DOCTOR OF PHILOSOPHY

Acetamiprid in the environment

The impact of commercial neonicotinoid formulations on soil function and ecology

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Acetamiprid in the environment: The impact of commercial neonicotinoid formulations on soil function and ecology

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Declaration

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

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With this in mind, I want to extend my thanks to everyone who has walked alongside me through the last five years, with celebration and laughter through the highs, and commiseration and support through the lows, I couldn't have done it without every single last one of you.

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And finally, to my family, and those we have lost along the way, this is for you.

Thesis Summary

Neonicotinoid pesticides have been used worldwide since the early 1990's. Despite their high target efficacy, and their low mammalian toxicity their use has been severely restricted across EU member countries and the UK. The use of neonicotinoid insecticides has been linked, on numerous occasions, to various deleterious effects in non-target populations, including reductions in honeybee queen production, increases in songbird mortalities, and decreases in earthworm activity. Despite neonicotinoid seed coatings leaving up to 90 % of the applied treatments in the soil, the effects of neonicotinoids on soil communities, functions and processes are vastly underrepresented in the literature. Even with the shift away from the use of seed dressings, systemic pesticides are still readily incorporated into the soil system.

The primary objective of this thesis was to assess the impact of the neonicotinoid acetamiprid on soil systems and soil ecology, accounting for realistic practices and agriculturally relevant management where possible. This thesis starts by presenting a review of our state of knowledge around neonicotinoids in the soil system, highlighting possible research areas and unanswered questions. It then leads into an analysis of the physicochemical behaviour and persistence of a selection of commercial formulations under different soil organic matter treatments (Chapter 2). Our findings demonstrated that both the different chemical formulation and organic matter treatment had significant influence on some of these behaviours within soil. Building upon these results we assess the biological influence of an agricultural formulation under true field conditions (Chapter 3), in this case finding that seasonal variation was a much larger driver in regulating soil-dwelling communities.

The studies presented in Chapter 4 & 5 continue to explore the biological responses to acetamiprid exposure, this time on a single target species (*Lumbricus terrestris*) under a mesocosm set-up. We employed a selection of commercial pesticide formulations whilst also including isolated active ingredients to allow for different chemical interactions to be

disentangled. Across these experiments we once again found differences in response across the chemical treatment, as well as an overall significant response to acetamiprid exposure. When combined, these findings begin to reveal the true consequences of neonicotinoid use, as well as highlighting the need to employ realistic and relevant conditions, chemicals, and test species.

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Introduction

Background

One third of the world's soils are now classified as degraded, and as global intensive agriculture continues to increase it is estimated that this number may increase to 90 % by 2050 (UNFAO, 2015). As a finite resource it is vital that we understand the consequences our agricultural practices could be having, especially on the long-term sustainability of food-productive agricultural land. These increases in reliance on agricultural resources can lead to changes in agricultural practices. In recent decades we have seen a shift towards systemic pesticides, lower mammalian toxicity and hugely efficient towards target pests, but unfortunately indiscriminate in their invertebrate toxicity. One family of systemic pesticides, neonicotinoids, was introduced to the commercial market in the 1990s, quickly becoming the most popular family of insecticides, accounting for 24% of the global market in 2016 (Woodcock et al., 2016). Unfortunately, as their use continued an increasing amount of research emerged suggesting their contribution to the global decreases in pollinators and other flying insects.

By 2015, three of the major neonicotinoid products were banned across the EU; the mounting evidence of their sublethal pollinator interactions and public outcry at their unintended effects led to this change in policy to become permanent in 2018 (European Commission, 2018a). Whilst policy and agricultural practices have changed based upon this evidence, there is still much about the effects and behaviours of neonicotinoids that we do not understand. Traditionally applied as a seed coating, neonicotinoids would often leave up to 90% of the chemical treatment in the soil (Goulson, 2013). Despite this level of possible contamination and exposure, research has vastly underrepresented the effects of these chemicals on the soil ecosystem.

Whilst pollinators have been quantified as contributing to every 1 in 3 mouthfuls of food, the contribution of soil invertebrates such as earthworms to the delivery of ecosystem services is vast (Blouin et al., 2013; Miglani and Bisht, 2019). Earthworms, along with other species of soil biota assist in the regulation and turnover of organic matter and nutrients within the soil, contributing to a vital soil function and a wide range of ecosystem services (Bhadauria and Saxena, 2010; George et al., 2017). Although there is relatively little research examining the impacts of neonicotinoids on soil mesofauna the current findings suggest that much like the pollinators, earthworms are suffering reduced growth rates, decreased reproductive outputs, and therefore their long-term population sustainability is at risk (Wang et al., 2015; Saggioro et al., 2019).

Broadly, this thesis aims to assess the impacts of the neonicotinoid acetamiprid on soil systems, investigating the physical behaviours of the chemical and ecological responses to its exposure (Fig. i).

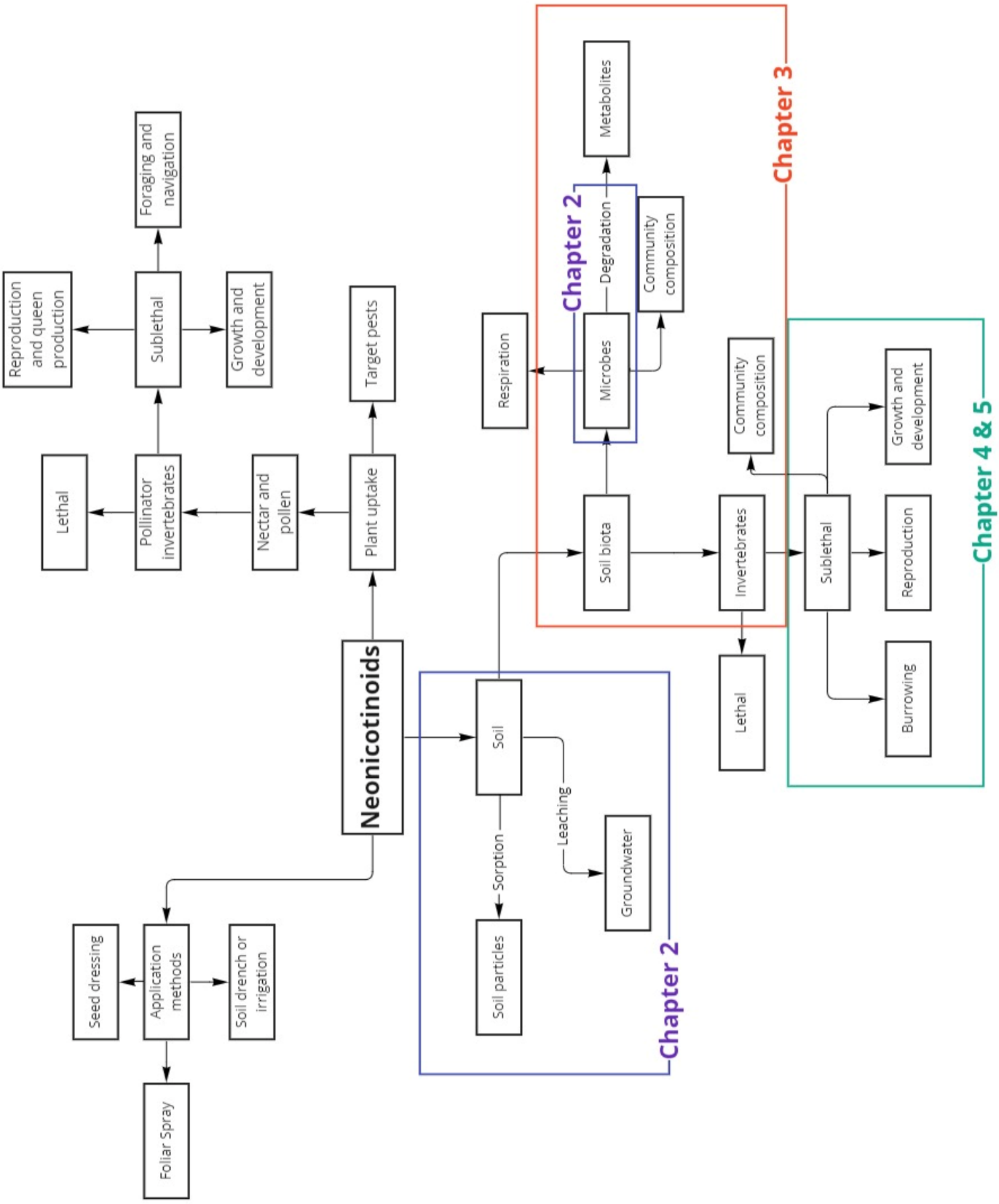


Figure i. Schematic of neonicotinoid exposure and interactions within the environment and how the chapters in this thesis will approach each area.

Research objectives

In order to assess the effects of the neonicotinoid acetamiprid in the environment, the main research objectives of this thesis were:

1. To investigate the influence of additional ingredients in commercial formulations, such as surfactants, adjuvants and other additives, on the behaviour and environmental persistence of acetamiprid products;
2. To assess how different farming management strategies and regimes can influence acetamiprid retention in the soil; and
3. To investigate the ecological impact of acetamiprid products on non-target soil fauna.

Since the key studies in this thesis were conducted after the moratorium and subsequent ban on certain neonicotinoid products in 2018, the product of primary focus for this PhD thesis was the neonicotinoid Acetamiprid.

To achieve the first objective, the studies featured in chapters two, four, and five investigated various commercial compounds containing acetamiprid and compared their outcomes to the pure active ingredients.

To achieve the second objective, we examined the influence of various soil organic matter regimes on the mobility and persistence of the neonicotinoid formulations in chapter two. We also ensured that all products were applied at or below standard application rate, therefore imitating real-life application rates and residue levels.

The third object was achieved across experiments featured in chapters three, four and five. The primary focus of these chapters were to investigate the impacts of acetamiprid application on the biological presence and function within soils. Chapter three presents a broad overview of the impacts of an agricultural formulation on mesofauna and microbial communities, whilst chapters four and five demonstrate a deeper investigation into differences in chemical

formulation on specific lethal and sub-lethal outputs on a single test organism, *Lumbricus terrestris*.

Experimental chapter information

The experimental chapters within this thesis have been written in the style of journal manuscripts. Each chapter's title page includes details of the authors and co-authors, author contributions, and current publication status of each manuscripts (published / accepted / submitted / not yet submitted). This thesis contains four experimental data chapters, located in chapters 2 - 5 of this document. For clarity the data chapters will be referred to in the order they appear in this thesis. The titles of these chapters are as follows:

Chapter 2: Acetamiprid transport and biodegradation in a sandy loam with contrasting soil organic matter contents: A comparison of commercial neonicotinoid formulations

Chapter 3: Seasonal variation is a bigger driver of soil faunal community composition than exposure to neonicotinoid pesticides

Chapter 4: Sub-lethal mass loss and food avoidance in earthworms (*Lumbricus terrestris*) in acetamiprid-treated soil

Chapter 5: Preferential attraction of earthworms (*Lumbricus terrestris*) to acetamiprid-treated soil

Chapter overviews

Chapter 1- Neonicotinoids in the environment: A review of the ecological implications and environmental fates of neonicotinoid insecticides.

An up-to-date review of the available literature, examining the impacts and behaviours of neonicotinoid pesticides within the environment. The first section introduces an overview of neonicotinoid compounds, examining their chemistry and current usage. Within the second section we introduce the environmental behaviours of the compounds, specifically their

persistence and mobility within soil. We then expand into exploring the influence of neonicotinoid exposure on soil biology, including a more in-depth review on the impacts on earthworm behaviours, mortality and alterations to ecosystem functions. This is then followed by an examination of the literature quantifying the degradation pathways and influence of soil microbiota in the metabolism of neonicotinoid products. In light of current legislation and product registrations, we then discuss the overall state of knowledge and identify any significant research gaps.

Chapter 2- Acetamiprid transport and biodegradation within agricultural soils: Comparison of commercial formulations under different soil organic matter regimes.

This chapter investigates the differences in mobility and biodegradation of different acetamiprid formulations under three soil organic matter regimes in a sandy-loam soil. Using radiolabelled acetamiprid, we collected data examining the biodegradation, sorption and leaching of three acetamiprid-containing mixtures, two commercially available formulations and the pure active ingredient. With this data we began to examine differences in chemical formulation as a result of the commercial additives, including surfactants, adjuvants and emulsifiers, and theorise how these differences may impact their environmental mobility and persistence. Our results demonstrate significant differences in chemical behaviours across the commercial formulations and organic matter contents; indicating the need to account for realistic farming practices and management within scientific research.

Chapter 3- Seasonal variation is a bigger driver of soil faunal community composition than exposure to neonicotinoid pesticides.

Building upon the findings from the previous chapter, this study begins to assess the impacts of neonicotinoids on soil biology; specifically under realistic field conditions. Employing the benefits of high-throughput 16S sequencing and traditional Tullgren funnel

invertebrate extractions, we identified no significant pesticide-driven changes, instead noting the importance of seasonal variation in regulating the shifts in soil communities. Combining these findings with un-targeted metabolomic analysis we concluded that a single exposure event of an acetamiprid-containing formulation does not have any significant influence on the biological function of soil.

This study truly highlights the need for chemical exposure studies to be conducted under conditions that are as close to true-practice as possible; including the use of agriculturally relevant formulations, applied at realistic rates and using realistic application methods

Chapter 4- Sub-lethal mass loss and food avoidance in earthworms (Lumbricus terrestris) in acetamiprid-treated soil.

In chapter four, I begin to explore the impacts of different acetamiprid formulations on a specific non-target organism. Using earthworms (*Lumbricus terrestris*), I examine the effects of a selection of commercially available pesticide formulations on the mortality, food avoidance and subsequent mass changes in the earthworms over a ten-week period. Using the corresponding active ingredients, solely and in combination, we were able to isolate any possible influence from the surfactants, adjuvants or emulsifiers featured in the commercial mixtures. We ensured that the application of all treatments was in the equivalent range of relevant residues identified within the available literature.

Findings from this study detected significant differences in both the mass change and the levels of food avoidance of the earthworms across the different chemical formulations tested. These results demonstrate that whilst the formulations may not induce any significant changes to mortality levels, the alterations to earthworm health and activity could have substantial consequences to sustaining soil health.

Chapter 5- Preferential attraction of earthworms (Lumbricus terrestris) to acetamiprid-treated soil.

Chapter five builds upon the findings drawn from chapter four; using the same treatment selection and test organism I monitored the effects of acetamiprid exposure on earthworm burrowing and avoidance behaviours. Using an adapted ISO recommended attraction/avoidance methodology, we assessed the influence of the commercial pesticide formulations and their constituent active ingredients (acetamiprid and triticonazole) on *Lumbricus terrestris,* finding that under our study conditions that the test specimens showed a preferential attraction towards the chemically treated soils. Modified Evan's boxes were used to monitor the burrowing behaviours of the earthworms once exposed to the same variety of pesticide treatments. Through the use of visual observations and measurements we were not able to determine any significant changes in burrowing behaviours or burrow formation. This data again demonstrates the need to better understand the effects of pesticides beyond direct mortality; especially when it concerns important keystone species such as earthworms.

"The balance of nature is not a status quo; it is fluid,

ever shifting, in a constant state of adjustment.

Man, too, is part of this balance."

Rachel Carson, The Silent Spring

Chapter I-

Neonicotinoids in the environment: A review of the ecological implications and environmental fates of neonicotinoid insecticides

1.1. Abstract

Neonicotinoids, once the most popular family of systemic insecticides, has over the last few years been associated with various unintended ecological losses. From reductions in pollinator and other invertebrate numbers, losses in songbird populations, through to changes in vital earthworm behaviours; neonicotinoid use has become synonymous with negative ecological and environmental consequences. This review aims to investigate the largely unexplored areas of neonicotinoid use, those felt and experienced below the surface of the soil. Neonicotinoids have frequently gone hand-in-hand with pollinator research; the systemic uptake of the pesticide into nectar and pollen providing a perfect exposure route to many beneficial insects. However, more recent findings have begun to suggest that large portions of the applied chemicals may actually remain within the soil and therefore be also interacting with soil-dwelling invertebrates.

The focus of this literature review is spread across two main areas, 1) to examine the physicochemical behaviour of neonicotinoid insecticides within the soil, and 2) to discuss the implications of these behaviours on non-target soil biota. We found that across all registered neonicotinoids the measures of persistence, and the length of the chemical half-life vary greatly, often influenced by a variety of abiotic factors such as soil type and texture, as well as biotic influences including rainfall and average temperatures. We have also identified substantial discrepancies across laboratory and field investigations, as well as evidence suggesting chemical influences from additional ingredients featured in commercial formulations, as well as chemical interactions between co-applied products.

These areas of question further provide opportunities for future research, indicating the real need for investigations conducted under realistic conditions with relevant agrochemical formulations, as well as the exploration of the ecological effects beyond that of pollinator species.

1.2. Introduction to neonicotinoids

Neonicotinoids are a family of new systemic agrochemicals, used for the protection of agricultural crops from biting and sucking pest insects (Tomizawa and Casida, 2005). Since the global commercial distribution of imidacloprid in the early 1990s, neonicotinoid insecticides have rapidly become one of the most popular agrochemicals worldwide (Jeschke et al., 2011; Woodcock et al., 2016). As of 2016, neonicotinoids accounted for 24 % of insecticide sales worldwide, with an average market value of $1.5 billion per year (Woodcock et al., 2016). The major drivers for the surge in popularity being their ease of application and high invertebrate toxicity.

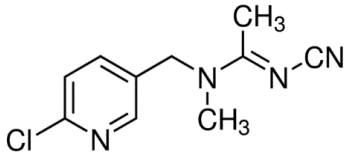
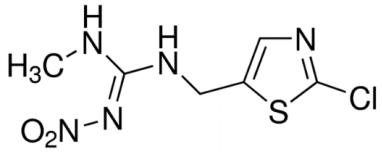
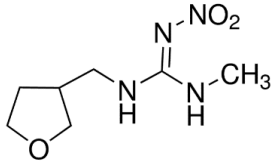
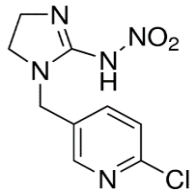
There are currently eight commonly used neonicotinoid agrochemicals on the global market registered for use. These active ingredients can be split between three major chemical groups; N-nitroguanidines (imidacloprid, clothianidin, thiamethoxam and dinotefuran), nitromethylenes (cycloxaprid and nitenpyram), and the N-cyanoguanidines (thiacloprid and acetamiprid) (Table 1.1). Each of these three chemical groups cause differing levels of toxicity, with the N-nitroguanidine imidacloprid being almost 800 times more toxic to honeybees than thiacloprid (N-cyanoguanidine) (Goulson, 2013; van Gestel et al., 2017). Despite their chemical differences, all neonicotinoids act as invertebrate nerve-disrupters (Maienfisch et al., 2001; Pandey and Mohanty, 2015). Upon contact or ingestion of the neonicotinoid, it binds strongly to the nicotinic acetylcholine receptors (nAChRs), disrupting the nerves of both target and non-target invertebrates (Downing and Grimwood, 2017; van Gestel et al., 2017).

Neonicotinoid pesticides are currently considered to be one of the major recent drivers of pollinator declines (Blacquière et al., 2012; Whitehorn et al., 2012; Rundlöf et al., 2015). The majority of neonicotinoids can be applied directly to the soil, either as a seed coating or as a soil drench in the form of water-dispersible granules, reducing the risk of human exposure (Žabar et al., 2012; Yong Li et al., 2018). The prophylactic use of neonicotinoid treated seeds

has offered significant resistance against agriculturally important pests, such as the peach potato aphid (*Myzus persicae*) and the cabbage stem flea beetle (*Psylliodes chrysoephala*), as well as being able to provide protection for the seed against soil dwelling organisms (Huseth and Groves, 2014; Scott and Bilsborrow, 2015).

Neonicotinoids are also applied as irrigation additives, foliar sprays or injections into the trunks of fruit trees (Banerjee et al., 2008; Bonmatin et al., 2015). According to packaging labels, neonicotinoid insecticides such as Redigo Deter contain 250 g L^{-1} clothianidin with a recommended application of 200 ml per 100 kg of seed. Despite these relatively low application levels, neonicotinoid treated seeds can contain some of the highest application concentrations of neonicotinoid chemicals within the field; with oilseed rape, beet and corn seeds being treated with up to 0.17, 0.9 and 1 mg of active ingredients respectively (Gibbons et al., 2015; Krischik et al., 2015). A single maize seed can be coated with up to 0.5 mg of clothianidin, which theoretically is enough active ingredient to kill 80,000 honeybees (Table 1.2; Krupke et al., 2012).

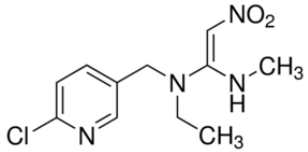
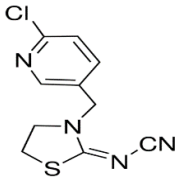
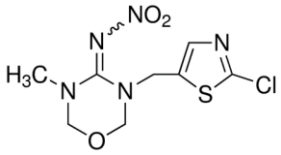
Table 1.1. Chemical and physical properties of the major neonicotinoid products

Compound	Structure [a]	Group and formulae	Agricultural products	Chemical properties [b]
Acetamiprid		N-cyanoguanidine $C_{10}H_{11}ClN_4$	-Mospilan -Assail -ChipcoTristar -Insyst	Solubility- 2950-4200 mg L^{-1} Melting point- 98.9 °C K_{OW}- $6.31x10^{00}$ pKa- 0.7 Vapour pressure- $1.73x10^{-04}$ mPa
Clothianidin*		N-nitroguanidine $C_6H_8ClN_5O_2S$	-Poncho -Dantosu -Dantop -Belay	Solubility- 340 mg L^{-1} Melting point- 176.8 °C K_{OW}- $8.04x10^{00}$ pKa- 11.1 Vapour pressure- $2.8x10^{-08}$ mPa
Dinotefuran		N-nitroguanidine $C_7H_{14}N_4O_3$	-Starkle -Safari -Venom	Solubility- 39830 mg L^{-1} Melting point- 107.5 °C K_{OW}- $2.82x10^{-01}$ pKa- 12.6 Vapour pressure- 0.0017 mPa
Imidacloprid*		N-nitroguanidine $C_9H_{10}ClN_5O_2$	-Confidor -Admire -Gaucho -Advocate	Solubility- 610 mg L^{-1} Melting point- 144 °C K_{OW}-$3.72x10^{00}$ pKa- Vapour pressure- $4.0x10^{-07}$ mPa

[a] sigmaaldrich.com

[b] Pesticide properties database, University of Hertfordshire

* Now banned for use in the EU

Nitenpyram		Nitromethylene $C_{11}H_{15}ClN_4O_2$	-Capstar -Guardian	Solubility- 59-84 ($x10^4$) mg L^{-1} Melting point- 82-84 °C K_{OW}- $2.19x10^{-01}$ pKa- 3.1 Vapour pressure- 0.0011 mPa
Thiacloprid		N-cyanoguanidine $C_{10}H_9ClN_4S$	-Calypso	Solubility- 184 mg L^{-1} Melting point- 136 °C K_{OW}- $1.82x10^{01}$ Vapour pressure-$3x10^{-07}$ mPa
Thiamethoxam*		N-nitroguanidine $C_8H_{10}ClN_5O_3S$	-Actara -Platinum -Cruiser	Solubility- 4100 mg L^{-1} Melting point- 139.1 °C K_{OW}- $7.41x10^{-01}$ Vapour pressure- $6.6x10^{-06}$ mPa

[a] sigmaaldrich.com

[b] Pesticide properties database, University of Hertfordshire

* Now banned for use in the EU

Table 1.2. Ecotoxicity values of neonicotinoid products to select terrestrial organisms. Data from The Pesticide Properties DataBase (2020).

	Acetamiprid		**Clothianidin**		**Dinotefuran**		**Imidacloprid**		**Thiacloprid**		**Thiamethoxam**	
Species	**Acute (LD_{50})**	**Chronic (NOEC)**	**Acute (LD_{50})**	**Chronic (NOEC)**	**Acute (LD_{50})**	**Chronic (NOEC)**	**Acute (LD_{50})**	**Chronic (NOEC)**	**Acute (LD_{50})**	**Chronic (NOEC)**	**Acute (LD_{50})**	**Chronic (NOEC)**
Rat (mammal)	146 mg kg^{-1}	> 51 mg kg^{-1} bw d^{-1}	> 500 mg kg^{-1}	32.7 mg kg^{-1} dw d^{-1}	> 2000 mg kg^{-1}	-	131 mg kg^{-1}	> 50 mg kg^{-1} bw d^{-1}	177 mg kg^{-1}	2.7 mg kg^{-1} bw d^{-1}	> 1563 mg kg^{-1}	62 mg kg^{-1} bw d^{-1}
Anas platyrhynchos (bird)	98 mg kg^{-1}	9.5 mg kg^{-1} bw d^{-1}	> 752 mg kg^{-1} bw d^{-1}	-	-	-	-	-	-	3.73 mg kg^{-1} bw d^{-1}	576 mg kg^{-1}	29.4 mg kg^{-1} bw d^{-1}
Eisenia fetida (earthworm)	9 mg kg^{-1}	1.26 mg kg^{-1}	13.21 mg kg^{-1}	2.5 mg kg^{-1}	4.9 mg kg^{-1}	-	10.7 mg kg^{-1}	≥ 0.178 mg kg^{-1}	105 mg kg^{-1}	0.185 mg kg^{-1}	> 1000 mg kg^{-1}	5.34 mg kg^{-1}
Folsomia candida (collembola)	-	0.27 mg kg^{-1}	-	-	-	-	-	-	-	10 mg kg^{-1}	-	-
Apis mellifera (honeybee)	Contact- 8.09 µg bee^{-1} Oral- 14.53 µg bee^{-1}	-	Contact- 0.044 µg bee^{-1} Oral- 0.004 µg bee^{-1}	-	Contact- > 0.023 µg bee^{-1}	-	Contact- 0.081 µg bee^{-1} Oral- 0.0037 µg bee^{-1}	-	Contact- 38.82 µg bee^{-1} Oral- 17.32 µg bee^{-1}	-	Contact- 0.024 µg bee^{-1} Oral- 0.005 µg bee^{-1}	-
Bombus terrestris (bumblebee)	Contact- > 100 µg bee^{-1} Oral- 22.2 µg bee^{-1}	-	Contact- 0.02 µg bee^{-1}	-	-	-	Contact- 0.218 µg bee^{-1} Oral- 0.038 µg bee^{-1}	-	Contact- > 100 µg bee^{-1}	-	Contact- 0.028 µg bee^{-1} Oral- 0.005 µg bee^{-1}	-
Osmia bicornis (mason bee)	Contact- 1.72 µg bee^{-1}	-	Oral- > 8.4 µg bee^{-1}		-	-	Contact- 0.031 µg bee^{-1}	-	Contact- 1.16 µg bee^{-1}	-	-	-

1.3. Soil

Up to 90 % of a neonicotinoid seed coating remains in the soil (Goulson, 2013), and the possibility exists for soil accumulation many times higher than the original concentration applied to the seed (Capowiez and Bérard, 2006; Goulson, 2013; De Lima e Silva et al., 2017). The contamination of soils and their associated fauna can be categorised via a combination of different exposure routes (Banerjee et al., 2008; Gupta et al., 2008a; Wang et al., 2015; Zhang et al., 2018). The localised area surrounding a treated seed will present a much higher level of acute exposure to soil organisms at the time of sowing (Girolami et al., 2009), with the compound leaching further through the soil profile over time (Liu et al., 2016; Rodríguez-Liébana et al., 2018). The sowing of neonicotinoid treated seeds can result in the production of contaminated dust through the use of seed drills (Marzaro et al., 2011; Girolami et al., 2013). This neonicotinoid dust can then be aerially deposited upon both soil and plants, increasing the spatial range of possible neonicotinoid contamination and exposure (Limay-Rios et al., 2016; Forero et al., 2017).

The residence time and risk of exposure for neonicotinoids in soils varies greatly, influenced by both changes in environmental conditions and soil characteristics (Karmakar et al., 2006; Liu et al., 2016; Castillo Diaz et al., 2017). Neonicotinoids are highly water soluble, intended to assist in the systemic uptake of the agrochemical in to all crop tissues (Huseth and Groves, 2014). This relatively high water solubility can lead to an increase in chemical mobility within the soil profile. Waterlogged and clay-rich soils could exhibit much higher residue levels due to their restricted mobility (Rexrode et al., 2003; Liu et al., 2015; Liu et al., 2016). The loss of neonicotinoids from a soil is considered as biphasic, commencing with a period of rapid loss, followed by a notably slower second phase, possibly illustrating the adsorption of the active substances to available soil particles (Papiernik et al., 2006; El-Hamady et al.,, 2009; Goulson, 2013).

The persistence of neonicotinoids within soil varies substantially across both compound used and soil type. 'Typical' reported half-life values range from a few weeks to three years. Despite many soil DT_{50} studies not mentioning the type of soils used for the investigations, there appears to be a wide and variable range of DT_{50} values across various spatial and chemical variables (Table 1.3). Post application, neonicotinoid pesticides can undergo various abiotic (chemical degradation, sorption, photo-degradation, volatilisation, surface runoff, leaching) and biotic (microbial degradation) processes, affecting their persistence and overall environmental impact (Oliver et al., 2005; Papiernik et al., 2006; Žabar et al., 2012; Phugare et al., 2013; Kurwadkar et al., 2014).

1.3.1. Soil processes

1.3.1.1. Chemical and biological degradation

Zhang et al. (2018) investigated the loss and degradation of three neonicotinoids (imidacloprid, clothianidin and thiacloprid) across four Chinese agricultural soil types (black soil, red soil, paddy soil and fluvo-aquic soil), attributing the majority of neonicotinoid loss to their use in respiration by soil microorganisms. Total degradation across all compound-soil combinations ranged from 25.4 % to 80.9 % with the highest degradation rates being recorded for thiacloprid throughout.

Work conducted by Liu et al. (2011), compared the degradation of four neonicotinoid compounds (acetamiprid, thiacloprid, imidacloprid and imidaclothiz) in sterilised and unsterilised soils. Their results suggested an increase in the rate and the level of degradation in unsterilised compared to sterilised soils, with 94 % and 98.8 % of acetamiprid and thiacloprid being degraded within 15 days in the unsterilised soils and only 21.4 % and 27.6 % of the two compounds being respectively degraded under sterile conditions.

The degradation of neonicotinoid compounds under chemically sterilised conditions was further investigated by Zhang et al. (2018). Their work demonstrated that, in the absence of microbial activity, neonicotinoids can be hydrolysed via amino and cyano hydrolysis. The rates of these mechanisms were strongly influenced by the pH and CEC (Cation Exchange Capacity) of the test soils, with the rate of hydrolysis increasing as the solution becomes alkaline (Liu et al., 2006; Zhang et al., 2018). Earlier work by Liu et al. (2006), found evidence to suggest that the presence of metal ions can act as a catalyst to the hydrolysis of neonicotinoids. Findings presented by Llorca and Cruz-Romero (1977), suggest that the presence of metal saturated clays, such as Na^{+}, Ca^{2+} and Al^{3+}clays, could react unstably, releasing OH^{-} ions, catalysing the hydrolysis of various chemicals. This release of ions could therefore increase the interlayer pH of the soil particles, further assisting the hydrolysis of neonicotinoids such as imidacloprid, clothianidin and thiamethoxam.

1.3.1.2. Sorption

As well as the chemical and biological degradation of neonicotinoid compounds, the insecticides can become unavailable as they are adsorbed onto particles within the soil (Papiernik et al., 2006; Carbo et al., 2007; Banerjee et al., 2008). Sorption of these compounds controls the amount available for transport. Neonicotinoids generally have a high solubility and a relatively low octanol-water partition coefficient, indicating a hydrophilic nature and a low potential for soil adsorption (Murano et al., 2018).

It is thought that the level of adsorption across different soils can be attributed to the organic matter and/or the clay content (Flores-Céspedes et al., 2002; Banerjee et al., 2008; Jin et al., 2016). Recent investigations have also shown that the addition of humic substances to the soils, can alter the sorption of certain compounds to the soil minerals (Murano et al., 2018). Murano et al. (2018), was able to show that the sorption of the neonicotinoid acetamiprid was

reduced through the addition of humic or fulvic acids. This reduction in sorption has been attributed to the hydrophobic interactions between humic/fulvic acid and humin, where the dissociated carboxyl and phenolic groups have reoriented to face the soil solution (Murano et al., 2018). Alternatively, work by Jin et al. (2016) found that the addition of organic bio-amendments, such as straw, biochar and manure increased the sorption capacity of the soil mixtures. The two studies indicate that whilst organic matter can evidently alter the transport and mobility of pesticides, only certain types can be used to stabilise soils contaminated with neonicotinoids.

1.3.1.3. Leaching

Neonicotinoids have relatively low octanol-water partition coefficients and high water solubility (Table 1.1), and have considerable potential to become water contaminants, through leaching and/or surface run-off. The sorption of these chemicals is one of the most important processes governing the rate of pesticide leaching within the soils.

As with other soil processes, leaching appears to be influenced by both the chemical composition of the neonicotinoid and environmental variables such as soil properties and climatic conditions (Huseth and Groves, 2014; de Perre et al., 2015; Fu et al., 2015). Neonicotinoid transport studies conducted by Radolinski et al. (2018) over a 33-day time period demonstrated that fine-particle soils transported over two orders of magnitude more thiamethoxam than coarse-texture soils. Radolinski et al. (2018) built upon findings from Karmakar et al. (2006), where sandy clay loam soils had the highest leaching potential (81.6 %) for thiamethoxam, compared to the lower potentials of sandy loam soils (69.5 %), silty clay loams (78.8 %) and loamy sands (55.7 %).

A large number of recent leaching studies have primarily focussed on the use of thiamethoxam (Gupta et al., 2008; Kurwadkar et al., 2013, 2014; Hilton et al., 2016; Wettstein

et al., 2016; Radolinski et al., 2018). As with sorption and degradation, the processing rates vary across the different neonicotinoid compounds, however, the leaching rates for neonicotinoids seem to show a strong correlation to their aqueous solubility values (Table 1.1) (Kurwadkar et al., 2014).

Aside from the chemical differences between the neonicotinoid compounds, their methods of application within agricultural systems are thought to be a major contributor to their leachability and rate of degradation (Wettstein et al., 2016). Compounds that are applied either directly to the soil or plant, such as soil drenches, irrigation additives or foliar sprays, may be more susceptible to run off and leaching as they are more readily incorporated into the aqueous phase of the soil. Topically applied compounds were significantly more vulnerable if applied directly before rainfall events (Anderson et al., 2015). In contrast, neonicotinoid compounds which are applied as seed dressings or in conjunction with a carrier matrix can be more persistent in the soil, with the kinetics dictating the release of active neonicotinoid ingredients from the mixture resulting in a < 50 % increase in persistence (Sarkar et al., 2012; Anderson et al., 2015; Wettstein et al., 2016). However, work by Wettstein et al. (2016) suggests that point-source applications, such as seed dressings, have a higher leaching potential when compared to diffuse (spray) applications, demonstrating that the significant differences in pesticide behaviours can be linked back to their initial application methods.

Table 1.3. Half-life studies for the major neonicotinoid compounds in a soil environment.

Compound	**DT_{50} (days)**	**Laboratory or Field**	**Soil type**	**Location**	**Reference**
Acetamiprid	1.6	Laboratory	NA	NA	University of Hertfordshire, 2017
Acetamiprid	3	Field	NA	NA	University of Hertfordshire, 2017
Acetamiprid	2.9	Field	NA	NA	Reported in Renaud et al. 2018
Acetamiprid	31-450	NA	NA	NA	Reported in Goulson, 2013
Clothianidin	545	Laboratory	NA	NA	University of Hertfordshire, 2017
Clothianidin	164	Field	Silt loam	Illinois, USA	de Perre et al. 2015
Clothianidin	955	Field	Silt loam	Illinois, USA	de Perre et al., 2015
Clothianidin	341	NA	NA	NA	Reported in de Perre et al. 2015
Clothianidin	277	Field	Sandy soil	Wisconsin, USA	USEPA, 2003

Clothianidin	1,386	Field	Clay loam	North Dakota, USA	USEPA, 2003
Clothianidin	Not determined due to limited dissipation during study (25 months)	Field	Silty clay loam	Saskatchewan, Canada	USEPA, 2003
Clothianidin	315	Field	Silt loam	Ohio, USA	USEPA, 2003
Clothianidin	365	Field	Silt loam	Ontario, Canada	USEPA, 2003
Clothianidin	121.1	Field	NA	NA	University of Hertfordshire, 2017
Dinotefuran	72	Field	NA	NA	University of Hertfordshire, 2017
Dinotefuran	75-82	NA	NA	NA	Reported in Goulson, 2013
Imidacloprid	187	Laboratory	NA	NA	University of Hertfordshire, 2017
Imidacloprid	174	Field	NA	NA	University of Hertfordshire, 2017

Imidacloprid	43.7	Laboratory	Coastal Alkaline	Canning, West Bengal	Sarkar et al. 2001
Imidacloprid	46.4	Laboratory	Lateric	Jhargram, West Bengal	Sarkar et al. 2001
Nitenpyram	8	NA	NA	NA	Reported in Goulson, 2013
Thiacloprid	1.3	Laboratory	NA	NA	University of Hertfordshire, 2017
Thiacloprid	18	Field	NA	NA	University of Hertfordshire, 2017
Thiacloprid	0.6-3.8	NA	NA	NA	Reported in EPA 2003
Thiacloprid	2.4-27.4	Field	NA	NA	Reported in EPA 2003
Thiacloprid	9-27	Field	NA	NA	Reported in Renaud et al. 2018
Thiacloprid	3.4 > 1,000	NA	NA	NA	Reported in Goulson, 2013

Thiamethoxam	121	Laboratory	NA	NA	University of Hertfordshire, 2017
Thiamethoxam	39	Field	NA	NA	University of Hertfordshire, 2017
Thiamethoxam	7-335	NA	NA	NA	Reported in Goulson, 2013
Thiamethoxam	40.9	Field	NA	Switzerland	Hilton et al. 2016
Thiamethoxam	22.2	Field	NA	Southern France	Hilton et al. 2016
Thiamethoxam	7.1	Field	NA	Switzerland	Hilton et al. 2016
Thiamethoxam	45.3	Field	NA	Sweden	Hilton et al. 2016
Thiamethoxam	60.9	Field	NA	Germany	Hilton et al. 2016

1.3.1.4. Photodegradation and solar volatilisation

The photodecomposition of neonicotinoids is a vitally important degradation pathway, as for compounds that are applied directly as sprays or drenches it is the first destructive process they undergo (Peña et al., 2011; Žabar et al., 2012; Vela et al., 2017; Aregahegn et al., 2018). The use of photodegradation has been investigated to assess its success in the removal of pesticides during waste water treatment (Peña et al., 2011). Peña et al. (2011) quantified the differences in degradation by photolysis between thiamethoxam and thiacloprid. Thiamethoxam showed a high intensity absorption band at 250-255 nm, meaning that it was able to absorb the tropospheric range of sunlight and was therefore susceptible to direct photolysis. In contrast thiacloprid does not exhibit any absorption above 290 nm, and was therefore not expected to undergo any direct photolysis (Peña et al, 2011).

More recent investigations have looked to assess the efficiency and feasibility of using solar heating techniques (solarisation and biosolarisation) as a restoration method for soils contaminated with neonicotinoids (Vela et al., 2017). Samples were taken for three neonicotinoids (acetamiprid, imidacloprid, thiamethoxam) across five different treatments (control, solarised, compost from sheep manure, meat-processing waste and sugar beet vinasse), sampled periodically during the 90 day study period. Across all treatment combinations, the highest degradation rates were found for the solarised treatment and the samples amended with meat-processing wastes, followed by the compost from sheep manure addition and sugar beet vinasse (both biosolarisation treatments). The results from this study suggest that the increase in temperatures caused by solarisation is the main factor influencing the increase in pesticide disappearance.

1.4. Soil biology

1.4.1. Soil mesofauna

Although neonicotinoids are intended to target sucking and biting crop pests (Simms et al., 2006), their use as seed treatments and soil additives, coupled with their long persistence times in soils (Goulson, 2013) implies that non-target soil fauna such as earthworms and collembola are likely to be at risk of chronic neonicotinoid exposure (Capowiez et al., 2006; Morrissey et al., 2015; Yang et al., 2017). Soil fauna plays a central role in the cycling of soil nutrients via bioturbation and assisting with decomposition. Any chemical contamination of their habitat can cause significant disruption in their contribution towards these vital ecosystem services (Capowiez and Bérard, 2006; Chagnon et al., 2015; Zaller et al., 2016; de Lima e Silva et al., 2017; Van Hoesel et al., 2017). The implications of pesticide-contaminated soils can range from variations in the physicochemical properties of the soil itself, to chronic, acute, lethal and sub-lethal effects exhibited by various soil organisms (Chagnon et al., 2015).

1.4.1.1. Earthworms

Earthworms in particular are often used as a bioindicator species, their high biomass in soils, ecological importance, and high sensitivity to environmental contaminants often making them suitable for testing at low realistic concentrations (Wang et al., 2015; Yang et al., 2017). Much like the effects of neonicotinoids on bees, the effects of neonicotinoid exposure on earthworms appears to be both physiological and behavioural, ranging across a variety of both lethal and sublethal effects (Capowiez et al., 2006; Zhang et al., 2014; Basley and Goulson, 2017; Chevillot et al., 2017; Liu et al., 2017; Van Hoesel et al., 2017).

When assessing the impact of chemical contamination, many primary investigations tend to begin by assessing how the contamination influences the survival of the test species. Whilst an assessment of mortality rates is an important factor in understanding the

environmental and ecological impacts of a chemical, it is not the only important parameter that should be measured and assessed. In this section we will explore the lethal and the sublethal effects recorded in earthworms when exposed to neonicotinoids. Whilst data is sparse in many of these areas, it will begin by examining the ecology and ecosystem services of earthworms, and then continue by discussing the influence neonicotinoid exposure can have on these provisions; ensuring to address both the differences in chemical compound and exposure methods, as well as differences in earthworm species and life history traits.

1.4.1.1.1. Ecology of earthworms in the British Isles

There are 31 species of earthworm recorded within the British Isles, 29 of which are known to be recorded within mainland Britain (Sims and Gerard, 1999; Carpenter et al., 2012; Sherlock, 2018). These species cover three main families Acanthrodrilidae, Lumbricidae, and Sparganophilidae. The most common is the Lumbricidae, accounting for 29 of these species (Table 1.4). Earthworms in general can be categorised by a combination of their burrowing and feeding habits, classifying them into specific ecotypes, as either epigeic, epi-endogeic, endogeic or anecic (Fig. 1.1; Bouché, 1977). Whilst these are recognised as the main ecological classifications for earthworms not all species will fall neatly into just one category, with some earthworms varying their burrowing behaviours or feeding preferences as a result of life stage and or environmental conditions within the soil (Fierer, 2019).

Epigeic individuals such as *Heliodrilus oculatus* generally reside in leaf litter, feeding on the decaying organic material. They have pigmented skin, as a result of living near/on the surface, and do not produce burrows as is normally characteristic of earthworms (Bouché, 1983; Fierer, 2019). Epi-endogeic species however are generally moderately larger than their true epigeic counterparts; they also generally reside slightly deeper in the soil, just beneath the

litter layer, and rarely produce deep burrows (Bouché, 1983; Bottinelli et al., 2020). True endogeic species such as *Allolobophora chlorotica* feed on and dwell in the soil (0-50 cm). They are also generally less pigmented than epigeic or epi-endogeic species due to residing deeper in the soil profile (Bouché, 1977; Bottinelli et al., 2020; Hallam and Hodson, 2020). Lastly, species such as *Aporrectodea longa* and *Lumbricus terrestris* are characterised as Anecic. Producing deep, permanent vertical burrows, they are often large in size and return to the surface in order to feed upon fresh litter and other organic materials (Table 1.4; Bouché, 1977; Hallam and Hodson, 2020).

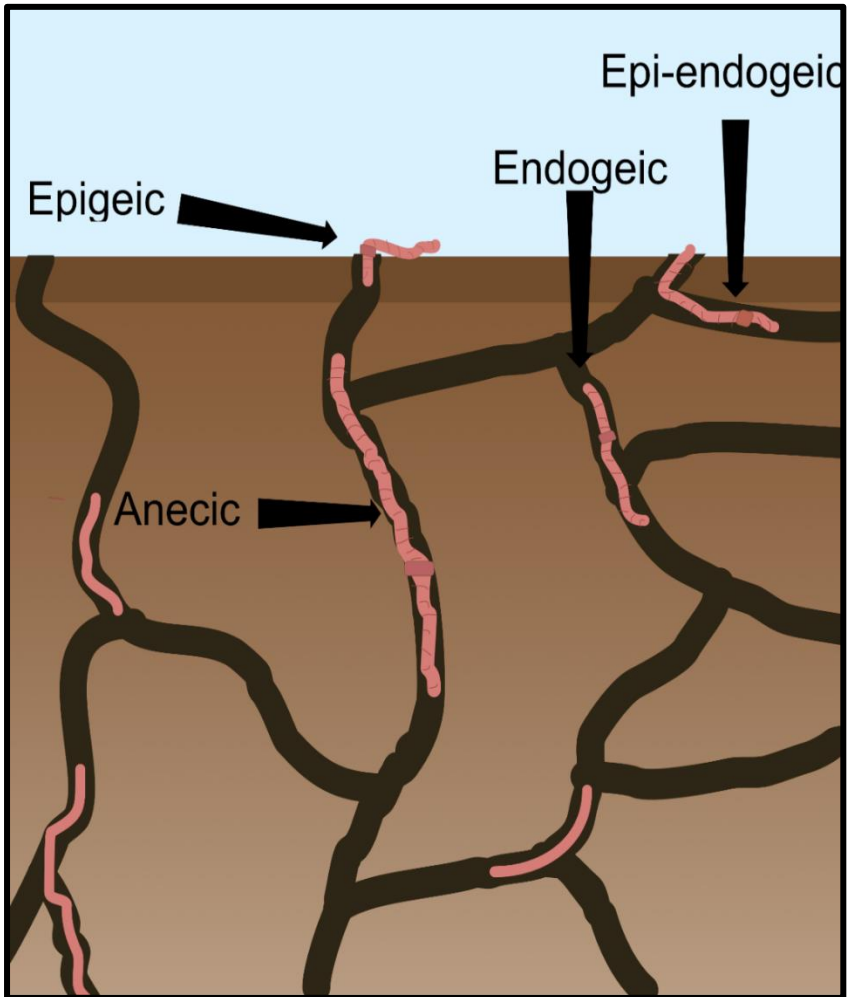


Figure 1.1. Burrowing behaviours of the primary earthworm ecotypes

Table 1.4. Identifying characteristics and behaviours of earthworm species recorded in the British Isles.

Species	Family	Type	Common habitat	Average size	Additional information
Microscolex phosphoreus	Acanthrodrilidae	endogric	Gardens and golf courses	10-35 mm	Very rare in UK, thought to originate from South America
Allolobophora chlorotica	Lumbricidae	endogeic	Woodlands, arable land, gardens	~50 mm	Two different colour morphs (pink and green) have different ecological preferences
Aporrectodea caliginosa	Lumbricidae	endogeic	-	60-85 mm	-
Aporrectodea cupulifera	Lumbricidae	-	-	-	Only found in Ireland and not mainland Britain
Aporrectodea icterica	Lumbricidae	endogeic	-	55-135 mm	-
Aporrectodea limicola	Lumbricidae	-	-	-	-
Aporrectodea longa	Lumbricidae	anecic	Alkaline soils and leaf litter	80-120 mm	Commonly known as the black-headed earthworm
Aporrectodea nocturna	Lumbricidae	anecic	-	-	-
Aporrectodea rosea	Lumbricidae	-	Fields, gardens and pasture	25-85 mm	-
Bimastos eiseni	Lumbricidae	-	-	-	Originally named *Allolobophoridella eiseni*
Bimastos rubidus	Lumbricidae	epigeic	Leaf litter and surface soil	20-100 mm	Originally named *Dendrodrilus rubidus*
Dendrobaena attemsi	Lumbricidae	epigeic	-	24-55 mm	-

Dendrobaena hortensis	Lumbricidae	-	-	-	-
Dendrobaena octaedra	Lumbricidae	epigeic	Leaf litter	20-40 mm	-
Dendrobaena pygmaea	Lumbricidae	epigeic	Well drained litter and moss	20-35 mm	-
Dendrobaena veneta	Lumbricidae	epigeic	Compost, leaf litter, forests	120-170 mm	Commonly known as the European nightcrawler
Eisenia andrei	Lumbricidae	epigeic	Compost, leaf litter	50-70 mm	
Eisenia fetida	Lumbricidae	epigeic	Surface soils	26-130 mm	Very common stripey earthworms
Eiseniella tetraedra	Lumbricidae	epigeic	Mud, river banks and other damp areas	20-80 mm	-
Helodrilus oculatus	Lumbricidae	epigeic	Leaf litter	-	-
Kenleenus armadas	Lumbricidae	-	-	~35 mm	Only found in Ireland, not mainland Britain
Lumbricus castaneus	Lumbricidae	epigeic	Leaf litter, surface soil	30-70 cm	-
Lumbricus festivus	Lumbricidae	epigeic	Leaf litter, compost, surface soil	50-100 mm	-
Lumbricus friendi	Lumbricidae	anecic	-	120-200 mm	Very rare in the UK
Lumbricus rubellus	Lumbricidae	epi-endogeic	-	100-150 mm	-
Lumbricus terrestris	Lumbricidae	anecic	All soils except coarse sands, bare rock and acidic peat	100-250 mm	-
Murchieona muldali	Lumbricidae	endogeic	-	-	-

Octolasion cyaneum	Lumbricidae	endogeic	-	65-108 mm	Distinguishable from *O. lacteum* due to yellow tail
Octolasion lacteum	Lumbricidae	endogeic	Shallow burrows, prefers moist environments	25-160 mm	-
Satchellius mammalis	Lumbricidae	epigeic	-	-	Monotypic genus
Sparganophilus tamesis	Sparganophilidae	-	Mud-dwelling species	60-200 mm	Thought to originate from North America

Information is sparse in some areas due to a limited number of verified recordings; therefore a complete information isn't available for all sections.

All species included have had verified recordings within the British Isles.

1.4.1.1.2. Ecosystem services of earthworms

Earthworms are often recognised as being important indicators of soil health and quality, providing significant levels of natural capital and influence over various vital ecosystem services (Fig. 1.2). The extent to which an earthworm provides these certain provisions is generally driven by the ecotype and diversity of ecotypes within the system, with geophagous endogeic species primarily responsible for aggregate formation, whilst through the production of permanent vertical shafts deeper burrowing anecic individuals have more influence over processes such as soil porosity (Bottinelli et al., 2010, 2020; Hallam and Hodson, 2020). In addition to the influence that earthworms can have over soil properties and processes, external drivers such as land-use change and management, and climate change can be significant drivers in the net influence of earthworms (Schon and Dominati, 2020). It is therefore important to understand that anything that can cause a significant decline in earthworm numbers, health or overall activity can lead to significant changes to or losses of particular ecosystem services.

1.4.1.1.3. Exposure methods

The level of chemical exposure felt by an earthworm is generally related to the ecotype and life history traits of the particular species. Earthworms that dwell near or on the surface of agricultural land are often at higher risk of direct chemical exposure; whereas earthworms that reside deeper in the soil profile may be less vulnerable to direct exposure. These anecic species, however, may become exposed through pesticide leaching within the soil profile, or through the physical agitation of the soil through mechanisms such as ploughing. In addition to direct exposure through the soil, earthworms could become exposed through the consumption of contaminated plant matter, ingesting the pesticide directly into their system (Basley and Goulson, 2017).

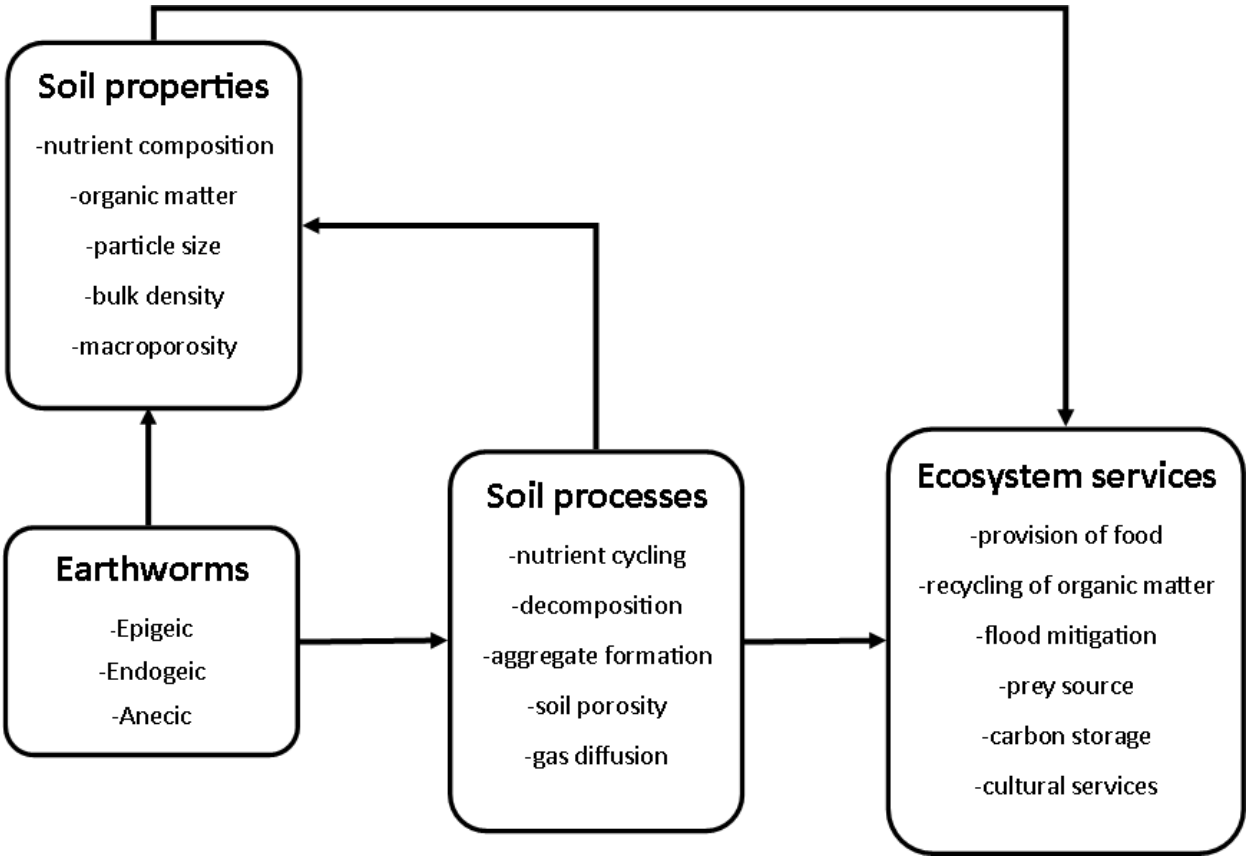


Figure 1.2. Contribution of earthworms to soil properties, soil processes and ecosystem services. Adapted from Schon and Dominati (2020).

1.4.1.1.4. Mortality and survival

Mortality and survival rates to chemical exposure are often quantified using standardised exposure studies such as LD_{50} (lethal dose for 50 % survival) and LC_{50} (lethal concentration for 50 % survival). The official LD_{50}'s and LC_{50}'s for currently registered neonicotinoid compounds can be found in Table 1.2. Whilst standardised lethality measures are helpful when proposing and producing agrochemical policies, it is important to account for a variety of factors that can affect the lethality levels. Differences in the soil- type, structure, texture, chemical composition and management strategies, as well as variations in life history

traits between earthworm species, and also the surfactants present in the commercial agrochemical mixtures can all have an impact on the physicochemical interactions and ecological results of neonicotinoid exposure in earthworms, producing a wide range of LD_{50} and LC_{50} results (Table 1.2). A review conducted by Pisa et al. (2015) provide an overview of neonicotinoid toxicity reporting a range of LC_{50} values for various earthworm species exposed to neonicotinoid contamination of 1.5 to 25.5 mg kg^{-1}, indicating the breadth of variation within these standardised results.

The method of chemical application can also impact the ecological exposure as point and diffuse applications (seed treatments Vs foliar sprays/irrigation treatments) will have different rates of leaching and thus have varying levels of mobility and bioavailability within the soil, all influenced by differing half-lives within the soil (half-lives for neonicotinoid compounds can be found in Table 1.3). These degradation rates are important as higher half-lives can lead to prolonged ecological exposure, which has been linked to an increase in toxicity as a result of chronic exposure.

However, when conducting experiments at field-relevant levels the rates of exposure tend to be many times lower than the levels used in the laboratory to calculate both LD_{50} and LC_{50} levels. For this reason, direct mortality as a result of field exposure to neonicotinoids isn't an outcome that is often observed, however, much like many other species, earthworms have been found to exhibit a wide variety of sublethal effects, altering their behaviours, health, reproduction and therefore impacting their long-term environmental sustainability (Capowiez and Bérard, 2006; Pereira et al., 2010; Miglani and Bisht, 2019; Saggioro et al., 2019).

1.4.1.1.5. Sublethal effects

1.4.1.1.5.1. Growth and health

Wang et al. (2015) used an artificial soil medium to assess the effects of imidacloprid on *E. fetida* over a 21 day exposure period. Imidacloprid adversely affected growth and damaged the epidermis of the worms. Both effects increased with increasing concentrations and contact times (Wang, et al., 2015). Growth rates of the earthworm *E. fetida* were significantly affected by imidacloprid exposure, with rates of 38.0 %, 42.0 %, 37.9 % and 48.9 % growth inhibition being recorded at 0.02, 0.1, 0.5 and 1.0 mg kg^{-1} imidacloprid at day 14 respectively (Wang et al., 2015). Conversely, experiments by Zaller et al. (2016) found that the use of imidacloprid treated seeds appeared to have no effect on earthworm activity, however the addition of earthworms to the study sites influenced unpredicted interactions such as a decrease in the wheat growth and a reduction in the total soil microbial biomass (Zaller et al., 2016).

De Lima Silva et al. (2017) assessed the variations in the survival and growth of adult *E. andrei* when exposed to differing levels of imidacloprid and thiacloprid contamination in soils. Their team estimated the LC_{50} of the two compounds to be 0.77 mg kg^{-1} dry soil for imidacloprid and 7.1 mg kg^{-1} dry soil for thiacloprid. In trials where survival remained at 100 % neither compound was found to significantly alter earthworm biomass, however 3.3 mg kg^{-1} dry soil thiacloprid produced a 23 % reduction in weight (De Lima Silva et al., 2017).

As well as variations in reactions across the different chemical compounds, stereoisomers of the same compounds have also been found to exhibit varied outcomes. Whilst investigating dinotefuran, Liu et al. (2018) found that both the growth and reproduction were seriously impacted by R-dinotefuran, S-dinotefuran and Rac-dinotefuran. Whilst all stereoisomers were shown to cause serious impact, the time taken for these outcomes to become

significant differed. Both Rac-dinotefuran and S-dinotefuran started by showing little influence on the biomass changes of the earthworms, however, after 14 days these treatments were now significantly different to those of the control group. R-dinotefuran also eventually exhibited significant differences, however these were only observed after 28 days (Liu et al., 2018).

1.4.1.1.5.2. Reproductive success

Reproductive successes can be assessed as the number of eggs/cocoons laid- noting variations in their physical size and weights as a result of exposure. An additional criterion for reproductive success is the hatchability of the cocoons and the survival rate of the juvenile hatchlings. Wang et al. (2015) assessed the effects of imidacloprid on reproduction over 56 days, finding that both the number of cocoons produced and their hatchability were significantly inhibited at 0.5 and 1.0 mg kg^{-1} imidacloprid (Wang et al., 2015). Under these conditions hatchability was reduced to 62.0 % and 49.6 % (0.5 mg kg^{-1} and 1.0 mg kg^{-1} respectively), significantly different compared to the average control hatchability of 78.5 %.

Conversely, studies performed by Chevillot et al. (2017) showed an increase in the number of cocoons and subsequent juvenile hatchlings from earthworms exposed to neonicotinoids. They also found evidence of earthworm reproduction responding in a similar way when exposed to another 54 different chemical pesticides (Chevillot et al., 2017).

Research conducted by Ge et al. (2018), concluded that cocoon and subsequent hatchability are usually two of the most sensitive endpoints of the parameters generally analysed in chronic exposure trials. Studies by Saggioro et al. (2019) further supported these results, finding that over the 45 days of the exposure trials there were no significant variations reported in earthworm biomass when exposed to low levels of acetamiprid. However, their study did show a significant ($p < 0.05$) decrease in the number of cocoons produced per

earthworm, reduced by 48.1 % and 67.5 % at 0.05 mg kg^{-1} and 0.1 mg kg^{-1} acetamiprid, respectively. Further to this, Saggioro et al. (2019) found that the number of young hatchlings per cocoon was also significantly impacted by acetamiprid exposure, 56.8 % and 64 % respectively for the same concentrations of acetamiprid.

Effects on reproductivity and fecundity have been found to be initiated through exposure to any of the currently registered neonicotinoid compounds. Wang et al. (2015) found that imidacloprid, acetamiprid, nitenpyram, clothianidin and thiacloprid could all seriously impact the fecundity of adult *E. fetida*, reducing their reproductive output by 84.0 %, 39.5 %, 54.3 %, 45.7 % and 39.5 % at the sub-lethal concentrations of 2.0, 1.5, 0.80, 2.0 and 1.5 mg kg^{-1} respectively for each of the respective neonicotinoid compounds.

On the whole, these results demonstrate that exposure to soil-borne neonicotinoid contamination can result a significant inhibition of the normal reproductive successes of earthworms. These observations are of concern, as earthworms represent a large portion of soil biomass, a key species in many terrestrial food-chains and are vital to the continuation of healthy and fertile soils.

1.4.1.1.5.3. Behavioural changes

In addition to physical changes such as mortality and reproductive successes, neonicotinoids have been linked to sub-lethal behavioural changes, specifically alterations to burrowing and food-linked behaviours. These behavioural changes include alterations to the physical burrows produced by earthworms post-exposure, including significantly smaller areas burrowed, shallower depths reached, lower total lengths and a general avoidance of neonicotinoid contaminated areas (Capowiez and Bérard, 2006; Capowiez et al. 2006). These behavioural changes, if left unchecked, could substantially impact earthworm survival, and

undermine the maintenance of fertile and productive arable land. Studies monitoring changes in burrowing behaviours are sparse, we hypothesise that this is due to their time and resource heavy requirements, with numerous set-ups required to produce statistically significant data.

In addition to physical burrow changes, shifts in earthworm behaviours can also be quantified through their attraction and/or avoidance of certain chemicals under test and field conditions. The use of ISO 17512-1 (2008) standard methodologies is common when assessing the influence of chemicals on earthworm avoidance. This methodology includes the use of either a two-compartment container, or a multi-compartment container. Simply, the containers allow for control and treatment soils (multi-compartment container may include soils with multiple measured levels of contamination) to be applied to either side, earthworms released down the centre line, and the abundance of earthworms on either side is assessed after a set time. This allows for a percentage avoidance/attraction net response measurement (NR value) to be calculated. Values above 80% are noted as being responsible for possible ecosystem function change (Hund-Rinke et al., 2003; Saggioro et al., 2019). Results compiled by Saggioro et al. (2019) showed significant avoidance behaviours in *E. andrei* after being exposed to 0.5 mg kg^{-1} and 1 mg kg^{-1} acetamiprid. These two levels of exposure resulted in NR values of 61.1 ± 10 % (0.5 mg kg^{-1}) and 78 ± 12 % (1 mg kg^{-1}), these results indicate a significant level of avoidance, nearing the 80 % boundary for functional change.

1.4.1.1.5.4. Other

Earthworms exposed to soil-borne neonicotinoids have been recorded as exhibiting DNA damage caused by oxidative stress (Wang et al., 2015; Liu et al., 2017; Zhang et al., 2017). Clothianidin at levels of 0.5 and 1.0 mg kg^{-1}, greatly enhance the levels of reactive oxygen species resulting in antioxidant enzyme activity, abnormal gene expression and damage

to macromolecules (Liu et al., 2017). Similar investigations were carried out by Zhang et al. (2017) where imidaclothiz was used in an artificial soil medium and exposed *E. fetida* to varying levels (0.3 and 1.0 mg kg^{-1}) for a 28 day test period. Using a comet assay, the olive tail moments (OTM) were used as an indication of DNA damage, with the OTM being significantly increased under treated conditions when compared to the control samples (Zhang et al., 2017). Comet assays were also conducted by Chevillot et al. (2017), exposing earthworms to a selection of seven neonicotinoid compounds. Their findings corroborate those of Zhang et al. (2017), as they detected significant increased DNA damage in the treated individuals in comparison to controls (Chevillot et al., 2017).

1.4.1.1.6. Species difference- life history trait differences

A consideration to keep in mind when comparing the respective toxicities of neonicotinoid compounds, and by extension any chemical- is the species being exposed. De Lima Silva et al. (2017), compared the toxicity of imidacloprid and thiacloprid on two species of the same Phylum: *E. crypticus* and *E. Andrei*. Despite being from the same Phylum (*Annelida*), these exposure tests yielded some interesting observations. The two species showed some distinct differences in their respective sensitivities to the neonicotinoids; imidacloprid was shown to be a factor of > 39 more toxic to the survival of *E. Andrei* and a factor 4-5 for impacts on its reproductive successes (De Lima Silva et al., 2017). The differences between these two species is thought to be as a result of differences in their metabolisms (De Lima Silva et al., 2017). Across earthworm species there is great variation in sizes, burrowing behaviour, and feeding strategies (Table 1.4). Accounting for these differences goes some way to explaining the variation in exposure responses, theorising that

smaller, surface dwelling, litter eating species may often be at higher risk of detrimental outcomes in comparison to the larger sub-surface earthworm species.

1.4.1.1.7. Knock-on impacts

Impacts to earthworm survival, behaviours and reproductive rates and successes can have direct environmental consequences, significantly altering biomechanisms within the soil and inhibiting soil fertility. Alterations to earthworm behaviours may directly impact organic matter decomposition, and reductions and changes in bioturbation could lead to changes in soil structure. These mechanisms are vital for maintaining soil fertility and ensuring that land remains productive and sustainable.

Earthworms, like many other soil invertebrates also form a key component of many food webs, providing a food source for many animals (Vermeulen et al., 2010). The contamination of earthworms by neonicotinoids, and various other agrochemicals, can provide a vector for the chemical exposure to progress up the food chain to higher trophic levels (Vermeulen et al., 2010; Douglas et al., 2015). Whilst neonicotinoids are classified as being hydrophilic and are therefore less likely to accumulate within the fatty tissue of an animal, there is still evidence of the biomagnification of neonicotinoids throughout the food chain (Douglas et al., 2015; Macdonald et al., 2018).

Chevillot et al. (2017) examined the accumulation of seven neonicotinoid compounds within both adult and juvenile earthworms, finding that six out of the seven had accumulated to levels higher than the original exposure level. Whilst comparing the accumulated concentrations in both adult and juvenile individuals it was found that the adults accumulated significantly higher concentrations, 200,000-fold more than the juveniles (Chevillot et al., 2017).

1.4.1.2. Other soil-invertebrates

Beyond the examination of earthworm responses there are relatively few studies assessing the impacts of neonicotinoids on other soil invertebrate species. Research by Peck (2009a,b) assessed the impacts of the preventative use of imidacloprid applications on the abundance of various non-target soil and surface fauna in a turf grass environment. Their results demonstrated a preferential decrease in soil-captured fauna to those captured in surface pitfall traps. Primarily, these decreases were present amongst the beetle populations, and were more often expressed across adult individuals, demonstrating the possibility of a knock-on driver to prey populations as a result of the suppression of predaceous adult beetle species (Peck, 2009a,b).

Other commonly assessed species and soil invertebrate groups include mites (*Acari*) and collembola (De Lima e Silva et al., 2017; Kristiansen et al., 2021; Pearsons and Tooker, 2021). These two groups play key roles in organic matter degradation and turnover within the soil ecosystem, and therefore any detrimental influences on their population diversity and abundance could lead to unexpected changes and losses of soil function (Rusek, 1998; Behan-Pelletier, 1999, 2003). Research from Mabubu et al. (2017) found that populations of the collembola *Folsomia candida* were significantly affected by the application of neonicotinoids. Under laboratory conditions, using OECD soil, they demonstrated that *F. candida* survival was adversely affected by imidacloprid, with an average LC_{50} lethal dosage of 0.84 mg kg^{-1} dry soil. Alternatively, there was a lower toxicity response to thiacloprid, with an LC_{50} of 3.5 mg kg^{-1} dry soil, however, thiacloprid exposure did exert adverse influence over the reproductive output of *F. candida.* These results demonstrate connections between neonicotinoid exposure and detrimental changes to the long term survival of vital populations of soil biota (Mabubu et al., 2017). In addition to this, findings from Kristiansen et al. (2021) showed that the

collembola species *Hypogastrura viatical* was much more sensitive to imidacloprid exposure than many of the other previously tested collembola species. When assessing the impact of exposure to reproductive outputs it was found that whilst at 0.01 mg kg^{-1} dry soil there was little to no change in total egg production, there was a substantial reduction in the number of eggs produced per batch as well as a reduction in successful hatchability. Sublethal responses such as these indicate the importance of quantifying the long-term effects of pesticide exposure in these previously under-investigated communities (Kristiansen et al., 2021). Across both of these studies the pure active ingredients were used, and therefore the results are possibly not truly indicative of realistic field conditions. Commercially available formulations often contain a mixture of emulsifiers, adjuvants, and surfactants, all of which could influence the environmental behaviour and subsequent ecological impact of the applied chemicals

1.4.2. Trophic progression and food chain movement

In 1962, Rachel Carson noted that '... for some reason, snail-like molluscs seem to be almost immune to the effects of insecticides...' (Carson, 1962), and this appears to continue to hold true for neonicotinoids. For example, imidacloprid only exhibits low toxicities to *Deroceras reticulatum* (Simms et al., 2006). Whilst molluscs do not appear to exhibit any ill effects from neonicotinoid exposure (lethal or sub-lethal) research by Douglas et al. (2015) found evidence to suggest that the toxicity of neonicotinoids could be transferred up the trophic food chain. Slugs are very likely to come into contact with neonicotinoids through the consumption of treated plants and crops. Using lab-based investigations, they found that whilst none of the slug pests (*D. reticulatum*) exhibited any negative effects (lethal or sub-lethal), over 60 % of the predatory beetle sample population, *Chalenius tricolor*, were physically impaired (twitching, mild motor difficulties and partial to extensive paralysis) or dead after

consuming the contaminated slugs (Douglas et al., 2015). Further residue analysis found that slugs collected from the field during the study period contained levels of thiamethoxam up to 500 ng g^{-1} , more than high enough to cause harm to insect predators higher up the trophic levels (Douglas et al., 2015).

Whilst neonicotinoids are highly selective towards invertebrates, and therefore unlikely to cause harm to mammals at low dosage levels, there is still concern as to whether certain mammals and birds associated with agricultural spaces come to harm through prolonged exposure to field levels of neonicotinoids. Most current research suggests that due to their high solubility and hydrophilic nature, neonicotinoids pass quickly through the mammalian digestive systems without negative impact (Yokota et al., 2003).

Yokota et al. (2003) orally administered one-off doses of ^{14}C labelled clothianidin to Wistar rats, to assess the effects of acute exposure. Within two hours of the initial exposure, the clothianidin was observed to be present within the tested organ tissues. The highest levels were recorded for the stomach, kidney and liver at levels of 7.17 ± 2.88, 5.69 ± 1.54 and 3.92 ± 0.64 µg clothianidin per gram of tissue respectively. These levels indicate that the process of metabolising and preparing to excrete the clothianidin occurred almost immediately following pesticide ingestion (Yokota et al., 2003).

Duzguner and Erdogan (2012) assessed the impacts of chronic low-dose exposure to imidacloprid over a period of 30 days. Rats in the treatment groups were exposed to 1 mg imidacloprid kg^{-1} body weight each day. After 30 days, the rats were euthanised and liver and brain samples were removed for analysis. Levels of nitric oxide production were quantified within the brain and liver samples, both showing significant increases in production ($p < 0.05$ and $p < 0.001$ respectively). The increased levels of nitric oxide production were used as indicators of increased oxidative stress because of the imidacloprid exposure.

The majority of neonicotinoid studies involving mammals are laboratory based, with the test subjects directly ingesting relatively large levels of pure active ingredients (Duzguner and Erdogan, 2012; Kapoor et al., 2014). Under realistic field conditions, exposure levels are likely to be much lower and would often be in the form of contaminated invertebrates or plant matter. Free-roaming species such as field mice (*Apodemus sylvaticus*), moles (*Talpa europaea*) and hedgehogs (*Erinaceus europaeus*), could be particularly at risk of secondary neonicotinoid exposure with their main food sources being readily contaminated within intensive agricultural environments. Whilst it is still unclear as to whether there are any bioaccumulation risks associated with neonicotinoids, the mole's and hedgehog's primary food sources (slugs, earthworms, beetles) appear to have the potential for pesticide accumulation (Douglas et al., 2015).

Aside from pollinator research, there are now mounting concerns regarding the impacts neonicotinoids may be having on granivorous bird health and population success (Van der Sluijs and Van Lexmond, 2014; Lopez-Antia et al., 2015). Granivorous birds, much like bees, appear to be susceptible to sub-lethal effects and biophysical changes as a result of low-level chronic neonicotinoid exposure; with reduced plasma levels and increased blood superoxide dismutase indicating neonicotinoid-induced stresses (Lopez-Antia et al., 2015).

1.4.3. Soil microbiota

Microbial communities are often considered as being one of the most sensitive bioindicators for quality change in soil, rapidly responding to their environments (Lau et al., 2012; Pescatore et al., 2020; Liu et al., 2021). Under adverse conditions, these changes in quality have been found to result in substantial changes in certain soil functions and ecosystem services (Lehman et al., 2015; Bünemann et al., 2018). The term soil microbiota encapsulates

numerous species of bacterial and fungal communities and is often representative of most of the biological life in the soil (Aislabie and Deslippe, 2013). The soil microbial community plays a pivotal role in regulating many soil processes including nutrient transformation and transfer, as well as assisting mesofauna with the degradation process of decaying organic materials (Harris, 2009; Aislabie and Deslippe, 2013).

The interactions between neonicotinoids, and any other xenobiotic or chemical contaminant, is two-fold, with communities and populations being impacted by the chemical, but also through changes in degradation and subsequent chemical persistence as a result of different microbial activity. Here in this subsection, we will explore the degradation pathways of neonicotinoid pesticides within the soil sphere, as well as examine the effects that neonicotinoid applications can have upon these microbial communities. We will then touch upon the environmental and ecological implications of these community changes, as well as theorise the possibilities of targeted microbial remediation techniques.

1.4.3.1. Neonicotinoid degradation

The degradation of chemicals is often different for each and every chemical; the same applies to neonicotinoids. Across the family of systemic insecticides, neonicotinoids have vastly different levels of environmental persistence (Table 1.3) and degradation pathways (Fig. 1.3-1.8). Also due to their differences in chemical structure they cannot all be metabolised and degraded by the same microbiota. Many studies have evaluated the degradation pathways, end points and metabolites of neonicotinoids using singular isolated bacterial strains, and whilst this information can be of use, especially for future specific remediation techniques, it is important to remember that under real-life field conditions chemicals are often exposed to large arrays of microbial consortia- working together, with different responsibilities for different

stages of the degradation (Hussain et al., 2016; Pang et al., 2020). Studies assessing the microbial degradation of chemicals can be categorised in two ways, 1) biodegradation by pure bacterial culture, or 2) microbial co-degradation. In pure bacterial degradation studies, the study chemical is the only source of carbon and nitrogen for the bacterial populations, whereas additional nutritional sources are provided during co-degradation studies (Table 1.5; Pang et al., 2020).

Table 1.5. Microbial degradation studies across the major neonicotinoid products

Microorganisms	Source	Mode of degradation	Optimal conditions	References
Imidacloprid				
Acinetobacter sp. TW	Solid tobacco waste	Catabolic (C, N)	-	Wang et al. (2011)
Aspergillus terreus YESM3 (fungus)	Agricultural wastewater	Catabolic (C)	28°C, pH 4	Mohammed and Badawy (2017)
Bacillus aerophilus	Sugarcane field soils	Co-metabolic; mixed culture	Soil slurry	Akoijam and Singh (2015)
Bacillus alkalinitrilicus	Sugarcane field	Catabolic (C, N)	Mixed culture of native soil	Sharma et al. (2014)
Bacillus sp.	Rhizospheric soil	Catabolic (C, N)	30-35°C, pH 7	Shaikh et al. (2007)
Bradyrhizobiaceae strain SG-6C	Soil	Catabolic (C)	-	Shettigar et al. (2012)
Brevundimonas sp.	Cotton field soil	Catabolic (C,N)	37°C, 120 rpm Liquid minimal medium	Shetti and Kaliwal (2012)
Bacillus weihenstephanensis	Soil	Catabolic (C,N)	22°C, pH 7	Shetti et al. (2014)
Burkholderia cepacian	Agriculture field soil	Catabolic	-	Gopal et al. (2011)
Hymenobacter latericoloratus CGMCC 16346	Water environment	Co-metabolic	-	Guo et al. (2020)
Klebsiella pneumoniae BCH-1	Pesticide-contaminated agricultural soil	Co-metabolic	pH 7, 30°C	Phugare et al. (2013)
Leifsonia sp. PC-21	Agricultural soil	Co-metabolic (glucose, succinate)	-	Anhalt et al. (2007)

Mycobacterium sp. Strain MK6	Agricultural soil	Catabolic (N)	Liquid minimal medium	Kandil et al. (2015)
Ocbrobactrum sp.	Tea rhizosphere soil	Catabolic (C)	30°C, pH 8	Hu et al. (2013)
Pseudoxanthomonas indica CGMCC 6648	Rhizospheric soil	Co-metabolic (glucose)	Liquid minimal medium	Ma et al. (2014)
Pseudomonas sp. 1G	Neonicotinoid-exposed golf course soil	Co-metabolic (glucose)	28°C, microaerophilic	Pandey et al. (2009)
Pseudomonas sp. RPT 52	Pesticide contaminated agricultural field	Catabolic (C)	-	Gupta et al. (2016)
Rhizobium sp.	Vegetable farming areas	Catabolic (C)	Liquid minimal medium	Sabourmoghaddam et al. (2015)
Sphingomonas sp. TY	Solid tobacco wate	Catabolic (C, N)	-	Wang et al. (2011)
Stenotrophomonas maltophila CGMCC 1.1788	Purchased	Co-metabolic	30°C, pH 7.2	Dai et al. (2006)
Acetamiprid				
Actinomycetes Streptomyces canus CGMCC 13662	Soil	Co-metabolic	pH 7, 30°C	Guo et al. (2019)
Ensifer meliloti CGMCC7333	Rhizosphere soils	Catabolic (N)	30°C	Zhou et al. (2014)
Fusarium sp. strain CS-3 (fungus)	Soil from pesticide factory	Co-metabolic	25-30°C, pH 5-7	Shi et al. (2018)
Ochrobactrum sp. D-12	Polluted agricultural soil	Catabolic	25-35°C, pH 6-8	Wang et al. (2013a)
Ochrobactum sp. D-12	Wastewater treatment pool	Catabolic	30-45°C, pH 5-10	Yang et al. (2013)
Phanerochaete sordida YK-624 (fungus)	Rotted wood	N-demethylated	-	Wang et al. (2012)
Pigmentiphaga sp. AAP-1	Pesticide contaminated factory soil	Catabolic	30°C, pH 7	Wang, et al. (2013b)
Pigmentiphaga sp. D-2	Wastewater from acetamiprid factory	Catabolic (C)	30-45°C, pH 5-10	Yang et al. (2013)

Pseudomonas sp. FH2	Sludge from pesticide factory	Co-metabolic	30°C, pH 7	Yao and Min (2006)
Pseudomonos sp. FH2	Agricultural field soil	Catabolic	-	Yao and Min (2006)
Pseudoxanthomonas sp. AAP-7	Pesticide contaminated factory soil	Co-metabolic	30°C, pH 7	Wang et al. (2013c)
Rhodococcus sp. BCH-2	Pesticide contaminated soil	Co-metabolic (6-CNA)	35°C, pH 7	Phugare and Jadhav (2015)
Rhodotorula mucilaginosa strain IM-2	Soil	-	-	Dai et al. (2010)
Strentrophomonas sp. THZ-XP	Sludge from an acetamiprid factory	Co-metabolic	30°C, pH 7	Tang et al. (2012)
Strentrophomonas maltophila CGMCC1.178	Purchased	Co-metabolic	30°C, pH 7.2	Chen et al. (2008)
Variovorax boronicumulans CGMCC 4969	Purchased	Co-metabolic	pH 7, 40°C	Sun et al. (2018)
Thiacloprid				
Ensifer meliloti CGMCC7333	Rhizosphere soils	Catabolic (N)	30°C	Ge et al. (2014)
Microvirga flocculans CGMCC 1.16731	Thiacloprid-contaminated soil	Co-metabolic	-	Zhao et al. (2019)
Stentrophomonas CGMCC1.178	Purchased	Co-metabolic	30°C, pH 7.2	Zhao et al. (2009)
Variovorax boronicumulans J1	Agricultural soil	Co-metabolic	30°C, pH 7.2	Zhang et al. (2012)
Thiamethoxam				
Acinetobacter sp. TW	Agricultural soil	Catabolic	pH 6-6.5, 37°C	Rana et al., (2015)
Bacillus aeromonas IMBL 5.2	Agricultural soil	Catabolic	pH 6-6.5, 37°C	Rana et al. (2015)
Ensifer adhaerens TMX-23	Rhizosphere soil	Catabolic (C,N)	30°C	Zhou et al. (2013)
Pseudomonas sp. 1G	Neonicotinoid exposed golf course soil	Co-metabolic	28°C, microaerophilic	Pandey et al. (2009)
Pseudomonas putida IMBL 5.2	Agricultural soil	Catabolic	pH 6-6.5, 37°C	Rana et al. (2015)
Sphingomonas sp. TY	Agricultural soils	Catabolic	pH 6-6.5, 37°C	Rana et al. (2015)

Clothianidin				
Phanerochaete sordida (fungus)	Rotted wood	N-demethylated	30°C	Mori et al. (2017)
Pseudomonas stuzeri smk	Agricultural soil	catabolic	pH 7, 30°C	Parte and Kharat (2019)
Nitenpyram				
Phanerochaete sordida YK-624 (fungus)	Rotted wood	Catabolic	-	Wang et al. (2019)
Dinotefuran				
Phanerochaete sordida YK-624 (fungus)	Rotted wood	Catabolic	-	Wang et al. (2019)

1.4.3.1.1. Imidacloprid

Over 20 isolated strains and microbial cultures have been reported as assist in the metabolism of imidacloprid (Table 1.5). The pathways for imidacloprid degradation can vary substantially across environmental conditions, as well as being dictated by the microbial communities involved (Ma et al., 2014; Hussain et al., 2016). For instance, the metabolism of imidacloprid using *Klebsiella pneumoniae* BCH-1 will degrade the compound following the imidacloprid → nitrosoguanidine metabolite → aminoguanidine metabolite pathway, whereas the isolated strain *Strentrophomonas maltophila* will, through the method of hydroxylation and dehydrogenation, produce the metabolite olefin (Dai et al., 2006; Phugare et al., 2013; Hussain et al., 2016; Pang et al., 2020).

The most frequent degradation pathway for the neonicotinoid insecticide imidacloprid starts with an initial deoxygenation, removing two oxygen atoms and producing nitrosoguanidine and aminoguanidine respectively (Fig. 1.3). Alternatively, the immediate cleavage of the N-N bond can transform imidacloprid into desnitro/guanidine derivatives, producing chemical intermediates with a ten-fold toxicity increase to the original imidacloprid compound. Under appropriate conditions this toxic metabolite is further oxidised into a non-

toxic urea-based derivative (Pandey et al., 2009; Phugare et al., 2013; Hussain et al., 2016). In addition, the dehydrogenation of several intermediate metabolites can spontaneously produce the olefin metabolite; the olefin metabolite has been found to have a significantly increased mammalian toxicity level (Nauen et al., 1999; Suchail et al., 2004; Hussain et al., 2016). Overall the oxygenation pathways for imidacloprid degradation are generally less responsive and therefore create relatively fewer metabolites (Hussain et al., 2016).

The total mineralisation of imidacloprid has also been characterised (Fig. 1.4), sharing a common starting point and pathway as the compound acetamiprid. Work by Shettigar et al. (2012) characterised this degradation pathway using the mineralising bacterium strain *Bradyrhizobiacae* SG-6C. This strain of 6-CNA degrading bacteria was isolated and cultured from soils previously contaminated with imidacloprid, indicating the possibility for full mineralisation of the pesticide within agricultural soils.

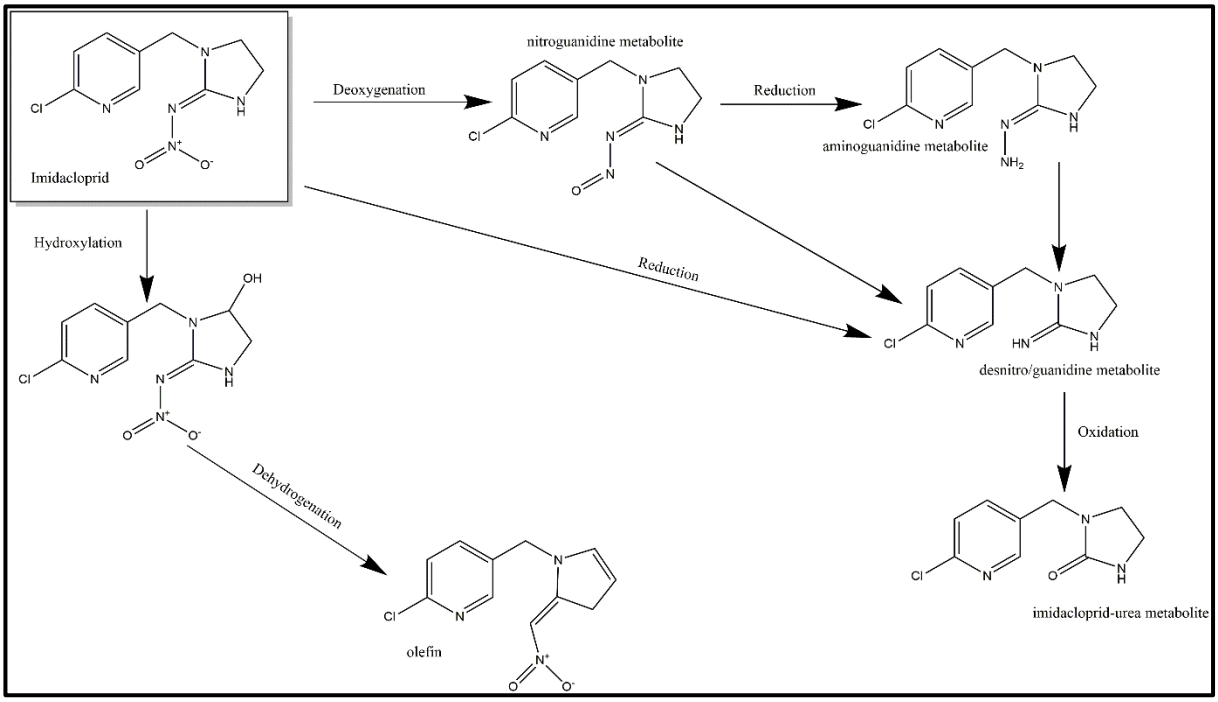


Figure 1.3. Partial microbial degradation pathways for imidacloprid. Based upon data from Sharma et al. (2014); Akoijam and Singh (2015); Sabourmoghaddam et al. (2015); Hussain et al. (2016); Pang et al. (2020).

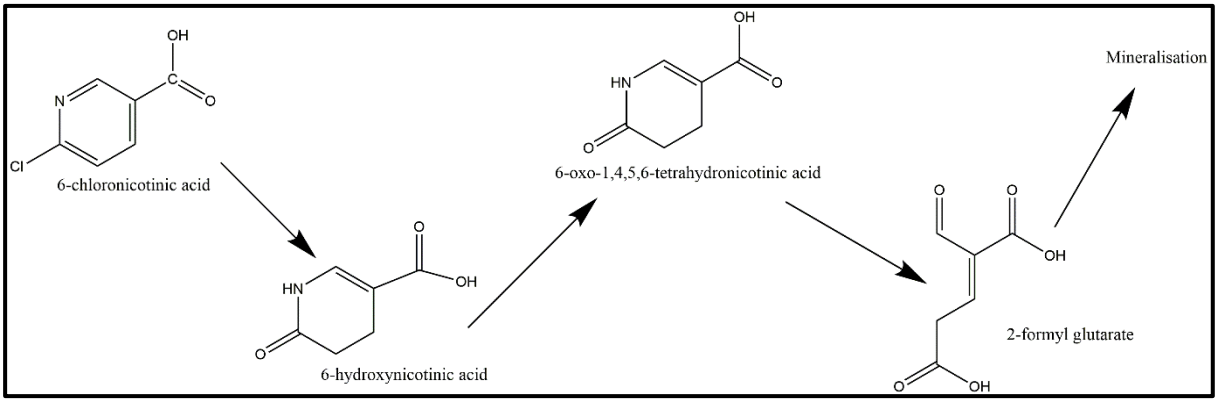


Figure 1.4. Final degradation pathway for 6-chloronicotinic acid (6-CNA), a shared metabolite for both imidacloprid and acetamiprid. Based upon data from Hussain et al. (2016); Pang et al., (2020).

1.4.3.1.2. Acetamiprid

The degradation of acetamiprid is often dependant on its =NC≡N functional group, with the C≡N bond often being oxidised to produce the N-amidoamide derivatives. Following asymmetric cleavage, this derivative is quickly degraded into N-methyl-(6-chloro-3-pyridyl) methaylamine and (Z)-1-ethylideneurea (Fig 1.5; Phugare and Jadhav, 2015; Hussain et al., 2016; Sun et al., 2018). These degradation pathways have been achieved using *Strentrophomonas* sp. THZ-XP (Tang et al., 2012) and Pigmentiphaga sp. (Wang et al., 2013; Yang et al., 2013). Both these pathways were found to transform the original acetamiprid compound faster than any other pure bacterial culture. These studies, however, relied upon acetamiprid utilisation as the sole energy source, resulting in slow bacterial growth (Wang 2013). Alternatively, following characterisation by GC-MS analysis, Phugare and Jadhav (2014) demonstrated that the co-metabolism of acetamiprid by *Rhodococcus* sp. BCH2 in combination with ammonium chloride and glucose (as additional nitrogen and carbon sources), resulted in the production of the metabolites N-methyl(6-chloro-3-pyridyl)methylamine and 6-CNA; indicating that in the presence of a co-metabolite that the start of a full metabolism

pathway could be achieved, with the further degradation pathway for 6-CNA demonstrated in figure 1.4, eventually resulting in the full mineralisation of the original compound. Similarly, a second co-metabolic pathway was described by Yang et al. (2013) using the bacterial strain *Pigmentiphaga* sp. D-2, resulting in the partial degradation of acetamiprid. Via N-deacetylation this co-metabolic pathway produced N-[(6-chloropyridin-3-yl)methyl]-N-methylacetamide and N-methyl-(6-chloro-3-pyridyl)methylamine (Yang et al., 2013).

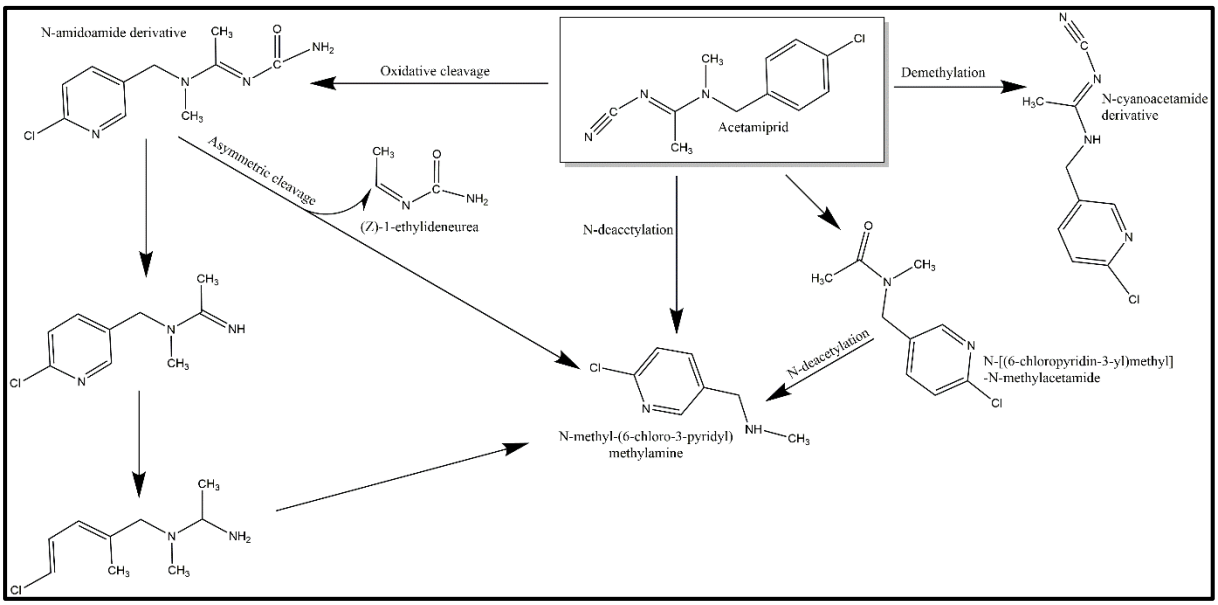


Figure 1.5. Partial microbial degradation pathways for acetamiprid. Based upon data from Phugare and Jadhav (2015); Hussain et al. (2016); Sun et al. (2018); Pang et al. (2020).

1.4.3.1.3. Clothianidin

In comparison to the number of studies conducted on the degradation of other neonicotinoid compounds, relatively few studies have assessed the metabolism of clothianidin. The cleavage of the C-N bonds between the guanidine moieties and thiazolyl methyl groups represent the primary degradation pathway for clothianidin (Fig. 1.6; Parte and Kharat, 2019; Pang et al., 2020a). Clothianidin is often recognised as being one of the more persistent

neonicotinoid products (Table 1.3), but through microbial denitrification and dehalogenation clothianidin eventually degrades to ((2-chlorothiazol-5-yl)methyl)-3-methylguanidine and methyl-3-((thiazol-5-yl)methyl) guanidine. To date, there appears to be no further detection of any other subsequent degradation products.

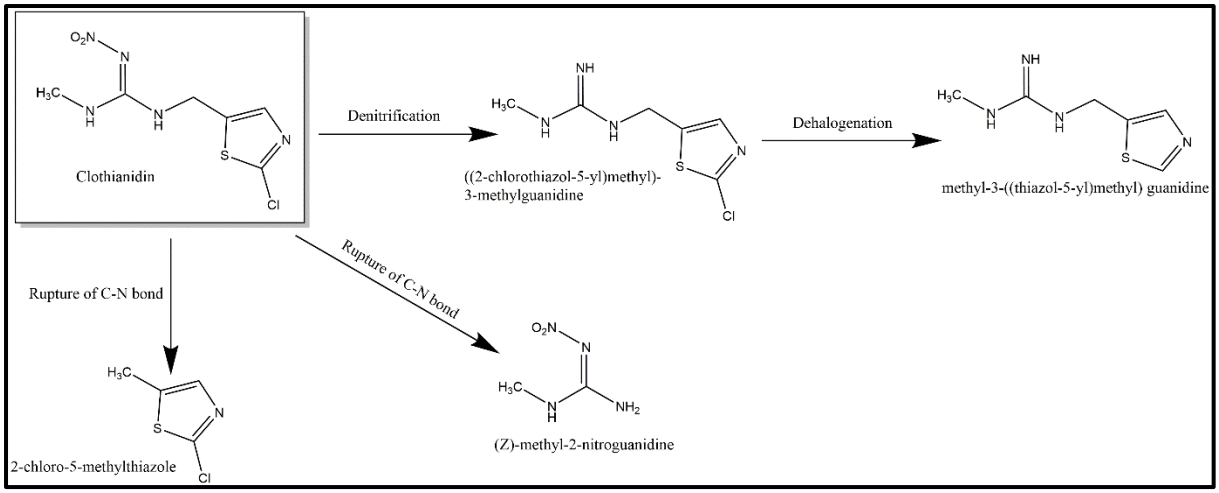


Figure 1.6. Partial degradation pathways for clothianidin. Based upon data from Hussain et al. (2016); Parte and Kharat (2019); Pang et al. (2020).

1.4.3.1.4. Thiamethoxam

The half-life of thiamethoxam varies greatly, with studies reporting between 7-335 days (Table 1.3; Goulson, 2013). Despite this environmental persistence there are relatively few studies assessing the biodegradation of thiamethoxam through targeted microbial interactions, there are however more reporting its transformation within plants and animals (Karmakar and Kulshrestha, 2009; Ge et al., 2017). The microbial degradation of thiamethoxam has been chacterised through the nitroreduction pathway, transforming thiamethoxam into metabolites such as nitrodoguanidne/nitrosamine, aminoguanidine, desnitro/guanidine/imine derivatives and urea (Fig. 1.7; Pandey et al., 2009; Zhou et al., 2013; Zhou et al., 2014; Hussain et al., 2016; Pang et al., 2020a).

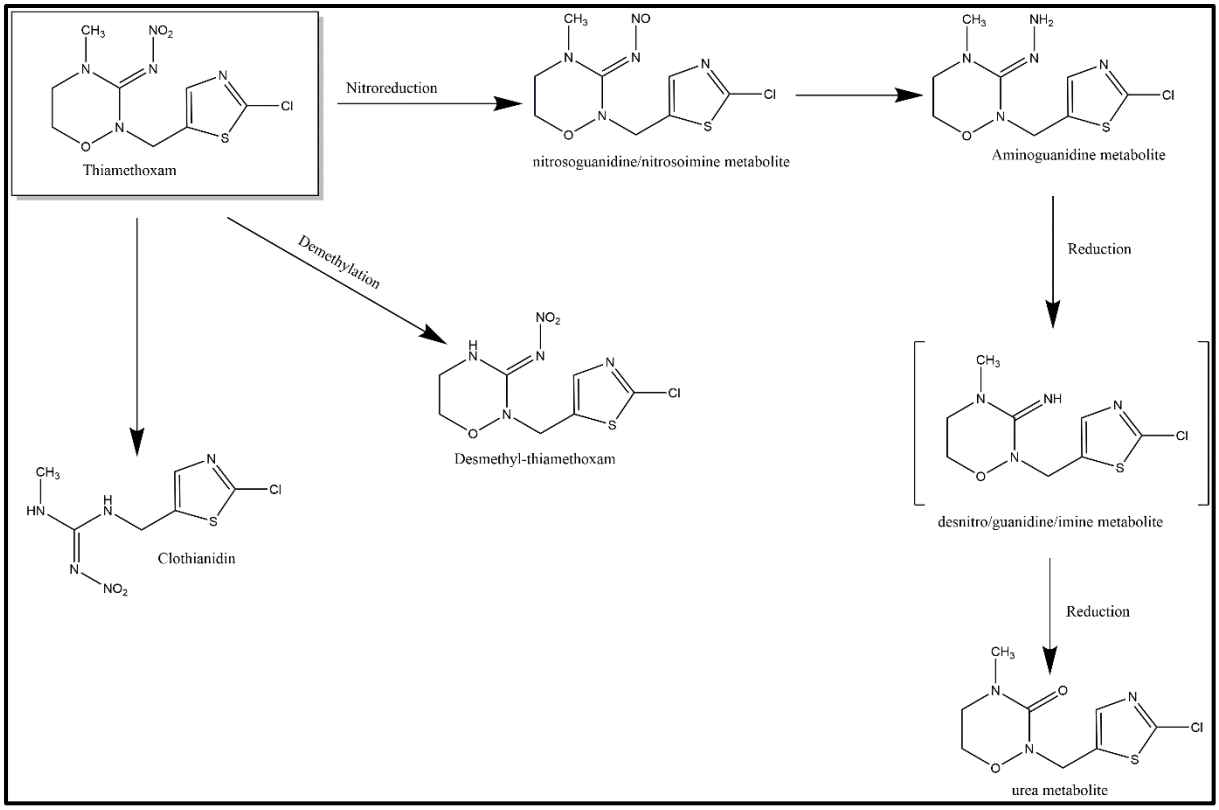


Figure 1.7. Partial degradation pathways for thiamethoxam. Based upon data from Pandey et al. (2009); Zhou et al. (2014); Hussain et al. (2016); Pang et al. (2020).

1.4.3.1.5. Thiacloprid

Whilst there are relatively few studies assessing the biodegradation pathways of thiacloprid, the microbial degradation of thiacloprid often rapidly results in the hydroxylation and subsequent decyanotation of the original compound, resulting in the formation of 4-hydroxy and 4-keto-imine metabolites (Fig. 1.8; Zhao et al., 2009). Additionally, studies conducted by Zhou et al. (2014) successfully characterised a degradation pathway for thiacloprid resulting in the production of thiacloprid amide (N-carbamoylimine derivative), via the biotransformation of thiacloprid by the nitrile hydratase enzyme *Ensifer meliloti* CGMCC 7333. The same enzyme is also able to transform acetamiprid to a similar N-amidoamide metabolite, however, the related enzyme *Ensifer* sp. SCL3-19 is only able to metabolise thiacloprid. This difference in bioactive success is thought to be as a result of structural

differences in the active sites of the two enzymes (Ge et al., 2014; Zhou et al., 2014; Pang et al., 2020). Furthermore, Ge et al. (2014) characterised the transformation of thiacloprid to thiacloprid amide via oxidative cleavage.

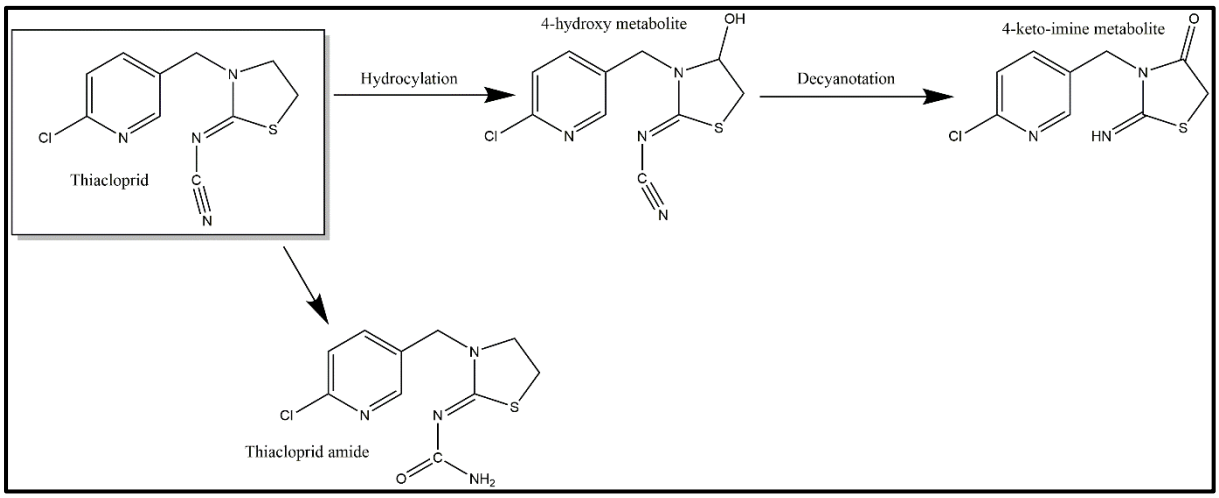


Figure 1.8. Partial degradation pathways for thiacloprid. Based upon data from Zhao et al. (2009); Thurman et al. (2013); Hussain et al. (2016); Pang et al. (2020).

Further to the degradation pathways discussed above, evidence from Shettigar et al. (2012), suggests that there are four commercial neonicotinoid products that can be metabolised through to 6-CNA. Via methylene hydroxylation Shettigar et al. (2012) demonstrated that imidacloprid, thiacloprid, nitenpyram and acetamiprid can all be metabolised to 6-CNA. This information, in combination with the metabolism pathway featured in Figure 1.4 could present a possible pathway for total mineralisation under field conditions for four neonicotinoid products.

1.4.3.2. Changes in microbial populations and diversity

Whilst degradation pathways, endpoints and the microbes responsible for catalysis can be quantified, and often remain similar across conditions, the wider impacts of neonicotinoids on the soil microbiome and its subsequent effects are much the opposite. Research can often

appear quite contradictory with some studies demonstrating significant detrimental effects on soil microbiota post-exposure, whilst others claim that no negative effect is felt. Much like the response of soil invertebrates, the biotic and abiotic conditions can affect the level of exposure and therefore play a significant role in impacting the outcome of the neonicotinoid applications.

Studies by Li et al. (2018) demonstrated that when applied as a seed coating, imidacloprid and clothianidin had no significant effect on microbial community or richness. Their studies also included the assessment of diversity indices for both the bacterial and fungal communities, finding that the β-diversity of both communities was suppressed during the initial seedling growth stages. Diversity levels did, however, revert to pre-treatment levels as the study progressed (Li et al., 2018). In opposition to these findings, research conducted by Wu et al. (2021) showed that under laboratory conditions that thiamethoxam had a significant influence on microbial abundance and diversity. The treatments were combined directly with the soil at three different dosage levels, 1.8 mg kg^{-1}, 18.0 mg kg^{-1} and 180 mg kg^{-1} ; under these conditions the treatments were found to significantly reduce microbial diversity and alter community structure. Changes in the microbial community showed decreases in the plant-growth promoting rhizosphere bacteria *Actinobacteria*, and increases in the pollutant-degrading bacteria *Firmicutes* (Wu et al., 2021). Zhang et al. (2021) presented similar findings, demonstrating that high concentrations of thiamethoxam reduced soil bacterial diversity, as well as reducing network complexity across the studied populations. Both these findings indicate the possible influence of neonicotinoid insecticides in producing a shift in soil functions.

It can be difficult to fully quantify the exact reasons for the differences identified above, however, we theorise that the additional chemical agents included in the seed dressings, such as polymer coatings to prevent loss of the chemical, prevent excess contact between the

chemical dressing and the localised area around the planted seeds (Ludwig et al., 2020), could further prevent increased effects on the microbial communities. However, foliar sprays often contain alternative ingredients to assist with the spread and integration of the agrochemical and are readily incorporated into the soil through rainfall or mechanical turnover. These studies, also highlight the differences between field experiments conducted under true and realistic conditions, in comparison to those conducted in laboratories utilising the pure active ingredients over the commercial formulations. In many cases we often see lower levels of effect in the field studies, suggesting that actual field systems may be much more complex than those studied and quantified under laboratory conditions. In addition, field studies also allow for the repopulation of soil communities from deeper un-exposed soil layers, offering a level of buffering capacity that is otherwise not available nor accounted for under laboratory mesocosm conditions.

The findings from the literature present a variety of data, with no conclusive results as to how neonicotinoids can influence microbial communities and any subsequent changes soil function. The gaps in this area also raise some interesting research questions-

- Firstly, the method of application appears to have substantial impact on the microbial responses of treated soils. This is especially important as many studies appear to directly incorporate the chemical into the soil, ensuring a thorough mixing. This method of application is not representative of true application methods, with foliar sprays, soil drenches, and irrigation additives, resulting in a change in chemical level across the soil profile (Wu et al., 2020).
- Secondly, many of the studies included in the literature use the pure active ingredients rather than the commercially available formulations. In contrast, other environmental studies have begun to examine the co-application of chemicals alongside

neonicotinoids, and the presence of additional ingredients in commercial formulations, with studies reporting substantial impacts (Peña et al., 2011; Gill et al., 2012; Cang et al., 2017; Pescatore et al., 2020). It is therefore possible that the same could happen within microbial communities, theorising a possibility of microbial preference towards one ingredient over another, or that the physicochemical properties of some ingredients make them more inaccessible for microbial interaction and degradation.

1.5. Politics, policy and legislation

As of 2013, the use of plant protection products containing imidacloprid, thiamethoxam, and clothianidin, within the EU, was restricted through a temporary ban following initial criticisms regarding neonicotinoid environmental safety (European Commission, 2013). The restrictions associated with the initial EU temporary ban limited the use of neonicotinoid chemicals to less "bee-attractive" crops, and prohibited their use between January and June as to limit the exposure to active pollinators (European Commission, 2013).

The temporary ban was initially intended to be in place for two years, and was up for review in December 2017, with a final decision made by the end of April 2018. A review of the ban was made with the intentions of increasing the moratorium to a total and more permanent ban on the three aforementioned neonicotinoid agrochemicals (Stokstad, 2018). The current legislation expanded upon the previous temporary ban, forbidding the use of the three stated neonicotinoids for any outdoor application, however there use is still allowed within permanent greenhouses under the constraint that the plant matter spends its entire lifecycle indoors (European Commission, 2018a). In February 2020, it was further decided, by the EU and the UK, to not renew the registration for the neonicotinoid thiacloprid, commencing a

phasing-out of its use and a *de facto* ban by the end of its registration period (Fig. 1.9; University of Hertfordshire, 2017).

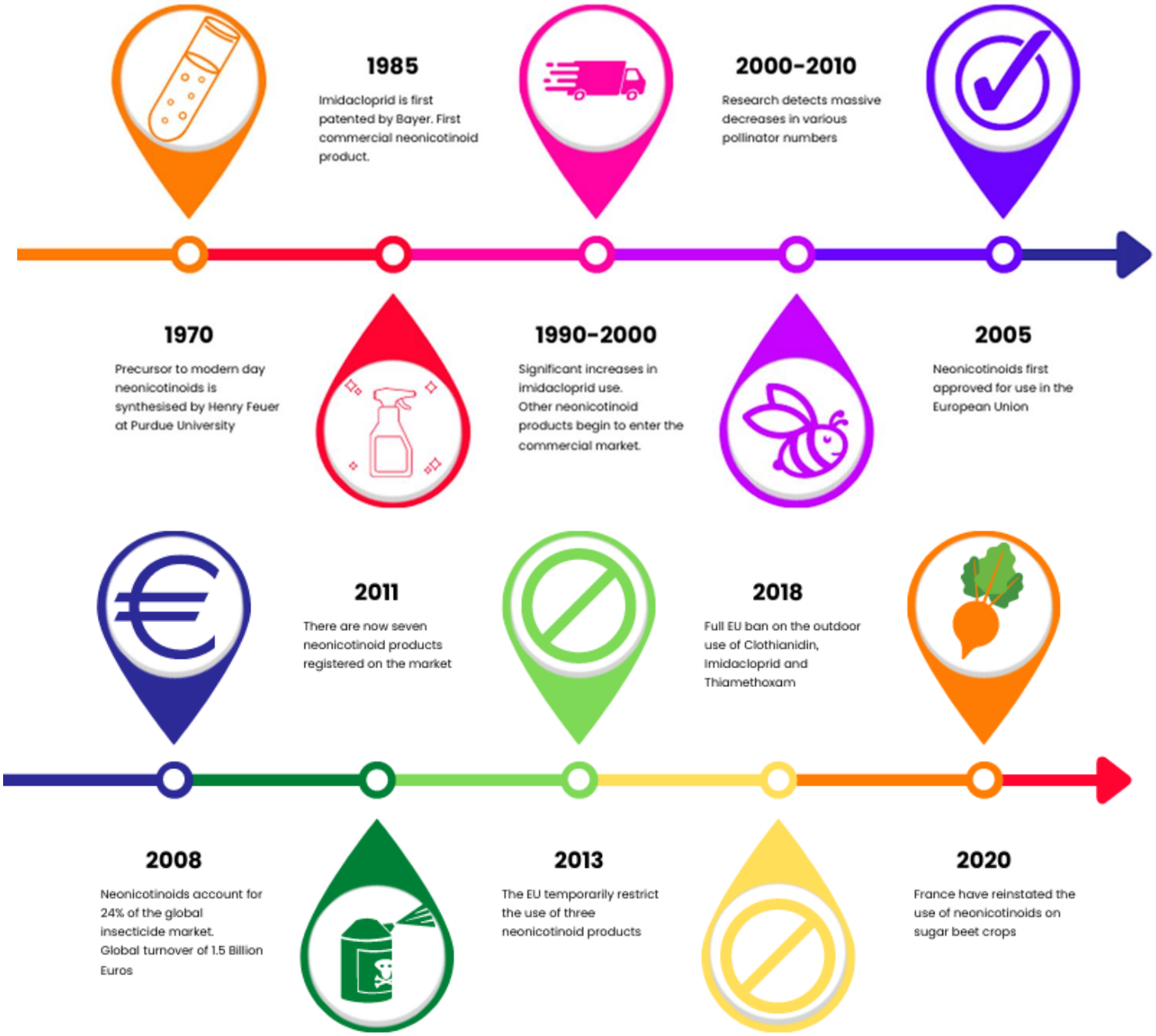


Figure 1.9. Timeline and overview of neonicotinoids, from registration through to current EU legislation.

The previous and current legislation builds upon the precautionary principle, with each previous temporary ban being instigated due to scientific evidence presenting some level of uncertainty in the environmental safety of neonicotinoid agrochemicals (Kriebel et al., 2001). However, the legislation has concentrated predominately on the impacts of neonicotinoids on pollinators, with little obvious focus on any other unwanted environmental effects, including those felt by fauna higher up the food chain (Uhl et al., 2015).

Since the EU ban in 2018, farmers across the affected areas have been allowed to apply for digressionary emergency permission to use neonicotinoids under exceptional circumstances. There have been over 200 such permissions were granted across the EU since 2016 (European Commission, 2022). These derogations have included 41 permissions for imidacloprid, 80 for thiamethoxam and 62 for clothianidin (European Commission, 2022). In many cases these continued permissions for emergency usage have allowed for almost continued 'normal' use since the EU ban was put in place. For instance, farmers in Denmark were granted permissions for the use of imidacloprid in 2019, 2020, and 2021, allowing for them to use imidacloprid-containing products on various crops including sugar beet across the growing season (European Commission, 2022). These special permissions have also been denied on numerous occasions, often stating that there were no exceptional needs for neonicotinoids specifically, and that the pesticide needs could be fulfilled by other registered products (Commission Implementing Decision (EU), 2020a,b).

Recent controversy has included the approval and registration of the chemical Sulfoxaflor. Sulfoxaflor, whilst technically classed as a sulfoxamine rather than a neonicotinoid, is classified as a Group 4 insecticide, the same commercial chemical grouping as neonicotinoids, acting upon similar systemic mechanisms, and affecting the insect nicotinic acetylcholine receptor (nAChR) in the same mode of action as that of neonicotinoids (Dow

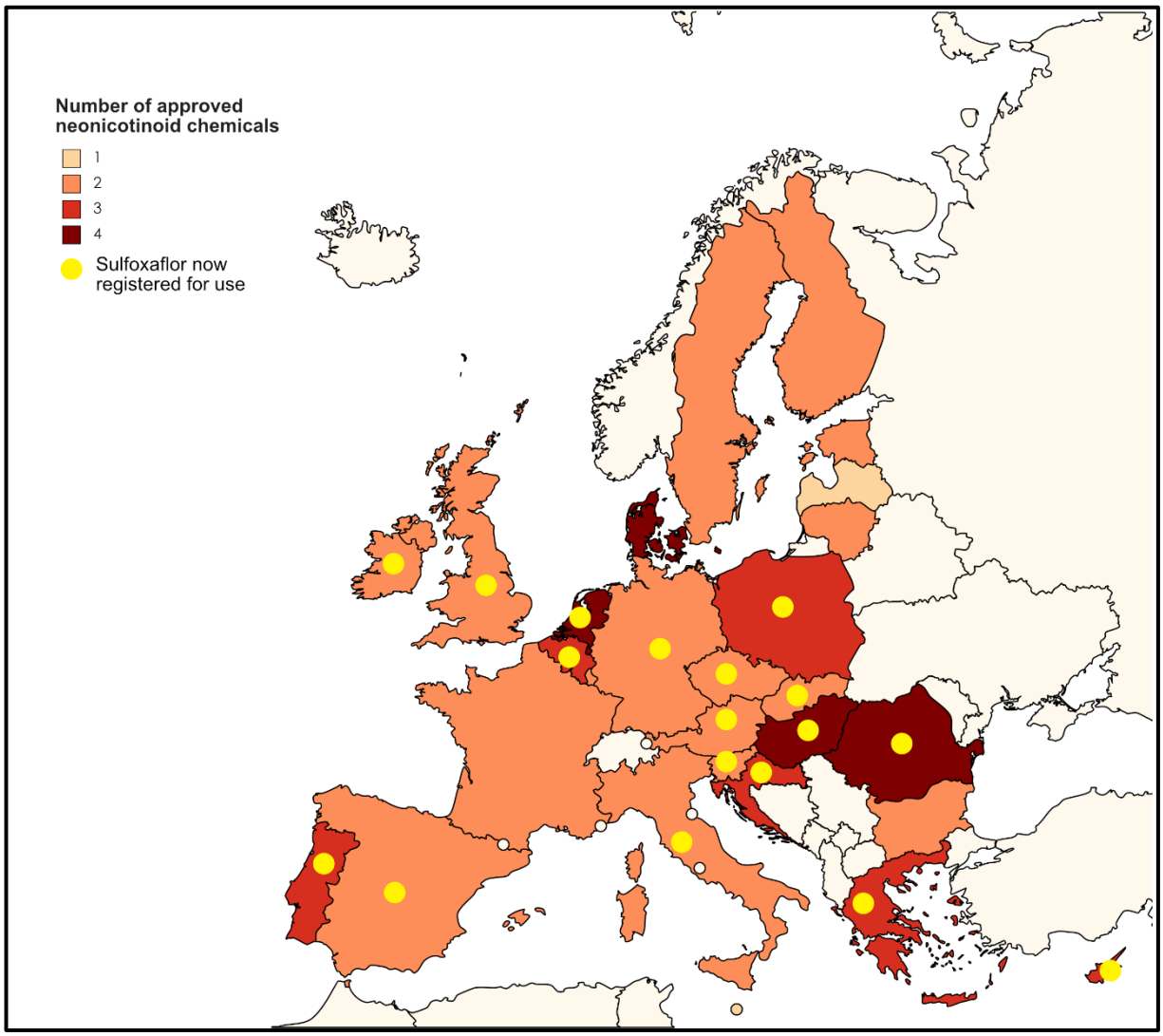


Figure 1.10. The number of registered neonicotinoid chemicals in EU member countries and UK. Data based off EC 1107/2009 from The Pesticide Property Database (Figure created using mapchart.net).

1.6. Conclusions

The research presented in this review offers a summary on the current state of knowledge regarding neonicotinoid usage and its impacts and interactions within the soil ecosystem. Whilst there have been recent political shifts, and changes to neonicotinoid product registration, we appear to continue to replace one pesticide with another (sulfoxaflor) without a clear understanding of their true environmental and ecological implications. As the evidence suggests, there is a growing knowledge base enhancing our understanding of the breadth of environmental impacts that can be traced back to the application of neonicotinoids, and other similar agrochemicals, however, it also highlights a number of knowledge gaps:

- The EU ban and subsequent phase-out of thiacloprid has left acetamiprid as the one remaining neonicotinoid product registered for use. Despite this, the available literature assessing the ecological impacts of acetamiprid as well as its mobility and persistence within the environment are seriously lacking.
- The majority of studies use the pure active ingredients in their research. This is not representative of realistic field conditions, and therefore it is recommended that future studies involve the investigation of the behavioural differences of commercially available formulations.
- The use of more realistic indicator species within ecological testing. *E. andrei* and *E. fetida* are often used due to their ease to rear, however, as composting-centric species they are rarely in contact with field-applied chemicals. It is therefore recommended that the use of a more ecologically relevant species, such as *Lumbricus terrestris* is used to better understand the impacts of agrochemical exposure.
- It had previously been quantified that the majority of responses exhibited by pollinator species post-exposure were sublethal, and therefore studies of direct and incidental

mortality only assess half of the problem. Further trials on other non-target species (i.e. earthworms) should involve the in-depth analysis of sublethal impacts and the subsequent environmental effects.

There is urgent need to be able to properly evaluate the impact of agrochemicals, specifically around how the behaviours and toxicities of chemicals change when in realistic commercial formulations, and how different application methods and farm management strategies can further influence these behavioural changes. All pesticides are harmful to a degree, but balancing the needs of food production and food security, with conserving biodiversity and ecosystem function is vital. Without reconciliation, the use of neonicotinoids, and any future pesticides, could pose a highly probably threat to the long-term sustainability of farming production and the wider environment.

Chapter II-

Acetamiprid fate in a sandy loam with contrasting soil organic matter contents: A comparison of the degradation, sorption and leaching of commercial neonicotinoid formulations

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2.1. Abstract

The impacts of neonicotinoids have generally focussed on the responses of the pure active ingredient. Using a selection of three acetamiprid mixtures, two commercial formulations and the active ingredient, we ran three laboratory studies using ^{14}C-labelled acetamiprid to study the leaching, sorption and mineralisation behaviours of the commercially available neonicotinoid formulations compared to the pure active ingredient. ^{14}C-spiked pesticide formulations were added to a sandy loam soil that had received long-term additions of farmyard manure at two rates (10 t/ha/yr and 25 t/ha/yr) and mineral fertilisers, as a control. We found significant differences in acetamiprid mineralisation across both the SOM and chemical treatments. Sorption was primarily impacted by changes in SOM and any differences in leachate recovery were much less significant across both treatment types. The mineralisation of all pesticide formulations was comparatively slow, with < 14.5 % of any given chemical/soil organic matter combination being mineralised over the experimental period. The highest mineralisation rates occurred in samples with the highest soil organic matter levels. The results also showed that 82.9 % ± 1.6 % of the acetamiprid applied was leached from the soil during repeated simulated rainfall events. This combined with the low sorption values, and the low rates of mineralisation, imply that acetamiprid is highly persistent and mobile within sandy soils. As a highly persistent neurotoxin with high invertebrate selectivity, the presence of neonicotinoids in soil presents a high toxicology risk to various beneficial soil organisms, including earthworms, as well as being at high risk of transfer to surrounding watercourses.

Keywords: Acetamiprid; Environmental fate; Persistence in soil; Soil management; Environmental risk

2.2. Introduction

The last fifty years has seen dramatic declines in insect species richness and population numbers worldwide (Hallmann et al., 2017). Drivers of decline include changes in land-use and management, agricultural intensification and the use of certain agrochemicals and pesticides (Goulson et al., 2015; Lima et al., 2016; Sánchez-Bayo et al., 2016). Pesticide use has led to many organisms experiencing long-term exposure to a diverse cocktail of synthetic chemicals, with neonicotinoids being strongly linked to insect pollinator decline (Blacquière et al., 2012; Whitehorn et al., 2012; Rundlöf et al., 2015). Neonicotinoids are a family of systemic agrochemicals, used primarily for the protection of crops from biting and sucking (saprivorous) pests (Tomizawa and Casida, 2005). Since the global commercialisation of imidacloprid in the early 1990s, neonicotinoids have become one of the most widely used agrochemicals worldwide (Jeschke et al., 2011; Woodcock et al., 2016). As of 2016, neonicotinoids accounted for 24% of all insecticide sales worldwide, with an average market value of $1.5 billion per year (Woodcock et al., 2016). The major drivers for their increased use were their ease of application and effectiveness at controlling invertebrate pests.

Neonicotinoids are acetylcholine antagonists, disrupting the nervous system of invertebrates on contact or through ingestion of treated plant matter (Maienfisch et al., 2001; Tomizawa and Casida, 2005; Downing and Grimwood, 2017; van Gestel et al., 2017). The mechanism of action of neonicotinoids is primarily attributed to their strong binding to nAChRs of the insect brain, however, they are indiscriminate between target pests and other non-target invertebrates (Tomizawa and Casida, 2005; Pisa et al., 2015; Botías et al., 2016; James et al., 2016). There is growing evidence that many other species are also impacted by neonicotinoids such as various soil invertebrates (Capowiez et al., 2006; Basley and Goulson, 2017; De Lima e Silva et al., 2017; Li et al., 2018). When applied as a seed dressing, up to 90% of the

neonicotinoid seed coating remains in the soil (Goulson, 2013), and the possibility therefore exists for soil accumulation many times higher than the original concentration applied to the seed (Capowiez and Bérard, 2006; Goulson, 2013; De Lima e Silva et al., 2017). Neonicotinoids are also applied as irrigation additives and foliar sprays by which they can also enter the soil. When originally applied as seed-dressings, the localised area surrounding a neonicotinoid-treated seed or plant will often present a much higher level of acute exposure to invertebrates (Girolami et al., 2009), with the compound leaching further through the soil profile over time (Liu et al., 2016; Rodríguez-Liébana et al., 2018). The persistence of these chemicals in soils can pose further ecotoxicological challenges to soil organisms (Zaller et al., 2016; Uwizeyimana et al., 2017; Renaud et al., 2018). A variety of impacts on soil fauna as a result of neonicotinoid contact or ingestion have been reported, including behavioural, reproductive and changes to community structures (Capowiez et al., 2006; Goulson, 2013; Pisa et al., 2015; Basley and Goulson, 2017; Saggioro et al., 2019). Alterations to the behaviours and reproductive rates and successes in vital ecosystem-engineer species such as earthworms, could imply wider changes to ecosystem functions because of neonicotinoid application.

Neonicotinoids are highly water soluble, facilitating their systemic uptake and transport to all crop tissues (Huseth and Groves, 2014). This solubility can lead to leaching and an increase in chemical mobility within the soil profile relative to other insecticides (Office of Pesticide Programs, 2003; Kurwadkar et al., 2013; Leiva et al., 2017). Waterlogged and heavy clay-rich soils have been shown to exhibit much higher residue levels due to their restricted mobility (Rexrode et al., 2003; Liu et al., 2015; Liu et al., 2016). The loss of neonicotinoids from a soil is considered biphasic, commencing with a period of rapid loss, followed by a notably slower secondary phase, possibly illustrating the sorption of the active substances to available soil particles (Papiernik et al., 2006; El-Hamady et al., 2009; Goulson, 2013). The

residence time and risk of exposure for neonicotinoids in soils varies greatly, influenced by both changes in environmental conditions and soil characteristics (Karmakar et al., 2006; Liu et al., 2016; Castillo Diaz et al., 2017).

In addition to chemical and biological degradation, neonicotinoid availability can be restricted through sorption to soil particles (Papiernik et al., 2006; Carbo et al., 2007; Banerjee et al., 2008). The rate and amount of sorption is thought to be dependent on the soil's clay and organic matter content (Flores-Céspedes et al., 2002; Banerjee et al., 2008; Jin et al., 2016). Neonicotinoids generally have a high solubility and a relatively low octanol-water partition coefficient, indicating a hydrophilic nature and a low potential for soil sorption (Murano et al., 2018). As with other soil processes, leaching appears to be influenced by both the chemical composition of the neonicotinoid and environmental variables, such as soil properties and climatic conditions (Jeschke et al., 2011; de Perre et al., 2015; Liu et al., 2017).

Typically, neonicotinoid studies have focused on the pure active ingredients acting in isolation, and as such ecotoxicity tests for the purpose of policy and regulation tend to follow the same methods (Bori et al., 2015). However, such agrochemicals are generally applied in either a carrier matrix or alongside a mixture of surfactants and emulsifiers combined within the commercial formulation (Malev et al., 2012; Anderson and Roberts, 1983; Neves et al., 2001). Consequently, studies that focus solely on the pure active ingredient may fail to capture the influence that surfactants and carrier matrices have on the fate and behaviour of neonicotinoids in soil (Bonmatin et al., 2015; Bori et al., 2015; Chagnon et al., 2015). Additionally, noenicitinoids are sometimes applied alongside other agrochemicals (Van der Sluijs et al., 2013; Goulson et al., 2015; Botías et al., 2017). Evaluating the synergistic and additive effects of these agrochemical mixtures is challenging due to the secondary effects on

soil biota, as the behaviours and persistence of the active ingredients may be altered when applied in conjunction with other agrochemicals.

We hypothesised that (i) increasing soil organic matter (SOM) content would promote acetamiprid sorption and biodegradation, whilst reducing leaching, and (ii) that this effect would be reduced in commercial formulations due to the presence of additional ingredients such as surfactants, adjuvants, and secondary active ingredients.

2.3. Materials and methods

2.3.1. Soil

The soil used in the experiments was obtained from selected treatments of the long-term Woburn Organic Manuring experiment (started in 1964) at Woburn Experimental Farm (Rothamsted Research), Bedfordshire, UK, in June 2018 (Mattingly 1974; Ma et al., 2020; Ma et al., 2021). The soil is classified as an Udipsamment (US Soil Taxonomy) or brown sand and has a sandy-loam texture (80% sand, 6% silt, and 14% clay) making it susceptible to pesticide leaching (Bromilow et al., 1999). The field was under winter rye (*Secale cereale* L.) at the time of sample collection. Soil samples (0-23 cm; Ahp horizon) were taken using a gouge auger from each replicate plot (n = 4) receiving either a high (25 t ha^{-1} y^{-1}) or low (10 t ha^{-1} y^{-1}) rate of farmyard manure (FYM) or control plots receiving mineral fertilisers only (Ma et al., 2020; Macdonald et al., 2018) since 2003. Hereafter, these treatments are referred to as SOM_{high}, SOM_{med} and SOM_{low}. The plots (with the exception of the SOM_{high} treatment) receive annual applications of triple superphosphate ($Ca(H_2PO_4)_2 \cdot H_2O$, 43-44% P_2O_5 97.5 kg ha^{-1} y^{-1}) and sulphate of potash (200 kg ha^{-1} y^{-1}, 50% K_2O, 45% SO_3). The treatment regimens for the field plots are further described in Table 2.1. The soil samples collected from these plots were passed through a 5 mm sieve and stored at 4 °C. Properties of the soil from the different treatments

are presented in Table 2.1. Soil moisture was calculated by drying soil at 105 °C for 24 h, average soil moisture was determined at 12.9 ± 0.3 %, hereto referred to as field moisture.

Table 2.1. Soil organic matter treatment regimes

	Treatment period		
	1966-1971	1981-1987	2003-2018
SOM_{low}	Mineral (P, K & Mg; equivalent to SOM_{high})	Mineral (P, K & Mg; equivalent to SOM_{high})	Mineral (P, K & Mg; equivalent to SOM_{high})
SOM_{med}	Mineral (P & K; 7.5 t ha^{-1} yr^{-1})*	No available data	FYM (10 t ha^{-1} yr^{-1})
SOM_{high}	FYM (50 t ha^{-1} yr^{-1})	FYM (50 t ha^{-1} yr^{-1})	FYM (25 t ha^{-1} yr^{-1})

* SOM_{med} mineral fertiliser-equivalent to a straw input of 7.5 t ha^{-1} yr^{-1}

Table 2.2. Soil physicochemical properties under long-term manure application.

	pH	Carbon (%)	Nitrogen (%)	DOC (mg C kg^{-1})	EON (mg N kg^{-1})	NO_3^- (mg kg^{-1})	NH_4^+ (mg kg^{-1})	MB-C (mg kg^{-1})	Basal respiration (mg CO_2 kg^{-1} h^{-1})
SOM_{low}	7.2 [a]	0.69 [c]	0.063 [c]	42.7 [b]	15.9 [a]	5.1 [b]	1.4 [c]	365 [c]	7.6 [b]
SOM_{med}	7.2 [a]	0.88 [b]	0.079 [b]	49.6 [b]	14.3 [a]	6.2 [b]	2.1 [b]	475 [b]	11.3 [a]
SOM_{high}	7.1 [a]	1.29 [a]	0.115 [a]	69.7 [a]	16.8 [a]	8.7 [a]	3.1 [a]	573 [a]	13.0 [a]

Values are means of four replicates. Different letters indicate significant differences between treatments at $P < 0.05$. EON: extractable organic nitrogen; DOC: dissolved organic carbon; MB: microbial biomass. Table adapted from Ma et al., (2020), soil samples collected in conjunction with both studies.

2.3.2. Neonicotinoid products

Two commercially available neonicotinoid products containing acetamiprid (N-[(6-chloropyridin-3-yl)methyl]-N'-cyano-N-methylethanimidamide) were tested. The first was a foliar spray product containing acetamiprid (0.05 g l^{-1}) within a mixture of ethanol and 1,2-benzisothiazolin-3-one (pH 6.62; Bug Clear Ultra®, Scotts Miracle-Gro Corp., Marysville, OH; hereon referred to as $Acet_{CF1;}$ Material Safety Data Sheet, 2008). The second was also a foliar

spray containing 0.05 g l^{-1} acetamiprid, but in combination with 0.15 g l^{-1} triticonazole fungicide and a range of emulsifiers and anti-bacterial preservatives (pH 5.22; Rose Clear Ultra®, Scotts Miracle-Gro Corp.; from here on in referred to as $Acet_{CF2}$; (Material Safety Data Sheet, 2010). A third solution of pure acetamiprid (0.05 g l^{-1}; Sigma-Aldrich) was used to account for any differences caused by any additional ingredients such as surfactants, adjuvants, emulsifiers, and fungicides present in the commercial mixtures (hereon referred to as $Acet_{Pure}$; Table 2.3).

Table 2.3. Physicochemical properties and additional information for chosen chemical treatments

	pH	DOC (mg L^{-1})	TN (mg L^{-1})	Supplier/Brand	Active ingredients	Additional known ingredients
Bug Clear Ultra® ($Acet_{CF1}$)	6.62	6686	20.49	Scotts Miracle-Gro Corporation	Acetamiprid- 0.05 g l^{-1}	Ethanol, benzisothiazolinone, glycerol, dipropylene
Rose Clear Ultra® ($Acet_{CF2}$)	5.22	3386	23.96	Scotts Miracle-Gro Corporation	Acetamiprid- 0.05 g l^{-1} Triticonazole- 0.15 g l^{-1}	Geraniol, Proxel GXL, citric acid mono hydrate
Pure Acetamiprid ($Acet_{Pure}$)	6.04	251	9.39	Sigma Aldrich	Acetamiprid- 0.05 g l^{-1}	

Values are means of three replicates. DOC, total dissolved organic C, TN, total dissolved N. Values are given for the prepared solutions, not the pure concentrates.

2.3.3. Pesticide movement

2.3.3.1. Acetamiprid mineralisation in soil

To determine acetamiprid degradation, 5 g of field moist soil from each SOM treatment were placed in individual sterile 50 cm^3 polypropylene centrifuge tubes and spiked with one of the three ^{14}C-labelled acetamiprid formulations ($Acet_{CF1}$, $Acet_{CF2}$ or $Acet_{Pure}$). ^{14}C-labelled acetamiprid [pyridyl-2,6-^{14}C; 1850 MBq $mmol^{-1}$] was purchased from the Institute of Isotopes

Co. Ltd., Hungary. This was spiked into the two commercial formulations and into a pure solution of acetamiprid. An aliquot of each spiked pesticide (0.5 ml, 0.05 g acetamiprid l^{-1}; 0.83 kBq $sample^{-1}$) was then applied to the soil surface. A 1 M NaOH trap (1 ml) was then suspended above the soil surface to capture any released $^{14}CO_2$ and the tubes were sealed. The NaOH traps were replaced periodically over 60 d (minimum of twice a week). Samples were incubated at 20 °C ± 1 °C. The amount of $^{14}CO_2$ within the traps was determined using Optiphase HiSafe 3 liquid scintillation fluid (PerkinElmer Inc., Waltham, MA) and analysed on a Wallac 1404 liquid scintillation counter (PerkinElmer Inc.) with automated quench correction. After 60 d, the soil was extracted with ethanol (20 ml; 30 min, 200 rev min^{-1}) to determine the amount of available pesticide residue remaining in the soil. The extracts were then centrifuged (18,000 *g*, 5 min) and the amount of ^{14}C in the supernatant determined by liquid scintillation fluid as described above.

2.3.3.2 Acetamiprid sorption in soil

To quantify acetamiprid sorption to the solid phase, 2 g of field moist soil from each SOM treatment was placed into individual 20 cm^3 polypropylene tubes. Subsequently, 10 ml of each ^{14}C-labelled acetamiprid formulation ($Acet_{CF1}$, $Acet_{CF2}$ or $Acet_{Pure}$; 0.05 g acetamiprid l^{-1}; 37 kBq $sample^{-1}$) was added to the tubes. The soil suspensions were then shaken (200 rev min^{-1}) for either 1, 2, 4, 24, 120 or 192 h. The soil suspensions were then centrifuged (18,000 *g*, 5 min) and the amount of acetamiprid remaining in solution determined by liquid scintillation counting as described above. The amount of sorption to the solid phase was calculated by difference.

As well as measuring the proportion of pesticide adsorbed to the soil, endpoint K_d partition values were also calculated for each treatment combination using the following formulae:

$$Cs = \frac{V(Ci-Ce)}{ms} \quad (1)$$

Calculating the concentration (g g^{-1}) in the solid phase (*Cs*), where *V* is the volume of water (ml), *Ci* is the initial concentration of chemical (g ml^{-1}), *Ce* is the equilibrium concentration (g ml^{-1}), and *ms* is the starting mass of soil (g).

K_d (g ml^{-1}) is then defined as the following-

$$K_d = {}^{Cs}/_{Ce} \quad (2)$$

K_d partition values, also known as the adsorption-desorption distribution coefficient, assist in our understand of a compound's mobility in the environment and how it distributes between the soils and solution phase

2.3.3.3 Acetamiprid leaching from soil

To determine the effects of SOM on acetamiprid leaching, field moist soil from each treatment (20 g) was placed into individual 20 cm^3 polypropylene syringe barrels (ø = 1.5 cm) to achieve a bulk density of 1.3 g cm^{-3}. The base of the syringe barrels was covered with a disk of glass microfiber filter paper (Whatman GF/C) to prevent soil loss. An aliquot of each ^{14}C-labelled acetamiprid formulation (0.5 ml, 0.05 g acetamiprid l^{-1}, 0.83 kBq $sample^{-1}$) was then applied to the soil surface and left to equilibrate for 1 h. The average total pore volume of the test samples was calculated via saturation, averaging 7.5 ml. Subsequently, the soil columns were leached by adding 7.5 ml of artificial rainwater to the soil surface (Jones and Edwards, 1993). This was repeated 6 times (i.e., 6 pore volumes), waiting for each pore volume to

percolate through before applying the next. Polypropylene vials were placed underneath each soil column to collect the leachate. The amount of ^{14}C-acetamiprid in the leachate was determined by liquid scintillation counting as described above.

2.3.4. Data analysis

All laboratory studies used a fully factorial experimental design, allowing for every combination of SOM level (n = 3) and pesticide formulation (n = 3) to be tested. All combinations were replicated four times, consistent with the replicated block design of the field experiment (Mattingly, 1974). The data for this study was analysed using the ANOVA and repeated measures packages in JASP (JASP Team (2020). JASP (Version 0.14.1) [Computer software]). For the purpose of this analyses, and due to the production of extreme outlying results, a single replicate from the $Acet_{CF1}$:SOM_{low} treatment combination was removed .

2.4. Results

2.4.1. Acetamiprid mineralisation in soil

The highest level of mineralisation was found in the $Acet_{CF1}$ formulation under the SOM_{high} treatment with 21.1 ± 0.81 % of the acetamiprid mineralised after the 60 day study period. Conversely, the $Acet_{CF2}$ formulation produced the lowest level of overall mineralisation across the study, with only 8.0 ± 0.83 % of the acetamiprid mineralised under the SOM_{low} treatment. Across all treatments, an average of 14.6 ± 0.71% of the applied acetamiprid was mineralised after 60 d of incubation. We analysed the results from across the three chemical treatments and found that they were significantly affecting the mineralisation of the test compound ($F_{(2,26)} = 31.117$, $p < 0.001$). The results from the ^{14}C- mineralisation study shows that all three formulations had a significantly different mineralisation behaviours to each other, with $Acet_{CF2}$ consistently producing lower levels and rates of degradation throughout the study

(Holm post-hoc comparisons- $Acet_{CF2}$:$Acet_{CF1}$ $p < 0.001$, $Acet_{CF2}$:$Acet_{Pure}$ $p < 0.001$, $Acet_{CF1}$:$Acet_{Pure}$ = 0.016). All three chemical treatments displayed the same pattern across the SOM treatments, with the highest rate and level of mineralisation found under the highest SOM treatment level and the lowest found under the lowest SOM treatment level (Fig. 2.1).

SOM level was also shown to produce significantly different results across the treatment levels, with the higher SOM levels producing higher total cumulative mineralisation values ($F_{(2,26)}$ =15.873, $p < 0.001$) , suggesting a higher rate of pesticide degradation (Mean ± SEM; SOM_{low}, 11.6 ± 1.2%, SOM_{med}, 14.5% ± 1.0%, SOM_{high}, 17.3% ± 1.0). The $Acet_{CF1}$ chemical formulation had the highest cumulative mineralisation across all three SOM levels (17.9 ± 1.0%), while $Acet_{CF2}$ had the lowest rates of mineralisation after 60 d (11.4 ± 1.0%) (Holm post-hoc analysis $p < 0.001$) (Fig. 2.1). The interaction between the two treatment regimes were also found to produce significant results ($F_{(4,26)}$ = 3.148, P = 0.031).

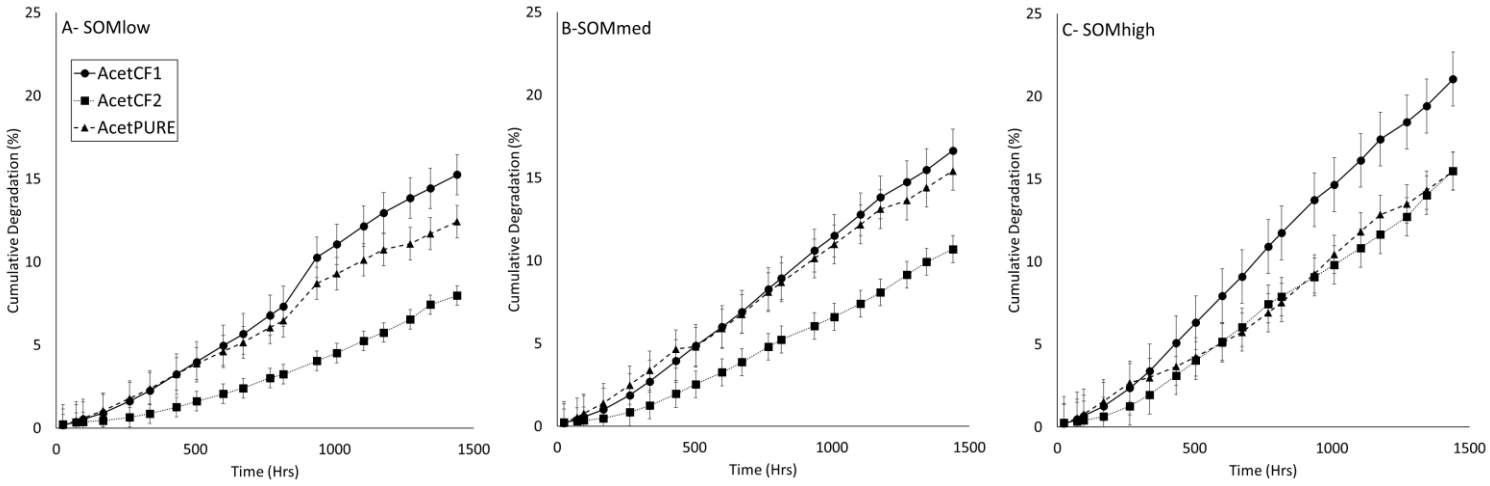

Figure 2.4. Cumulative mineralisation (by relative percentage) of acetamiprid in the three chemical formulations under the different SOM treatments. Values represent means ± SEM (n = 4).

2.4.2. Acetamiprid sorption to soil

The highest levels of sorption (15.9 ± 0.78 %) by the end of the study were found in $Acet_{Pure}$ under the SOM_{high} treatment. Under the SOM_{low} treatment the lowest levels of sorption were also found in the $Acet_{Pure}$ chemical solution, with only 9.3 ± 1.17 % of the acetamiprid being adsorbed by the end of the study period (Fig. 2.2). Both experimental treatments, the chemical mixture and the SOM level significantly affected the sorption behaviour of acetamiprid within the agricultural soils (formulation: $F_{(2,27)} = 9.590$, $p < 0.001$, SOM: $F_{(2,27)} = 159.007$, $p < 0.001$). The interaction between the two treatments also had a significant influence on the sorption behaviour ($F_{(4,27)} = 11.725$, $p = 0.031$). Significant differences in sorption behaviour occurred under each of the SOM treatments, with $Acet_{CF2}$ behaving significantly differently to the other two formulations ($Acet_{CF2}$: $Acet_{CF1}$ $P < 0.001$, $Acet_{CF2}$: $Acet_{Pure}$ $p = 0.013$; Fig. 2.2).

When solely analysing the endpoint data, the chemical treatment no longer exhibited significant differences in total sorption with an average of 12.3% ± 0.45% being adsorbed to the soil after 192 h. However, the SOM levels still had a significant influence on the sorption of acetamiprid (SOM: $F_{(2,27)} = 65.273$, $p < 0.001$), with the SOM_{high} treatments having a higher final proportion adsorbed to the soil relative to the other treatments (Mean ± SEM; SOM_{high} = 14.8 ± 0.7%, SOM_{med} = 12.0% ± 0.5%, SOM_{low} = 10.1% ± 0.4%; Fig. 2.2).

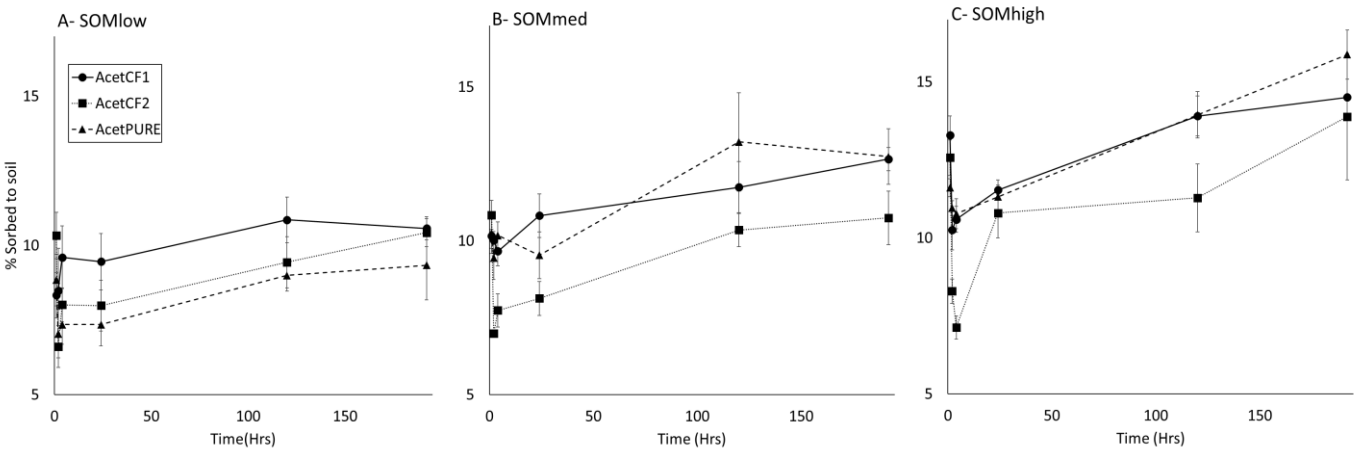

Figure 5.2. Sorption of acetamiprid in the three chemical formulations under different SOM treatment levels. Measured by relative percentage adsorbed within the soil. Values represent means ± SEM (n = 4).

Our K_d values show a significant increase alongside SOM treatment increase ($F_{(2,27)}$ = 16.641, $p < 0.001$; Table 2.4), with SOM_{high} exhibiting significantly different values across all three chemical treatments (Tukey post-hoc analysis: High:Low $p < 0.001$, High:Med p = 0.005). Unlike the initial sorption analysis, when assessing the endpoint K_d values only the level of SOM was shown to be significant; with none of the chemical treatments being recorded as distributing significantly different from the others.

Table 2.4. Endpoint K_d partition coefficient values (g ml^{-1}) for the three different acetamiprid formulations under three different SOM levels.

	SOM_{low} [a]	SOM_{med} [a]	SOM_{high} [b]
Bug Clear Ultra® ($Acet_{CF1}$)	0.591	0.725	0.849
Rose Clear Ultra® ($Acet_{CF2}$)	0.583	0.604	0.816
Pure Acetamiprid ($Acet_{Pure}$)	0.518	0.732	0.945

2.4.3. Acetamiprid leaching from soil

The lowest level of acetamiprid recovery was found for the $Acet_{Pure}$ and SOM_{med} combination with 75.4 ± 5.0 % of the acetamiprid detected by the end of the study. The highest levels of recovery were for the $Acet_{CF2}$ formulation under the SOM_{low} treatment with 92.8 ± 0.96 % of the applied acetamiprid recovered. Across all treatments, an average of 82.9% ± 1.6% of the applied acetamiprid was recovered in the leachate throughout the experiment. When comparing the endpoint data for this study, there were no significant differences in the percentage recovery of acetamiprid between the different chemical mixtures used (Fig. 2.3).

Previous research has shown that an increase in SOM can reduce the leaching of organic pollutants (Bollag et al., 1992), due to an increase in sorption and therefore less being available for leaching. Our results support this, as SOM had a significant impact on the level of acetamiprid recovered within the leachate ($F_{(2,27)} = 3.516$, $p = 0.044$). We also found that the chemical mixture and the interaction between the chemical and SOM treatments did not have a significant effect on the leachability and behaviour of the neonicotinoid compound (Chemical formulation: $F_{(2,27)} = 2.439$, $p = 0.106$, Chemical formulation:SOM: $F_{(4,27)} = 0.969$, $p = 0.440$) (Fig. 2.3)

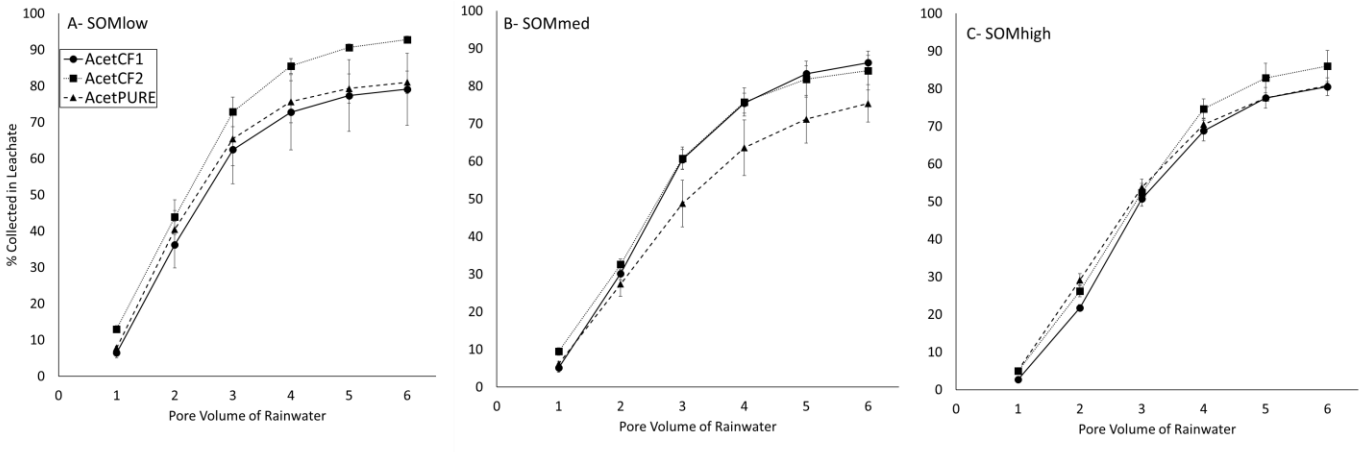

Figure 2.6. Cumulative percentage of acetamiprid in three different formulations recovered in leachate under three different SOM levels. Values represent means ± SEM (n = 4).

2.5. Discussion

2.5.1. Soil organic matter treatment effects

2.5.1.1. Acetamiprid mineralisation in soil

The differences in chemical fate across the varying levels of SOM could most likely be attributed to the increasing microbial biomass found to accompany the soils with increased SOM. Long term manure applications have previously been shown to be a primary driver for regulating and altering microbial communities within agricultural soils (Lin et al., 2019). Selected plots on the Woburn Organic Manuring Experiment (Rothamsted Research, UK), have received different manure applications since the experiment started in 1964 (Mattingly, 1974). The different treatments have resulted in significantly different levels of SOM and microbial biomass in each of the three test soils; these changes in microbial biomass are further reflected by an increase in basal respiration rate in soils treated with FYM in comparison to the mineral fertiliser treatment (Table 2.2). These increases and changes in microbial biomass and activity could be linked to the increases in acetamiprid degradation in the FYM treated soils (SOM_{high} and SOM_{med}). Though despite the increases in microbial biomass across these soils (Table 2.2), previous work, using the same study soils, has shown that the structure of the microbial communities remain similar, irrespective of SOM level (Ma et al., 2020), indicating that it is the growth in community rather than any changes in composition that are having an effect.

Within our ^{14}C mineralisation experiment we are accounting for total C mineralisation, rather than just the metabolism of the original compound into a secondary or tertiary metabolite. This means that whilst we were able to track how much of the original compound had been degraded, we were unable to quantify the levels of other intermediate metabolites generated. Whilst known to be produced in much lower quantities, some metabolites have

similar and sometimes higher invertebrate toxicities than the original neonicotinoid compound (Suchail et al., 2001). The compounds will also degrade and metabolise differently under different soil conditions (aerobic/anaerobic), and the metabolites will also vary depending on the biological communities in the soil (Liu et al., 2011, 2015; Rana et al., 2015; Hilton et al., 2016; Zhang et al., 2018).

The degradation of neonicotinoid compounds has previously also been investigated under chemically sterilised conditions. Zhang et al. (2018) demonstrated that, in the absence of microbial activity, neonicotinoids can be hydrolysed via amino and cyano hydrolysis. The rates of these mechanisms were strongly influenced by the pH and cation exchange capacity of the tested soils, with the rate of hydrolysis increasing as the solution becomes alkaline (Liu et al., 2006; Zhang et al., 2018). The soils used within this experiment all had similar pH levels (7.1-7.2) (Table 2.2), suggesting that pH level had, in this case, little influence over the fate of the applied formulations. The formulations themselves were found to have substantially different pH levels (Table 2.3). However, when accounting for the absolute amount of chemical applied and soils innate buffering capacity these differences are unlikely to have been responsible for any noticeable differences.

2.5.1.2 Acetamiprid sorption to soil

The fate of agrochemicals in soil are affected by various physical and chemical properties, although generally sorption is viewed as being one of the most important controlling processes (Pietrzak et al., 2020). The level of sorption exhibited by a chemical will impact all other processes within the soil such as the chemical's persistence, and biological or chemical degradation, as well as a pesticides' migration pathway (Pietrzak et al., 2020). The rate and extent of sorption exhibited by a chemical are controlled by the physico-chemical properties of

the chemical itself and also by environmental factors such as pH, temperature, soil type and organic matter persistence and amount (Bollag et al., 1992; Stefanakis et al., 2014). In our case the chemical treatment with the highest pH ($Acet_{CF1}$) exhibited the highest levels of mineralisation (Fig. 2.1), whilst the most acidic chemical treatment ($Acet_{CF2}$) had the lowest mineralisation end point (Fig. 2.1) and highest percentage leachate recovery (Fig. 2.3).

When analysing the sorption results from this study there were no significant differences between our commercial formulation with just one active ingredient ($Acet_{CF1}$) and the sorption of the pure acetamiprid ($Acet_{pure}$), however, the commercial formulation with two active ingredients ($Acet_{CF2}$) had significantly different sorption behaviour from the other two test chemicals; indicating a possible interaction between the active ingredients. We attribute this to the blocking of sorption sites by triticonazole which is known to readily bind to soil (Beigel and Pietro, 1999), therefore leaving fewer active sorption sites for the remaining acetamiprid. In this case the addition of additional ingredients (including secondary active ingredients, surfactants and adjuvants) alone did not appear to significantly impact the sorption of our test chemicals. This is also consistent with the results for triticonazole which also showed no impact of surfactant on its sorption to the soil's solid phase (Beigel and Barriuso, 2020).

The high water-solubility and low adsorption potential of neonicotinoid pesticides makes them highly susceptible to loss into the wider environment (Leiva et al., 2015; Morrissey et al., 2015). K_d values, also known as the adsorption-desorption distribution coefficient, assist in our understanding of a compound's mobility in the environment and how it distributes between the solid and solution phase. Throughout the literature, acetamiprid is generally noted as exhibiting K_d partition values as below 1.0 g ml^{-1} (Carbo et al., 2007; Dankyi et al., 2018; Murano et al., 2018). Studies by Carbo et al. (2007) present evidence suggesting that K_d values increase with soil horizons of increasing depth; associating soil sorption not only to the

chemical differences of the applied formulations but also to the physicochemical attributes of the soil itself. Since our experiment tested soil collected from the top 0-23 cm (Ahp horizon) our values support those collected in previous studies using soils from similar depths (Carbo et al., 2007; Murano et al., 2018).

The level of adsorption across different soils can often be attributed to the organic matter content (Flores-Céspedes et al., 2002; Banerjee et al., 2008; Jin et al., 2016; Mörtl et al., 2016). Our results support these previous findings, as with the higher levels of FYM application we found higher levels of sorption. Recent studies have shown that the addition of humic substances to the soils, can alter the sorption of certain compounds to the soil mineral phase (Murano et al., 2018). Murano et al. (2018) were able to show that the sorption of the neonicotinoid acetamiprid was reduced through the addition of humic or fulvic acids. This reduction in sorption has been attributed to the hydrophobic interactions between humic/fulvic acid and humin, where the dissociated carboxyl and phenolic groups have reoriented to face the soil solution (Murano et al., 2018). Alternatively, work by Jin et al. (2016) investigated the sorption of the neonicotinoid imidacloprid, found that the addition of organic bio-amendments, such as biochar from straw and manure increased the sorption capacity of the soil mixtures. These two studies indicate that, whilst organic matter can alter the transport and mobility of pesticides, only certain organic materials can be used to stabilise soils contaminated with neonicotinoids.

2.5.1.3 Acetamiprid leaching from soil

The increases in SOM investigated within our study have shown significant changes to the level of acetamiprid recovered in the leachate, with the highest level of recovery found in the lowest SOM treatments. These results correspond well to findings from Bollag et al. (1992),

who found that the increases in SOM level often reduced the amounts of chemical available for leaching. The further differences in behaviour exhibited by the different chemical mixtures could be due to the presence or absence of the additional surfactants added during manufacture. These additional chemicals are thought not to affect the designed action of the active ingredient, but in this study, it appears they may be involved in either an interaction with the active ingredient and/or the surrounding soil matrix, and as such are causing unexpected changes to acetamiprid fate in soil.

The ability of neonicotinoid pesticides to enter agricultural runoff and groundwater systems further extends the reach of their insecticide toxicity, increasing the risk to aquatic and marine invertebrates and other organisms (Morrissey et al., 2015). To date, there has been little research reporting the leaching behaviour of acetamiprid. However, when compared to other neonicotinoid compounds our results fall within the expected range. Gupta et al. (2008), reported thiamethoxam leachate recovery rates of between 66-79%, with zero detectable residue left within the soil. When combined with other research findings we conclude that all currently registered neonicotinoid compounds have a high leaching potential (Gupta et al., 2008; Liu et al., 2016; Wettstein et al., 2016; Aseperi et al., 2020; Pietrzak et al., 2020).

The methods of agricultural pesticide application are also considered major contributors to neonicotinoid leachability and rate of degradation (Wettstein et al., 2016). Compounds that are applied either directly to the soil or plant, such as soil drenches, irrigation additives or foliar sprays, may be more susceptible to run-off and leaching as they are readily incorporated into the aqueous phase of the soil. However, topically applied compounds (foliar sprays) were significantly more vulnerable to entering soil if applied directly before rainfall events (Anderson et al., 2015). In contrast, neonicotinoid compounds that are applied as seed dressings or in conjunction with a carrier matrix can be more persistent in soil, as the kinetics

dictating the release of active neonicotinoid ingredients from the mixture can result in <50% increase in persistence (Sarkar et al., 2012; Anderson et al., 2015; Wettstein et al., 2016). However, Wettstein et al. (2016) suggest that point source applications, such as seed dressings, have a higher leaching potential when compared to diffuse (spray) applications.

Previous studies have also shown that various soil properties, such as structure and texture, can also have significant impacts on the persistence of agrochemicals such as neonicotinoids (Bollag et al., 1992; Yang et al., 2013; Castillo Diaz et al., 2017; Murano et al., 2018; Rodríguez-Liébana et al., 2018; Pietrzak et al., 2020). The need to fully understand previous land management strategies and practices, as well as underlying historic soil contamination, are of paramount importance to better understand how these chemicals behave in the soil. Even the presence of heavy metal residues has been found to act as a catalyst to the degradation of certain neonicotinoids (Tariq et al., 2016). Further, many recent studies have used residual analysis, allowing for refined estimates of the timings and rates the degradation of the original active ingredient. However, many of these studies do not consider secondary and tertiary metabolites, and consequently published field and laboratory half-lives may underestimate long-term persistence of the possible breakdown products.

2.5.2. Chemical formulation differences

The differences in the behaviours of the tested chemicals could most likely be attributed to the presence of additives (including surfactants and adjuvants), and other secondary active ingredients, such as those present in *Acet*$_{CF2}$. These differences could suggest a chemical interaction either between the active ingredients or the additional ingredients (including surfactants, adjuvants, and emulsifiers) featured within the commercial products. From the information available there appears to be little difference in the carrier formulations for the two

commercial products, therefore these results possibly highlight a microbial preference to the secondary active ingredients (triticonazole) present in *Acet*$_{CF2}$, therefore resulting in a slower rate of mineralisation and lower final percentage degradation end point. Whilst this study restricted itself to comparing two commercial products, a study by Van der Sluijs et al. (2013) has suggested a synergistic relationship between neonicotinoids and azole-based fungicides, influencing both their environmental persistence but also their ecotoxicity. This combination of agrochemicals also impacts the decomposition of plant matter by influencing soil organisms (Zaller et al., 2016). It is uncommon for a single agrochemical to be applied in isolation as a pure ingredient. As well as additional active ingredients, many commercial pesticides are premixed with a combination of additional ingredients such as surfactants, preservatives (e.g., benzisothiazolinone), adjuvants, and other additives such as geraniol. These assist in ease of application or for aesthetic or perfumed purposes and can alter the natural behaviour of the pure ingredient. These changes can occur through a chemical relationship (i.e., synergistic, or additive) or through assisting with the physical dispersion of the pesticides.

As well as altering the physical and chemical behaviours of the pesticide action, the addition of surfactants and secondary active ingredients can have impacts on their interactions with the microbial communities within the soils (Pescatore et al., 2020), which in turn can alter the chemical's environmental persistence and resilience. In the case of *Acet*$_{CF1}$, the presence of additional ingredients within the commercial formulation produced a high level of soluble C (Table 2.3). *Acet*$_{CF1}$ underwent the highest level of biodegradation across all three SOM treatments. We therefore hypothesise that the extra C provided by the additional ingredients encouraged additional microbial growth within the soils, thus increasing the rate of microbial degradation (Fig. 2.1). This is supported by studies on other pesticides, where the degradation of trifluralin was reduced when in the presence of surfactants (Mata-Sandoval et al., 2001).

However, with *Acet*$_{CF2}$, despite an increase in TOC compared to the pure acetamiprid the opposite results were found (Fig. 2.1). In this case it is possible that the additional active ingredient, triticonazole, was more attractive or more easily utilised, than the acetamiprid; ultimately resulting in a lower degradation of the acetamiprid. Alternatively, the anti-fungal effect of triticonazole, and the antibacterial properties of certain additives, may have reduced the components of the microbial community responsible for acetamiprid transformation in soil.

2.6. Conclusions

Our findings show that the chemical carrier matrix and surfactants used in commercial formulations have a significant impact on the behaviour of acetamiprid when applied to agricultural soil. These findings imply that these additional ingredients, including secondary active ingredients, surfactants, and other additives may have adverse effects on the microbial communities influencing rates of mineralisation and altering degradation pathways. We also theorise that in the presence of additional ingredients, the additives could be being preferentially adsorbed on to the soil and targeted by microbial communities, reducing their interaction with acetamiprid. We also found that the addition of farmyard manure also affected the sorption of acetamiprid, and mineralisation and leaching when measured in combination with the differences in chemical formulation.

These findings are of particular interest when considering that many pesticide policies and regulations are supported by experiments focussing solely on the behaviour of the pure active ingredient. In the case of our pure active ingredient treatment (*Acet*$_{Pure}$), it often yielded significantly different results than those produced by the commercial formulations. These findings were particularly obvious when assessing the rate of mineralisation, with *Acet*$_{Pure}$ continually falling in between the two commercial formulations. These differences imply that

the experimental practice of using the active ingredient in isolation could provide unrealistic results, and thus may misrepresent the rate of persistence and associated risks to soil biota. This is especially pertinent when considering the ecotoxicological risk from contact or ingestion of contaminated material to various beneficial organisms, including pollinators and earthworms.

There are major knowledge gaps as to how neonicotinoids interact when applied in combination with other agrochemicals. Further work is required to improve our understanding of the major chemical interactions and evaluate the risks that these may pose. Overall, there is a need to better understand how land management (including gardening habits and farming practices) can influence the persistence and toxicity of neonicotinoid insecticides.

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Author Contribution

Davey L. Jones, Paul Cross, Jessica Potts and Andrew Macdonald conceived the study. Jessica Potts and Qingxu Ma performed the field sampling and soil characterisation. Jessica Potts undertook the radiotracer studies and data analysis and wrote the first draft of the manuscript. All authors contributed to revisions of the manuscript and approved the final version of the manuscript.

Chapter III-

Seasonal variation is a bigger driver of soil faunal community composition than exposure to a single dose of neonicotinoid pesticides over a growing season

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3.1. Abstract

Neonicotinoid pesticides are widely used within agroecosystems. Due to their systemic nature and high solubility, neonicotinoids are frequently recorded in soil, water, untreated plant matter and non-target organisms. Studies have demonstrated their capacity to induce invertebrate mortality, however, very little research has been conducted beyond pollinator exposure, particularly under field conditions. Typically, many neonicotinoids are applied via seed-dressings, reducing their direct contact with pollinators, but offering an unintended soil-exposure pathway. Soil biology underpins many vital functions, from regulating water and gas flow, to maintaining physical soil structure. In this study we investigated the effect of a commercial neonicotinoid pesticide (Insyst®, applied at a rate of 250 g ha^{-1}) on the abundance, richness, and community composition of both meso- and microfauna within soil (sandy clay loam textured Eutric Cambisol). Our results showed that over a single growing season, foliar application of Insyst® had no significant effect ($p > 0.05$) on measured soil biological indexes, including mesofauna count, alpha and beta diversity indices, as well as microbial taxonomic composition. We determined that seasonal variation was a significantly greater driver in regulating biological communities within the soil than the presence of pesticide. In addition, we showed that the active ingredient (acetamiprid) was rapidly degraded by the soil microbial community (average of 0.42 % day^{-1}). These results help highlight the need for realistic field studies, as agricultural pesticides are never pure, often containing surfactants, adjuvants, or emulsifiers. Understanding the biological interactions of vital soil fauna with necessary pesticide usage will enable proper risk assessments to maintain soil biological and ecological health.

Keywords- Soil quality; Ecological impact; Soil microbiology; Insecticide exposure; Acetamiprid

3.2. Introduction

Soil biology underpins many essential soil functions and processes, from maintaining organic matter utilisation and turnover, improving soil structure, and regulating air and water flow through the soil profile (Behan-Pelletier, 1999; Bottinelli et al., 2010; Lin et al., 2019; Wu et al., 2021). The quality and health of soil is often described by its functional capacity, and ability to provide vital ecosystem services (Karlen et al., 1997; Hou et al., 2020). Sustainable soil health is at the cornerstone of maintaining global food production (Comerford et al., 2013; Kopittke et al., 2019), however, achieving this remains a major challenge. The drive to improve crop production in the face of a rising population continues to drive our reliance on crop protection agents. Recent studies have shown that increased agrochemical usage can greatly affect soil-dwelling communities, from altering earthworm survival and longevity (Cang et al., 2017; Fang et al., 2018), to influencing keystone microbial taxa (e.g. mycorrhizas, nitrifiers; Yu et al., 2020; Wu et al., 2021). Changes in these populations within the soil have been shown to alter various biologically driven processes and ecoservices within the soil; for example, reductions in earthworm populations have been linked to reductions in litter decomposition in agricultural settings, which can subsequently lead to the immobilisation of nutrients, reduction in crop emergence and potential increases in the prevalence of crop pests (Basley and Goulson, 2017; Pearsons and Tooker, 2021)

Whilst there has been increasing comprehension of the impacts of farm management strategies (e.g. tillage) and their possible deleterious impacts on soil function, our understanding of how agrochemicals affect biochemical interactions in soil communities remains relatively low (Pisa et al., 2017). Changes to the relative abundances and composition of organisms in soil after exposure to agrochemicals can be difficult to quantify due to a wide range of external and confounding factors, including but not limited to – soil type, climate,

chemical used (including surfactants, additives, adjuvants, emulsifiers, and active ingredients), as well as historic land-use and underlying geogenic properties (Horswell et al., 2014; George et al., 2017, 2019; Uwizeyimana et al., 2017).

Recent years have seen an upsurge in the use of highly selective and systemic insecticides such as neonicotinoids. These types of pesticides accounted for approximately one third of the global insecticide market in 2015 (Simon-Delso et al., 2015) and have been at the forefront of pest management practices for the last 30 years, with over half of soybean seeds and almost all maize seeds being treated with neonicotinoids in the United States of America (Douglas and Tooker, 2015). Their highly selective neurotoxic mechanism, targeting the acetylcholine receptors, initially made neonicotinoids a staple insecticide, as due to their lower mammalian toxicity levels they were deemed environmentally safe and low risk for human contact (Tomizawa et al., 2007; Kimura-Kuroda et al., 2016; Casida, 2018). However, ever since their release in the early 1990's neonicotinoids have been continually linked to declines in pollinator insects, as well as songbird mortalities and ground water contamination events (Whitehorn et al., 2012; Gilburn et al., 2015; Lopez-Antia et al., 2015; Schaafsma et al., 2015; Mogren and Lundgren, 2016).

Often incorporated in soil through seed dressings, soil drenches, irrigation or secondary ploughing of treated crop stubble (Jones et al, 2014; Bonmatin et al., 2015; Zaller et al., 2016), neonicotinoids are highly water soluble and have been shown to persist in soil for over 1000 days (Baskaran et al., 1999; Sarkar et al., 2001; Rexrode et al., 2003; European Commission, 2004; Gupta et al., 2008; Fernández-Bayo et al., 2009; DeCant and Barrett, 2010; European Chemicals Agency, 2015). This prolonged level of contact provides the perfect conditions for soil-borne neonicotinoids to interact with and influence soil biology, on both a macro, meso and micro scale. To date, most neonicotinoid research has focussed on above-ground pollinator

impacts and the use of pure active ingredients, where the negative effects of neonicotinoid exposure have been well documented (Jin et al., 2015; Williams et al., 2015; Sánchez-Bayo et al, 2017; Tavares et al., 2017). However, the consequences of subterranean exposure to neonicotinoids remains largely undocumented.

Whilst developed to protect plants against biting and sucking insects such as aphids and weevils (Homoptera) (Jeschke et al., 2011), neonicotinoids have been widely documented to have similar deleterious impacts on non-target invertebrate species (Vijver and Van Den Brink, 2014; Douglas et al, 2015; Pisa et al., 2015; Zaller et al., 2016). Soils contain highly diverse biological communities of meso- and micro-organisms that are responsible for maintaining vital soil functions. Disruption of these soil biological communities can therefore have detrimental impacts on soil health, quality and function.

To date there have been very few studies focussing on the impact of acetamiprid on soil systems, particularly using commercial formulations under field conditions. Here we conducted a field experiment to assess the impact of an acetamiprid-based foliar spray on soil meso- and microbial communities. Tullgren funnel extractions, 16S rRNA high-throughput sequencing combined with metabolomic analysis were applied to investigate this exposure. This field-based study aimed to assess the impacts of a single application of the neonicotinoid acetamiprid formulation Insyst® on key biological groups and indicators in the soil of an arable cropping system. We were especially interested in the influence that neonicotinoid pesticides have on the abundance and composition of mesofauna groups such as Collembola and Acari. These two groups of soil-dwelling mesofauna play important roles in maintaining soil functions such as their involvement in litter decomposition and supporting soil microstructures (Rusek, 1998), and their abundance and diversity have been well documented to be impacted by various

human activities, making them useful indicator species (Rusek, 1998; Pearsons and Tooker, 2021).

The aims of this present study were to test i) the degradation rate of a field relevant level of Insyst® under field conditions, ii) quantify the production of any significant metabolites as a result of their degradation or changes in soil biochemical pathways and to iii) monitor any significant changes in the abundance and community composition of soil mesofauna and microbial communities. We hypothesised that the pesticide treatment will i) have a negative impact on mesofauna abundance, and ii) significantly alter community composition of both mesofauna groups and microbial communities.

3.3. Materials and methods

3.3.1. Site

The experimental field site was located at Henfaes Agricultural Research Station, Bangor University, Abergwyngregyn, North Wales, UK (53°14′N, 4°01′W). The trial was undertaken from May-Sept 2019. The field site has a temperate oceanic climate with mean average temperature of 10 °C and an average annual rainfall of 1060 mm. The soil is classified as a sandy clay loam textured Eutric Cambisol developed on a mixed glacial till parent material. Prior to the study, the site was under grass. The only recorded pesticide usage is the application of glyphosate for old sward destruction a rate of 6 L ha^{-1} pre-cultivation. The area has no previous record of neonicotinoid use.

3.3.2. Field design

In March 2019, a split-plot design was established creating four replicated split-plots, each half was randomly assigned either treatment or control (n = 4). Each combined plot (3 × 3 m) was contained by plastic boards sunk 20 cm into the ground in order to prevent lateral

water flow and movement of chemicals and soil fauna between plots, each board extended 10 cm above the soil surface to deter surface invertebrate migration (experimental field layout shown in Appendix 2). All plots were subsequently hand-sown with spring oilseed rape (OSR; *Brassica napa* L.) at a rate of 150 seeds m^{-2}, the plots were later thinned to average 80 plants m^{-2} (Roques and Berry, 2016). Fertiliser was applied in accordance with national guidelines (RB209; AHDB, 2019), with 50 kg N ha^{-1} (NH_4NO_3) applied.

3.3.3. Pesticide treatment

One commercially available neonicotinoid product containing acetamiprid (N-[(6-chloropyridin-3-yl)methyl]-N'-cyano-N-methylethanimidamide) was tested. Insyst® (Certis UK Crop Protection, Great Abington, UK) is a specialist agricultural formulation sold as a dissolvable powder with application rates of 200-250 g ha^{-1} (liquid dose 200-600 L ha^{-1}). Insyst® is a formulation of 20% w/w acetamiprid in combination with benzenesulfonic acid, mono-C10-13-alkyl derivatives, and sodium salts. The Insyst® insecticide treatment was mixed to a final concentration by dissolving in ultrapure water (resistivity = 18.2 MΩ-cm; total organic carbon < 5 ppb). The treatment was applied using a knapsack hand-held sprayer at a rate of 250 g ha^{-1} (equivalent to maximum application rates), protecting the control plots with plastic sheeting to avoid direct spray-drift.

3.3.4. Experimental protocols

3.3.4.1. Mesofauna extraction and identification

Soil mesofauna were extracted from soil cores using the Tullgren funnel methodology (Rusek, 1998; Behan-Pelletier, 1999). Soil cores (ø = 10 cm, depth = 10 cm) were left to run for seven days and extracted samples were collected in tubes containing 70% IMS (industrial methylated spirit). This process was repeated at monthly intervals throughout the durations of

the experiment (Sampling regime in supplementary information, Appendix 2). Invertebrate samples were refrigerated until ready for visual identification. Upon identification, individuals were separated into Collembola (springtails), Acari (mites), Coleoptera (beetles), Diptera (flies), Nematodes (roundworms), and "Other" (which included unidentifiable larvae and one-off individuals). Further consideration was given to the mesofauna samples of Acari and Collembola, sub-dividing further into orders and families, allowing for further examination due to their importance and proportional dominance within the soil communities. Acari were identified following Crotty and Shepherd (2014), whilst Collembola were identified following Hopkin (2007); initial identifications were verified by a competent colleague.

3.3.4.2. 16S Microbial analyses

Soil samples were collected periodically (6 collection points; sampling schedule in Appendix 2). Using an auger (0-10 cm) four samples were taken per plot and aggregated, and then stored at -80 °C until ready for further analysis. The samples (n = 48) were then freeze-dried, ground using a stainless-steel ball mill and shipped with dry ice (-78.5 °C) to Microbiome Insights (Vancouver, British Columbia, Canada) to conduct the 16S analyses.

3.3.4.2.1. DNA extraction, PCR, sequencing and sequence processing

Specimens were placed into a MoBio PowerMag Soil DNA Isolation Bead Plate. DNA was extracted following MoBio's instructions on a KingFisher robot. Bacterial 16S rRNA genes were PCR-amplified with dual-barcoded primers targeting the V4 region (515F 5'-GTGCCAGCMGCCGCGGTAA-3', and 806R 5'-GGACTACHVGGGTWTCTAAT-3'), as per the protocol of Kozich et al. (2013). Amplicons were sequenced with an Illumina MiSeq using the 300-bp paired-end kit (v.3). Sequences were denoised, taxonomically classified using Silva (v. 138) as the reference database, and clustered into 97%-similarity operational

taxonomic units (OTUs) with the mothur software package (v. 1.44.1) (Schloss et al. 2009), following the recommended procedure (https://www.mothur.org/wiki/MiSeq_SOP; accessed Nov 2020).

3.3.4.2.2. Quality control

The potential for contamination was addressed by co-sequencing DNA amplified from specimens and from template-free controls (negative control) and extraction kit reagents processed the same way as the specimens. A positive control from 'S00Z1-' samples consisting of cloned SUP05 DNA, was also included. Operational taxonomic unit were considered putative contaminants (and were removed) if their mean abundance in controls reached or exceeded 25 % of their mean abundance in specimens.

3.3.4.3. Metabolomic analysis

Additional soil samples for metabolomic analyses were gathered at the same time as those used for the 16S analysis (6 time points; Appendix 2). The four samples were randomly taken from across each plot and homogenised to obtain a representative sample from each plot (n = 8). These samples (n = 48) were stored in clip-top glass jars at -80°C until ready for analysis. The samples were then prepared in the same manner as those for 16S, freeze drying and ball milling. Samples were then shipped with dry ice (-78.5 °C) to the West Coast Metabolomics Center (UC David Genome Center, Davis. California, USA) for untargeted primary metabolic analysis. The analysis was conducted using automated liner exchange cold injection system gas chromatography time of flight mass spectrometry (ALEX-CIS GCTOF MS).

The extraction of the untargeted primary metabolites involved vortexing a 1:0.025 (w/v) soil-to-3:3:2 (v/v/v) MeCN/IPA/H_2O solution, followed by shaking for 5 min at 4 °C.

The sample solutions were then centrifuged, and an aliquot of the supernatant removed for analysis. Metabolomic analysis was achieved using a 689- GC (Agilent Technologies) coupled to a Pegasus IV TOF MS (Leco Corporation, St. Joseph, MI, USA), injected via a Gerstel CIS4 with dual MPS Injector (Gerstel, Muehlheim, Germany), following the parameters laid out by Fiehn et al. (2008). Data pre-processing was conducted without smoothing, using; 3 s peak width, baseline subtraction just above the noise level, and automatic mass spectral deconvolution and peak detection at signal/noise levels of 5:1 throughout the chromatogram using ChromaTOF vs.2.32. The data set was then validated, aligned and filtered using the BinBase algorithm as described in Fiehn et al. (2008) and Fiehn, (2016). The final compiled results were reported as peak heights, the data set also included internal standards for quality control and peak correction purposes. As is common practice for untargeted analyses the data presented in this study are therefore qualitative, and the compounds are tentatively identified (Gertsman and Barshop, 2018).

3.3.4.4. ^{14}C-labeled pesticide mineralisation

To determine the primary pesticide degradation rate, small areas of each treatment plot were encased within small plastic tubes and further treated with radiolabelled ^{14}C-acetamiprid mixtures (Experimental set-up diagram in Appendix 2). ^{14}C-labelled acetamiprid [pyridyl-2,6-^{14}C; 1850 MBq mmol^{-1}] was purchased from the Institute of Isotopes Co. Ltd., Hungary. This was spiked into the prepared Insyst® solutions (3.5 kBq sample^{-1}). A 4 M NaOH trap (1 ml) was placed within each of these tubes and then capped, allowing for the respired $^{14}CO_2$ to be captured and used to calculate total mineralisation within the field. The tubes were sampled and replaced periodically over nine weeks (sampling regime in Appendix 2).

The amount of $^{14}CO_2$ within the traps was determined by taking 0.25 ml from each NaOH trap and combining with 4 ml Optiphase HiSafe 3 liquid scintillation fluid (PerkinElmer Inc., Waltham, MA) and analysed on a Wallac 1404 liquid scintillation counter (PerkinElmer Inc.) with automated quench correction.

3.3.4.5. Nutrient analysis

Nitrate and ammonium content of the soils were analysed by combining fresh soil samples (5 g) with 0.5 M K_2SO_4 (25 ml) and shaking for 1 h. The extracts were analysed colorimetrically using the salicylate procedure of Mulvaney (1996) for NH_4^+ and vanadate procedure of Miranda et al. (2001) for NO_3^- using a PowerWave microplate reader. The same procedure, but using 0.5 M acetic acid, was used to extract available phosphate from the soils (Ron Vaz et al., 1993) with P analysed colorimetrically using the molybdate blue method of Murphy and Riley (1962). The moisture content of the soils was determined by oven drying the soils at 105 °C for 24 h. pH and EC (electrical conductivity) were determined by combining 10 g of the treatment soil with 25 ml of deionised water and testing with standard electrodes.

3.3.4.6. Data analysis

The 16S and metabolomic analyses were conducted in the R environment (R core team, 2022), with graphical analysis being performed using the '*ggplot2*' package (Wickham et al., 2021). Mesofauna data was analysed using ANOVA (repeated measures and one-way as appropriate) and post-hoc packages in JASP (JASP Team (2020). JASP (Version 0.14.1) [Computer software]), unless stated otherwise. Alpha diversity was estimated with the Shannon index on raw OTU abundance tables after filtering out contaminants. Operational taxonomic unit (OTUs) are mathematical unit used to classify groups of closely related sequences and are therefore used as proxies for microbial 'species'. OTUs defined at 97% sequence similarity are

loosely estimated as a species. The significance of diversity differences was tested with ANOVA. To estimate beta diversity across samples, we excluded OTUs occurring with a count of less than 3 in at least 10 % of the samples and then computed Bray-Curtis indices using the '*vegan*' package (Oksanen et al., 2020). We visualized beta diversity, emphasizing differences across samples, using non-metric multidimensional scaling (NMDS) ordination of the OTU community composition. Variation in community structure was assessed with permutational multivariate analyses of variance (PERMANOVA; Anderson et al, 2013) with treatment group as the main fixed factor and using 999 permutations for significance testing.

3.4. Results

3.4.1 Mesofauna Tullgren funnel extracts

A total of 4250 invertebrate individuals were counted and identified throughout this study period. There was a significant increase ($F_{(2,18)} = 21.598$, $p < 0.001$) in total invertebrate abundances across the sampling season with 382, 1412, and 2456 individuals extracted in May, July, and August respectively. There was no significant difference in total invertebrate counts between the two treatment scenarios ($F_{(1,18)} = 0.193$, $p = 0.665$).

3.4.1.1. Mesofauna abundance

Across all sampled invertebrate groups pesticide exposure was found to have no significant effect on the number of any measured invertebrate groups. We categorised the invertebrate samples into six distinct but broad groups, Collembola (springtails), Acari (mites), Coleoptera (beetles), Diptera (flies), Nematodes (roundworms), and "Other" (which included unidentifiable larvae and one-off individuals). Mites and Collembola were then further identified and sub-divided into the Acari order Mesostigmata, and the sub-orders Astigmata,

Oribatida; Collembola were further sub-divided into the order Symphypleona, and superfamilies Entomobryoidea and Poduridae.

Collembola were generally found to be the most common mesofauna group, on average accounting for between 34.5 – 50.3% of the mesofauna samples across the sampling season. Coleoptera were the least represented group in both treatment scenarios across the sampling season, accounting for between 0.7 – 1.3 % of recorded individuals (Fig. 3.1). Across the sampling season there was an increase in the number of individual Collembola extracted from the samples. An average of 47.8 ± 13.06, 176.5 ± 16.2, and 307 ± 40.2 individuals were recorded in May, July, and August respectively.

The two major mesofauna groups, Collembola and Acari, were then further sub-divided into identifiable orders/families. Collembola numbers are dominated by Entomobryoidea, (Fig. 3.2). Mesostigmata are the most commonly identified group of Acari collected from the Tullgren funnel extracts throughout this study, accounting for an average of 65 ± 4.7 % of collected Acari throughout the study across both treatment scenarios (Fig. 3.3). Astigmata mites were constantly the least recorded Acari order throughout the experiment accounting for on average 14 ± 4.0 % of Acari counts. However, the Astigmata counts remain relatively similar across both the treatment and control plots. Conversely, Mesostigmata count, and abundance increase significantly across the season (Count- $F_{(2,12)} = 8.414$, $p = 0.005$; Proportional abundance- $F_{(2,12)} = 12.570$, $p = 0.001$; Fig. 3.3).

Across all the broad invertebrate groups identified and measured within this study, there is no indication of any significant changes in individual numbers as a result of the pesticide treatment (Table 3.1). Many of the results do, however, show a significant change in absolute abundance across the study period, indicating that seasonal variance may play a much bigger role in biological composition than external contamination sources. Additionally, the results

from the nematode counts show a significant interaction between time and pesticide treatment ($F_{(2,10)} = 4.195$, $p = 0.048$), with the treatment producing a reduction in nematode numbers over time, whereas individual numbers increased in the control plots across the study period (Fig. 3.4B).

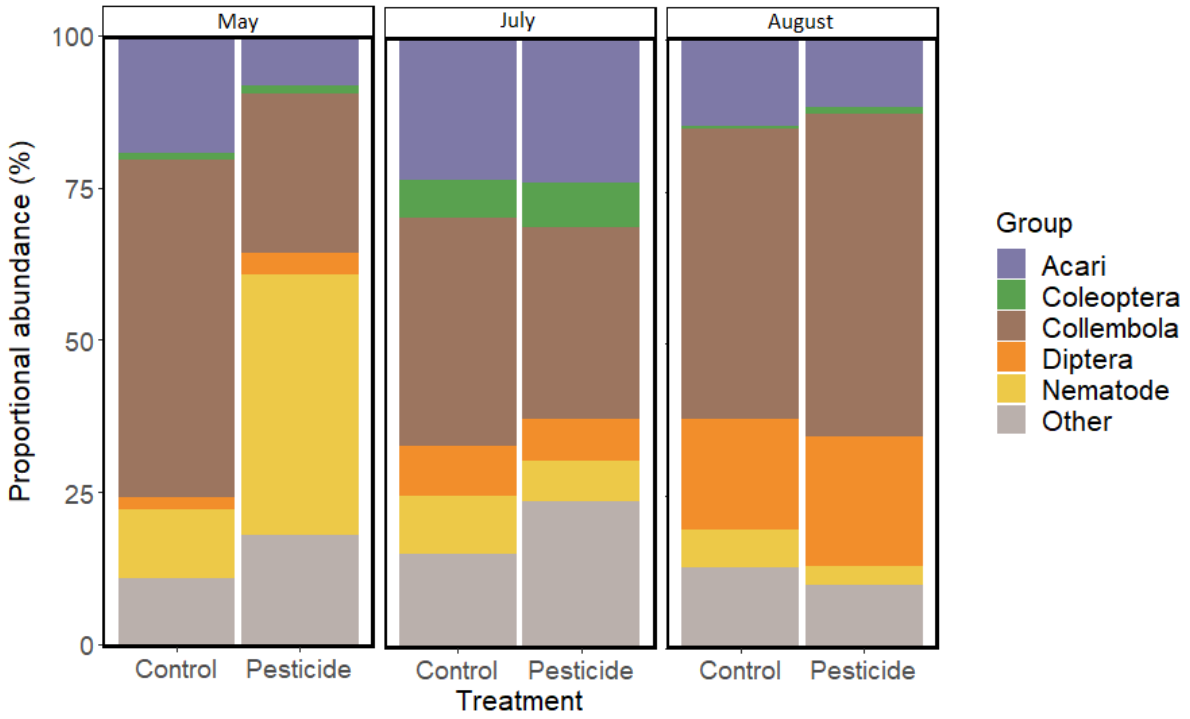

Figure 3.1. Proportional abundance of the major mesofauna groups collected from Tullgren funnel extractions in response to the addition of neonicotinoid pesticide. Values are averaged across the replicated field plots ($n = 4$).

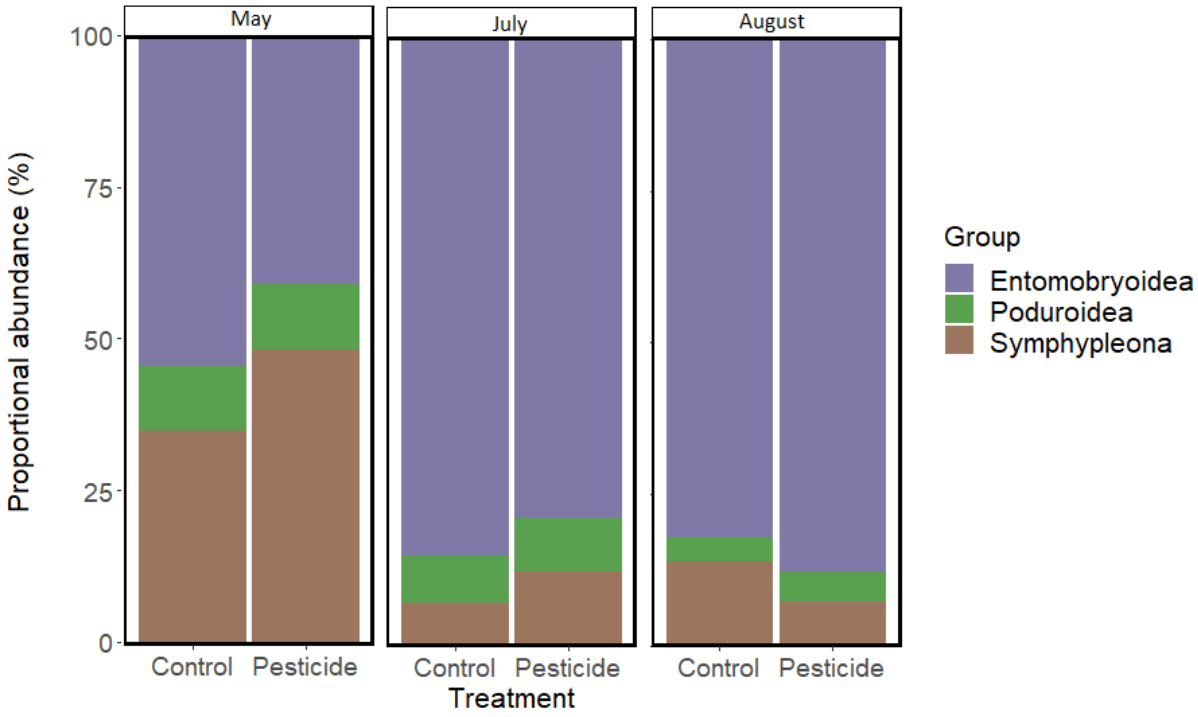

Figure 3.2. Proportional abundance of the major Collembola families collected from Tullgren funnels in response to the addition of neonicotinoid pesticide. Values are averaged across the replicated field plots (n = 4).

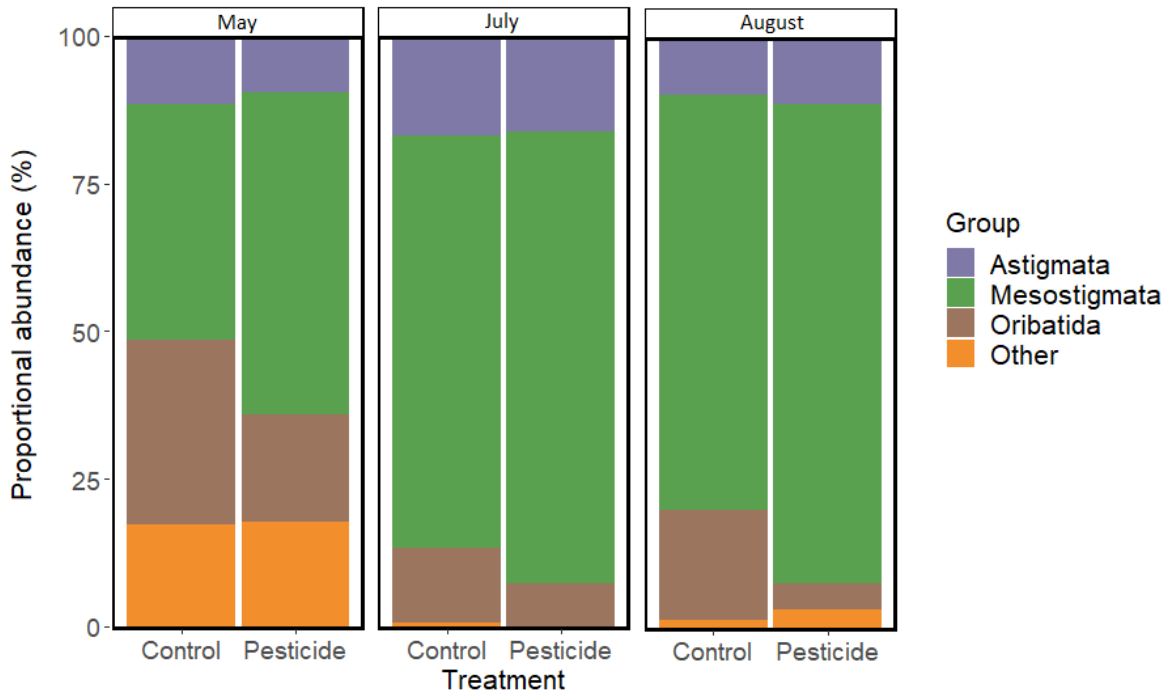

Figure 3.3. Proportional abundance of the major Acari orders from Tullgren funnels in response to the addition of neonicotinoid pesticide. Values are averaged across the replicated field plots (n = 4).

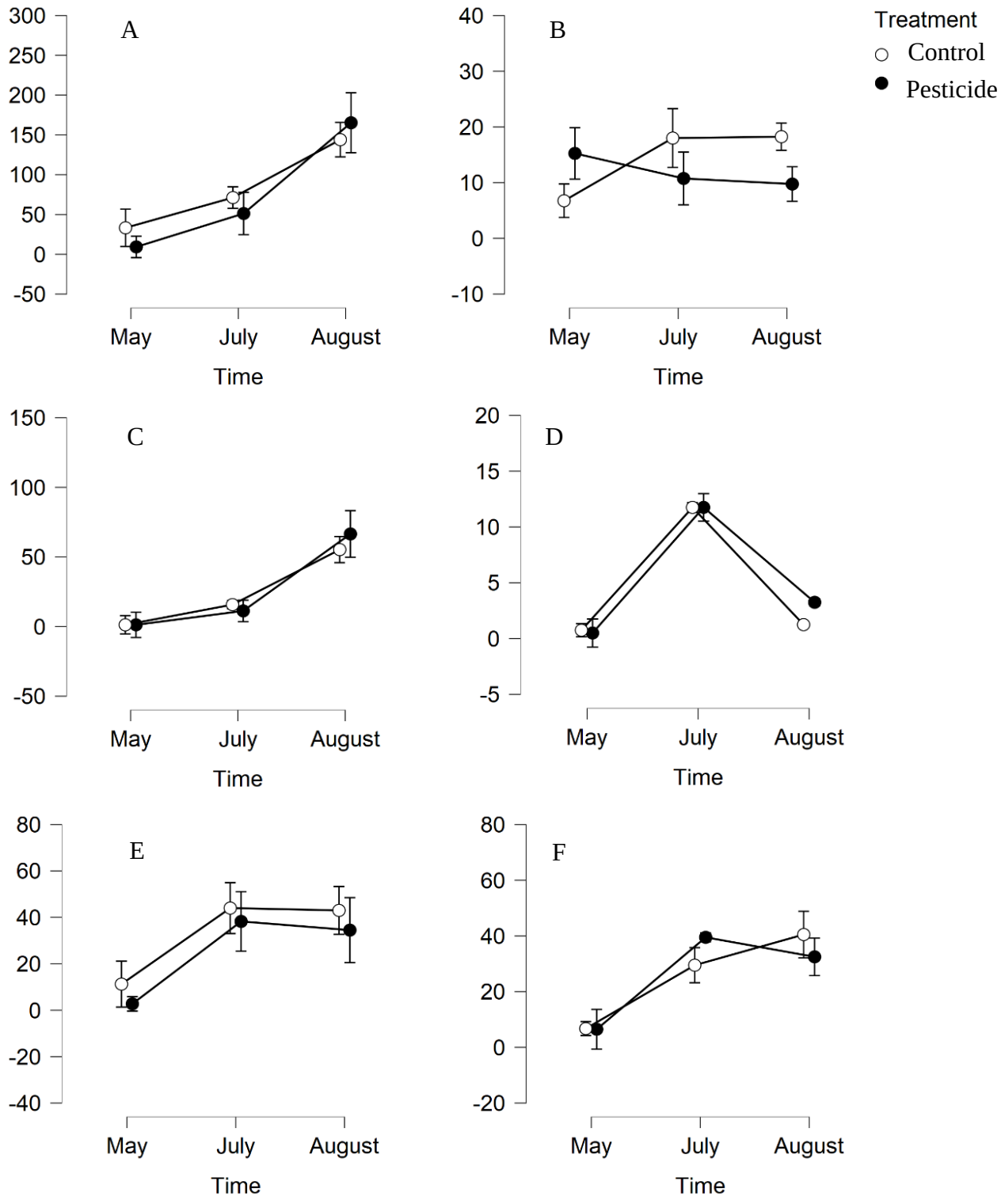

Figure 3.4. Changes in individual numbers in response to the addition of neonicotinoid pesticide throughout the growing season. A: Collembola, B: Nematode, C: Diptera, D: Coleoptera, E: Acari, F: Other. Mean ± SEM (n = 4). Note different scales on the y-axis

Table 3.1. Repeated measures ANOVA outputs across the major mesofauna groups

	Time		Treatment		
Species	$F_{(2,10)}$	p	$F_{(1,5)}$	p	**Other significant data** [a]
Collembola	1.51	0.267	0.239	0.646	May:August = 0.001 July:August = 0.009
Nematode	4.074	0.051	0.461	0.527	Month:Treatment $F_{(2,10)}$ = 4.195 P = 0.048
Diptera	1.59	0.263	0.061	0.815	May:August < 0.001 July:August = 0.001
Coleoptera	21.70	0.004	0.191	0.68	May:July < 0.001 July:August < 0.001
Acari	0.15	0.864	1.628	0.258	May:August = 0.031 May:July = 0.031
Other	3.712	0.062	0.017	0.9	May:July < 0.001 May:August < 0.001

a- post-hoc analyses conducted using Holm analysis

3.4.1.2. Mesofauna community composition and diversity

3.4.1.2.1. Alpha diversity

Shannon diversity values significantly changed across the sampling season ($F_{(2,18)}$ = 7.624, p = 0.004), with the values in July being significantly higher than those of the other two months (Tukey post-hoc analysis- July:August, p = 0.013; July:May, p = 0.007). Pesticide treatment was not found to have any significant influence on the diversity values ($F_{(1,18)}$ = 0.895, p = 0.426; Fig. 3.5). Average Shannon diversity values were calculated to be 1.28 ± 0.07, 1.56 ± 0.03, and 1.31 ± 0.05 for May, July, and August respectively (Mean ± SEM).

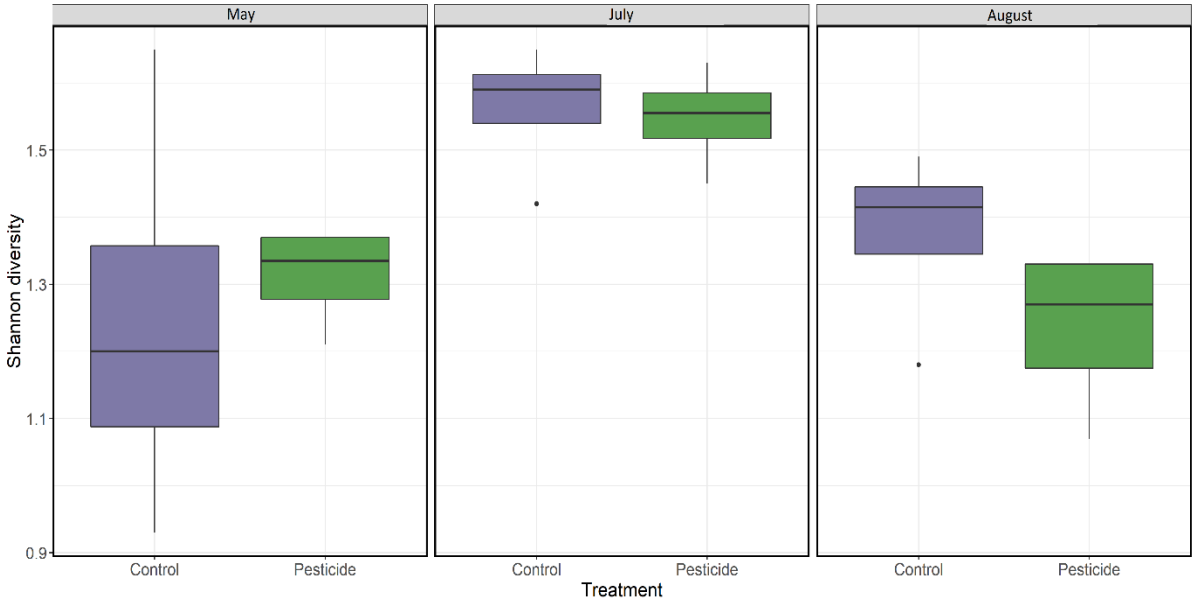

Figure 3.5. Shannon diversity averaged across replicated plots (n = 4) in response to the addition of neonicotinoid pesticide measured across the growing season. Boxes are bounded on the first and third quartiles; horizontal lines denote medians, and black crosses denote mean values. Black dots are outliers beyond the whiskers.

3.4.1.2.2. Beta diversity

The NMDS ordination shows no clear separation in mesofauna communities between the soils treated with the Insyst® insecticide and the control field plots (Fig. 3.6). The mesofauna communities were also more closely grouped in the July and August sampling dates. Through PERMANOVA analysis we showed that whilst pesticide treatment had no significant effect on the mesofauna β-diversity ($F_{(1,23)}$ = 0.5595, p = 0.751); β-diversity did change significantly across the sampling season ($F_{(2,23)}$ = 6.6809, p < 0.001).

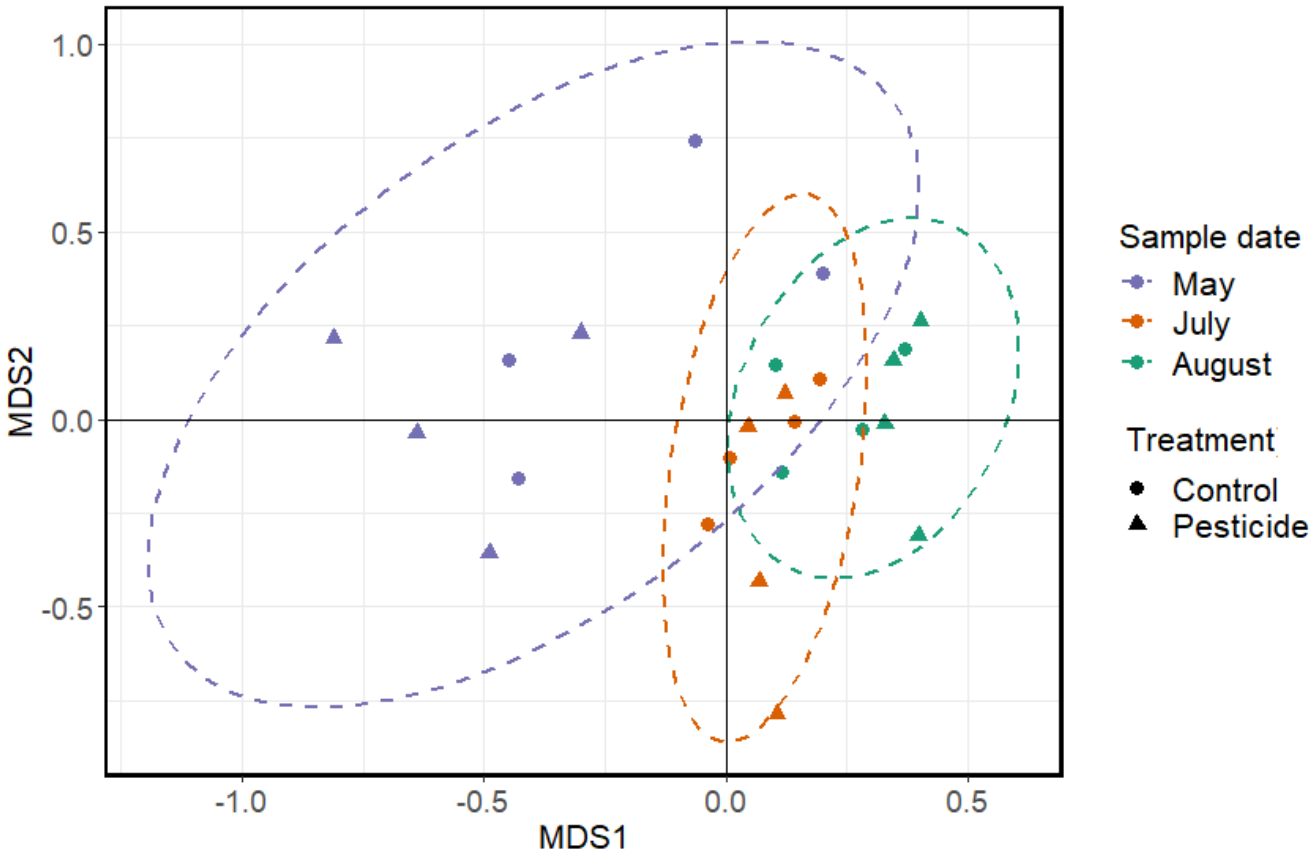

Figure 3.6. Non-metric multidimensional scaling (NMDS) ordination plot of major mesofauna group composition in response to the addition of neonicotinoid pesticide measured across the growing season.

3.4.2 16S Microbial analysis

3.4.2.1. Taxonomic composition

In total, 13594 operational taxonomic units (OTUs) were identified from the 16S reads. Twenty distinct phyla were identified, with seven distinct phyla (Firmicutes, Verrucomicrobiota, Proteobacteria, Actinobacteriota, Acidobacteriota, Chloroflexi, and Planctomycetota) accounting for 75.8 ± 0.3 % of these counts. We therefore categorised the OTUs into eight distinct groups, seven of which were the aforementioned phyla, and "Other", which consisted of unclassified bacteria and phyla with < 2.5 % average abundance.

Pesticide exposure was found to have no significant impact on any of the distinct microbial phyla, although it did have a significant effect on the "other" category ($F_{(1,36)} = 4.654$, $p = 0.038$) with the proportion of OTUs classified as "other" increasing significantly across the first three sampling points (Fig. 3.7). However, since 72.5 ± 1.0 % of the "other" category is dominated by unclassified bacteria, we are unable to specify the exact impacts of the pesticide exposure on this group. Of the major identified phyla, the most abundant across the season was Proteobacteria, accounting for 22.3 ± 0.3 % of OTUs. Chloroflexi was constantly the least identified of the major microbial phyla across this study, accounting for an average of 4.1 ± 0.15 % of OTU counts across the season (Fig. 3.7).

Across all the major microbial phyla identified and measured within this study, there was no indication of any significant changes in individual numbers as a result of the pesticide treatment (Table 3.2). Many of the results do, however, show a significant change in absolute abundance across the study period, highlighting the importance of seasonal variation in regulating microbial communities..

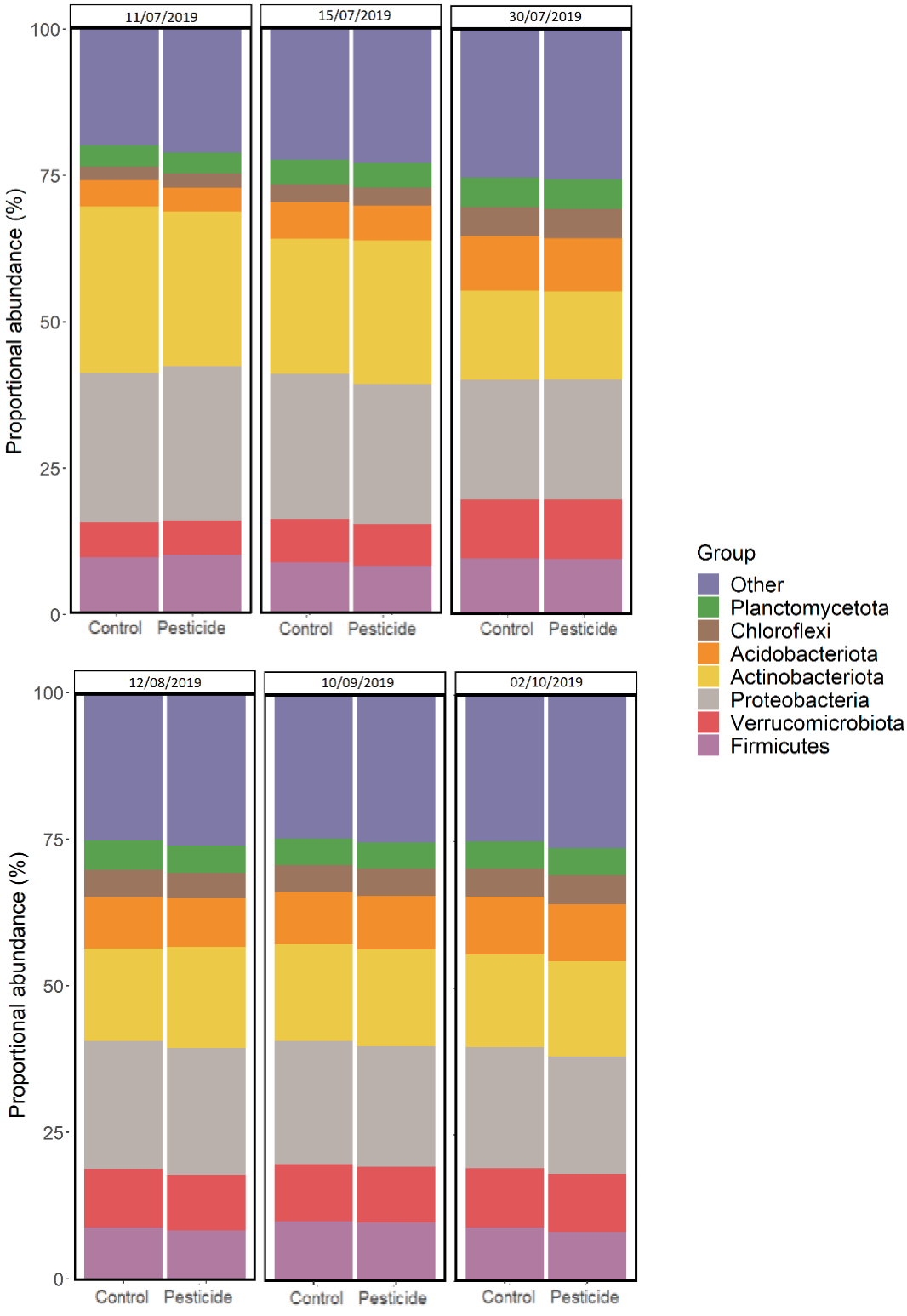

Figure 3.7. Proportional abundances of OTUs for major microbial phyla across the six sampling periods in response to the addition of neonicotinoid pesticide measured across the growing season.

Table 3.2. ANOVA outputs across the major microbial phyla

	Time		Treatment	
Phyla	$F_{(5,36)}$	p	$F_{(1,36)}$	p
Firmicutes	3.700	0.071	0.626	0.434
Verrucomicrobiota	24.475	< 0.001	0.457	0.503
Proteobacteria	37.515	< 0.001	0.567	0.456
Actinobacteriota	33.512	< 0.001	0.084	0.774
Acidobacteriota	59.093	< 0.001	1.027	0.318
Chloroflexi	83.199	< 0.001	0.134	0.717
Planctomycetota	8.124	< 0.001	0.159	0.693
Other	20.292	< 0.001	4.654	0.038

3.4.2.2. Diversity

Diversity within a sample, is referred to as alpha diversity, and diversity between samples referred to beta diversity. Measures of diversity are derived from tables of relative abundance and/or prevalence.

3.4.2.2.1. Alpha diversity

Richness is the sum of unique OTUs found in each sample. Shannon diversity (Shannon 1949) utilizes the richness of a sample along with the evenness (how evenly distributed the OTUs are) of the present OTUs to calculate a diversity index.

Shannon diversity values changed across the sampling season ($F_{(5,36)}$ = 32.681, p < 0.001), with the baseline values in May being significantly lower than those across the rest of the growing season (Tukey post-hoc analysis- Baseline values: Rest of season p < 0.001).

Pesticide treatment was not found to have any significant influence on the diversity values ($F_{(1,36)}$ = 0.021, p = 0.884; Fig. 3.8). Average Shannon diversity values were calculated to be 5.62 ± 0.03, 5.88 ± 0.01, 5.93 ± 0.01, 5.93 ± 0.02, 5.85 ± 0.1, and 5.88 ± 0.02 for each of the sampling points respectively (Mean ± SEM).

3.4.2.2.2. Beta diversity

All profiles are inter-compared in a pair-wise fashion to determine a dissimilarity score and store it in a distance dissimilarity matrix. Distance functions produce low dissimilarity scores when comparing similar samples. Abundance-weighted sample pair-wise differences were calculated using the Bray-Curtis dissimilarity. Bray-Curtis dissimilarity is calculated by the ratio of the summed absolute differences in counts to the sum of abundances in the two samples (Bray and Curtis 1957).

NMDS analysis was used to show the clustering of soil-borne microbial communities, under the two treatment scenarios across the six sampling points. Overall, there was no clear separation between the two treatment scenarios, but there was a clear cluster separation between the first two sampling dates (11/07/2019 and 15/07/2019) and the further four sampling points (Fig. 3.9). This was confirmed through PERMANOVA analysis, finding that pesticide treatment had no significant effect on bacterial β-diversity ($F_{(1,47)}$ = 0.0859, p = 0.923). In addition, β-diversity did change significantly across the sampling season ($F_{(5,47)}$ = 38.301, p < 0.001).

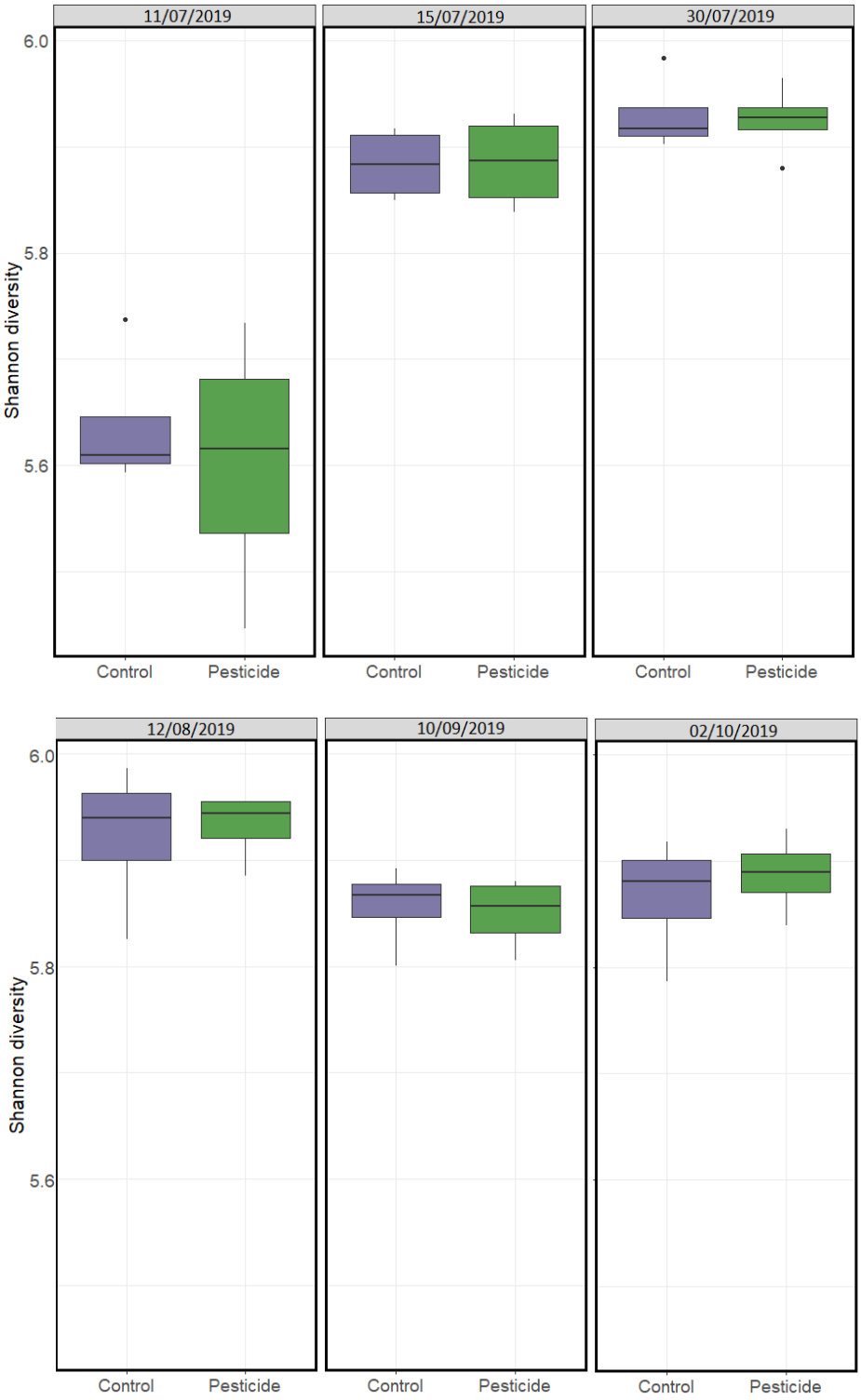

Figure 3.8. Shannon diversity for OTUs averaged across replicated plots (n= 4) in response to the addition of neonicotinoid pesticide measured across the growing season. Boxes are bounded on the first and third quartiles; horizontal lines denote medians, and black crosses denote mean

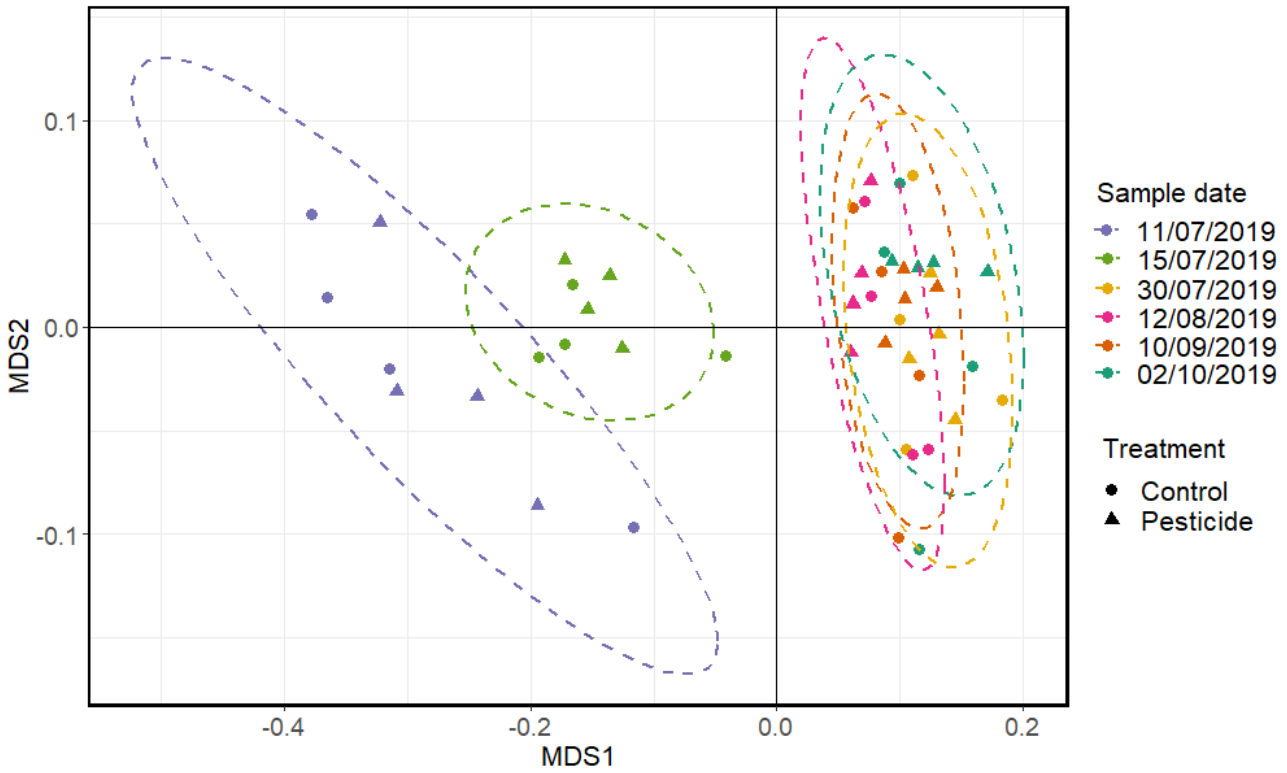

Figure 3.9. Non-metric multidimensional scaling (NMDS) ordination plot community composition of OTUs separated by date across the growing season for the two treatment pesticide scenarios. Overlapping areas indicate no significant differences in community composition.

3.4.2. Untargeted metabolomics

We identified 87 distinct chemical compounds from the metabolomic analysis, including a selection of amino acids, fatty acids, and saccharides. Using visual analysis there are no distinguishable differences in the compounds and amounts produced between the treated and control plots (Fig. 3.10). There is however an obvious shift in metabolomic composition across the sampling dates, predominately between 15/07/2019 and 30/07/2019.

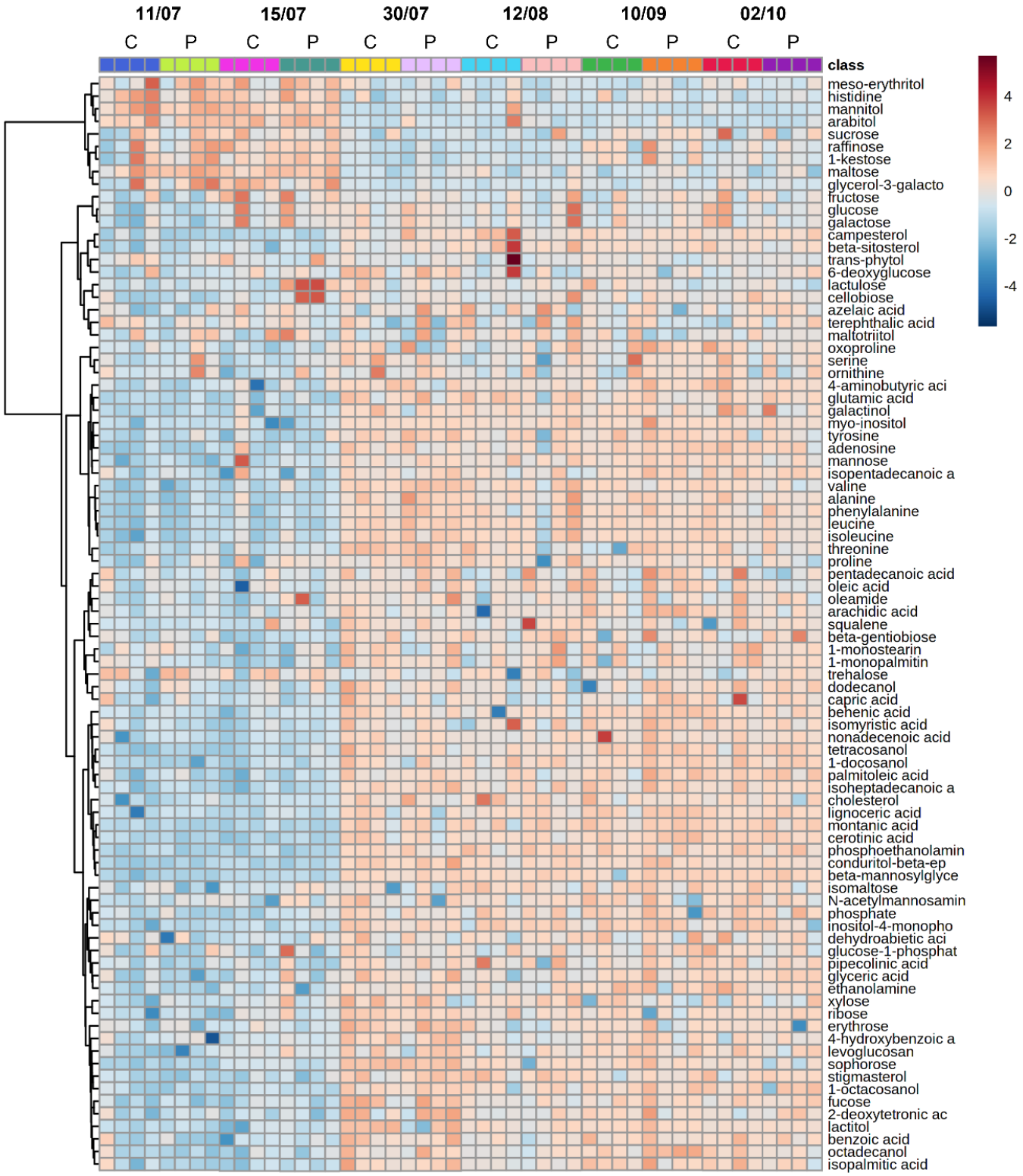

Figure 3.10. Heat map of relative changes in chemical prevalence in control and pesticide plots across the sampling season. C- control, P- pesticide. The colour of samples ranges from red to blue, indicating metabolite concentration z-score; numbers 4 to −4 on the scale bar indicate the number of standard deviations from the mean.

3.4.3. Soil analysis

3.4.3.1. Field mineralisation of radio-labelled acetamiprid

The level of mineralisation of ^{14}C-labelled acetamiprid across all plots increased significantly across the study period ($F_{(9,27)}$ = 178.74, p < 0.001; Fig. 3.11). The rate of mineralisation was also significantly different between the field plots ($F_{(3,8)}$ = 6.022, p = 0.019). Using Tukey Post-hoc analysis the primary significant differences were shown between plot 1 and plot 3 (p = 0.023).

An average of 33.1 ± 3.4 % of the ^{14}C-labelled acetamiprid was mineralised across the study period. Assuming a linear rate of mineralisation, with no external confounding factors, this equates to an average mineralisation rate of 0.42 % day^{-1}, presenting a theoretical half-life of 119.3 days.

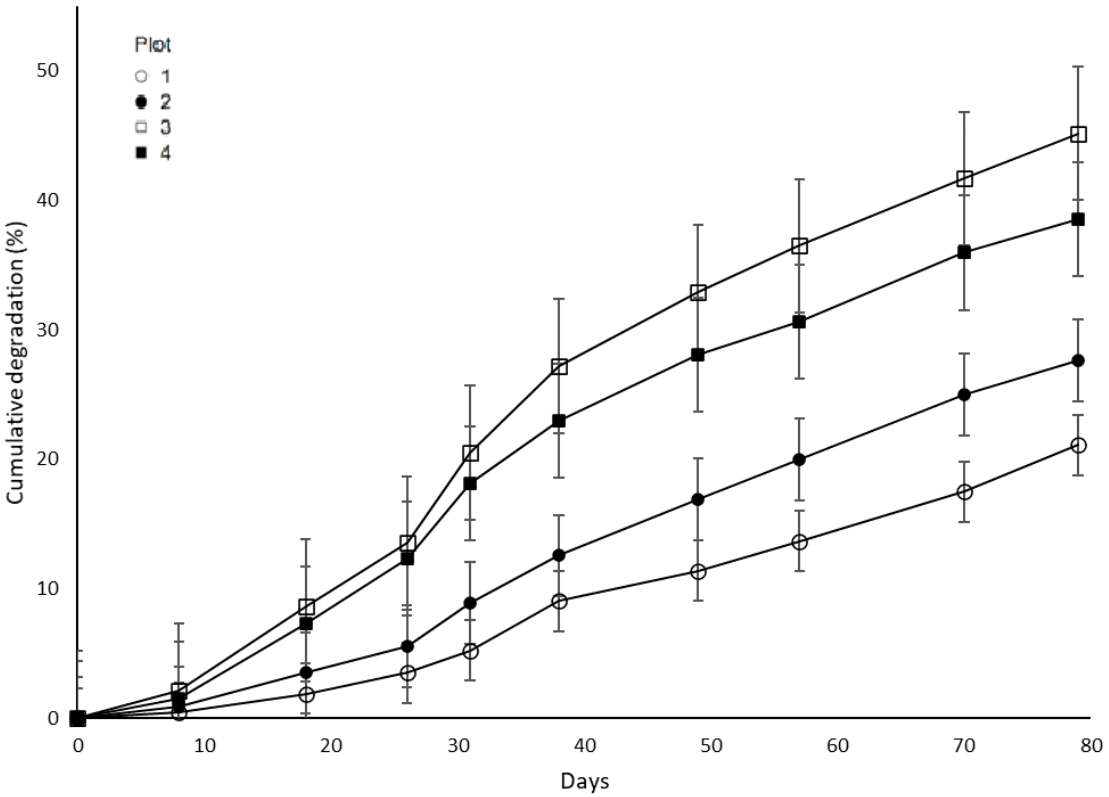

Figure 3.11. Cumulative degradation of ^{14}C-spiked acetamiprid in the field over a 79-day period. Mean ± SEM, n = 3 per plot.

3.4.3.2. Changes in nutrient levels

Neonicotinoid treatment was found to have no significant effect on the level of any of the analysed nutrients within this study (ammonium; $F_{(1,6)} = 3.903$, $p = 0.096$, nitrate; $F_{(1,6)} = 1.924$, $p = 0.215$, phosphate; $F_{(1,6)} = 0.103$, $p = 0.759$). All nutrients were found to change significantly over time across the study season (ammonium; $F_{(4,24)} = 4.556$, $p = 0.007$, nitrate; $F_{(4,24)} = 5.808$, $p = 0.002$, phosphate; $F_{(4,24)} = 50.069$, $p < 0.001$; Fig. 3.12).

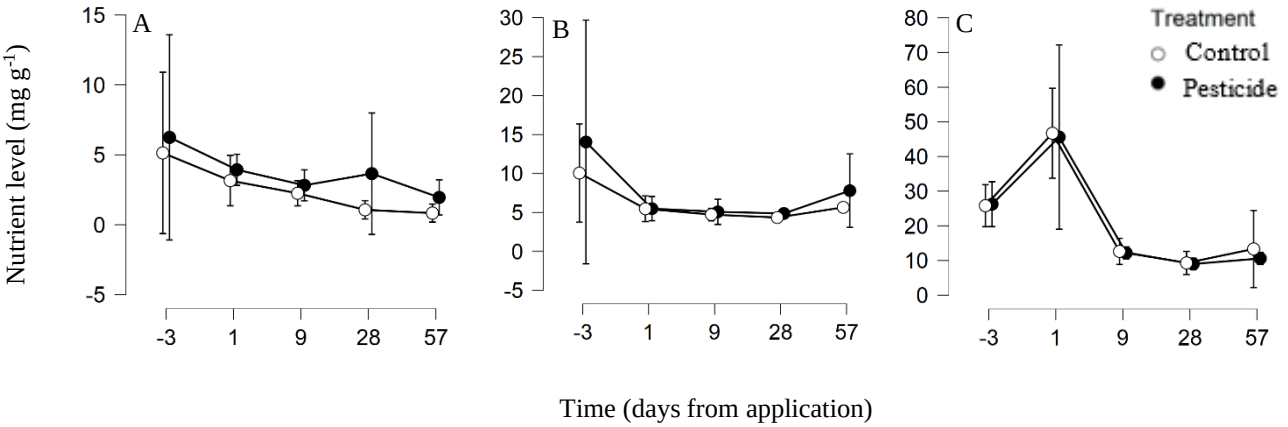

Figure 3.12. Nutrient levels in soil in response to the addition of neonicotinoid pesticide. Mean ± SEM, $n = 4$. A = ammonium, B = nitrate, C = phosphate.

3.4.3.3. pH and electrical conductivity

Soil pH did not vary significantly over time or across treatment scenarios (Treatment: $F_{(1,30)} = 0.113$, $p = 0.739$; Time: $F_{(4,30)} = 1.869$, $p = 0.142$). Average soil pH was found to be 7.07 ± 0.06. Electrical conductivity was found to significantly change throughout the study period ($F_{(1,30)} = 5.151$, $p = 0.003$), dropping significantly between 30th July and 12th August (Tukey post-hoc $p = 0.023$; Appendix 2, Table S3).

3.5. Discussion

3.5.1. Mesofauna communities

Total mesofauna abundance and diversity values were both lower in the early season samples. However, whilst total abundance values rose across the season, and were highest in the August samples; the diversity values were significantly highest in the July, then dropping down to levels similar those from the baseline samples. Total abundance values were primarily dominated by Collembola individuals. Collembola numbers continued to increase throughout the sampling season, with the August count values being significantly higher than any of the previous sampling points. These results appear to counter the accepted collembola behaviour strategy, as Collembola are often noted as reaching their lowest numbers during the driest part of the summer (Rusek, 1998). This change in sampled numbers may be as a response to the presence of intensive vegetation cover of the OSR crop, with the Collembola utilising areas of refuge provided by this cover. Collembola are regarded as highly specialised feeders, with mouth parts and related prey/food sources varying across and amongst species (Rusek, 1998; Kristiansen et al., 2021). Significant shifts in Collembola family composition, could therefore be as a result of available food and resources changing across the season, with increases in Entomobryoidea numbers and decreases in Symphypleona counts responding in kind.

Unlike Collembola counts, total abundance of Acari individuals reached their highest point in the July, with total numbers decreasing slightly between July and August. Across the sampling season the general composition of the Acari samples extracted remained relatively similar, with Mesostigmata accounting for the majority of the samples throughout. Overall, treatment was not found to play a significant role in affecting the total count of Acari, nor their proportional abundance in the total mesofauna samples. Though not statistically significant, the proportion of Oribatid mites decreased substantially across the season in samples exposed

to the insecticide treatment. Oribatid mites are generally sensitive to agricultural practices and disturbances, primarily due to their low fecundity and relatively long generational times (Behan-Pelletier, 1999; George et al., 2017). Despite these decreases, research from De Lima e Silva et al., (2017) found that when exposed to the neonicotinoids imidacloprid and thiacloprid that a species of Oribatid mite (*Oppia nitens*) appeared insensitive to their exposure. *Oppia nitens* was shown to be essentially tolerant to levels of neonicotinoids exceeding 1000 mg kg^{-1} in soil over 35 days (De Lima e Silva et al., 2017). It was also found that in this case thiacloprid was found to be more toxic to the survival of *Oppia nitens* than imidacloprid. The results of De Lima e Silva et al. (2017) are further supported by research from Akeju (2014) finding that a species of predatory mite had moderately low sensitivity to both thiacloprid (EC_{10} = 968 mg kg^{-1}, EC_{50} = 3674 mg kg^{-1}) and acetamiprid (EC_{10} = 447 mg kg^{-1}, EC_{50} = 651 mg kg^{-1}). These results support our findings of no total abundance changes in Acari numbers as a result of neonicotinoid treatment and suggest that reductions in Oribatida numbers may be due to something else rather than direct lethal toxicity.

Whilst the pesticide treatment may have no apparent direct impact on the survival and mortality levels of mesofauna groups across this study, it is possible that the additional ingredients present in the insecticide mix could be influencing the surrounding soil ecosystem and produce changes in the abundance of certain specialist families or orders as a result. Most additional ingredients can be categorised as either additives, adjuvants, emulsifiers, or surfactants, often assisting in the mode of action, ease of application or even improving the aesthetics or smell of the commercial formulation (Peña et al., 2011; Pescatore et al., 2020). The addition of these ingredients can sometimes be linked to changes in the available microflora in the soil (Pescatore et al., 2020). Oribatid mites are fungivores and it is therefore

suggested that the decrease in their presence could be as a result of a decrease in suitable available food resources, possibly as a result of the additional ingredients.

Despite significant changes in mesofauna counts, abundances and community composition across the sampling season, there is no evidence in this study of any significant changes to soil mesofauna populations as a result of exposure to the neonicotinoid spray Insyst®. These results are in opposition to those of Penn and Dale (2017), who found that when ants were exposed to imidacloprid-coated seeds, significant changes in locomotion and mortality were found, demonstrating the importance of assessing the sublethal impacts of pesticide exposure as well as quantifying the lethal effects. Despite this, there is much research to suggest that acetamiprid is recognised as being a "less toxic" neonicotinoid than its forebears, namely imidacloprid, clothianidin and thiamethoxam (Grimm et al., 2012; Amirzade et al., 2014; Pang et al., 2020). These differences in chemical behaviour and insecticidal mode of action could partially explain the apparent lack of response to treatment across this study. However, the results from Penn and Dale (2017) used treated seeds when assessing the impacts on soil mesofauna and biological activity. When applying neonicotinoids as seed dressings it is generally likely for no more than 2% of the applied pesticides to be systemically absorbed by the target plant. The remaining < 98% of the chemical is often transferred into the surrounding soil (Tapparo et al., 2012). In these cases, areas of high localised exposure are expected, with changes in biological numbers and activity mirroring this level of exposure. However, due to changes in EU regulations regarding neonicotinoid use, only certain neonicotinoids, including acetamiprid and thiacloprid, are still registered for outdoor use (European Commission, 2004, 2018a, 2018b). Both acetamiprid and thiacloprid are seldom used in seed coatings and are instead often applied through a foliar spray, as shown in this study. The use of a foliar spray therefore decreases the amount and incidence of chemical contact and incorporation into the soil.

Therefore, whilst the field application levels were representative of maximum rates, the amount that eventually contacts with the soil and soil-borne communities could be negligible.

In addition to the differences in chemical behaviour, field-based studies also allow for a more realistic response from the test organisms. Under controlled laboratory conditions, test organisms are unable to escape further into the soil profile and are therefore exposed to an "unrealistic" extent. Controlled laboratory mesocosm studies also focus exclusively on a finite number of test individuals, whereas under field conditions the organisms are often able to repopulate from unexposed subsoil, demonstrating soil's biological buffering capacity.

3.5.2. Microbial composition

Soil microorganisms are often considered to be the most sensitive bioindicator to changes in soil quality (Lau et al., 2012; Pescatore et al., 2020; Liu et al., 2021). Their ability to rapidly respond to changes in their environment can often result in substantial changes in ecosystem function and quality (Lehman et al., 2015; Bünemann et al., 2018). The results from this study demonstrate that changes in microbial composition and therefore the function of soil ecosystems are more significantly regulated by seasonal changes than by their exposure to neonicotinoid pesticides.

Previous studies in this area have often yielded conflicting data, with results regularly offering contradicting outcomes. Findings from Wu et al. (2021) showed that the direct incorporation of thiamethoxam in soil significantly altered the abundance, diversity and community structure of bacterial species over a period of 60 days under controlled indoor conditions. They demonstrated substantial decreases in the growth promoting rhizosphere bacteria, actinobacteria, implying possible future challenges in sustaining soil health (Wu et al., 2021). Whereas a study conducted by Li et al. (2018), under realistic field conditions, found

that the use of imidacloprid and clothianidin treated seeds did not negatively impact the richness nor diversity of the rhizosphere microbial communities. They did, however, find that species richness across both the bacterial and fungal communities were suppressed during the seedling stage due to neonicotinoid treatment. Despite the community being shown to revive further on in the growing period, this shift in early community composition could indicate a shift in ecologic function, and possibly influence early-season growth or seed setting (Li et al., 2018).

Our microbial results support the findings from Li et al. (2018), however, this array of contrasting outcomes clearly demonstrates the variability in environmental and microbial responses. It also further highlights the discrepancies found between the results of studies using pure active ingredients and direct integration into the soil matrix, and those which utilise agriculturally relevant formulations, applied at realistic rates under realistic field conditions.

3.5.3. Metabolomic analysis

The results from the metabolomic analysis appear to strongly correspond with the rest of the data collated from this study, demonstrating a strong influence of seasonal change on the biological capacity and function of the soil, whilst revealing no discernible differences due to acetamiprid exposure. Overall, the seasonal shift in primary metabolites appear to be as a response to the changes in plant growth and development across the season (Tarpley et al., 2005; Blancaflor et al., 2014). There was also a substantial increase in a range of amino acids (e.g., serine, ornithine, valine) across the sampling season. The increases in these compounds are often used as proxies for increases in bacterial growth (Bastviken and Tranvik, 2001; Sasse et al., 2018; Zampieri et al., 2019; Braissant et al., 2020). These findings correlate well with the 16S data from this study; we therefore theorise that these changes in chemical pathways

and detected compounds are strongly influenced by and correlate with the changes in microbial abundance and community composition.

3.6. Conclusion

This study showed that a single spray application of acetamiprid-containing insecticide (Insyst®) applied to maximum legal rates to a field with no history of previous neonicotinoid use, had no significant effect on soil communities, on both a meso- and micro-scale, over a single growing season. These field-based results demonstrated that seasonal variation played a more significant role in influencing mesofauna and microbial communities within agricultural arable soils than the presence of neonicotinoid pesticide. This can be ascribed to the relatively rapid degradation of the pesticide which limits its toxicological potential. The findings from the metabolomic analyses further support these conclusions, showing no changes in compound abundance as a result of pesticide exposure, and instead demonstrating a clear shift in response to seasonal variations and plant development stages. Our results demonstrate a clear need to study realistic rates of relevant formulations under true agricultural conditions. Future work in this area should involve the monitoring of a multi-season, multi-application design, as this would assess the capability of soil ecosystems to deal with a potential accumulation of pesticides and metabolites, drawing out the true capability of the soil system buffer against a continued level of exposure.

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Author contributions

Davey L. Jones, Paul Cross, Jessica Potts conceived the study. Jessica Potts performed the field sampling and soil characterisation. Jessica Potts undertook the invertebrate extractions and identifications. Jessica Potts and Robert Brown prepared samples for metabolomic and 16S analysis, and analysed the data. Jessica Potts the first draft of the manuscript. All authors contributed to revisions of the manuscript and approved the final version of the manuscript.

Chapter IV-

Sub-lethal mass loss and food avoidance in earthworms (*Lumbricus terrestris*) in acetamiprid-treated soil

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4.1. Abstract

Earthworms are ecosystem engineers and provide a host of vital ecosystem functions, however knowledge of how pesticides influence their ecological functions remains limited. Many neonicotinoid studies focus on the impacts of pure active ingredients on above-ground biota and ecosystems, but there is substantial underrepresentation of their influence on below-ground systems and functions. An earthworm mesocosm study was carried out to determine several sub-lethal and toxicity endpoints for the earthworm, *Lumbricus terrestris*. Seven chemical mixtures were administered (applied at a rate of 1.25 mg acetamiprid L^{-1}), representing a range of commercially available formulations with sole and dual-active ingredients (a.i.- acetamiprid and tritconazole). These mixtures represented commercial formulations as well as the individual pure active ingredients without the commercial surfactants and additives. Earthworm survival, percentage mass change, and relative food removal were used to assess the non-target impact to pesticide exposure in soil. Post-treatment differences in nutrient and organic matter status were also quantified. The results suggest that whilst survival was not significantly affected by chemical treatment type, earthworm mass change ($p < 0.001$), food removal ($p < 0.001$) and subsequent changes to soil characteristics (ammonium $p < 0.001$; phosphate $p < 0.001$) were all influenced by the neonicotinoid formulation. The agricultural formulation Insyst® (acetamiprid- 20% w/w) produced significant losses in earthworms' mass, falling to 73.6 % ± 0.42 % within the first four weeks of the experiment, and failing to regain starting mass by the end of the tenth week. These results indicate that some agro-chemical application strategies can influence changes in earthworm activity, possibly leading to further changes in soil health and crop nutrition.

Keywords: Neonicotinoid; Invertebrate; Sublethal impacts; Ecological risk; Commercial formulations

4.2. Introduction

Neonicotinoids are one of the most used families of plant protection chemicals worldwide (Tomizawa and Casida, 2005; Jeschke et al., 2011; Woodcock et al., 2016). They can be divided into two major groups: N-nitroguanidines (imidacloprid, clothianidin, dinotefuran and thiamethoxam) and N-cyanoamidines (thiacloprid and acetamiprid) (Goulson, 2013; van Gestel et al., 2017). In 2018, three of the major neonicotinoid compounds (imidacloprid, thiamethoxam and clothianidin) were banned from outdoor use across the EU (European Commission, 2018), however N-cyanoamidine compounds such as thiacloprid and acetamiprid are still registered for use and are commercially available, both for agricultural and domestic use (University of Hertfordshire, 2017; European Commission, 2018b).

Most studies on these insecticides have focussed on the use of the pure active ingredients and their impacts on above ground fauna, primarily pollinators such as honeybees and bumblebees (Girolami et al., 2009; Krupke et al., 2012; Bonmatin et al., 2015; Drummond et al., 2018). There is growing evidence, however, to suggest that for seed treatments, up to 90% of the active ingredient remains in the soil (Sur and Stork, 2003; Tapparo et al., 2012). Neonicotinoids have relatively long half-lives in soils, from a few to over 1000 days, 28-1250 days for imidacloprid (Baskaran et al., 1999; Sarkar et al., 2001; Fernández-Bayo et al., 2009); 148-6931 days for clothianidin (Rexrode et al., 2003; DeCant and Barrett, 2010); 7-353 days for thiamethoxam (National Registration Authority for Agricultural and Veterinary Chemicals, 2001; Gupta et al., 2008); 31-450 days for acetamiprid (European Commission, 2004) and 3-74 days for thiacloprid (National Registration Authority for Agricultural and Veterinary Chemicals, 2001a; European Chemicals Agency, 2015). The persistence of these insecticides raises concerns over their environmental fate and ecotoxic impacts on other non-target fauna,

especially those below ground (Capowiez et al., 2006; Peck, 2009a,b; Wang et al., 2015; Zaller et al., 2016).

Earthworms are a vital component in maintaining soil structure and fertility and are therefore crucial to preserving soil quality and promoting sustainable agriculture (Capowiez et al., 2006; Bhadauria and Saxena, 2010; Zaller et al., 2016; Capowiez et al., 2021). Earthworms are also a primary food source for many animals including birds, reptiles, and mammals (Bhadauria and Saxena, 2010; Chevillot et al., 2017). The contamination of earthworms can be a significant vector in the biomagnification of various soil contaminants, affecting organisms throughout the food chain (Vermeulen et al., 2010; Douglas et al., 2015; Frank and Tooker, 2020). Earthworms can become exposed to neonicotinoid pesticides through various application routes, including seed coatings, direct drip irrigation, incorporation of contaminated plant matter or through other soil contamination (de Perre et al., 2015; Simon-Delso et al., 2015; Huff Hartz et al., 2017; Rodríguez-Liébana et al., 2018). Soil invertebrates are most at risk of soil contamination, through physical dermal contact and/or the ingestion of contaminated soil particles (Liu et al., 2015; Basley and Goulson, 2017; Wood and Goulson, 2017). Neonicotinoid molecules can be adsorbed on to soil particles, posing an extended risk to soil-dwelling invertebrates (Anderson et al., 2015; Bonmatin et al., 2015; Singh et al., 2016; Castillo Diaz et al., 2017). Earthworm behaviour, mortality rates and reproductive success can be altered via neonicotinoid exposure (Capowiez and Bérard, 2006; Capowiez et al., 2006; Saggioro et al., 2019).

Most neonicotinoid toxicity studies have investigated the effect of the pure active ingredients in isolation (Žabar et al., 2012; Mörtl et al., 2016; Leiva et al., 2017; Anderson and Harmon-Threatt, 2019), although De Lima e Silva et al. (2017, 2020) did asses the toxicity of selected neonicotinoids and their corresponding commercial formulations. This is important,

as many commercial formulations contain surfactants and/or additional active ingredients which may affect their behaviour and fate in soil. Research from De Lima e Silva et al. (2017, 2020) found that there was little difference in toxicity outputs between the pure active substances and the commercial formulations when tested on the earthworm *Eisenia andrei,* however the springtail *Folsomia candida* exhibited differences in toxicology reactions when exposed to pure acetamiprid and imidacloprid and their corresponding formulations (De Lima e Silva et al, 2020). Their findings show that pure acetamiprid was three times more toxic to survival, and pure imidacloprid four times more toxic to reproduction for *F.candida* than their corresponding commercial formulations (De Lima e Silva et al., 2020). These outcome differences are critical to understand, as most toxicity experiments and case studies are conducted using only pure active substances.

Whilst many pesticide studies have used *E.andrei*; *Lumbricus terrestris* was chosen for this experiment as a more representative species of those found in gardens, agricultural fields, and field margins across the United Kingdom. *E.andrei,* is more common in compost heaps and leaf litter and therefore has less agricultural relevance (Boström, 1995; Basley and Goulson, 2017; Zaller et al., 2021).

We aimed to determine how the toxicity of neonicotinoids to *L. terrestris* is altered through the joint use of conazole-based fungicides, both as pure active ingredients and their corresponding commercial formulations. We exposed *L.terrestris* to field-relevant levels of the neonicotinoid acetamiprid and the fungicide triticonazole, as pure active substances and their respective commercial formulations. We assessed: 1) the toxicity of pure active substances and their corresponding commercial mixtures, and 2) the sensitivity of various biometrics of *L. terrestris* to these chemicals through analysis of mass loss, food avoidance and survival, and

3) differences in soil physicochemical composition as a result of the chemical exposure and changes to earthworm activity.

4.3. Materials and methods

4.3.1. Soil

The test soil consisted of 97 % sterilised Kettering loam (24 % clay, 18 % silt, 58 % sand) combined with 3 % composted bark chipping. Composted bark chipping was added to ensure the friability of the soil material once wet (Hooper et al., 2011). Kettering loam has been used as a reliable earthworm culture by many researchers and has been proposed as a standard medium for toxicological assessments (Sizmur et al., 2011; Basley and Goulson, 2017; Elliston and Oliver, 2020; Turner et al., 2021). Soil characteristics and particle size distribution for Kettering loam (prior to the integration of composted bark chippings) can be found in Table 4.1.

4.3.2. Chemical treatments

Four commercially available pesticide products were tested; two contained acetamiprid only ((N-[(6-chloropyridin-3-yl)methyl]-N'-cyano-N-methylethanimidamide), one contained triticonazole only (RAC-(1R,5E)-5-((4-chlorophenyl)methylidene)-2,2-dimethyl-1-(1H-1,2,4,triazol-1-ylmethyl)cyclopentan-1-ol), and one contained both. Three of these products are marketed as domestic foliar sprays (*Acet*$_{CF1}$, *Acet*$_{CF2}$, and *Trit*$_{CF1}$), and one is a specialist agricultural formulation sold as a dissolvable powder (*Acet*$_{CF3}$). Chemical properties and suppliers of all the test chemicals are presented in Table 4.2. We also tested comparable mixtures of pure acetamiprid and triticonazole (Sigma-Aldrich Ltd., Poole, UK), singularly and in combination. Stock solutions for all the neonicotinoids were prepared in milliQ water. All acetamiprid treatments were applied at a rate of 1.25 mg L^{-1}, with the triticonazole treatments

applied to correspond with the equivalent level found in *Acet*$_{CF2}$ (commercial formulation with two active ingredients, namely acetamiprid and triticonazole). Contaminated soil test mixtures were created by combining 1.5 kg of oven-dried soil substrate with 300 ml of the pesticide stock solution. For comparison, the equivalent highest agricultural application rates used in the UK are for formulations such as Insyst which is applied at a rate of 200-250 g ha^{-1} (liquid dose 200-600 L ha^{-1}; equivalent max rate of 1.25 g L^{-1} ha^{-1}). We also included a control treatment (water), as well as a mixture featuring *Acet*$_{Pure}$ and *Trit*$_{Pure}$ hereto referred to as *Mix*$_{Pure}$. This was used as the corresponding pure active ingredient mixture to *Acet*$_{CF2}$.

Table 4.1. Physicochemical characteristics of Kettering loam as provided by Pitchcare.

Assessment		Value
Organic matter	Total organic matter (%)	6.72
Nutrients (mg kg^{-1})	Available P	0
	Available K	0
	Available Mg	0
% Particle size distribution (particle diameter in µm)	Stones(>10,000)	0
	Coarse gravel (10,000 > 5,000)	0
	Fine gravel (5,000 > 2,000)	0
	Very coarse sand (2,000 > 1,000)	1
	Coarse sand (1,000 > 500)	4
	Medium sand (500 > 250)	29
	Fine sand (250 > 125)	20
	Very fine sand (125 >63)	4
	Coarse silt (63 >20)	6
	Fine silt (20 > 2)	12
	Clay (<2)	24
pH	pH (1:1, soil : water)	6.9

Table 4.2. Physicochemical properties and additional information for the chosen chemical treatments.

	pH	EC	Supplier/Brand	Active ingredients	Additional known ingredients
Bug Clear Ultra® (*Acet*$_{CF1}$)	6.43^{b}	23.2^{b}	Scotts Miracle-Gro Corporation	Acetamiprid- 0.05 g l^{-1}	Ethanol, benzisothiazolinone, glycerol, dipropylene
Rose Clear Ultra® (*Acet*$_{CF2}$)	6.25^{c}	20.1^{b}	Scotts Miracle-Gro Corporation	Acetamiprid- 0.05 g l^{-1} Triticonazole- 0.15 g l^{-1}	Geraniol, Proxel GXL, citric acid mono hydrate
Fungus Clear Ultra® (*Trit*$_{CF1}$)	6.04^{a}	6.8^{a}	Scotts Miracle-Gro Corporation	Triticonazole- 10.2 g l^{-1}	
Insyst® (*Acet*$_{CF3}$)	6.44^{b}	4.1^{a}	Certis	Acetamiprid- 20% w/w	Benzenesulfonic acid, mono-C10-13-alkyl derivatives, sodium salts
Pure Acetamiprid (*Acet*$_{Pure}$)	6.04^{a}	3.7^{a}	Sigma Aldrich	Acetamiprid- 0.05 g l^{-1}	
Pure Triticonazole (*Trit*$_{Pure}$)	5.86^{d}	3.5^{a}	Sigma Aldrich	Triticonazole- 0.15 g l^{-1}	

Values are mean of four replicates. Different letters indicate significant differences between treatments at $p < 0.05$. EC indicates electrical conductivity.

4.3.3. Test organisms

Lumbricus terrestris earthworms were purchased from Worms Direct (Maldon, Essex, UK). They underwent a 14-day soil substrate acclimatisation period prior to data collection. Subsequently, individual adult *L.terrestris* with visible clitellum weighing between 3.4-6.9 g (4.77 ± 0.07 g) were randomly selected and allocated to each treatment replicate. The sum mass of earthworms (n = 4) within each replicate was 19.1 ± 0.3 g.

4.3.4. Mesocosm experiment

Thirty-two microcosms were established using 4 L plastic containers (221 (H) x 158 (W) x 150 (D) mm) filled with 1.5 kg of contaminated soil mixture. Four replicates for each of

the seven pesticide treatments and a distilled water control were used. The soil was carefully packed in to ensure that no large air pockets were created, as this could alter the normal behaviours of the earthworms by providing unnecessary refuges.

Four adult earthworms were collectively weighed and then added to each microcosm. Horse manure (used as food for the earthworms) was sterilised before use by drying at 80 °C for 24 hours and then rewetting with agrochemical-spiked water to five times its dry weight. Care was taken to ensure that no worming treatments had been given to the horses at least 4-6 weeks prior to dung collection as this could interfere with the survivability of the test organisms. The sterilised horse manure was used as an organic food substitute for the test organisms, applied at a rate of 15 g dry weight per mesocosm. Food was removed and replaced weekly from each mesocosm. New food was freshly spiked each week to ensure no deterioration in chemical exposure. Old food was redried in the oven at 80 °C, and weekly food removal per replicate was calculated.

Each microcosm was then covered with fine insect mesh to prevent worms escaping, whilst allowing for continued air flow. Microcosms were checked on a weekly basis to maintain moisture content. During these maintenance checks any dead earthworms were noted and removed from the microcosm.

Each microcosm was emptied weekly, the worms collected, carefully washed, blotted dry and weighed. The collective mass of earthworms in each microcosm was then calculated, as well as an average weight for an individual earthworm. The mass fluctuations of an individual earthworm over time were not measured as we were unable to mark individual earthworms for continued identification. We decided against voiding the gut content of the earthworms as this could cause additional undue stress to the earthworms. We also made note of any mortalities throughout the experiment, all dead earthworms were removed from the

experiment. We then weighed the remaining soil (minus the worms) and determined the water loss for the week; this loss was then corrected through the addition of replacement water. The same worms and soil were then returned to the same container and the experiment continued. The experiment ran for a total of ten weeks and was sampled weekly.

At the end of the test period all samples were sieved to separate remaining earthworms and cocoons from the soil matrix. All earthworms were counted and weighed, following the same procedure as before. No cocoons or juveniles were observed and thus recorded during the study.

4.3.5. Soil analysis

Once all earthworms and remaining horse manure were removed, the soils underwent a variety of analyses to determine any changes due to the chemical treatments' exposure and earthworm activity.

Nitrate and ammonium content of the soils were analysed by combining fresh soil samples (5 g) with 0.5 M K_2SO_4 (25 ml) and shaking for 1 h. The extracts were analysed colorimetrically using the salicylate procedure of Mulvaney (1996) for NH_4^+ and vanadate procedure of Miranda et al. (2001) for NO_3^- using a PowerWave microplate reader. The same procedure, but using 0.5 M acetic acid, was used to extract available phosphate from the soils (Ron Vaz et al., 1993) with P analysed colorimetrically using the molybdate blue method of Murphy and Riley (1962). The moisture content of the soils was determined by oven drying the soils at 105 °C for 24 h. Loss-on-ignition was determined on the oven dried samples by ignition at 400 °C for 16 h. pH and EC were determined by combining 10 g of the treatment soil with 25 ml of deionised water and testing with standard electrodes.

4.3.6. Data analysis

We averaged the mass change results from all four earthworms within each replicate. The data for each treatment was then analysed using ANOVA (repeated measures and one-way) and Tukey post-hoc packages in JASP (JASP Team (2020). JASP (Version 0.14.1) [Computer software]).

4.4. Results

4.4.1. Pesticide influence on changes to earthworm mass

The mean starting mass of an individual earthworm was 4.77 g ± 0.07 g, increasing to 5.34 g ± 0.09 for the surviving 118 earthworms at the end of the sampling period. Exposure of the earthworms to the different pesticides had a significant effect on the mean cumulative mass ($F_{(7,23)}$ = 15.64, p < 0.001) with two chemical treatments being significantly different to the control. Treatment $Trit_{CF1}$ (commercial fungicide formulation- triticonazole) produced mass gains significantly larger than five of the other treatments (Tukey post-hoc p-values: $Acet_{CF1}$ = 0.043, $Acet_{CF2}$ = 0.004, $Acet_{CF3}$ < 0.001, $Acet_{Pure}$ = 0.014, Mix_{Pure} = 0.003). Earthworms in this treatment had a mean mass of 5.94 g ± 0.23 g, equivalent to 128.1 % of the original starting mass. Conversely, treatment $Acet_{CF3}$ (agricultural insecticide formulation – acetamiprid) produced significant losses in mass compared to all other treatments (Tukey post-hoc: p < 0.001). By the end of the ten-week study period, the mean mass for this treatment was 4.24 g ± 0.19 g, equivalent to 89.7 % of the original starting mass.

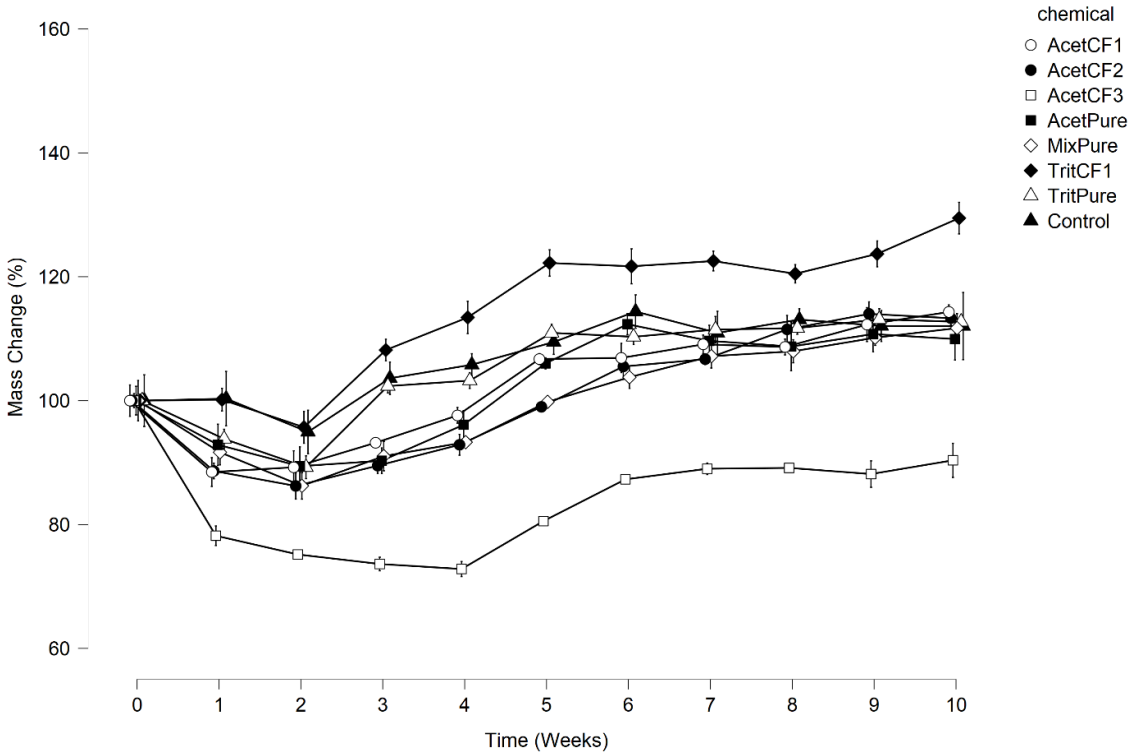

Figure 4.1. Cumulative change in earthworm mass (by relative percentage) under the different chemical treatments. Values represent means ± SEM (n=4).

All treatments demonstrated similar patterns, with an initial decrease in mass over the first two weeks. By week four all treatments exhibited relative mass gain and after week six stabilised, with minimal mass increase (Fig. 4.1).

4.4.2. Food removal rates

The rate of food removal was calculated as a sensitivity endpoint to assess the impact of pesticide exposure on earthworm health and activity. The weekly mass of food remaining was significantly affected by chemical treatment ($F_{(7,23)} = 5.58$, $p < 0.001$). The chemical treatment $Acet_{CF3}$ (agricultural insecticide formulation – acetamiprid) produced significantly lower levels of food consumption than four of the other treatments (Tukey post-hoc *p*-values: $Acet_{CF1} = 0.039$, $Trit_{CF1} < 0.001$, $Trit_{Pure} = 0.011$, Control = 0.002) (Fig. 4.2).

Food removal was calculated as the mean mass of food removed per week per earthworm, adjusting for any variations in earthworm numbers due to deaths. The rate of food removal followed a similar pattern to that of the mass changes, with the first two weeks showing relatively high levels of food removal, followed by a substantial reduction in removal by week three. The lowest levels of food removed were found in week three (Fig. 4.2), one week after most treatments experience the largest loss in mass (Fig. 4.1). We determined that the rate of food removal changed significantly over time throughout the experiment ($F_{(9,207)}$ = 129.92, $p < 0.001$). The highest levels of food removal were during the first two weeks (week 1: 1.71 ± 0.07 g earthworm^{-1}, week 2: 1.91 ± 0.08 g earthworm^{-1}). These levels of removal then decreased significantly during the third sampling period to 0.66 ± 0.07 g earthworm^{-1} ($p <$ 0.001). The mean then increased to the 2nd highest rate of average food removed at week five, 1.69 ± 0.06 g earthworm^{-1} (Fig. 4.2). This corresponds to the point where most of the earthworm mass increases begin to level off (Fig. 4.1). By the end of the tenth week, the average level of food removed had reduced to 0.57 ± 0.05 g earthworm^{-1}. Food removal values calculated across this study may be overestimating the actual levels of food consumed as *L. terrestris* are known to take food into burrows and bury it.

4.4.3. Mortality and survival

There were no statistically significant treatment factors influencing the levels of earthworm mortality within this study. Whilst chemical treatment was found to be non-significant, there were earthworm mortalities in five of the chemical treatments, including the control. By the end of the ten-week sampling period the following treatments had experienced earthworm losses (total absolute losses across all four replicates) $Acet_{CF1}$ = 5 (four of which

were all from one replicate), $Trit_{CF1}$ = 1, $Trit_{Pure}$ = 1, Mix_{Pure} = 1, Control = 2 (both from one replicate). The earthworm mortality rates also did not vary significantly over time.

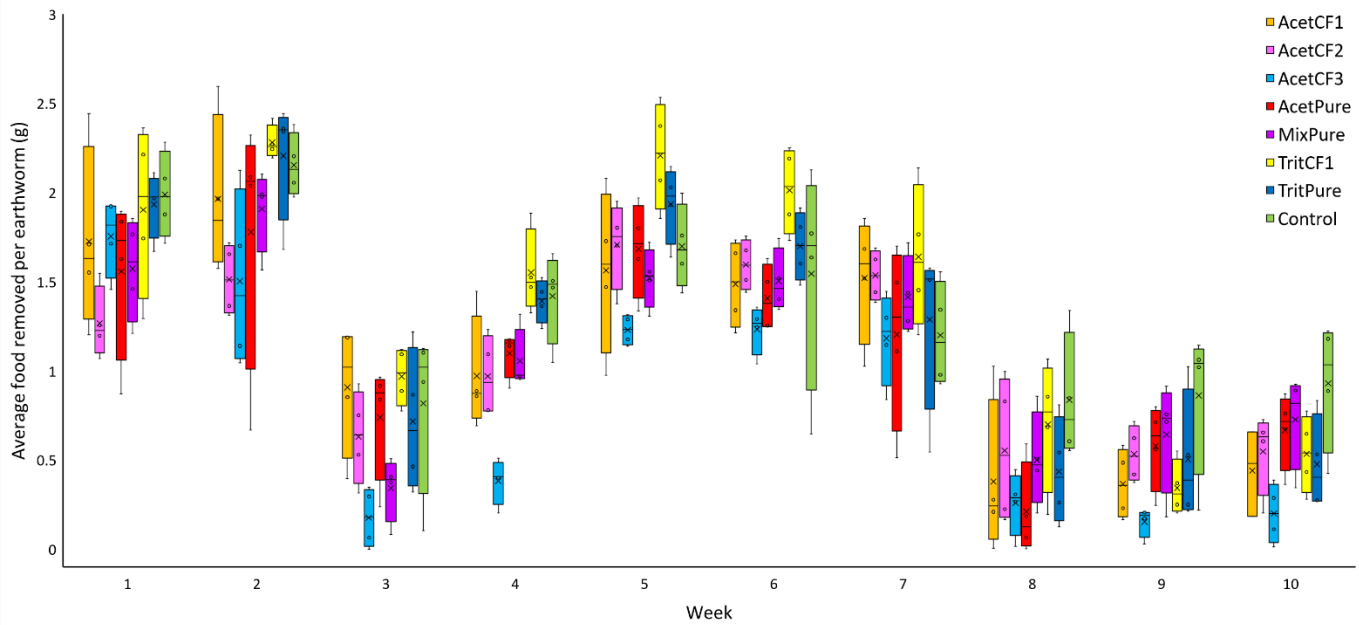

Figure 4.2. Mean mass of food (horse manure) removed per earthworm, adjusted for the number of remaining earthworms per replicate per week (n = 4).

4.4.4. Variations in nutrient levels after pesticide exposure

A mean of 20.9 ± 4.6 mg nitrate kg^{-1} of dry soil was recorded across all samples (Fig. 4.3). There were no significant changes in nitrate levels as a result of the either the chemical treatment or generalised earthworm activity in comparison to the baseline samples taken at the start of the experiment.

There was a significant change in ammonium levels due to chemical treatment ($F_{(8,27)}$ = 12.707, p < 0.001). Across all treatments (inclusive of control) the average ammonium concentration was 7.43 ± 0.38 mg ammonium kg^{-1} (Fig. 4.4). Baseline ammonium levels were significantly higher than those of the chemically exposed soils (17.0 ± 0.6 mg kg^{-1}) (p < 0.001).

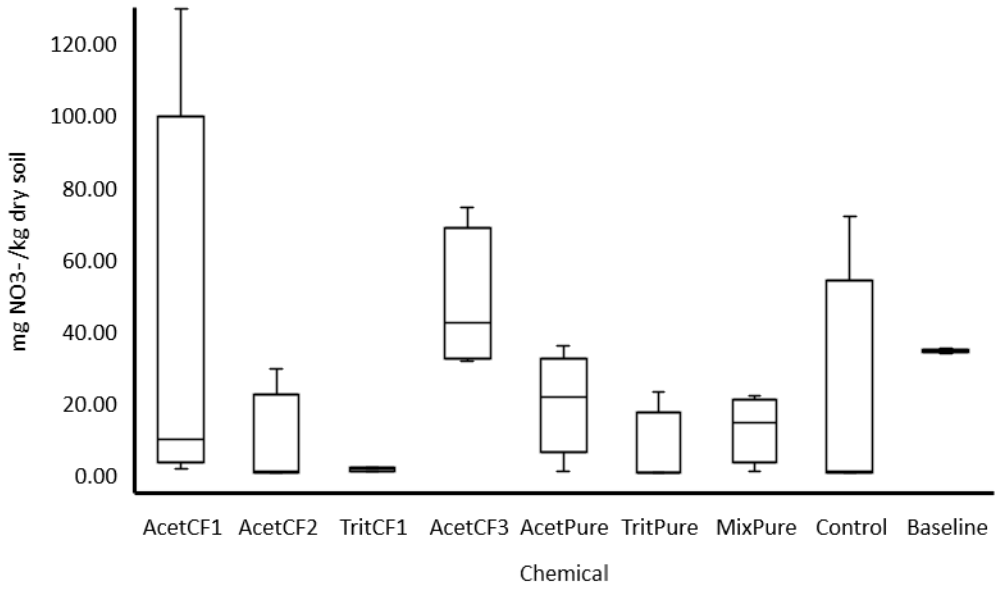

Figure 4.3. Level of nitrate (mg/kg dry soil) in test soils at the end of the ten-week experiment (*n* = 4). Baseline samples are taken before the chemical exposure and earthworm additions.

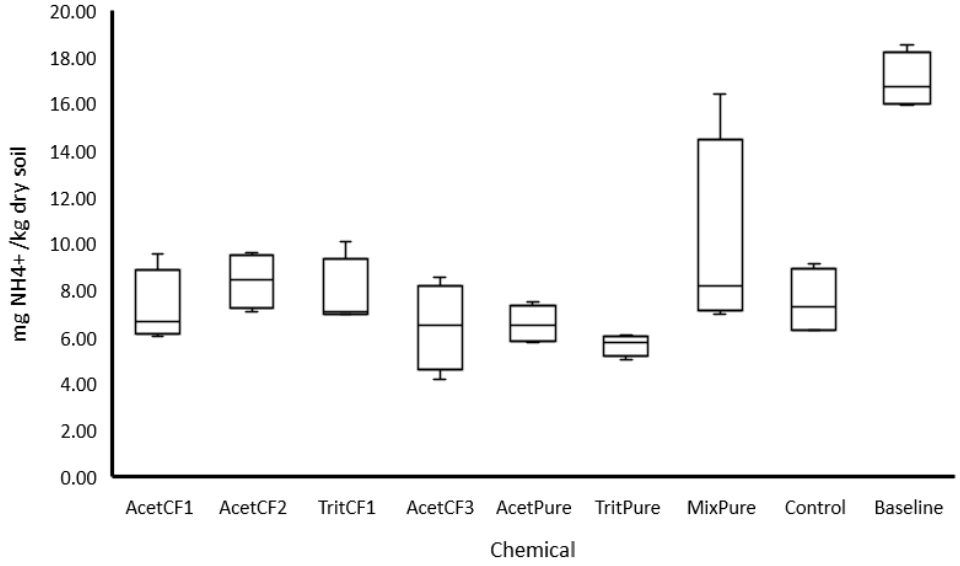

Figure 4.4. Level of ammonium (mg/kg dry soil) in test soils at the end of the ten-week experiment (*n* = 4). Baseline samples are taken before the chemical exposure and earthworm additions.

Available P significantly increased during the experiment, relative to the baseline measurements ($p < 0.001$) (Fig. 4.5). Chemical treatment was found to be a significant factor in measured phosphate levels ($F_{(8,27)} = 14.423$, $p < 0.001$) with two chemical treatments producing significantly lower results relative to the control (Tukey post-hoc analysis: $Acet_{CF1}$: 23.8 ± 3.05 mg kg^{-1}, $p = 0.017$, $Acet_{CF3}$: 22.74 ± 1.26 mg kg^{-1}, $p = 0.009$).

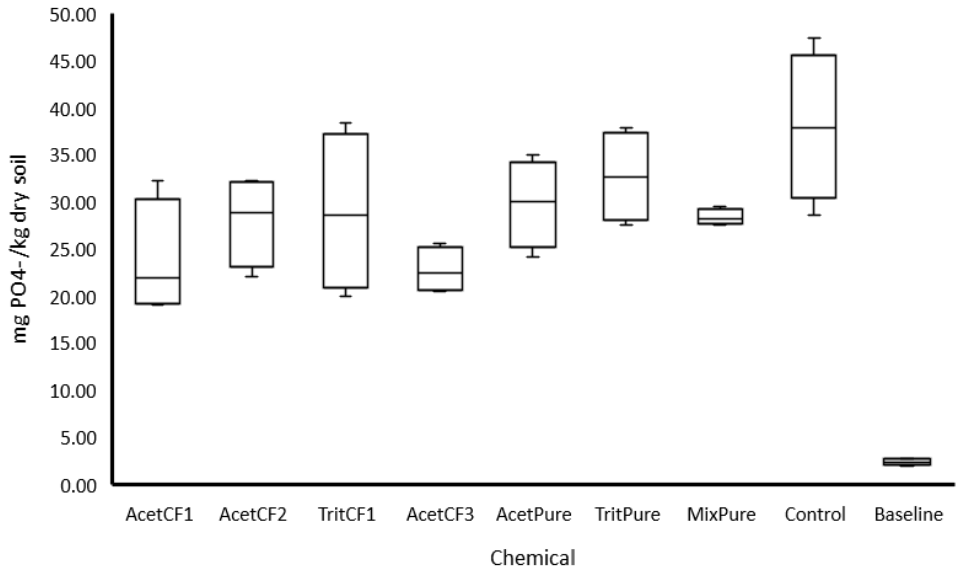

Figure 4.5. Level of phosphate (mg/kg dry soil) in test soils at the end of the ten-week experiment ($n = 4$). Baseline samples are taken before the chemical exposure and earthworm additions.

A mean of 10.6 ± 0.1 % organic matter content was found across the treated soils (inclusive of control), which whilst non-significant, did show an increase when compared to the 9.9 % ± 0.9 % found in the baseline sample soils (Fig. 4.6).

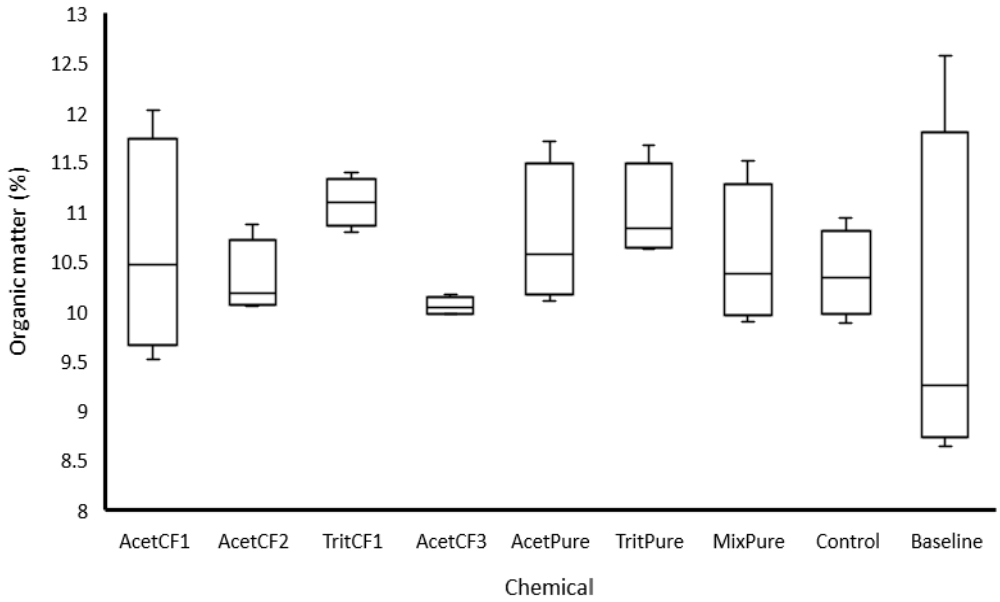

Figure 4.6. Organic matter content (%) in test soils at the end of the ten-week experiment (n = 4). Baseline samples are taken before the chemical exposure and earthworm additions.

4.5. Discussion

Variations in earthworm mass losses can be attributed to the differences in chemical composition of the pesticide formulations. The greatest mass loss was found in earthworms exposed to *Acet*$_{CF3}$ with a mass equivalent of 89.7 % of the original starting mass by the end of the study. Whilst the earthworms exposed to this chemical treatment had the lowest final mass and lowest rate of food consumption, none of the individuals died across all four replicates, (Fig. 4.1 & 4.2). In contrast, earthworms exposed to the *Acet*$_{CF1}$ formulation had a mass gain and a level of food consumption similar to the unamended control. However, they also experienced the highest mortality level across the study. These differences in ecological interaction could be linked to surfactants within the pesticide mixtures. The agricultural mixture *Acet*$_{CF3}$ could contain chemicals that interact with the earthworm's olfactory senses

deterring it from consuming the pesticide-spiked food, causing them to lose a significant level of mass, but remaining alive.

Alternatively, *Acet*$_{CF1}$ may attract the earthworms, or counteract whatever chemosense would otherwise deter its consumption, subsequently causing a notably higher mortality rate. All mixtures containing acetamiprid produced final masses and rates of gain below that of the control. The only mixture that produced mass gain exceeding that of the control was *Trit*$_{CF1}$ (commercial fungicide formulation – triticonazole). *Trit*$_{CF1}$ followed a similar mass gain pattern to the control for the first two weeks, until deviating at week three. From this point the earthworms exposed to *Trit*$_{CF1}$ continued to gain mass, reaching a mean of 128.06 % of their original body mass by the end of the study. The fact that this treatment produced mass results substantially higher than those of the control suggests that the specific commercial formulation interacted differently than the pure triticonazole mixture (*Trit*$_{Pure}$). This suggests that there may be an additive or synergistic effect between the active ingredients (triticonazole) and the surfactants found in *Trit*$_{Cf1}$. This effect could be protecting the earthworms from fungal infections that would otherwise have reduced their immunocompetence, influenced their survival, and reduced their mass gain and final mass.

Whilst little research has been conducted to assess the impacts of the neonicotinoid acetamiprid on earthworm survival and behaviour, Saggioro et al. (2019) found significant decreases in *Eisenia andrei* reproductive rates and success under acetamiprid concentrations of 0.05 mg and 0.1 mg kg^{-1}. It is understood that different species of earthworms will have different life history traits and may therefore react and interact differently to pesticide contamination within their environment (Syers and Springett, 1983; Boström, 1995; Aira et al., 2003). Some of these differences can be attributed to the typical burrowing depth of the species. For example, *L. terrestris* is characterised as an epi-anecic species, meaning that their

burrowing habits tend to consist of the creation of permanent vertical burrowing shafts which the individual earthworms reuse to reach the surface to feed on organic matter. Other UK relevant species such as *Eisenia andrei* and *Eisenia fetida* typically create smaller more transient burrows within the topsoil, generally preferring areas rich with organic matter such as manure, leaf mould and compost heaps (Boström, 1995; Basley and Goulson, 2017; Zaller et al., 2021).

Basley and Goulson (2017) investigated the impacts of clothianidin, another neonicotinoid formulation, on *Lumbricus terrestris*. Their results indicated significant decreases in both food consumption rates on survival after chronic exposure to field-realistic levels of clothianidin. Zaller et al. (2016) found similar results in response to pesticide seed dressings, concluding that the adverse impacts on earthworm activity were found to have further knock-on effects, including the decrease in decomposition rates of organic materials in treated fields. The study did not show any significant loss in body mass as a result of exposure to clothianidin. Clothianidin was recently included in the European Moratorium which banned several neonicotinoid active ingredients from use on outside crops (European Commission, 2013, 2018a). This ban however did not include acetamiprid, and our results suggest that the application of certain acetamiprid mixtures can have significant adverse effects on earthworm biomass, health, and survival, possibly leading to subsequent decreases in both the health of the soils and the nutrition status of the crops produced.

When first commencing the study we also intended to assess and quantify the impacts of pesticide exposure to both reproductive output and successes as well as quality and quantity of earthworm casts produced. However, neither of these variables were able to be measured in the final experimental design. Despite this, these two variables remain some of the most important ecological outputs used to assess environmental factors on an earthworm's

ecological function. Cast production, or the production of vermicompost, is recognised as vital process for the maintenance of both organic matter and nutrient status within agriculturally relevant soils. Assessments of both the nutrient composition and subsequent influence on enzyme abundance and activity within and around sites of earthworm castings, concluded that the sustainable production of earthworm casts are vital in maintaining the nutrient status within soils (Syers and Springett, 1983; Parkin and Berry, 1994; Mcinerney and Bolger, 2000; Chaoui et al., 2003; Sheehan et al., 2006; Sanchez-Hernandez et al., 2014)

Quantifying the changes in nutrient levels post-study allowed us to further understand how the pesticide impacted on earthworm survival and activity, and how this could affect the nutrient composition of the soils. Whilst there were no significant differences in nitrate values across the chemical treatments, the nitrate values measured in the soils treated with $Acet_{CF1}$ had a much larger range of values across the replicates. This included the replicate with the highest number of earthworm deaths, all four dying by the start of week nine, presenting the highest nitrate levels of all treatment replicates. Additionally, treatment $Acet_{CF3}$ had a nitrate level similar to the original baseline value; the earthworms in these treatment replicates had the lowest overall mass, food consumption and observed activity. Conversely, the treatment $Trit_{CF1}$ produced earthworms with significantly greater mass gains and had a substantially lower nitrate level and smaller range in values across the replicates (Fig. 4.3).

Nitrate levels in soil are vital for sustained productive agricultural output, with many farmers relying upon artificial fertiliser inputs. Naturally occurring nitrate is regulated and cycled by a host of nitrogen-specialist microfauna, as well as litter-feeding soil macrofauna and geophages such as earthworms. Nutrient inputs from earthworm activity are often the result of incorporation of earthworm casts (Aira et al., 2003; Chaoui et al., 2003; Sanchez-Hernandez et al., 2014). These casts have been recorded as being a significant source of both nutrients and

specialist enzyme communities, with nitrogen levels similar to many traditional soil additions (Chaoui et al., 2003; Sheehan et al., 2006). The variations in nitrate levels recorded in this study, whilst non-significant, have been linked to the relative level of earthworm activity and survival within each treatment, this could be further attributed to the quantity and quality of earthworm castings produced under each chemical treatment.

Understanding ammonium levels in soils is vital for productive crop growth and sustainable soil health and fertility. There were no significant differences in ammonium between each of the applied chemical treatments, except the post-study measurements and the baseline samples (Fig. 4.4). Reductions in ammonium levels due to earthworm involvement can be linked to an increase in nitrifying microfauna found in the earthworm castings, transforming the ammonium into nitrate. Results from Parle (1963) suggested that freshly deposited casts were often higher in ammonium, with levels rapidly decreasing over time, whilst nitrate levels increased indicating higher levels of microbial nitrification. The increases in nitrate however do not fully account for the losses in ammonium, indicating substantial levels of immobilisation and denitrification within the cast-incorporated soils. Eventually a stable level of both nitrogen forms is reached, as further transformation is prevented due to the organic matter protection within the dried earthworm casts (Parle, 1963; Chaoui et al., 2003; Sheehan et al., 2006; Ferlian et al., 2019).

Phosphate is a vital plant nutrient, used in seed germination, photosynthesis, metabolism and overall plant growth (Wan and Wong, 2004; Mayilswami and Reid, 2010). A large fraction of naturally occurring phosphate comes in the form of rock phosphate, which without further metabolism is unavailable for plant uptake (Wan and Wong, 2004). Specialist enzymes and microflora such as *Bacillus megateriumi*, assist with the solubilisation of phosphate within the soil (Wan and Wong, 2004). These specialist bacteria have been recorded

at high levels within earthworm casts, indicating a valuable association between earthworm activity and soil and plant nutrition.

Within this study, we recorded elevated levels of phosphate in all samples with earthworms after the ten weeks. Much like with the nitrate samples we quantified substantial treatment differences in both $Acet_{CF1}$ and $Acet_{CF3}$, respectively the treatment with the highest relative level of mortality and the treatment with the highest mass loss. These two treatments produced significantly lower phosphate levels, indicating that their reduction in earthworm survival, activity and total mass could have an influence on the quantity and quality of casts produced and therefore have a significant impact on the nutrient status of the soil.

4.6. Conclusions

Our findings show significant ecological changes as a result of variances in the carrier matrix and surfactants, these differences suggest a significant interaction between the additional and active ingredients within the mixture. These results demonstrate that significant environmental changes to the soil can be induced even when there is no evidence of mortality as a result of pesticide exposure, therefore demonstrating the need to understand and consider the sub-lethal effects of these pesticide formulations.

These findings underline the importance of understanding how realistic agrochemical 'cocktails' interact, as most pesticide regulations have focussed solely on the effects of active ingredients applied in isolation. This study demonstrated the importance of understanding how the same active ingredient, applied at the same concentration, can have significantly different ecological impacts due to the accompanying surfactants. In the case of our agriculturally relevant formulation (Insyst®) it did not drive direct mortality. However, it induced significant

mass loss, food avoidance and further significant alterations to the nutrient status of the soil, therefore demonstrating the importance in considering sub-lethal and knock-on impacts.

There continues to be extensive knowledge gaps in understanding how neonicotinoids interact when applied in conjunction with other agrochemicals, as well understanding how the combination of specialist surfactants can alter the responses of non-target soil fauna. Further investigation is necessary to increase our understanding of these influences, and the possible detriment they could have to the sustainable health of our soils.

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Author contributions

Davey L. Jones, Paul Cross, Jessica Potts conceived the study. Jessica Potts performed the earthworm monitoring study and data analysis and wrote the first draft of the manuscript. All authors contributed to revisions of the manuscript and approved the final version of the manuscript.

Chapter V-

Preferential attraction of earthworms (*Lumbricus terrestris*) to acetamiprid-treated soil

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5.1. Abstract

Intensive agriculture has increased the use of pesticides, with many persisting in the environment, interacting with various biogeochemical processes, as well as coming in to contact with a range of organisms. Chemicals entering the soil ecosystem can have detrimental impacts on soil health and fertility. Earthworms can be exposed to soil-borne chemicals, through dermal contact or ingestion of contaminated organic matter. This study explored the influence of pesticides on avoidance and burrowing strategies of the earthworm (*Lumbricus terrestris)*. To understand the influence of commercial-added surfactants and the interactions between co-applied active ingredients, we conducted a replicated ($n = 4$) earthworm mesocosm study featuring seven chemical mixtures (from a range of acetamiprid and triticonazole formulations) applied at a rate of 1.25 mg acetamiprid L^{-1}, to Kettering loam . The results indicated a significant preferential attraction ($p = 0.025$) towards soils treated with the pesticide formulations, possibly increasing the frequency of earthworm exposure to long-term chronic contamination. Whilst the burrowing behaviours of the earthworms were not significantly affected by the chemical treatments, they did exhibit significant losses in mass during the study. The agricultural formulation Insyst® (acetamiprid – 20 % w/w) produced earthworm mass losses of 26.7 ± 3.3 % across the 5-day study. The results from this study contrast those using the standard ecotoxicology earthworm species *Eisenia andrei*. In conclusion, we present evidence showing that some neonicotinoid formulations negatively affect earthworm health. Further, we highlight the importance of understanding the ecological and behavioural differences between earthworm species and emphasising how a “one size fits all” approach is not suitable when assessing ecotoxicological responses.

Keywords: Neonicotinoid; Invertebrate; Behavioural changes; Ecological risk; Commercial formulations

5.2. Introduction

The global expansion of intensive agriculture has led to an increase in pesticides use and accompanying environmental risks to non-target organisms. Agrochemical pollution has become increasingly widespread, affecting both agricultural land and neighbouring wildlife habitats (Douglas et al., 2015; David et al., 2016; Miglani and Bisht, 2019; Sharma et al., 2019). Consequently, terrestrial organisms in these surrounding areas are at increased exposure to both the agrochemicals and their associated metabolites, often interrupting vital ecosystem services (Capowiez and Bérard, 2006; Bori et al., 2015; Zaller et al., 2016; Miglani and Bisht, 2019; Capowiez et al., 2021). For example, it is well known that agrochemicals such as neonicotinoids can negatively affect pollinators (Marzaro et al., 2011; Sandrock et al., 2014; Kessler et al., 2015; Mach et al., 2018), however, their impact on below-ground ecosystem services remains less well understood. A large proportion of applied chemicals accumulate in the soil system, subjecting many soil organisms to unintendedly high levels of contamination (Capowiez and Bérard, 2006; Goulson, 2013; De Lima et al., 2017).

Earthworms are vital to the maintenance and sustainability of healthy and fertile soils, and underpin the food supply chain (Bhadauria and Saxena, 2010; Chevillot et al., 2017). They are known to influence many soil processes, from the biological interactions with other soil biota, the physical transfer of decaying matter, to the biogeochemical processes that determine the soil-based various nutrient cycles (Capowiez et al., 2006; Bhadauria and Saxena, 2010; Capowiez et al., 2021; Zaller et al., 2021). Earthworms are a key biomarker of soil health, being sensitive to changes within their environments, responding through both physiological and behavioural changes, resulting in both lethal and sub-lethal endpoints (Blouin et al., 2013; Zhang et al., 2014; Brami et al., 2017; Miglani and Bisht, 2019; Saggioro et al., 2019). Due to their ecological significance, alongside their relatively short life cycles, high fecundity, and

relative ease of cultivation, earthworms are often used in chronic and acute ecotoxicity assessments (Brami et al., 2017).

Earthworms are often exposed to soil-borne pesticides either through direct dermal contact or through the ingestion of contaminated matter (de Perre et al., 2015; Liu et al., 2015; Basley and Goulson, 2017; Huff Hartz et al., 2017; Wood and Goulson, 2017; Rodríguez-Liébana et al., 2018). Studies have found that various agrochemicals can have detrimental impacts on earthworms, including increased mortality rates (Pisa et al., 2015; Liu et al., 2018), reducing rates of growth (Wang et al., 2015; Zaller et al., 2016; De Lima e Silva et al., 2017), altering burrowing behaviours (Capowiez and Bérard, 2006; Capowiez et al., 2006) and diminished reproductive success (Hackenberger et al., 2018; Miglani and Bisht, 2019; Saggioro et al., 2019).

Acetamiprid is a systemic insecticide belonging to the neonicotinoid family of pesticides. Neonicotinoid pesticides can be divided into two main chemical groups, N-nitroguanidines, which includes imidacloprid, clothianidin and thiamethoxam, and N-cyanoamidines consisting of thiacloprid and acetamiprid (Goulson, 2013; van Gestel et al., 2017). Neonicotinoids work through the disruption of acetylcholine receptors, inducing paralysis and death for target species (Tomizawa and Casida, 2005; Downing and Grimwood, 2017; van Gestel et al., 2017). Acetamiprid was chosen for this study as in 2013 the N-nitroguanidine neonicotinoids imidacloprid, thiamethoxam and clothianidin were banned from outdoor usage in the European Union under a European Commission moratorium (European Commission, 2013, 2018a).

Toxicity research for neonicotinoids has generally involved the use of the pure active ingredients at levels far above the rates of field applications. Such studies are unrealistic, as the use of pure active ingredient in the field is improbable. In this study, we explored the

interactions between active ingredients (acetamiprid and triticonazole) and surfactants in commercial formulations and evaluated the subsequent changes in behavioural outputs of the earthworm *Lumbriscus terrestris*. *L. terrestris* presents a more field-relevant earthworm species for UK agricultural studies, often being found in both field margins and agricultural soils (Guild, 1948; Boström, 1995; Basley and Goulson, 2017; Zaller et al., 2021).

Here we evaluated the use of several pesticide formulations on earthworm behaviour. These included two commercial pesticide formulations containing the neonicotinoid acetamiprid, one commercial fungicide formulation containing triticonazole, and one commercial preparation containing both acetamiprid and triticonazole. We compared these against the pure active ingredients without commercially added surfactants.

Studies have shown that acetamiprid can significantly alter chemical avoidance behaviours, survival and reproductive success of the earthworm *Eisenia andrei* (Saggioro et al., 2019). Additionally, Basley and Goulson (2017) showed that field-relevant concentrations of the neonicotinoid clothianidin can significantly alter the rates of food consumption for *L. terrestris*.

Triticonazole is the second active ingredient investigated in this study. Occurring in conjunction with acetamiprid in one of the commercial formulations, triticonazole is a widely used fungicide in both horticultural, domestic, and agricultural practices. Several studies suggest that the application of both azole-based fungicides and neonicotinoids in unison can induce synergistic interactions, changing the original interaction pathways of both chemicals (Haas and Nauen, 2021). Commercial formulations, containing both the above active ingredients, biodegrade at a significantly reduced rate in comparison to the pure active compounds, and consequently may persist for longer in the soil (Kucharski and Sadowski, 2011).

In the present study, the objective was to find out whether different formulations, and their active ingredients in isolation, alter the behaviours of the earthworm *L. terrestris.* We quantified avoidance behaviours when exposed to pesticide formulations using a modified version of the standardised test recommended by ISO 17512-1 (2008) (Hund-Rinke et al., 2003; Rastetter and Gerhardt, 2018; Saggioro et al., 2019), and tracked burrow formation and morphology using 2D terraria.

5.3. Materials and methods

5.3.1. Soil

The soil used for these tests consisted of 97 % sterilised Kettering loam (24 % clay, 18 % silt, 58 % sand) combined with 3 % composted bark chipping. Composted bark chipping was added to ensure the friability of the soil material once wet (Hooper et al 2011). Kettering loam has been used as a reliable earthworm culture and has been proposed as a standard medium for toxicological assessments (Sizmur et al., 2011; Basley and Goulson, 2017; Elliston and Oliver, 2020; Turner et al., 2021). Further soil characteristics and particle size distribution for Kettering loam (prior to the integration of composted bark chippings) are presented in Table 5.1.

5.3.2. Chemical treatments

Four commercially available pesticide products were assessed, two of which solely contained acetamiprid ((N-[(6-chloropyridin-3-yl)methyl]-N'-cyano-N-methylethanimidamide), one containing just triticonazole (RAC-(1R,5E)-5-((4-chlorophenyl)methylidene)-2,2-dimethyl-1-(1H-1,2,4,triazol-1-ylmethyl)cyclopentan-1-ol) as an active ingredient, and one containing both. Three of these pesticide products are marketed as domestic foliar sprays (*Acet*$_{CF1}$, *Acet*$_{CF2}$, and *Trit*$_{CF1}$), and one is a specialist agricultural formulation sold as a

dissolvable powder (*Acet*$_{CF3}$) (Table 5.2). We also assessed comparable mixtures of pure acetamiprid and triticonazole (Sigma-Aldrich Ltd, Poole, UK), singularly and in combination. Stock solutions for all the neonicotinoids were prepared in milliQ water. All acetamiprid treatments were applied at a rate of 1.25 mg acetamiprid L^{-1}, with the triticonazole treatments corresponding to the equivalent level found in *Acet*$_{CF2}$ (commercial formulation with two active ingredients- acetamiprid and triticonazole). Contaminated soil test mixtures were created by combining 0.75 kg of oven-dried soil substrate with 150 ml of the pesticide stock solution. For comparison, the equivalent highest agricultural application rates used in the UK are for formulations such as Insyst which is applied at a rate of 200-250 g ha^{-1} (liquid dose 200-600 L ha^{-1}; equivalent max rate of 1.25 g L^{-1} ha^{-1}). We also included a control treatment (water), as well as a mixture featuring *Acet*$_{Pure}$ and *Trit*$_{Pure}$ hereto referred to as *Mix*$_{Pure}$, used as the corresponding pure active ingredient mixture to *Acet*$_{CF2}$.

Table 5.1. Physicochemical characteristics of Kettering loam as provided by Pitchcare.

Assessment		Value
Organic Matter	Total organic matter (%)	6.72
Nutrients (mg kg^{-1})	Available P	0
	Available K	0
	Available Mg	0
% Particle size distribution (particle diameter in µm)	Stones(>10 mm)	0
	Coarse gravel (10,000 > 5,000)	0
	Fine gravel (5,000 > 2,000)	0
	Very coarse sand (2,000 > 1,000)	1
	Coarse sand (1,000 > 500)	4
	Medium sand (500 > 250)	29
	Fine sand (250 > 125)	20
	Very fine sand (125 >63)	4
	Coarse silt (63 >20)	6
	Fine silt (20 > 2)	12
	Clay (<2)	24
pH	pH (1:1, soil : water)	6.9

5.3.3. Test organisms

Lumbricus terrestris earthworms were purchased from Worms Direct (Maldon, Essex, UK). The earthworms underwent a 14-day bedding-in period to acclimatise to the new soil substrate. Post bedding-in, individual adult *L.terrestris* with visible clitellum were randomly selected and allocated to each treatment replicate.

Table 5.2. Physicochemical properties and additional information for the chosen chemical treatments

	pH	EC (ms cm^{-1})	Supplier/Brand	Active ingredients	Additional known ingredients
Bug Clear Ultra® (*Acet*$_{CF1}$)	6.43^{b}	23.2^{b}	Scotts Miracle-Gro Corporation	Acetamiprid- 0.05 g l^{-1}	Ethanol, benzisothiazolinone, glycerol, dipropylene
Rose Clear Ultra® (*Acet*$_{CF2}$)	6.25^{c}	20.1^{b}	Scotts Miracle-Gro Corporation	Acetamiprid- 0.05 g l^{-1} Triticonazole- 0.15 g l^{-1}	Geraniol, Proxel GXL, citric acid mono hydrate
Fungus Clear Ultra® (*Trit*$_{CF1}$)	6.04^{a}	6.8^{a}	Scotts Miracle-Gro Corporation	Triticonazole- 10.2 g l^{-1}	
Insyst® (*Acet*$_{CF3}$)	6.44^{b}	4.1^{a}	Certis	Acetamiprid- 20% w/w	Benzenesulfonic acid, mono-C10-13-alkyl derivatives, sodium salts
Pure Acetamiprid (*Acet*$_{Pure}$)	6.04^{a}	3.7^{a}	Sigma Aldrich	Acetamiprid- 0.05 g l^{-1}	
Pure Triticonazole (*Trit*$_{Pure}$)	5.86^{d}	3.5^{a}	Sigma Aldrich	Triticonazole- 0.15 g l^{-1}	

pH and EC values are mean of four replicates. Different letters indicate significant differences between treatments at $p < 0.05$.

5.3.4. Avoidance test

Avoidance observations were performed using an adapted version of the experimental protocol set out in ISO 17512-1 (2008). Thirty-two 4 L plastic containers (221 (H) x 158 (W) x 150 (D) mm) were used. Each unit was divided into two sections, separated using a thin

removable plastic sheet, one side filled with 0.75 kg of uncontaminated control soil, and the other with an equal amount of treated soil. The central separator was then removed, and ten earthworms were placed on the central line. The units were then covered with ultra-fine insect mesh, preventing escapees. The tests were run for 48 hours, when control soil sides were separated and the number of earthworms on either side were counted. Controls tests with two sides of clean uncontaminated soils were also carried out. No food was provided during this study, as the addition of a food source could be used to skew the attraction of the earthworms. All treatments comprised four replicates.

The avoidance responses for each of the acetamiprid treatments were calculated using the following equation:

$$NR = \left(\frac{(C-T)}{10}\right) \times 100 \quad (1)$$

where *NR* = Net Response; *C* = total of earthworms in the control soil; *T* = total of earthworms in the contaminated soil; and 10 = total earthworms per replicate. A positive *NR* value indicates avoidance, while a negative *NR* value indicates "no response" or "attraction" to the compound.

5.3.5. Burrowing behaviour

Changes in burrowing behaviours were estimated using two-dimensional terrariums, otherwise known as Evans' boxes (Grigoropoulou et al, 2008, 2009). The use of these containers allowed observation of the earthworms trapped between the two acrylic sheets. The boxes were constructed of 4mm thick clear acrylic and external dimensions of 500 mm (H) × 300 mm (W) × 14 mm (D), and internal dimensions of 480 mm (H) × 260 mm (W) × 6 mm (D). The space between the acrylic sheets was filled with soil substrates prepared in the same

manner as those as used in the avoidance study. No food was provided during this experiment, as the addition of food can alter normal burrowing behaviours.

One adult earthworm was weighed and placed on the soil surface of each terrarium. The 2-D terrariums were kept indoors in the dark for the duration of the experiment. Burrowing behaviours, including the progress of the burrows and the location of the earthworm, were marked on the front of the terrarium as well as burrow length at each observation point. Observations were made four times a day over five days (every six hours), for a total of 108 hours (18 observation points). The accumulated observation records were summarised at the end of the study period.

We measured changes to burrow morphology where maximal width was defined as the width between the two most extreme points of the burrowing system; total depth was measured as the distance between the surface of the soil and the furthest point in the burrow system, and total length was measured as the total length of the burrows within the system (example burrow trace in Appendix 3). Burrow branching was also noted and was defined as any burrows additional to the main burrow. Observable activity was measured by marking the location of the earthworm at each sampling point and calculating how far the earthworm had moved from its previous marked time point. Measurements were taken from the tip of the earthworm's head. Mass change throughout the experiment was calculated for each individual and across each chemical treatment.

5.3.6. Data analysis

Data was analysed using ANOVA (repeated measures and one-way) and Tukey post-hoc packages in JASP (JASP Team (2020). JASP (Version 0.14.1) [Computer software]).

5.4. Results

5.4.1. Avoidance

There was a significant change in net response (*NR*) across the chemical treatments ($F_{(7,24)} = 2.868$, $p = 0.025$). A positive *NR* value indicates avoidance, while a negative *NR* value indicates "no response" or "attraction" to the compound. Using the average *NR* value, six of the eight chemical treatments produced negative *NR* values, exhibiting a level of attraction response. Mix_{Pure} presented a net avoidance response (*NR* = 20), and the control produced a neutral net response (Fig. 5.1). There was a 100 % survival rate of individuals across this study. Avoidance tests are classed as valid if < 10 % earthworms are lost (ISO 17512-1, 2008).

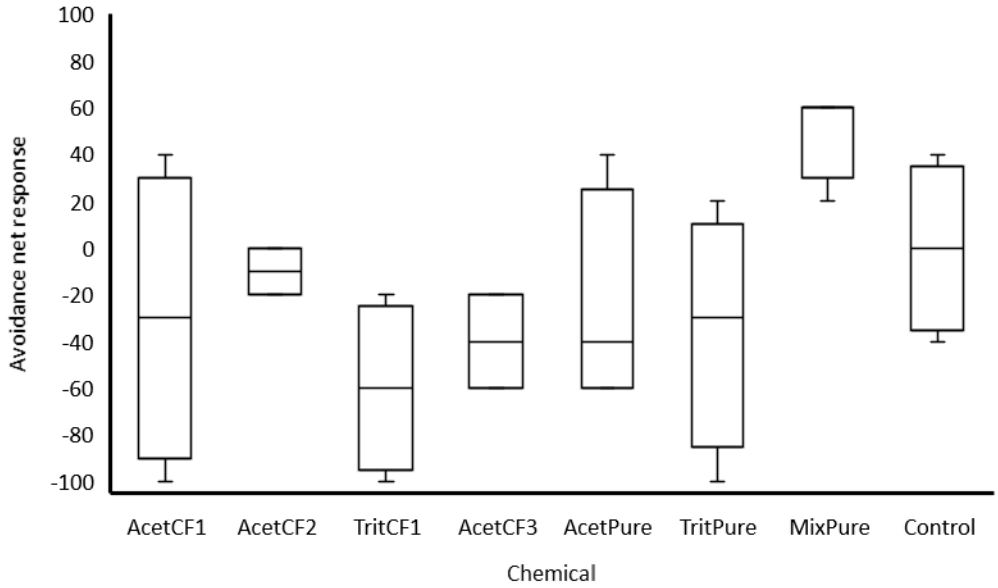

Figure 5.1. Average avoidance response (net response) for each chemical treatment (*n* = 4). Negative values represent an attraction response, whilst positive values represent an avoidance response.

($F_{(7,24)} = 1.817$, $p = 0.13$), total length ($F_{(7,24)} = 1.242$, $p = 0.32$), nor maximal width ($F_{(7,24)} = 1.038$, $p = 0.431$), were affected. The average burrow had a total depth of 17.6 ± 2.1 cm, a length of 28.8 ± 3.3 cm, and a maximal width of 11.5 ± 1.4 cm.

5.4.2.2. Burrow branching

The number of branches ranged from 0 – 4 per burrow, with an mean of 1 ± 0.2 branches. Chemical treatment had no significant effect on the number of branches observed ($F_{(7,24)}$ = 1.279, p = 0.302).

5.4.2.3. Observable earthworm activity

Chemical treatment had no significant impact on the total movement of the earthworms, $F_{(7,24)}$ = 1.406, p = 0.249. The average total observed movement was 72.3 ± 9.5 cm (Fig. 5.2).

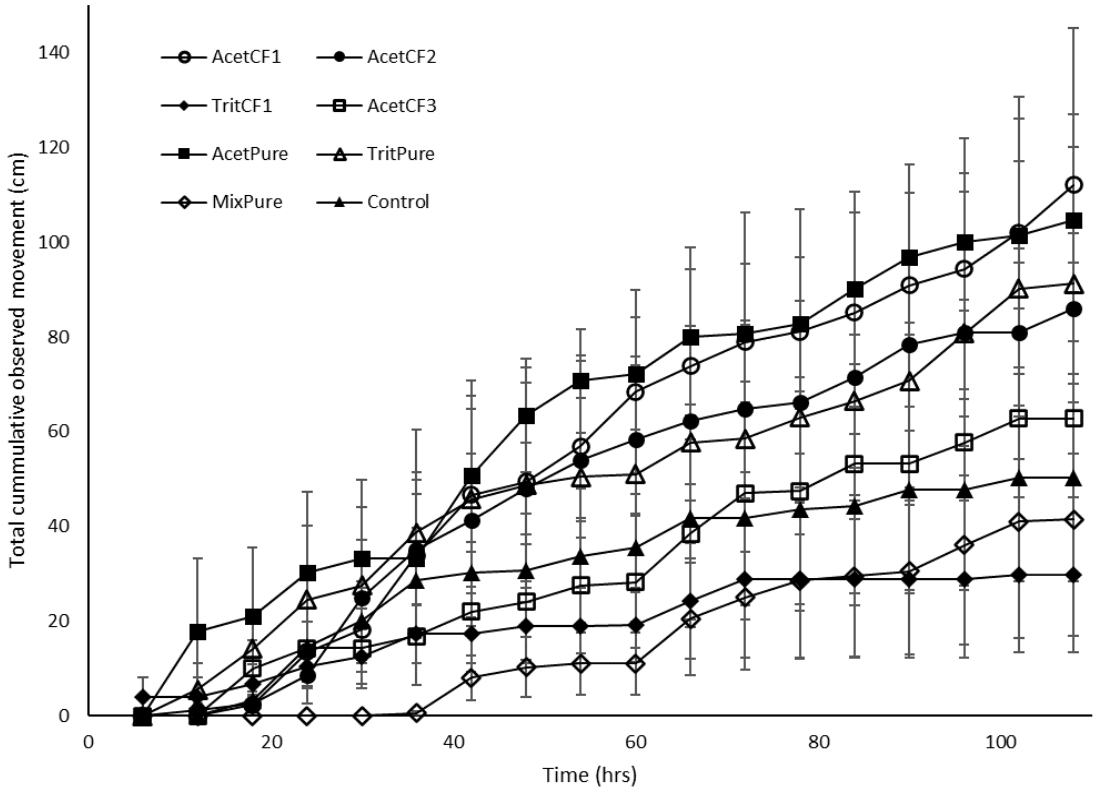

Figure 5.2. Cumulative total observed earthworm movement across the chemical treatments. Values represent means ± SEM (n = 4)

5.4.2.4. Mass change

Absolute mass differences were converted into relative percentage mass change to allow for results to be comparable across the treatments, taking into account he differences in starting earthworm mass. Chemical treatment was found to have a significant effect on the mass change of the earthworms throughout the burrowing study ($F_{(7,24)}$ = 3.967, p = 0.005). Significant differences were also noted between the chemical treatment $Acet_{CF3}$ (agricultural acetamiprid formulation) and $Acet_{CF1}$ (commercial acetamiprid formulation, p = 0.014) and $Trit_{Pure}$ (pure active ingredient triticonazole, p = 0.004), with mass loss from earthworms treated with $Acet_{CF3}$ being significantly higher, 26.7 ± 3.3 % mass loss over the study period. We recorded no significant correlation between the mass change of the individual and the total length of the burrows formed (R^2 = 0.14), there is however some suggestion that smaller earthworms produce shorter burrowing systems, and *vice versa* (Fig. 5.3). There were no earthworm losses, escapees, or deaths, throughout this study.

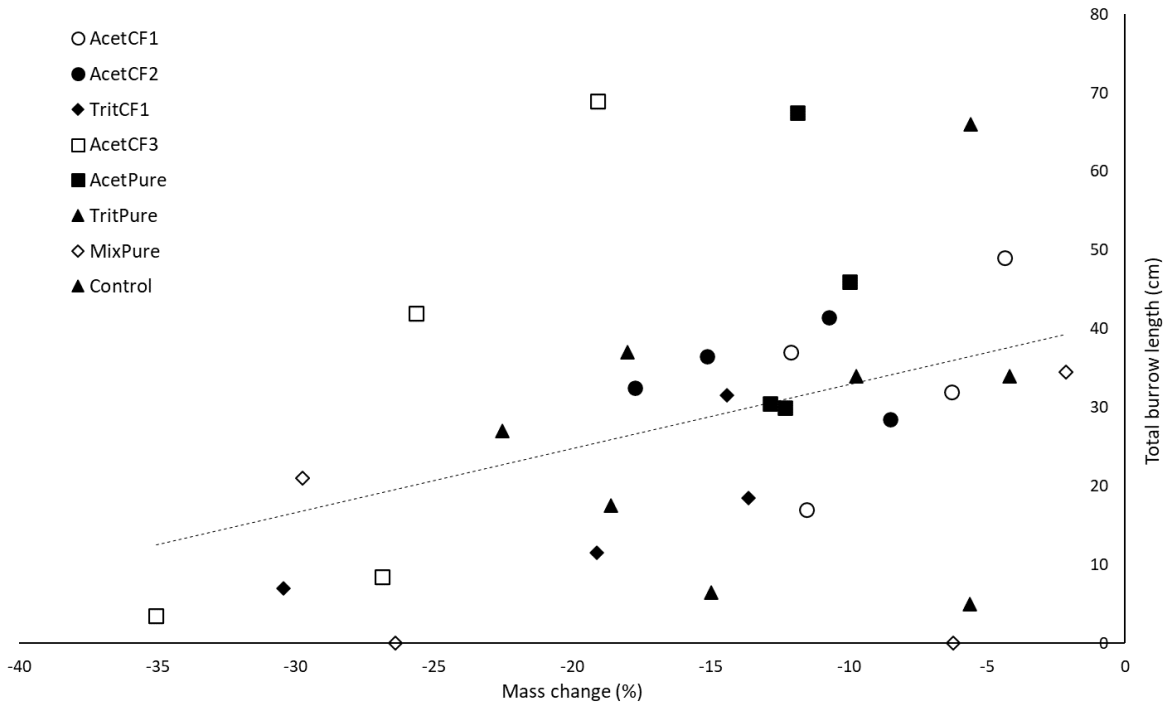

Figure 5.3. Weight change and burrow length for each individual earthworm across the chemical treatments. Mass loss is represented by a negative value on the x-axis.

5.5. Discussion

5.5.1. Attraction and avoidance

Whilst our study showed a significant behavioural change in response to chemical formulation, it was the opposite response to what we expected. Overall, our results show a positive attraction to the formulations (Fig. 5.1). The results from this study show no obvious pattern in behavioural responses between the commercial formulation and the corresponding pure ingredient mixture ($Acet_{CF1}$:$Acet_{Pure}$, $Acet_{CF2}$:Mix_{Pure}, $Trit_{CF1}$:$Trit_{Pure}$).

Under ISO 17512-1 (2008) recommendations, *NR* values up to 20 % indicate no difference in behaviour response, values between 20 to 80 % present noticeable avoidance, and values above 80 % begin to present behavioural changes resulting in habitat function losses. Mix_{Pure} was the only treatment chemical producing an avoidance response (*NR* = 50 ± 10 %, Fig. 5.1). Conversely, Saggioro et al., (2019) showed significant avoidance behaviours by *E. andrei* to acetamiprid at 0.5 mg kg^{-1} (*NR* value = 61.1 ± 10.0 %) and 1 mg kg^{-1} (*NR* = 78.0 ± 12.0 %), whilst our results are for responses at 0.25 mg kg^{-1}. At the lower acetamiprid level, 0.1 mg kg^{-1}, Saggioro et al., (2019) observed levels of subtle attraction (*NR* value = -10.3 ± 7.1 %) similar to those seen in this study (Fig. 5.1). Agricultural formulations such as $Acet_{CF3}$ can be applied at rates of 200-250 g ha^{-1}, in 200-600 L ha^{-1}, indicating that residue levels in treated fields could potentially be many times higher than the rates analysed in this study.

Our attraction net responses mirror those exhibited by other species such as bees. In work conducted by Kessler et al. (2015) it was found that bees (*Apis mellifera* and *Bombus terrestris*) consumed more of, and were more significantly attracted to, sucrose solutions containing neonicotinoid pesticides. These results may indicate that some typically functioning behaviours have been incapacitated through the pesticides' neurotoxic pathways, or that a

surfactant within the pesticide formulations is overriding the olfactory chemosenses and are therefore producing a preferential attraction response (Kessler et al., 2015).

5.5.2. Burrowing behaviour

There were no significant behavioural differences as a response to pesticide exposure. The results suggest that when applied at levels similar to standard field rates, acetamiprid-based pesticides have no significant impacts on the burrowing habits of the earthworm *L. terrestris.* Most burrowing behaviour studies of earthworms have primarily focussed on imidacloprid, using shallow burrowing species such as *Eisenia spp.* (Bori et al., 2015; Brami et al., 2017; De Lima e Silva et al., 2017). There are no previous studies examining the influence of acetamiprid, and acetamiprid-based formulations, on the burrowing behaviours of earthworms.

When examining the effects of imidacloprid on the earthworms *Allolobophora icterica* and *Aporrectodea nocturna*, Capowiez and Bérard (2006) found that whilst there was no evidence of avoidance behaviours, there were several modifications to burrowing activity and morphology. They also determined that when exposed to imidacloprid at rates of 0.5 or 1 mg kg^{-1} individuals of *A. icterica* stopped burrowing after 24 hours. Conversely, in our study there were no significant differences for the length of time spent burrowing or moving, with individuals still observed moving/burrowing an average of 83.8 ± 5.5 hours into the experiment. Results such as these highlight the importance of understanding the ecological and behavioural differences between earthworm species and emphasise how a "one size fits all" approach is inappropriate when assessing ecotoxicological responses.

Protocols for environmental toxicology assessments often prefer the use of artificial soils over natural soils, allowing for both uniformity of properties and comparisons across results. The use of standardised artificial soils removes the added factors of unwanted

organisms and pollutants that could otherwise influence the assessments. The original ISO recommendations for the avoidance studies suggest the use of OECD artificial soil (OECD, 2000). OECD artificial soil substrate is defined as having the following properties: sand content: 50 – 75 % ; pH: 5.5 - 7.5; organic carbon content: 0.5 - 1.5 %; and a carbon content of at least 1 % of the total soil organic carbon (OECD, 2000). Despite these recommendations there are no strict guidelines on the specific properties for each of the constituent parts required to make up the recommended soils substrate (Brami et al., 2017). It is also very common for earthworm studies to use Kettering loam as an alternative standardised soil substrate (Sizmur et al., 2011; Basley and Goulson, 2017; Brami et al., 2017; Elliston and Oliver, 2020; Turner et al., 2021). Brami et al. (2017) compared the differences in earthworm response to varying ratios between OECD artificial soil and Kettering loam. Their findings showed that there were significant differences in behaviours and preferences between the two soil substrates. Their results recorded preferential attraction towards sections containing higher proportions of Kettering loam relative to the artificial OECD soil (Brami et al., 2017), demonstrating that differences in soil properties can significantly influence the behavioural responses of earthworms.

Despite the non-significant differences in burrowing behaviours, we assessed that there were significant differences in earthworm mass loss across the chemical treatments. The earthworms were not provided with a food source during the burrowing study to prevent any bias in burrowing towards the food. The mass loss was assessed as a relative percentage of the original earthworm mass. Individuals exposed to $Acet_{CF3}$ experienced the largest mass loss of across the 5-day burrowing study. This significant mass loss coupled with the evidence of attraction towards the pesticide, could indicate a chronic issue with long-term viable earthworm populations. Mass loss in earthworms is often seen as an important indicator of general health

in earthworms (Capowiez and Bérard, 2006). The total mass of an earthworm is a combination of its body biomass and its intestinal contents. As all earthworms were unfed for the duration of the burrowing study, the reductions in mass can be directly attributed to decreases in the biomass of the earthworm. Mass loss in earthworms can directly reduce the health and efficiency of their hydraulic skeletal system, therefore impeding there locomotion and burrowing abilities (Quillin, 1999; Capowiez and Bérard, 2006).

Deep burrowing anecic earthworm species such as *L. terrestris* can significantly alter soil porosity and aggregation, controlling both water filtration rates and aeration levels within the soil (Bottinelli et al., 2010; Brami et al., 2017). We have demonstrated a preferential attraction towards areas treated with acetamiprid-based pesticides and their associated ingredients (Fig. 5.1). Whilst this attraction level suggests that earthworm ecosystem services could remain unaffected by the use of these chemicals, it is possible that the long-term population survival could be affected, and therefore have long-term consequences for sustaining soil health. Saggioro et al., (2019) demonstrated reduced reproductive output and success in *Eisenia andrei* earthworms over a 45-day period when exposed to acetamiprid at 0.05 and 0.1 mg kg^{-1}

5.6. Conclusion

This is the first study to assess the behavioural changes of *L. terrestris* in response to a range of acetamiprid- and triticonazole-based pesticide formulations. Even when applied at low concentrations, they produced behavioural changes in earthworm activity, most notably a preferential attraction to soils treated with the test formulations. The quantification of sub-lethal behavioural changes often requires long-term monitoring and assessment of chronic exposure responses. The results in this experiment, whilst interesting, may not fully represent

the magnitude of responses exhibited under true field conditions. Whilst the use of sterilised and standardised substrates, either OECD artificial soil or Kettering loam, allows for the confounding factors induced from the unaccounted-for soil properties, it does not necessarily allow for a 'real-life' response. Accounting for these differences and variations in biotic and abiotic soil properties could allow for more accurate determination of pesticide persistence and biotic interactions. Further work is therefore required to assess the ecotoxicity of commercial formulations on ecologically relevant non-target biota, developing an understanding of the heterogeneity in responses across species of the same taxa under field conditions.

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Author contributions

Davey L. Jones, Paul Cross, Jessica Potts conceived the study. Jessica performed the earthworm burrow monitoring, data analysis, and wrote the first draft of the manuscript. All authors contributed to revisions of the manuscript and approved the final version of the manuscript.

Chapter VI-

Discussion and future work

6.1. Introduction

As the global human population continues to rise, and the pressure on the natural environment, and resources such as agricultural land and food production increases, the need to properly understand our influence on these systems is urgent. None of these vital systems operate in isolation, meaning that the management of one area can often be felt and identified in another. Neonicotinoids provide such an example. Whilst proper pesticide practice and management is an important component of modern farming regimes, the non-target effects of these chemicals are still poorly understood. In addition to protecting plants from biting and sucking pests, neonicotinoids are frequently linked to population decreases and behavioural changes in a variety of invertebrate communities.

In this thesis I discussed the interactions and implications of neonicotinoid application and exposure across the soil ecosystem, from changes in physiochemical behaviour when applied under different carrier matrices, to significant reductions in earthworm mass and activity. These findings reveal the extent of the influence of neonicotinoids on the wider natural environment.

A detailed discussion of the results from each experiment are found in Chapters 2-5. In this chapter I will synthesise the major findings from the previous chapters, discussing them in relation to the main aims of this thesis, drawing out parallels, wider thematic issues, and future implications of this work. I will also comment on any significant challenges faced during the completion of this thesis, and how they were tackled and overcome. I will then conclude by

suggesting future research pathways, building upon the questions that have arisen throughout the process of this work.

6.2. Synthesis of findings

The aims of this thesis were to;

1. investigate the influence of additional ingredients in commercial formulations, such as surfactants, adjuvants and other additives, on the behaviour and environmental persistence of acetamiprid products;
2. assess how different farming management strategies and regimes can influence acetamiprid retention in the soil; and
3. investigate the ecological impact of acetamiprid products on non-target soil fauna.

I will now progress through each of the main aims and objectives of this thesis by examining the main findings. The experiments in this thesis have begun to address and disentangle some of impacts of neonicotinoid use. This thesis offers an insight into understanding the differences in physicochemical behaviours between pure active ingredients and those of commercially available and agrochemical formulations, as well as the impact these formulations can play in altering soil biology.

6.2.1. Commercial formulations can produce significantly different outputs

One of the primary outcomes from this research relates to the difference that the carrier matrix in commercial formulations can make on their environmental behaviour. All experimental chapters in this thesis have used realistic commercial formulations in their assessment of their behaviours and interactions. Chapters 2, 4, and 5, used both a selection of commercially available formulations as well as the pure active ingredient, acetamiprid. This combination of chemicals allowed for direct comparisons to be drawn between them, with any

differences in analysis inferred to be as a result of the differences in chemical formulation. For clarification, the same insecticide formulations were used throughout the experiments featured in this thesis, with the exception of Fungus Clear which is only featured in Chapters 4 and 5; this formulation was included to represent the effects of triticonazole (as the primary active ingredient) in formulation with commercial additives, and to separate out the effects caused by triticonazole over the carrier matrices.

There remains comparatively little research for neonicotinoid insecticides and the influence of co-applied agrochemicals and additive mixtures, especially regarding their fate and impact under realistic agricultural conditions. Material safety data sheets provided the necessary environmental, chemical and ecotoxicity data for the individual potential risk components, however, there little evidence exists as to whether these assessments have been run for the commercial formulations. Whilst there is little specific research on the co-application of neonicotinoid pesticides, what is available has tended to discuss the possibility that the chemicals may react, either additively or synergistically, ultimately altering the desired end-point effect. For example, Heneberg et al. (2020) demonstrated that the co-application of the neonicotinoids imidacloprid and thiamethoxam with the -azole based fungicides increased the ecotoxicity of the applied chemicals. Whilst I have not run any specific toxicity tests, such as LD_{50}, EC_{50} or NOEC assessments, I did collate several studies that demonstrated differences in both physiochemical behaviours and biological impact of different acetamiprid-based commercial formulations. Chief amongst these differences is demonstrated in Chapter 4, where earthworm mass changes over a 10-week period significantly increased mass loss in comparison to the other formulations. As part of these investigations, I also assessed the influence of the pure active ingredients, acetamiprid, triticonazole, and acetamiprid and triticonazole in combination. I found that in the case of triticonazole, the commercial additives

had a significant influence on their interactions with earthworms, with the commercial formulation (*Trit*$_{CF1}$) significantly increased mass gain across the study.

Throughout this thesis, information from Material Safety Data Sheets (MSDS) were used to inform our understanding of the commercial formulations being used. However, each MSDS only offered environmental and ecological data for a limited number of the constituent chemicals. There appeared to be no available information on the formulations as a whole. Whilst it is arguable that comprehensive testing is not feasible for every single agrochemical formulation that comes to market, a greater understanding of how these chemicals interact when under the conditions of their applied environments, would be beneficial. Further testing and the isolation of individual components within the carrier matrices (surfactants, adjuvants, and additives), would also increase our understanding of these interactions. It is possible that certain additives can have, for example, more favourable sorption capacities and therefore reduce the capacity of soils to sorb the intended active ingredient. It is also feasible for there to be microbial preferences towards certain additives, and therefore reduce the rate of decomposition of the active ingredients, thus increasing the possible residue levels within the system.

6.2.2. Understanding land management practices is a significant piece of the puzzle

Agrochemicals such as neonicotinoids are designed for agriculturally relevant conditions, and therefore assessments of their toxicity and impact should be undertaken in the same conditions. Whilst I understand the benefits of controlled and cost-effective laboratory experiments, they do not allow for the full characterisation of environmental responses to pesticide exposure. All experiments conducted in this thesis used chemicals applied at or below the recommended application rate (Chapters 2 and 3), or at rates equivalent to residue levels

recorded in the literature (Chapters 4 and 5), with the intention of providing field-realistic data. The application levels used were often many times lower than those featured across the literature, where higher dose rates are often employed to ensure that a noticeable and measurable response is exhibited. I also ensured that, where possible, I used commercially available pesticide formulations, often including them alongside the pure active ingredients, to differentiate between the responses of the pure chemical and the additives within the formulations. Again, this contrasts with many published studies, where the use of the pure active ingredients is often common practice. Whilst this preference towards the sole use of the active ingredient allows for more controlled treatment parameters and removes any confounding factors produced as a result of the chemical mixtures, it is not field-realistic or a reliable representation of contemporary agricultural or horticultural practices.

Whilst not explicitly measured in this study, changes in biological communities as result of insecticide treatments and/or farm management strategies such as ploughing, can alter ecosystem services such as decomposition rates and nutrient turnover. While conservation-based farming regimes have been shown to increase invertebrate abundance and activity (House and Parmelee, 1985; House and Stinner, 1987; Reeleder et al., 2006; Mbuthia et al., 2015; Jabbour et al., 2016), continued use of neonicotinoid insecticides may counteract these increases, inadvertently slowing the breakdown of plant residues. This excess of undecomposed plant residues can subsequently immobilize nutrients, reduce seedling emergence, as well as exacerbate certain pest problems, such as slugs (Hendrix et al., 1986; Williams et al., 1998). Therefore, it is possible that a pesticide, intended to reduce pest presence, and decrease the need for additional agrochemical applications, could produce the opposite effects over the long-term. Some of this evidence now suggests that the unintended consequences of neonicotinoid may include decreases in pollinator numbers as well as

reductions in earthworm populations and activity, and long-term alterations to beneficial soil microbial communities. All of these changes, when combined, could begin to have knock-on impacts on food production, countering the original intention of the pesticide formulations.

6.2.3. The influence of acetamiprid in regulating soil biology

Understanding the holistic, system-wide nature of pesticide movement is vital. The changes in biological presence and function described in this work need to be further considered in the wider context of the environment and systems in which they are present. Focussing on the results produced in Chapter 3, I demonstrated that the measurable lethal impacts of realistic acetamiprid use may be negligible, compared to other studies which detail the often-significant influences that neonicotinoids can have on soil biology (Cang et al., 2017; Parizadeh, Mimee and Kembel, 2021; Wu et al., 2021). However, this study only presents the results from a single growing season containing one foliar pesticide application. It is possible that under these conditions that the buffering capacity of the soil was great enough to mitigate any detrimental effects that would have otherwise been recorded within the soil biological communities. Neonicotinoids have often been shown to accumulate in soils, potentially increasing with each season of treatment as the previous season's residues are combined with the current season's application. This is supported by the degradation results provided in Chapter 2. Whilst I did not investigate the exact formulation used in Chapter 3 (Insyst), I did show that under laboratory conditions the half-life for total mineralisation averaged at 207 days, with only < 14.5 % of the applied formulations mineralising across the 60 day study. Under field conditions these figures are much lower, as shown in the field ^{14}C-labelled pesticide data (Chapter 3), but it does present the possibility for accumulation across the seasons. The slow rates of total mineralisation allow the possibility of secondary and/or tertiary metabolite

production. Neonicotinoid metabolites have occasionally been characterised as being more cytotoxic than the original compounds (Nauen et al., 1999; Suchail et al., 2004; Hussain et al., 2016). Consequently, it can be challenging to fully quantify the ecotoxicological impact of repeated neonicotinoid applications.

In addition to recognising the limitations of a singular season of treatment and sampling, the use of Tullgren funnels as a key part of my methods also presented limitations. Tullgren funnel extractions are only able to extract and account for active invertebrate stages, and therefore eggs, pre-larval, and dormant pre-moult instar stages remain in the soil samples unaccounted (Behan-Pelletier, 1999). This sampling bias presents serious challenges in understanding community composition as the approach may underrepresent total species counts and abundances. Total abundance of the other major mesofauna groups changed significantly across the sampling season. This change in seasonal dominance of certain mesofauna groups could be biased by the extraction techniques used. Tullgren funnel extraction analyses do not account for "inactive" instar stages leading to a potential underestimate of the total number of individuals of a species.

Chapter 3 allowed for an examination of community changes under realistic agricultural conditions, however, the sampling and analysis methods only permitted the quantification of changes driven by lethal exposure. Neonicotinoids have been linked to sub-lethal changes and interactions in exposed invertebrates, most commonly recorded in pollinators such as honey and bumblebees (Blacquière et al., 2012; Williamson et al., 2014; Muth and Leonard, 2019). Chapters 4 and 5 provided the opportunity to investigate this area of study and directly measure and assess a selected cohort of sub-lethal responses in the earthworm *Lumbricus terrestris*. Sub-lethal responses, such as those measured in chapters 4 and 5, were not possible in Chapter 3 as larger invertebrate species such as earthworms were often not captured within the Tullgren

funnel systems. Sub-lethal responses would have also been difficult to quantify in the study presented in Chapter 2 due to the difficulty in controlling confounding factors within a realistic field study. Chapters 4 and 5 allowed the quantification of various acetamiprid and triticonazole formulations across both lethal and sub-lethal responses in *Lumbricus terrestris*. The findings from this study align with previous studies on pollinators, demonstrating a stronger sub-lethal rather than lethal response following neonicotinoid exposure (Williamson et al., 2014; Doublet et al., 2015). No individuals died in the study presented in Chapter 5, and there was only an 8.3 % mortality rate over 10 weeks for the study presented in Chapter 4. Despite these low mortality levels, the significant reduction in earthworm masses demonstrate a clear deleterious impact of acetamiprid exposure. In Chapter 4 these reductions in mass have been linked to changes in the nutrient status of the soil, whilst in Chapter 5 there is possible suggestion that mass loss may be linked to reductions in total burrow lengths. When combined these findings demonstrate a clear link between mass reduction and the possible loss of beneficial ecological function of *Lumbricus terrestris*.

6.3. Challenges and methodological justifications

During this research there have been both political and experimental events that presented hurdles to the successful completion of this study. In order to address many of these challenges I implemented changes to both the methods and research questions in this thesis.

The first challenge was the European ban on three of the main neonicotinoid products, formally registered for use within the UK. This policy change meant that the original plan to investigate the impact of imidacloprid seed coatings could no longer be undertaken. Instead I focussed on studying the influence of acetamiprid-based formulations. I chose to use acetamiprid, as it was the one neonicotinoid formulation I was able to procure alongside a range of different commercial formulations.

6.3.1. Chapter III

In addition to the initial policy changes, I altered the field design part-way through the experiment featured in Chapter 3. The original design consisted of 12 field plots, each measuring 3 m x 3 m, each with OSR growing. Across these 12 plots, I planned to have a control (n = 4), a single application treatment (n = 4), and a pulse application treatment (n = 4) where over the course of two or three applications the plots would receive the same level of acetamiprid as the single application. This experimental design was compromised by an unexpected drought in the summer of 2019, resulting in a reduction in rainfall across the region which inhibited the growth of the OSR in eight of the planned plots. This left four viable plots which were split in two, and the second pulse application treatment type was removed.

Additional samples were to be taken the following year. These would have allowed for a direct comparison across the seasons, allowing the separation of the influence of seasonal variation from longer-term changes as a result of the pesticide treatment. Due to Covid-19 restrictions and lockdowns, this work was not permissible. Any future studies investigating microbial communities *in-situ* would be advised to plan follow-up sampling over the coming seasons, as currently seasonal variation in community composition and microbial abundance appears to have a significantly larger impact than the applied treatments.

The types of analyses planned for Chapter 3 were modified due to Covid-19 restrictions. Original plans included residue and metabolite analysis, due to be performed by the Centre for Environmental Biotechnology (CEB) at Bangor University. However, this laboratory group became involved in Covid-19 research and analysis and therefore were no longer able to accommodate our research. Additional funds were sought from STARS (Soil Training and Research Studentships) which allowed for samples to be sent to UC Davis (California, USA) for metabolomic analysis, and Microbiome Insights (Vancouver, Canada) for 16S analysis.

6.4. Future research

This study presents new knowledge and data on the influence of both the biotic and abiotic environmental factors on neonicotinoid behaviours. The study has also assessed some of the sub-lethal impacts of neonicotinoid exposure to non-target organisms, such as earthworms. The research process has raised further questions and identified further knowledge gaps, some of which are detailed below.

6.4.1. Further investigation in to the differences in response to exposure across different indicator species

Across the available literature only a few species are repeatedly selected as model organisms to assess the ecological impacts of agrochemicals. In the UK there are over 270 identified species of bee, with around 250 of them classified as solitary species (Falk, 2015). Despite this, neonicotinoid pollinator assessments have focussed on a small handful of species, such as the honeybee (*Apis mellifera*), common bumblebee (*Bombus terrestris*) and the red mason bee (*Osmia bicornis*) (Sandrock et al., 2014; Williamson et al., 2014; Peters et al., 2016). Even across this small selection of bee species, significant differences in life-history traits and chemical exposure responses have been recorded (Heard et al., 2017; Woodcock et al., 2017; Dietzsch et al., 2019). Similarly, soil invertebrate studies have used a limited pool of species, including *Folsomia candida* and the earthworms *Eisenia andrei* and *Lumbricus terrestris*. Multiple studies have recorded substantial differences between species of the same genera (De Lima e Silva et al., 2017). I suggest that future investigations assess a much larger range of species within specific genera to fully understand the biological impact of pesticides.

6.4.2. To quantifying differences in chemical retention and persistence as a response of different soil characteristics

Differences in the organic matter level of soil can have a significant impact on the physicochemical behaviours of applied chemicals. When assessing the literature, it is apparent that other soil characteristics are responsible for the regulation of chemical retention, degradation and movement (Tariq et al., 2016; Zhang et al., 2018). Physical characteristics such as the soil texture and particle size distribution, have been found to have a significant influence on chemical movement, and soils with higher clay levels often display higher sorption capacity and retention of neonicotinoids (Flores-Céspedes et al., 2002; Banerjee et al., 2008; Jin et al., 2016). In addition to the physical composition and structure of the soils, previous contamination residues can also influence the chemical behaviour of insecticides such as neonicotinoids. Whilst it is not possible to investigate every possible combination of soil factors, a series of experiments investigating different soil types (e.g. clay-rich pelosols vs. sandy textured brown soils) accounting for differences in physical, chemical, and biological characteristics, could allow for the development of a model to estimate degradation and retention of neonicotinoids and similar chemicals.

6.4.3. Assess the influence of soil management strategies

An area of interest that deserves additional investigation is how soil management techniques can influence neonicotinoid exposure and response. With the recent changes in European policy, there are currently no neonicotinoid seed treatments registered for use within the EU, therefore the primary application method is now foliar spray (European Commission, 2013, 2018a). However, across much of the world seed treatments are commonly used, and/or pesticides are applied through the use of irrigation additives or soil drenches (Leiva et al., 2015; Langdon et al., 2017).

When applied as a foliar spray comparatively little of the applied pesticide reaches the ground, and the amount that does will merely touch the surface. In contrast, when the pesticide is applied via an irrigation additive or soil drench, the application method often facilitates a wider dispersal within the soil profile. Understanding the influence of these application methods, along with other soil management practices (e.g. ploughing) can assist with our understanding of how farming practices can influence pesticide retention.

6.4.4. Other areas of interest

In addition to the research areas stated above, there are a number of other questions that I believe would be interesting avenues to explore further including;

6.4.4.1. To assess the long-term impacts of neonicotinoid application

Neonicotinoids, like many agrochemicals, can accumulate in the environment, either in their original form or as a secondary or tertiary metabolites. Due to the systemic nature of neonicotinoids they are also present in all areas of the plant, including crop residues. The reapplication of these plant products could act as a source of neonicotinoid application. The half-lives for neonicotinoid pesticides vary greatly, especially when considered in their commercial formulations, therefore with each subsequent growing season the reapplication of pesticide treatments could lead to further build up in the environment resulting in levels which far exceed a single field dose. I therefore recommend the implementation of a multi-season study under realistic management conditions to begin to quantify these legacy impacts. Quantifying the ecotoxicological effect of the breakdown products and their fate and persistence would also be useful.

6.4.4.2. Further analysis of microbial differences in soil

As shown here (Chapters 4 & 5), neonicotinoids can negatively affect soil biological functioning. However, what these studies did not assess was the knock-on influence these changes had on microbial communities and systems within the soil. I hypothesise that altering the mass, activity and longevity of keystone species such as earthworms will have a major effect on microbial richness and community compositions, and by extension further influence vital soil processes.

6.4.4.3. 2nd stage growth study on contaminated soils

If, as the literature and the research in this thesis suggest, neonicotinoids can have long-term effects on soil biology, it is possible that even after the pesticide has been banned that residual effects may still have quantifiable effects on soil quality and function. It is therefore important to understand how long a soil takes to recover from pesticide exposure, whether they recover to the same starting point, or whether the biological community is irreversibly altered. With changes in both mesofaunal and microbial communities, it is possible that the chemical pathways in soil are also altered. Therefore growth studies on pre-exposed soils, at different time points since exposure, could aid our understanding of the legacy of noenicitinoids.

6.5. Concluding remarks

As the global human population continues to increase, research such as this is necessary to inform practice and policy in finding ways to develop sustainable food security and maintain practical crop protection strategies that present minimise risk to the environment. Further work needs to build upon these findings and develop our understanding of pesticide interactions under real-world scenarios and conditions. I believe that these approaches can be utilised beyond neonicotinoids, developing a more critical understanding of the effects of different

agrochemicals, especially in the form of commercial formulations, and applying our understanding to their environmental persistence and ecological interactions.

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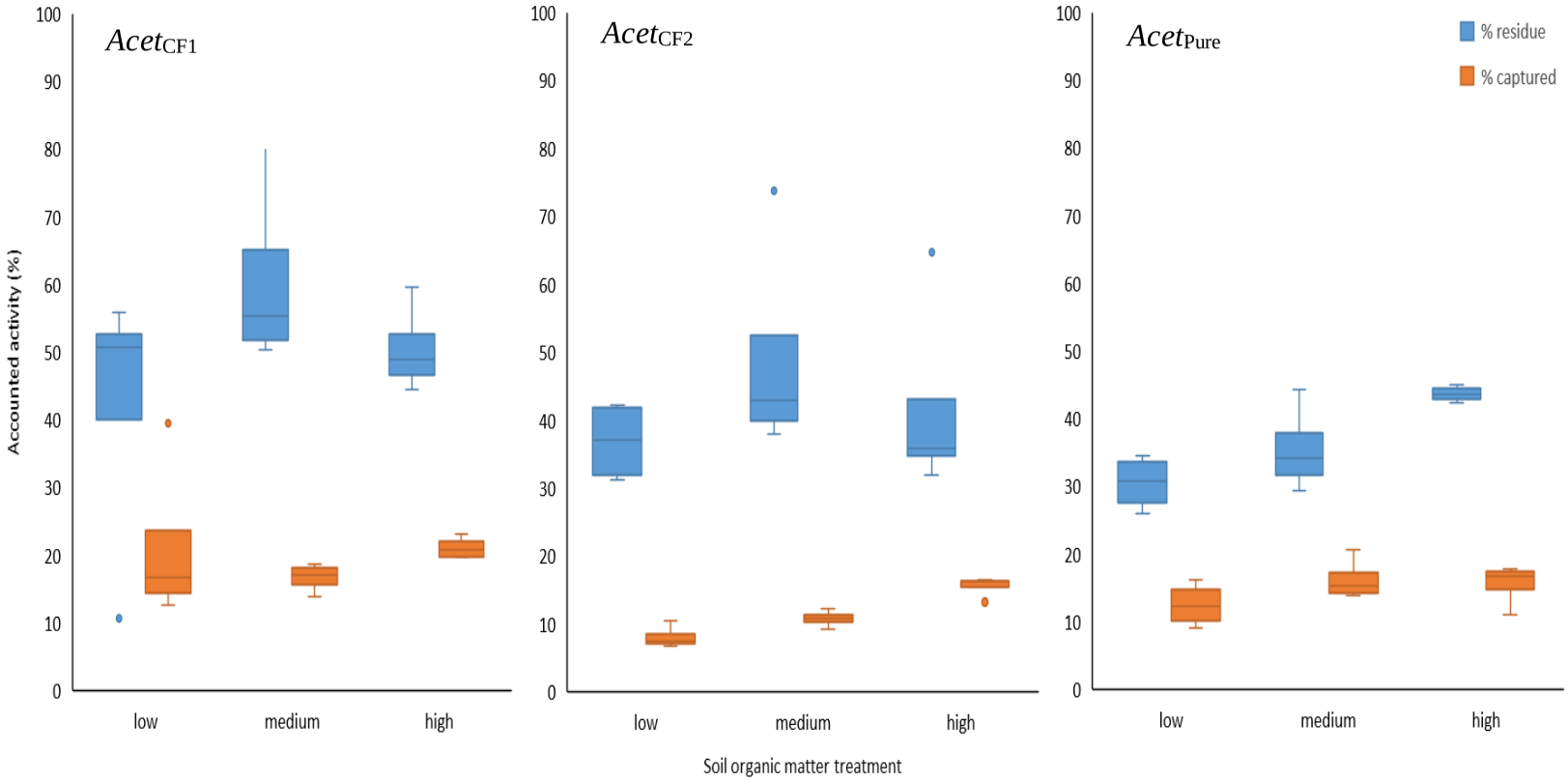
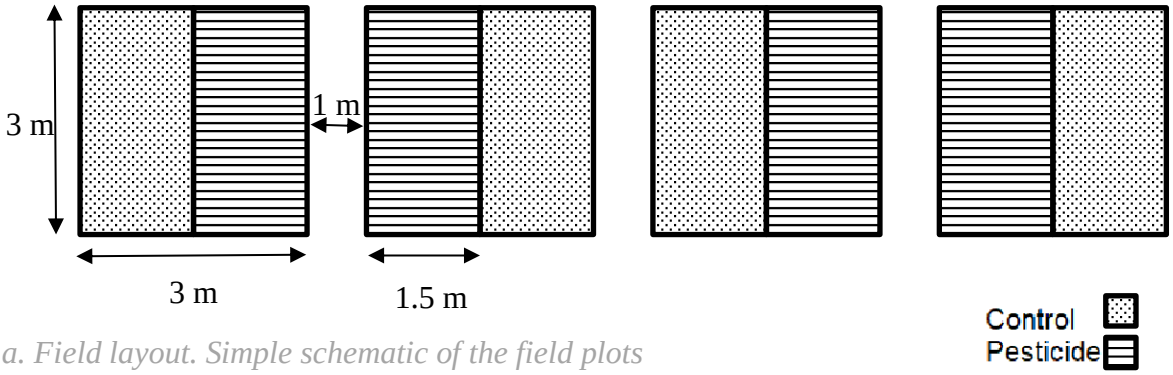


Percentage of radiolabelled acetamiprid accounted for in both the captured and remaining soil residues Boxes are bounded on the first and third quartiles; horizontal lines denote medians, and black crosses denote mean values. Coloured dots are outliers beyond the whiskers.

Appendix 2

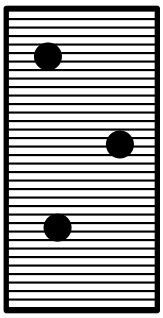
Supplementary material from Chapter 3

S1. Field and experimental layout



S1a. Field layout. Simple schematic of the field plots

S1b. Layout of radioactive traps



NaOH trap for $^{14}CO_2$ capture, random layout across each pesticide treated plot (n = 4). Black dots are representative of individual NaOH traps (n = 3).

S1c. Photograph of initial field layout

S2. Mesofauna Tulgren Extractions- Raw data

		May 2019- baseline samples								July 2019- post planting/pretreatment								August 2019- Post treatment							
		1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Collembla	symphypleona	9	1	1	2	3	13	3	33	2	1	8	1	3	6	10	14	9	4	8	18	11	10	27	43
	poduroidea	10	1	2	0	1	1	0	3	3	2	0	3	5	13	3	11	3	10	1	10	4	7	14	6
	entomobryoidea	2	6	4	4	9	1	4	57	32	10	58	73	94	70	24	44	35	221	38	121	186	161	160	130
Mites	mesostigmata	6	1	1	2	0	3	1	10	9	7	32	24	42	41	37	48	11	55	4	26	22	29	24	62
	oribatida	8	0	1	0	0	1	0	6	1	0	7	2	10	4	1	9	1	3	1	8	2	2	0	21
	astigmata	0	0	0	1	1	1	0	3	2	0	5	5	19	12	7	3	3	7	0	2	3	3	5	8
	Ticks	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	1	0	1	1	1	0
	unidentified mite	0	0	0	4	0	0	2	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beetle	rove beetle	1	0	0	2	0	1	0	0	0	1	0	1	0	0	0	2	0	2	0	2	0	2	2	1
	other beetle (small)	0	0	0	0	0	0	0	0	8	16	8	12	13	10	11	9	0	0	0	0	0	0	0	0
	Other beetle (big, mix)	0	1	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	5	2	0	2	0	0	0
Other	centipede	2	0	0	0	1	0	0	0	2	0	0	1	0	1	0	0	1	5	0	1	3	0	5	4
	millipede	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	larvae (mix,unidentified)	2	10	3	2	2	4	2	2	0	1	4	0	0	2	1	3	2	0	0	0	1	0	0	14
	Diptera (fly)	1	1	0	1	2	2	2	1	10	12	9	22	11	9	15	20	39	48	58	88	31	32	128	63
	nemotode (worm)	0	5	5	5	16	36	15	6	25	8	15	23	10	13	7	14	13	1	3	15	25	16	19	20
	weevil	0	0	0	1	0	0	2	5	32	31	27	15	29	25	53	29	13	7	28	46	19	26	52	42
	symphyla	0	1	0	0	0	0	0	2	0	0	2	0	0	0	0	0	4	0	1	1	6	1	2	2
	spider	0	0	0	3	0	0	1	2	0	0	0	0	0	1	2	0	0	0	0	0	0	0	1	2
	moth	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	minature wasp	1	2	1	0	2	0	0	0	2	2	4	3	2	1	0	0	0	1	1	1	0	0	0	0

S3. pH and electrical conductivity data

Average pH and EC values in response to the addition of neonicotinoid pesticide calculated across the four replicated field plots (n = 4) across the sampling season. Mean ± SEM

	pH		**Electrical conductivity (µS cm^{-1})**	
Sampling Date	**Pesticide**	**Control**	**Pesticide**	**Control**
11/07/2019	6.9 ± 0.04	6.9 ± 0.24	65.2 ± 7.33	72.6 ± 14.12
15/07/2019	7.1 ± 0.14	7.2 ± 0.19	56.4 ± 6.39	62.7 ±7.29
30/07/2019	7.4 ± 0.29	7.3 ± 0.26	65.7 ± 2.72	68.5 ± 3.88
12/08/2019	7.0 ± 0.17	6.9 ± 0.15	47.3 ± 3.63	44.4 ± 1.48
10/09/2019	7.0 ± 0.10	6.9 ± 0.19	46.5 ± 0.70	50.0 ± 6.81

S4. Soil moisture data

	Moisture content per plot (%)			
Days	**1**	**2**	**3**	**4**
1	17.08	16.74	54.52	16.38
8	19.15	18.34	18.55	18.35
22	20.23	19.67	23.11	21.40
26	27.85	29.35	28.25	29.99
57	27.84	25.27	27.62	27.85

S4. Sampling regime

Month	Week	Tullgren Funnel	16S	Metabolomics	^{14}C Degradation	Nutrient Extracts
May	3	-	-	-	-	-
	4	OSR planting				
	5	✓	-	-	-	-
June	1	-	-	-	-	-
	2	-	-	-	-	-
	3	-	-	-	-	-
	4	-	-	-	-	-
July	1	-	-	-	-	-
	2	-	✓	✓	-	✓
		Insyst neonicotinoid treatment application 14/07/2019				
	3	✓	✓	✓	✓	✓
	4	-	✓	✓	-	✓
August	1	-	-	-	✓	-
	2	✓	✓	✓	✓	✓
	3	-	-	-	✓	-
	4	-	-	-	✓	-
September	1	-	-	-	✓	-
	2	-	✓	✓	✓	✓
	3	-	-	-	-	-
	4	-	-	-	✓	-
October	1	-	✓	✓	✓	-

Appendix 3

Supplementary material from Chapter 5

S1. Example burrow trace