the toxicity of acetamiprid to the soil invertebrates *Folsomia candida*, *Eisenia andrei*, *Enchytraeus crypticus* and *Hypoaspis aculeifer* by determining its acute and chronic toxicities to these organisms using survival and reproduction endpoints. In addition, the relative sensitivities of the invertebrates to acetamiprid are evaluated and implications for terrestrial ecological risk assessment are discussed.

3.3 MATERIALS AND METHODS

3.3.1 Test organisms

Springtails (*Folsomia candida*) were cultured in plastic containers lined with an 11:1 mixture of plaster of Paris (POP) and activated charcoal. Food was added twice a week as small amounts of dry yeast to avoid fungal spoilage. Earthworms (*Eisenia andrei*) were bred in aerated plastic containers with horse manure and peat mixture as substrate and fed twice a month with oat porridge. *Enchytraeus crypticus* were cultured in agar and fed *ad-libitum* with oatmeal twice a week. *Hypoaspis aculeifer* were cultured in plastic containers lined with an 8:1 ratio of plaster of Paris and activated charcoal. The predatory mites were fed with cheese mites (*Tyrophagus putrescentiae*) 2-3 times a week. Test organisms were cultured at 20^{o}C ±2^{o}C, 16h: 8h light-dark cycle and 400 - 800 Lux illumination.

3.3.2 Test soil

Tests were conducted using artificial soil prepared from the constituent mixture (75% sand, 5% peat and 20% kaolin clay) according to OECD (1984) guideline. Though the tested invertebrates possess a world-wide distribution, the OECD artificial soil was chosen to standardize soil properties and to facilitate easy interpretation and comparison of results obtained. According to EPPO (2003), a typical agricultural soil has a maximum organic matter content of 5%. Therefore, peat content of the artificial soil was decreased to 5% and sand content increased to 75% accordingly. Peat was sieved through 5-mm mesh for *E. andrei*, *F. candida*, *E. crypticus* and through 2-mm mesh for *H. aculeifer*. $CaCO_3$ was added to adjust the pH of the artificial soil to 6.0±0.5. Water holding capacity (WHC) was determined to ensure that soil moisture reached 50% of the maximum WHC.

3.3.3 Test substance

Bioassays were based on nominal concentrations of acetamiprid (Table 6) – IUPAC name – (E)-N1-[(6-chloro-3-pyridyl) methyl]-N2-cyano-N1-methylacetamidine, the active ingredient in the water soluble granulated commercial formulation known as Epik® (composition: acetamiprid 20% w/w).

Table 6: Physicochemical properties of Acetamiprid

Molecular formula	$C_{10}H_{11}ClN_4$
Molecular mass	222.68
Specific gravity (20 °C/20 °C)	1.330 (99.7%)
Log K_{ow} (25 °C)	0.80 (>99%)
Mean $DT50_{lab}$ (20 °C, aerobic)	2.6 days
Mean $DT90_{field}$	20.2 days
Henry's law constant (25 °C)	<5.3x10-8 Pa m^3 mol^{-1}
Vapour pressure (25 °C)	<1x10-6 Pa (expected)

3.3.4 Toxicity assessments

Toxicity assays were performed according to OECD and ISO[2] guidelines on assessing the effects of chemicals on the reproduction of the test invertebrates. Moisture content of artificial OECD soil was adjusted to 50% of the maximum water-holding capacity (WHCmax) at the start of each toxicity test.

Treatment concentrations: to obtain the desired test concentrations, dilutions from a stock solution were mixed into artificial soil at a volume equivalent to the corresponding 50% WHC for that amount of soil. Range-finding tests with mortality as the only endpoint were conducted for each of the test species – except *Eisenia andrei* – to refine the range of concentrations chosen for the definitive reproduction tests. The definitive reproduction test for *E. andrei* was based on the 14-day acute toxicity (LC_{50}) value obtained from the European Commission review report on acetamiprid (EC 2004). For each range-finding test, artificial soils were spiked with nominal acetamiprid concentrations of 0.1, 1, 10, 100, 1000 mg kg^{-1}. Eight test concentrations (including the control) were used in definitive testing with five replicates (Collembola, enchytraeid and predatory mite) or four replicates (earthworm). Nominal concentrations of thiacloprid for each of the test species include: 0, 0.3, 0.6, 1.6, 3.9, 9.8, 24.4 and 61.0 mg kg^{-1} (*F. candida*); 0, 0.5, 1.15, 2.60, 6.10, 14.0, 32.2 and 74 mg kg^{-1} (*E. andrei*); 0, 0.5, 1.3, 3.1, 7.8, 19.5, 48.8 and 122.0 mg kg^{-1} (*E. crypticus*); 0, 23, 49, 103, 216, 454, 952 and 2000 mg kg^{-1} (*H. aculeifer*).

3.3.5 Definitive reproduction test

Folsomia candida: The collembolan reproduction test (ISO, 1999) was chosen to assess adult survival and juvenile production by *Folsomia candida*. Test duration was 28 days. Each glass test vessel was filled with 30 g of artificial soil (wet mass). At the start of the experiment, 10 synchronized juveniles (10 – 12 days) were added to each vessel and fed with 2 – 10 mg of granulated dry yeast. Thereafter, test vessels were aerated once every week, while dry yeast was added and water content was adjusted after two weeks.. On day 28, surviving adults and

[2] International organization for standardization

juveniles were photographed and counted using the Image Tool software (Wilcox et al. 2002) after flotation.

Enchytraeus crypticus: Though the ERT guidelines ISO 16387 (ISO, 2003) and OECD 220 (OECD, 2004) recommended the use of *Enchytraeus albidus*, *E. crypticus*, an alternative testing species was used due to practical advantages. 20 g of artificial soil (dry weight) and 10 adults of *E. crypticus* with well-developed clitella including 50 mg of finely ground dry oats were supplied into each test vessel. The ERT was terminated after 28 days and organisms in each test vessels were fixed and stained with alcohol and Bengal red. Surviving adults and juveniles were counted under a stereomicroscope.

Hypoaspis (Geolaelaps) aculeifer: Test was conducted according to the guideline OECD 226 (OECD, 2008). Ten adult females of 28–35 days old were introduced into test vessels containing 20 g of artificial soil (dry mass) and then fed with cheese mites. Test vessels were weighed to serve as reference for soil moisture loss check-up. After 14 days, test was terminated and *H. aculeifer* adults and juveniles were separated from the soil substrate by heat extraction. Finally, surviving adult and juvenile mites were then counted under a dissecting microscope.

Eisenia andrei: The epigeic earthworm *Eisenia andrei* was selected for the earthworm reproduction test and test procedures followed the international guideline ISO 11268-2 (ISO, 1998). Before testing, synchronized adult worms (2 to 12 months old) with clitella were selected and acclimatized for 24 hours in OECD soil. 500 – 600 g (dry mass) of artificial soil was measured in each test container and 10 adult worms (250 – 600 mg) were added to each test container. Adult survival was determined after 28 days and test was terminated after 56 days by the counting of the juveniles hatched from the cocoons.

For each test performed, soil pH and moisture content for each treatment concentration – including control – were measured at the beginning and end of each experiment. All test vessels were incubated under controlled laboratory conditions: temperature of 20 ± 2ºC, light-dark cycle of 16h: 8h and an illumination of 400 - 800 Lux.

3.3.6 Estimation of predicted environmental concentrations (PECs)

Initial (PEC_{intial}) and time-weighted average (PEC_{twa}) exposure concentrations of acetamiprid under worst-case scenarios were calculated based on the use of the formulation acetamiprid 20% SP through foliar spray on young or non-producing citrus trees during spring and summer. This estimation was done by assuming a uniform distribution in the upper soil layer of 5 cm depth and a dry soil bulk density of 1.5 g cm^{-3}. Given that maximum dissipation time ($DT50_{lab}$) of acetamiprid is 5 days, maximum application rate of the pesticide was taken to be 100 g a.i ha^{-1} (EC 2004) (see Table 7). An interception factor (F = 0.5) for citrus crops specifically under the inflorescence emergence growth phase (BBCH code = 50 – 59) was adopted from the proposed table of harmonized interception factors for various crops (Linders et al. 2000)

Table 7: Estimated predicted environmental concentrations for acetamiprid in the top soil

Initial (mg/kg)	Time-weighted (mg/kg)	
	14 days	28 days
0.067	0.04	0.02

3.3.7 Statistical analyses

For the definitive reproduction tests, mortality (LC_{50}) and reproduction (EC_{10} & EC_{50}) endpoint values were calculated by fitting various nonlinear regression models in the R package 'drc' used for the analysis of dose-response curve data (Ritz & Streibig 2005). Generally, the three-parameter logistic model provided the best fit for all toxicity data (except *E. andrei*) as given below:

$$Y = f(x) = \frac{d}{1 + \exp(b(\log(x) - \log(e)))} \quad (1)$$

Where Y is the number of juveniles or adult survival (fraction), x is the nominal test concentration (mg a.i kg^{-1} dry soil), b is the maximal slope of the logistic function, d is the maximum response in the controls (upper limit) and e is the LC_{50} or EC_{50} value. The exponential model (see Equation 4) was used to estimate EC_{50} for *E. andrei*. *ED.drc* function was used to estimate 10%, 20% and 50% lethal and effective concentrations (LC/EC) respectively. Equation 2 gives the initial soil exposure concentration (*Ci*) of acetamiprid for the assessment of acute effects (EPPO 2003a).

$$Ci = \frac{A \times (1 - F)}{L \times 10^2 \times D} \quad (2)$$

The time-weighted average concentration (*TWAC*) of acetamiprid for chronic toxicity assessment was calculated using the following equation (EPPO 2003a):

$$TWAC = Ci \times \left(\frac{DT50}{t.In2}\right) \times \left[1 - \exp\left(-t \times \frac{In2}{DT50}\right)\right] \quad (3)$$

Where A = application rate (kg ha^{-1}); F = interception factor; L = soil layer depth (cm); D = soil bulk density (g cm^{-3}); t = duration of toxicity test and *DT50* = laboratory half-life of acetamiprid. To determine NOEC[3] and LOEC[4] values, the normality and homogeneity of reproduction, survival and earthworm weight loss data were tested using Shapiro-Wilk and Levene's test before statistical analysis. Where normality and homoscedascity assumptions are satisfied, significant differences between treatment response means were tested using one-way ANOVA analysis followed by post-hoc trend (Tukey HSD) or pair-wise comparison (Dunnett) tests

[3] NOEC is the highest concentration used in a toxicity test that causes a toxic effect which is not significantly different (at $P \leq 0.05$) from the control

[4] LOEC is the lowest concentration used in a toxicity test that causes a toxic effect which is significantly different (at $P \leq 0.05$) from the control.

(significance: *p-value* < 0.05). Otherwise, appropriate functions in the R package 'pgirmess' (Giraudoux 2013) were applied to determine NOECs/LOECs using the non-parametric Kruskal-Wallis test followed by post-hoc multiple comparisons (significance: *p-value* < 0.05) as described by Siegel & Castellan (1988).

For acute range-finding tests, the exponential decay model as obtained in the 'drc' R package was chosen as it provided the best LC_{50} estimates with lowest-bound confidence intervals. The model equation is given below:

$$Y = f(x) = c + (d - c)(\exp(-x/e)) \quad (4)$$

Where d is the estimated survival rate at $x = 0$, c is the estimated survival rate at $x \sim \infty$ and $e > 0$ determines the steepness of the decay curve. All statistical analyses were performed according to OECD standard guidelines (OECD 2006) using R (R Core Team 2014) and Microsoft Excel.

3.4 RESULTS

3.4.1 Test validation

Validity requirements in the controls were satisfied in the rangefinding and definitive tests for all organisms (except *E. andrei)* as prescribed by test guidelines. The average adult survival for *F. candida*, *E. crypticus* and H. aculeifer in the definitive reproduction tests was < 20%. Additionally, mean reproduction rate for *F. candida* was 421 juveniles while *H. aculeifer* produced 190 juveniles per replicate. *E. crypticus* produced an average of 577 juveniles per replicate. Coefficient of variation (CV) for all test organisms was lower or approximately 30% except for *E. andrei* where CV = 55%. There was also less than 30 juveniles per replicate for *E. andrei*. For the acute range-finding tests with *F. candida* and *E. crypticu*s, adult mortality was << 20%.

3.4.2 Acute (range-finding) toxicity

Range-finding tests (see Table 8 below) showed that acetamiprid was extremely lethal to *F. candida* (LC_{50} = 0.52 mg kg^{-1}). In contrast, *E. crypticus* seemed resistant with an estimated LC_{50} value of 371.12 mg kg^{-1}. For *H. aculeifer*, average mortality in test concentrations up to 1000 mg kg^{-1} was very low and therefore insufficient to allow LC_{50} extrapolation from the model.

Table 8: Acute toxicity (LC_{10} & LC_{50}) of acetamiprid to *F. candida* and *E. crypticus*

Organism	Parameter (mg/kg)	Estimate	95% CI
F. candida	LC_{10}	0.08	0.018 – 0.14
	LC_{50}	0.52	0.12 – 0.92
E. crypticus	LC_{10}	56.41	39.05 – 73.77
	LC_{50}	371.12	256.91 – 485.33

3.4.3 Chronic toxicity: lethal and sublethal effects

Definitive reproduction test results (Table 9, Figure 5) shows that acetamiprid causes lethal and sublethal effects in *F. candida* at similar concentrations. The 50% lethal and effect concentrations (LC_{50} and EC_{50}) values for *F. candida* are 0.40 and 0.29 mg kg^{-1} respectively while determined NOEC & LOEC values for both adult survival and reproduction respectively are approximately < 0.3 and 0.3 mg kg^{-1}. It was however a different case for *E. crypticus* as lethal and sublethal effects occurred at different concentrations of acetamiprid (see Table 9). For instance, LC_{50} estimate of 12.34 mg kg^{-1} for adult survival was greater than EC_{50} (1.90 mg kg^{-1}) by a factor of 6 and LOEC (19.5 mg kg^{-1}) for adult survival was greater than LOEC (1.60 mg kg^{-1}) for reproduction by a factor of 12. *H. aculeifer* was evidently the least sensitive invertebrate tested with bounded NOEC and LOEC values of 454 and 952 mg kg^{-1} respectively. Also, EC_{10} and EC_{50} values were calculated to be 447.27 mg kg^{-1} and 650.60 mg kg^{-1}, correspondingly. As in the range-finding test results stated above, adult mortality was too low to allow for LC_{50} estimation.

For *Eisenia andrei*, acetamiprid seems to cause sublethal effects at lower concentrations compared to lethal effects as LC_{10} and LC_{50} values of 1.94 and 2.31 mg kg^{-1} respectively were greater than EC_{10} and EC_{50} values of 0.13 and 0.18 mg kg^{-1}. This situation holds true for hypothesis testing as adult survival NOEC (1.15 mg kg^{-1}) & LOEC (2.60 mg kg^{-1}) were greater than the NOEC (0.5 mg kg^{-1}) & LOEC (1.15 mg kg^{-1}) for reproduction. However, it is essential to note here that the validity criteria was not fully satisfied (number of juveniles per replicate in the controls was < 30). Hence, results obtained for *E. andrei* cannot be taken with absolutely confidence. The sensitivity of growth as an endpoint in *E. andrei* was measured as percentage weight loss in 28 days during the reproduction test. When compared to the controls, weight loss in treatment concentrations was found to be significant starting from 2.60 mg kg^{-1} as shown in Figure 6.

In adherence to the EU technical guidance document on the risk assessment of chemicals (EC 2003), a predicted no effect concentration in soil ($PNEC_{soil}$) was derived by dividing lowest 10% effect concentration (EC_{10}) value by an assessment factor of 50. Among the three[5] test organisms from two different trophic levels that were considered, EC_{10} value for *F. candida* was used as it was the lowest. Hence, derived $PNEC_{soil}$ for acetamiprid = 0.004 mg kg^{-1}.

3.5 DISCUSSION

3.5.1 Relative sensitivity of the tested soil invertebrates

Comparing the sensitivities of the invertebrates using appropriate lethal and sublethal parameters, i.e. the median lethal and effect concentrations, the relative sensitivity for the test

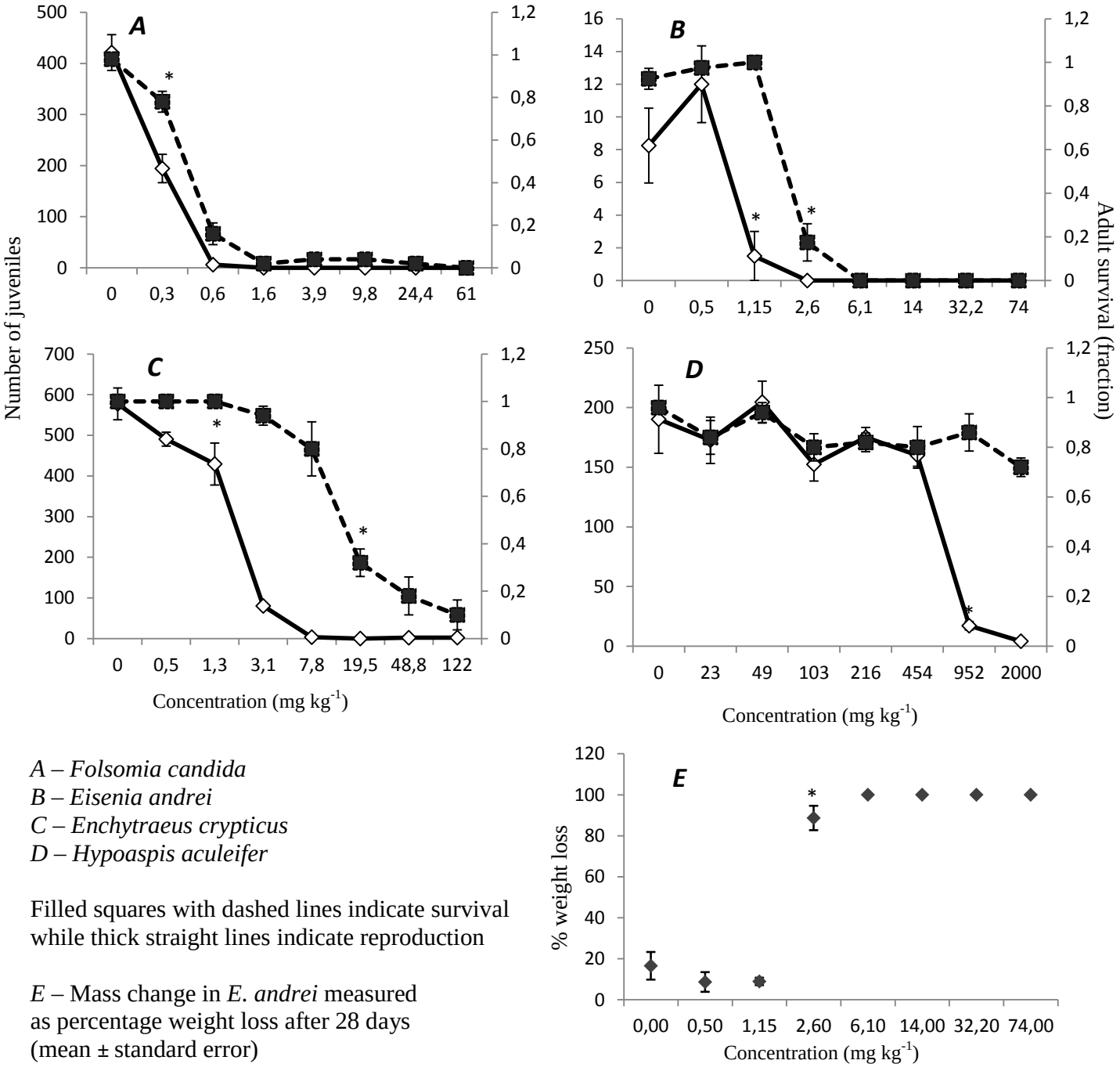
[5] *E. andrei* EC10 value not considered due to the invalidity of reproduction test

species can be expressed as: *F. candida* > *E. andrei* > *E. crypticus* > *H. aculeifer*. *F. candida* is easily the most sensitive species tested as expected, and laboratory studies have repeatedly confirmed the higher sensitivity of *F. candida* especially to pesticides with broad-spectrum insecticidal properties which also applies to acetamiprid (Daam et al. 2011). *Eisenia andrei*: The 28-day LC_{50} of 2.31 mg kg^{-1} and reproductive NOEC (0.5 mg kg^{-1}) as obtained in this study is very low and this could imply that acetamiprid exhibits a higher toxicity to *E. andrei* compared to more than 95% of other pesticides (Pelosi et al. 2013).

In addition, the comparative higher sensitivity of *E. andrei* to acetamiprid which is second only to *F. candida* agrees with similar comparative sensitivities to other non-neonicotinoid insecticides as reviewed by Frampton et al. (2006). Though the lower relative sensitivity of *E. crypticus* compared to *F. candida* and *E. andrei* as obtained in this study is similar to that obtained for dimethoate in a previous study (Martikainen 1996), relative sensitivity of enchytraeids could vary among other insecticides as explained in a review by Rombke (2003), that "enchytraeids are not less sensitive than either earthworms or collembolans in general".

H. aculeifer is unarguably the least sensitive invertebrate tested. For all that is known, *H. aculeifer* seems to exhibit lower or intermediate relative sensitivity to certain chemicals when compared to other soil invertebrates (Owojori et al. 2014). However, generalizations cannot be made now as information concerning the toxicity of many other chemicals to *H. aculeifer* is scarce in scientific literature. Though *H. aculeifer* is thought to be potentially sensitive to chemicals (EC_{50} ranges for dimethoate = 3.0 – 7.0 mg kg^{-1}; boric acid = 71 – 402 mg kg^{-1}) (Smit et al. 2012), it has been found to exhibit lower sensitivity to natural and synthetic naphthoquinones (Whitaker et al. 2009), chemicals that possess certain similar physico-chemical properties with neonicotinoids.

The extremely low sensitivity or insensitivity of *H. aculeifer* obtained in this study could be attributed to fact that exposure through cheese mites (prey) is likely to be negligible due to the low Log K_{ow} (0.80) of acetamiprid which suggest a low potential for bioconcentration (Bezchlebová et al. 2007). The high toxicity of acetamiprid to *F. candida* may be due to the highly selective neurotoxic mode of action as acetamiprid binds strongly and irreversibly to insect nicotinic acetylcholine receptors (nAChRs), coupled with the fact that the thin-walled ventral tube located in the segmented abdominal area of *F. candida* might serve as a major exposure route to dissolved thiacloprid in pore soil water (Fountain & Hopkin 2005). Soft-bodied oligochaetes i.e. *E. crypticus* and *E. andrei* are likely to be exposed to thiacloprid mainly through pore soil water. Exposure through food or ingestion of soil particles is likely to be negligible especially for chemicals like acetamiprid with Log K_{ow} < 5 (Bezchlebová et al. 2007). As a result, an explanation for the intermediate relative sensitivities of *E. crypticus* and *E. andrei* may be that acetamiprid and other neonicotinoids are less selective for the nAChRs of oligochaetes compared to that of arthropods.



A – Folsomia candida
B – Eisenia andrei
C – Enchytraeus crypticus
D – Hypoaspis aculeifer

Filled squares with dashed lines indicate survival while thick straight lines indicate reproduction

E – Mass change in *E. andrei* measured as percentage weight loss after 28 days (mean ± standard error)

Figure 5: Number of juveniles (reproduction) and adult survival for the four (A – D) test invertebrate species (mean ± standard error) as a response to different treatment concentrations of acetamiprid in OECD artificial soil. In addition, growth as a sub-lethal endpoint was measured in *E. andrei* during the reproduction test as shown (E). *Asterisk denotes that response means from that treatment concentration and above are significantly different from respective controls ($P < 0.05$) (see Table 4)

Table 9: Ecotoxicity parameters based on adult survival and reproduction endpoints for four soil invertebrate species exposed to acetamiprid in artificial OECD soil.

Organism	Endpoint	Parameter[a] (mg kg^{-1})	Estimate with 95% CI[b]	SSI[c]
F. candida[e]	Adult survival	LC_{10}	0.24 (0.22 – 0.26)	2
		LC_{50}	0.40 (0.37 – 0.43)	
		NOEC	< 0.3	
		LOEC	0.3	
	Reproduction	EC_{10}	0.20 (0.10 – 0.30)	
		EC_{50}	0.29 (0.27 – 0.31)	
		NOEC	< 0.3	
		LOEC	0.3	
E. andrei[f]	Adult survival	LC_{10}	1.94 (0.19 – 3.70)	17.77
		LC_{50}	2.31 (1.46 – 3.16)	
		NOEC	1.15	
		LOEC	2.6	
	Reproduction	EC_{10}	0.13 (0.06 – 0.21)	
		EC_{50}	0.88 (0.37 – 1.88)	
		NOEC	0.5	
		LOEC	1.15	
E. crypticus[g]	Adult survival	LC_{10}	5.06 (2.84 – 7.28)	12.22
		LC_{50}	12.34 (9.37 – 15.31)	
		NOEC	7.8	
		LOEC	19.5	
	Reproduction	EC_{10}	1.01 (0.74 – 1.28)	
		EC_{50}	1.90 (1.64 – 2.16)	
		NOEC	0.5	
		LOEC	1.6	
H. aculebifer[h]	Reproduction	EC_{10}	447.27 (292.42 – 602.11)	NA[d]
		EC_{50}	650.60 (493.37 – 807.83)	
		NOEC	454	
		LOEC	952	

[a]Parameter ⇛ LC_{10} = 10% lethal concentration; LC_{50} = median lethal concentration; EC_{10} = 10% effective concentration; EC_{50} = median effective concentration; LOEC = lowest-observed-effect concentration; NOEC = no-observed-effect concentration. [b]CI = Confidence intervals. [c]SSI = Sublethal sensitivity index (LC_{50}/EC_{10}). NA[d] = SSI could not be calculated due to inability to estimate LC_{50}. NOEC/LOEC for reproduction: [e]determined by One-way ANOVA followed by Tukey's HSD; [f]determined by Kruskal's test, followed by Siegel-Castellan multiple comparisons; [g]determined in the same way as for *E. andrei*; [h]determined by One-way ANOVA, followed by Dunnett pairwise comparison.

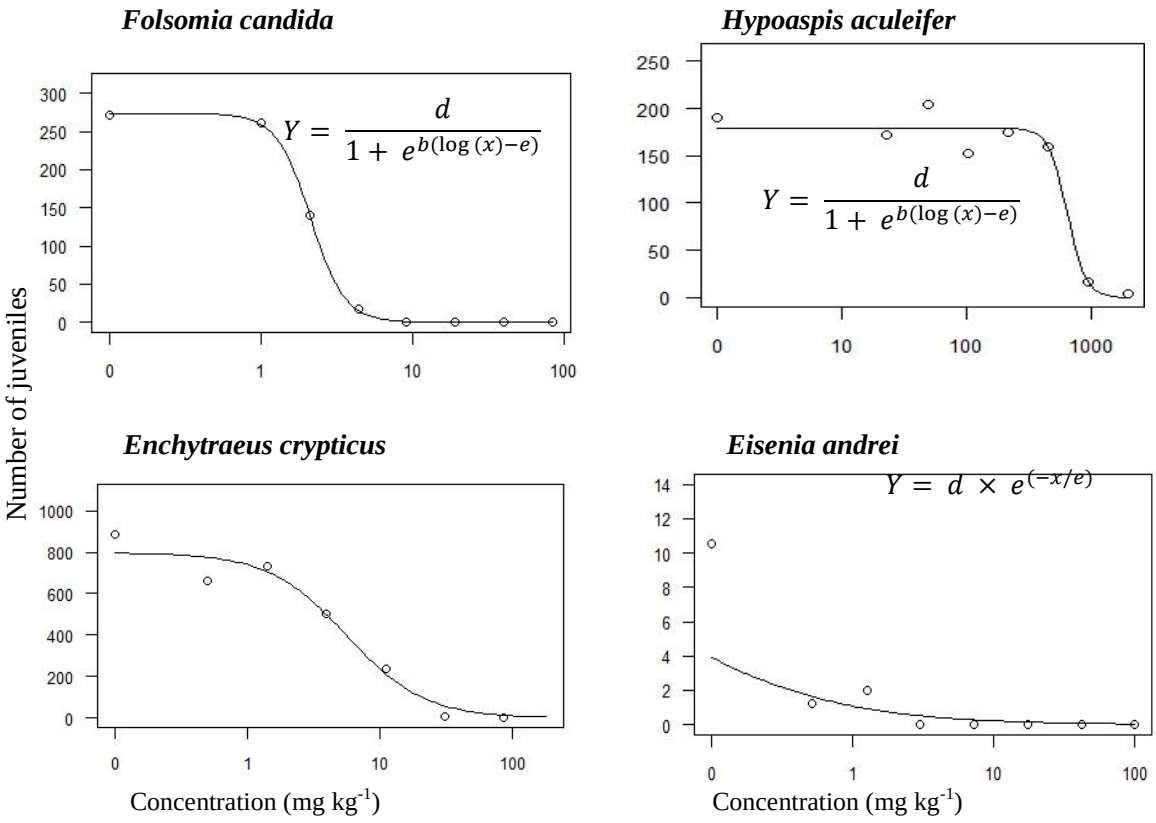


Figure 6: Sublethal effects of acetamiprid on *F. candida*, *E. crypticus*, *E. andrei* and *H. aculeifer* using reproduction as endpoint. Graphs show concentration-response relationships as predicted by the model

3.5.2 Relating Lethal and Sublethal effects

Generally for the four invertebrates tested, reproduction appears to be more sensitive than survival for all estimated endpoint parameters as presented in Figure 3 and Table 4, which is the consensus for most ecotoxicity studies given the relevance of reproductive endpoints for assessing population-level effects of pesticides (Stark & Banks 2003; van Gestel 2012). When chronic LC_{50}/EC_{10} ratios, also called sublethal sensitivity index (SSI), are compared among these organisms, *F. candida* has the lowest value meaning survival and reproduction is adversely affected at similar concentrations of acetamiprid. It could then be inferred that *F. candida* maintains population growth up till the occurrence of lethal effects when exposed to concentrations of acetamiprid. However, for *E. crypticus* and *H. aculeifer*, we can conclude that from their high SSI values, survival takes on higher priority than reproduction when exposed to increasing acetamiprid concentrations (Crommentuijn et al. 1995).

3.5.3 Comparing concentration-response modeling with hypothesis testing

The use of hypothesis testing in generating ecotoxicity data for regulatory testing have been criticized and the replacement of NOEC/LOEC with point estimates have been proposed due to problems in the statistical and testing procedures used in determining its values (Fox 2008; Warne & Dam 2008). In most cases, NOECs/LOECs are highly variable and inaccurate; NOECs normally correspond to 10 – 30% effect concentrations, while LOECs correspond to > 30% effect concentrations (Warne & Dam 2008). However, this study found NOEC and LOEC values to correspond to EC_{10} & EC_{50} values respectively (see Table 4) though estimated EC values were more conservative and hence more accurate. These results show that NOEC/LOEC values can still be generated as an alternative to regression-based EC_X method provided eco-toxicity tests are properly designed and conducted with a high degree of precision. This could be achieved through increased replication, choosing an appropriate lowest treatment concentration, choosing a spacing factor not exceeding 3.2, etc.

3.5.4 Initial considerations for Environmental Risk Assessment

Following the risk assessment scheme defined by the European and Mediterranean Plant Protection Organization (EPPO 2003b) in order to assess the chronic toxicity of acetamiprid to soil fauna, predicted environmental concentrations, calculated as 14 & 28-day TWAC, was compared to EC_{10} values (PEC/EC_{10}) to obtain exposure-toxicity ratios (ETRs) for all test invertebrates except *E. andrei*. ETRs obtained were found to be less than the trigger value of 0.2 preset by the EU (EC 1991). It can therefore be maintained with some measure of confidence that formulated acetamiprid (20% SG) poses low risk to field populations of *F. candida*, *E. crypticus* and *H. aculeifer* following a single maximum application rate of 100 g a.i ha^{-1} by foliar spraying to young citrus plants.

Regarding risk characterization, since *F. candida* may not be the most sensitive species in the soil ecosystem, and calculated hazard quotient i.e. PEC/PNEC ratio was found to be greater than 1, it can be concluded initially that the effect of acetamiprid in the soil compartment is unacceptable pending further ecotoxicity testing that can lead to the refinement of the PEC and PNEC. The PEC/PNEC ratio as defined by the EU Technical guidance document on the risk assessment of chemicals (EC 2003) was used to extrapolate from laboratory results to other soil organisms since derived PNEC can be taken as the concentration of acetamiprid below which an unacceptable effect is unlikely to occur in the soil ecosystem.

3.6 CONCLUSION

Both survival and reproduction endpoint parameters showed that *Folsomia candida* was highly sensitive to acetamiprid (LC_{50}: 0.40 mg kg^{-1}; EC_{50}: 0.29 mg kg^{-1}). In a general manner, survival endpoint parameters proved to be less sensitive compared to reproduction as expected, except for

F. candida where survival and reproduction occurred at very similar concentrations (SSI = 2). The use of hypothesis testing in ecotoxicological procedures is also supported, as determined NOEC/LOEC values corresponded with estimated LC/EC values in this study. Finally, while the risk of acetamiprid to *F. candida*, *E. crypticus* and *H. aculeifer* was shown to be low following a single maximum application of 100 g a.i ha^{-1} (Epik 20% SG), hazard quotient of > 1 indicates the unacceptable risk of acetamiprid to the soil compartment.

3.7 REFERENCES

Bezchlebová, J., Cernohlavkova, J., Lana, J., Sochova, I., Kobeticova, K. & Hofman, J. (2007). Effects of toxaphene on soil organisms. *Ecotoxicology and Environmental Safety*, 68(3): 326–334.

Crommentuijn, T., Doodeman, C.J.A.M., Van Der Pol, J.J.C., Doornekamp, A., Rademaker, M.C.J., Van Gestel, C.A.M. (1995). Sublethal sensitivity index as an ecotoxicity parameter measuring energy allocation under toxicant stress: application to cadmium in soil arthropods. *Ecotoxicology and Environmental Safety*, 31: 192 – 200.

Daam, M.A., Leitao, S., Cerejeira, M.J., Sousa, J.P. (2011). Comparing the sensitivity of soil invertebrates to pesticides with that of *Eisenia fetida*. *Chemosphere*, 85(6): 1040–1047.

EC (1991). Council Directive of July 1991 concerning the placing of plant protection products on the market.

EC (2004). SANCO/1392/2001 – Final. Review report for the active substance acetamiprid. , 6(February 2000), pp.1–34.

EC (2003). Technical Guidance Document on Risk Assessment.

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

EPPO (2003a). EPPO Standards. Environmental risk assessment scheme for plant protection products. Chapter 4: Soil. *OEPP/EPP0 Bulletin*, 33: 147–149.

EPPO (2003b). EPPO Standards. Environmental Risk Assessment Scheme for Plant Protection Products. Chapter 8: Soil Organisms and Functions. *Bulletin OEPP/EPPO*, 33: 147–149.

Fountain, M.T. & Hopkin, S.P. (2005). *Folsomia candida* (Collembola): A "standard" soil arthropod. *Annual review of Entomology*, 50: .201–222.

Fox, D.R. (2008). NECS , NOECS and the ECx. *Australasian Journal of Ecotoxicology*, 14: 7–9.

Frampton, G.K. et al., (2006). Effects of Pesticides on Soil Invertebrates in Laboratory Studies: A review and analysis using species sensitivity distributions. *Environmental Toxicology and Chemistry*, 25(9): 2480–2489.

Giraudoux, P. (2013). Pgirmess: Data analysis in Ecology. Available at: http://cran.r-project.org/package=pgirmess.

Goulson, D. (2013). REVIEW: An overview of the environmental risks posed by neonicotinoid insecticides D. Kleijn, ed. *Journal of Applied Ecology*, 50(4): 977–987.

El Hassani, A.K., Dacher, M., Vincent, G., Michel, L., Monique, G. & Catherine, A. (2008). Effects of sublethal doses of acetamiprid and thiamethoxam on the behavior of the honeybee (*Apis mellifera*). *Archives of Environmental Contamination and Toxicology*, 54(4): 653–661.

ISO (1998). Soil quality - Effects of pollutants on earthworms (Eisenia fetida). Part 2: Determination of Effects on Reproduction, No 11268-2. ISO, Geneve. , (I).

ISO (2003). Soil quality - Effects of pollutants on Enchytraeidae (Enchytraeus sp.) - Determination of effects on reproduction and survival. , 2003.

ISO (1999). Soil quality - Inhibition of reproduction of Collembola (Folsomia candida) by soil pollutants, No. 11267. ISO, Geneve. , pp.1–16.

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(7): 2897–2908.

Jeschke, P. & Nauen, R. (2010). Neonicotinoid Insecticides. In L. Gilbert & S. Gill, eds. *Insect Control: Biological and Synthetic agents*. San Diego, USA: Elsevier B.V., pp. 61–103.

Linders, J. et al.,(2000). IUPAC Technical Report: Foliar interception and retention values after pesticide application. A proposal for standardized values for environmental risk assessment. *Pure and Applied Chemistry*, 72(11): 2199–2218.

Martikainen, E. (1996). Toxicity of dimethoate to some soil animal species in different soil types. *Ecotoxicology and Environmental Safety*, 33(2): 128–136.

OECD (1984). Earthworm Acute Toxicity Tests. OECD guideline for testing of chemicals (207). Paris, pp.1–9.

OECD (2006). Current approaches in the statistical analysis of ecotoxicity data: a guidance to application, OECD Series On Testing And Assessment, No 54, ENV/JM/MONO(2006)18.

OECD (2008). Predatory mite (*Hypoaspis (Geolaelaps) aculeifer*) reproduction test in soil. OECD Guidelines for Testing Chemicals 226, Paris.

Owojori, O.J., Waszak, K. & Roembke, J. (2014). Avoidance and reproduction tests with the predatory mite *Hypoaspis aculeifer*: effects of different chemical substances. *Environmental Toxicology and Chemistry / SETAC*, 33(1): 230–237.

Palumbo, J., Horowitz, A. & Prabhaker, N. (2001). Insecticidal control and resistance management for *Bemisia tabaci*. *Crop Protection*, 20(9): 739–765.

Pelosi, C., Barot, S., Capowiez, Y., Mickael, H. & Vandenbulcke (2013). Pesticides and earthworms. A review. *Agronomy for Sustainable Development*, 34(1): 199–228.

R Core Team (2014). R - A language and environment for statistical computing. Available at: http://www.r-project.org/.

Racke, K.D. (2003). Release of pesticides into the environment and initial concentrations in soil, water, and plants. *Pure and Applied Chemistry*, 75(11-12): 1905–1916.

Ritz, C. & Streibig, J.C. (2005). Bioassay Analysis using R. *Journal of Statistical Software*, 12(5).

Rombke, J. (2003). Ecotoxicological laboratory tests with enchytraeids : *Pedo*, 47: 607–616.

Siegel, S. & Castellan, J.N. (1988). *Non-parametric statistics for the behavioural sciences*, New York: MacGraw Hill International.

Smit, C.E., Moser, T. & Römbke, J. (2012). A new OECD test guideline for the predatory soil mite *Hypoaspis aculeifer*: results of an international ring test. *Ecotoxicology and Environmental Safety*, 82: 56–62.

Stark, J.D. & Banks, J.E. (2003). Population-level effects of pesticides and other toxicants on arthropods. *Annual Review of Entomology*, 48: 505–519.

Szczepaniec, A., Raupp, M.J., Parker, R.D., Kerns, D. & Eubanks, M.D. (2013). Neonicotinoid insecticides alter induced defenses and increase susceptibility to spider mites in distantly related crop plants. *PloS one*, 8(5), p.e62620.

Van Gestel, C.A.M. (2012). Soil ecotoxicology: state of the art and future directions. *ZooKeys*, 296(176): 275–296.

Warne, M.S.J. & Dam, R.V. (2008). NOEC and LOEC data should no longer be generated or used. *Australasian Journal of Ecotoxicology*, 14: 1–5.

Whitaker, J., Chaplow, J.S., Potter, E., Scott, W.A., Hopkin, S., Harman, M., Sims, I. & Sorokin, N. (2009). The comparative toxicity to soil invertebrates of natural chemicals and their synthetic analogues. *Chemosphere*, 76(3): 345–352.

Wilcox, D.C., Dove, B.S., McDavid, D.W. & Greer, D.B. (2002). UTHSCSA Image Tool version 3.00. *The University of Texas Health Science Center, San Antonio, Texas*. Available at: http://compdent.uthscsa.edu/dig/itdesc.html#What.

Zalom, F.G., Toscano, N.C. & Byrne, F.J. (2005). Managing resistance is critical to future use of pyrethroids and neonicotinoids. *California Agriculture*, 59(1): 11–15.

Chapter 4

4 GENERAL DISCUSSION

Jeschke et al (2011) stated that all neonicotinoid insecticides are grouped into the same mode of action class (Group 4A) as agonists of nicotinic acetylcholine receptors (nAChRs) by the insecticide resistance action committee (IRAC). Among the major categories of insecticides with the exception of pyrethroids, neonicotinoids show the highest selectivity for insect nAChRs compared to mammalian ones (Sánchez-Bayo 2012a). Neonicotinoids are also known to exhibit high and moderate toxicities to the survival of arthropods and worms respectively (Sánchez-Bayo 2012b). From the range-finding (14 days) and definitive (28 days) tests conducted in this study, the toxicity of acetamiprid and thiacloprid especially to *Folsomia candida* and *Enchytraeus crypticus* was shown to augment with an increase in exposure time. Although this situation is just as expected for systemic pesticides, acetamiprid and thiacloprid are non-persistent insecticides with average half-lives of 5 days and their metabolites have low toxicity to non-target soil invertebrates (EC 2004a; EC 2004b). However, constant uptake from pore water concentration and the irreversible neurotoxic mode of action of neonicotinoid insecticides can be a reasonable explanation for this observation (Sanchez-Bayo 2013).

Relative sensitivities of the four test invertebrates to thiacloprid and acetamiprid using both reproduction and survival endpoints were the same and can be expressed as *F. candida* > *E. andrei* > *E. crypticus* > *H. aculeifer* where *F. candida* was the most sensitive organism. This outcome is probably due to the fact that the route of exposure and mode of action of these two neonicotinoids to each of the tested invertebrates are similar. Compared to thiacloprid, acetamiprid showed higher toxicity to the test invertebrates. For instance, based on median lethal concentrations in the range-finding tests, acetamiprid was 82 times more toxic to *F. candida*. Similarly for the chronic reproduction tests, acetamiprid showed higher toxicity to all test invertebrates except for *E. andrei* where the 8-week reproduction test was invalid.

It is important to note that while the definite cause(s) responsible for the existing differential toxicity between acetamiprid and thiacloprid is not fully known, several reasons can be suggested using relevant information from scientific literature. Most times, these reasons often originate from differences present in their chemical structures. The chemical structure of a particular toxicant affects its toxicity as the chemical structure is normally responsible for the mechanism of action, physico-chemical, toxicokinetic and toxicodynamic properties of a particular compound (Blaauboer, 2003). For instance, the rate of uptake of acetamiprid in pore water by soil invertebrates is bound to exceed that of thiacloprid. This is because acetamiprid,

like other non-cyclic compounds is more hydrophilic than thiacloprid which has a cyclic structure. In addition, the presence of a sulphur atom in the ringed structure of thiacloprid may contribute to its lower toxicity to the test invertebrates. This is because of the presence of non-carbon atoms in ringed compounds may affect toxicity. The relative potency of these atoms is as follows: Nitrogen > Carbon > Sulphur > Oxygen (Matsuda *et al*. 2001).

Regarding the mechanism of action, Tan *et al* (2007), in a study on the agonist actions of neonicotinoids on nAChRs receptors of cockroaches discovered that non-cyclic neonicotinoids (e.g. acetamiprid, clothianidin, dinotefuran) were more effective agonists compared with heterocyclic neonicotinoids (e.g. thiacloprid, imidacloprid, etc). This finding could be a reasonable explanation for the outcome of this study where acetamiprid was generally more toxic to the four soil invertebrates.

4.1 REFERENCES

Blaauboer, B.J., (2003). The integration of data on physico-chemical properties, in vitro-derived toxicity data and physiologically based kinetic and dynamic as modelling a tool in hazard and risk assessment. A commentary. *Toxicology letters*, 138(1-2): 161–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12559700.

EC (2004a). European Commission review report for the active substance thiacloprid SANCO/4347/2000 - Final. *Commission Working Document.*

EC (2004b). European Commission review report for the active substance acetamiprid SANCO/1392/2001 - Final. *Commission Working Document.*

Matsuda, K., Buckingham, S.D., Kleier, D., Rauh, J.J., Grauso, M. & Sattelle, D.B., (2001). Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends in pharmacological sciences*, 22(11): 573–80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11698101.

Sánchez-Bayo, F. (2012a). Ecological Impacts of Insecticides, Insecticides - Advances in Integrated Pest Management, Dr. Farzana Perveen (Ed.), ISBN: 978-953-307-780-2, InTech, Available from: http://www.intechopen.com/books/insecticides-advances-in-integrated-pest-management/ecological-impacts- of-insecticide

Sánchez-Bayo, F. (2012b) Insecticides Mode of Action in Relation to Their Toxicity to Non-Target Organisms. Journal of Environmental and Analytical Toxicology S4:002. doi:10.4172/2161-0525.S4-002

Sánchez-bayo, F., Tennekes, H.A. & Goka, K., (2013). Impact of Systemic Insecticides on Organisms and Ecosystems. In S. Trdan, ed. *Insecticides - Development of Safer and More Effective Technologies*. InTech, pp. 367 – 416. Available at:

http://www.intechopen.com/books/insecticides-development-of-safer-and-more-effective-technologies/impact-of-systemic-insecticides-on-organisms-and-ecosystems.

Tan, J., Galligan, J.J. & Hollingworth, R.M. (, 2007). Agonist actions of neonicotinoids on nicotinic acetylcholine receptors expressed by cockroach neurons. *Neurotoxicology*, 28(4): 829–842. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17561262 [Accessed July 22, 2014].