Australian National University

Effects of Neonicotinoid Insecticides on Soil Microbial Communities and Their Interactions with Selected Soil Properties

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A thesis submitted for the degree of Doctor of Philosophy of
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Candidate's Declaration

I declare that the work presented in this thesis is original and was carried out during my candidature at the Australian National University. It has not been submitted, in whole or in part, for the award of any other degree or diploma at any university. Except where explicitly stated in the Statement of Contribution for multi-author papers included in the thesis, all work is my own. To the best of my knowledge, this thesis contains no material previously published or written by another person, and all results are derived solely from this research, except where proper acknowledgment and referencing have been provided.

(Sharmin Akter)
18 August 2026

Statement of Contribution

Preface

This thesis is presented as a traditional PhD thesis. It begins with an introductory chapter outlining the background, aims, and conceptual framing of the research. A dedicated materials and methods chapter consolidates the core experimental design, laboratory procedures, sequencing workflows, bioinformatics processing, and statistical analyses used throughout the thesis. Each results chapter includes concise, chapter-specific methodological details and cross-references to the comprehensive methods chapter where appropriate to avoid unnecessary repetition. A conceptual chapter integrates the theoretical and applied insights that emerge across the research. The thesis concludes with a final conclusion chapter that summarises the major findings, discusses their significance, and highlights directions for future work.

Formatting, terminology, and units have been standardised across all chapters to ensure consistency. Australian English has been used throughout.

I was actively involved in all aspects of the research, including conducting literature reviews, formulating research questions and hypotheses, designing experiments, performing laboratory work, processing data, carrying out statistical analyses, interpreting results, and writing manuscripts. Throughout the research and publication process, I received guidance and support from my supervisors: Dr Craig Strong (CLS), Dr Julia Jasonsmith (JJ), and Dr Nilantha Hulugalle (NRH).

Dedication

This thesis is dedicated to my family and parents, with love and gratitude. Their prayers, sacrifices, and encouragement made this journey possible.

Acknowledgements

The PhD journey has been one of the most demanding and transformative experiences of my life. It tested my patience, resilience, and belief in myself in ways I could never have predicted. There were times of frustration, exhaustion, and uncertainty, especially during the COVID-19 pandemic, which disrupted my plans and delayed the start of my on-campus research. Yet these challenges became part of my growth, teaching me perseverance and balance. Above all, I am sincerely grateful to Almighty Allah for guiding me through every stage of this journey. His mercy and wisdom gave me the strength to keep moving forward when things were difficult and the clarity to appreciate every small achievement along the way.

Through every stage of this journey, I was never alone. I was surrounded by people whose guidance, kindness, and encouragement made the path easier and the experience richer. Their presence reminded me that progress is never a solitary achievement, but a shared effort built on the support and goodwill of others.

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I am especially thankful to the Soil Resource Development Institute (SRDI), Ministry of Agriculture, Bangladesh, my home institution, for granting me study leave and supporting my research journey abroad.

I carry the memory of my beloved mother, who passed away during my PhD. Her strength and faith continue to inspire me every day. She faced her illness with courage and grace and taught me the meaning of patience and perseverance. I pray that almighty Allah grants her eternal peace and the highest place in Jannah (heaven). I am also deeply thankful to my father, whose patience, prayers, and quiet strength have been a constant source of comfort and motivation. His hope to see me complete this PhD has been one of the greatest reasons I kept going. I am grateful to my brother for his care and quiet encouragement throughout this journey, even from afar.

Finally, to my husband and daughter, thank you for your endless patience, understanding, and love. Your encouragement gave me the strength to move forward and the joy that made even the hardest days worthwhile. This achievement is as much yours as it is mine.

Abstract

Neonicotinoids are globally dominant systemic insecticides, yet their effects on belowground microbial communities remain incompletely characterised. Soil microorganisms regulate nutrient cycling, organic matter decomposition, and agroecosystem resilience, making their responses to pesticide exposure central to understanding long-term soil health. This thesis investigated how neonicotinoid seed treatments shape soil bacterial and fungal communities across multiple timescales in two soil types, Luvisol and Vertisol with contrasting textures. The research included a systematic review, a short-term experiment with three neonicotinoids (imidacloprid, thiamethoxam, clothianidin), and a longer experiment focusing on soil textures (loamy sand, sandy loam, clay).

A systematic review that synthesised global research on neonicotinoid–microbe interactions was conducted. It highlighted that the outcomes were highly variable and major methodological gaps, such as limited field studies, poor representation of fungal communities, and lack of integration with soil physicochemical data existed. These findings provided the rationale for the experimental design, which addressed the identified gaps by including both bacterial and fungal communities and examining their responses across different soil textures together with soil physicochemical properties.

Microbial communities were characterised using 16S rRNA gene and ITS amplicon sequencing, enabling simultaneous evaluation of bacterial and fungal diversity, taxonomic composition, co-occurrence networks, and predicted functional profiles. Short-term microcosm experiments (10 days) demonstrated that bacterial responses were characterised by selective changes in community composition, without a consistent overall shift in diversity. Transient increases in bacterial diversity occurred under thiamethoxam and clothianidin, whereas imidacloprid reduced diversity by Day 10. Taxonomic shifts were largely compound-specific, with *Mesorhizobium* consistently enriched across all neonicotinoids, whereas nitrifying and other bacterial taxa responded differentially among treatments. Fungal responses were characterised by largely stable diversity despite temporally variable community composition, with clothianidin generating the greatest number of discriminatory fungal taxa together with increased saprotrophs and reduced symbiotrophs.

A 28-day texture-stratified experiment with imidacloprid demonstrated that soil physicochemical context strongly mediated microbial outcomes. Clay soils exhibited reduced evenness, less cohesive co-occurrence networks, and broad predicted functional suppression. Loamy sand showed intermediate patterns, with increased network integration despite reduced diversity. Sandy loam maintained stable richness, evenness, and

phylogenetic diversity and developed denser, more modular networks, suggesting reorganisation rather than collapse. Loamy sand showed intermediate patterns, with increased network integration despite reduced diversity. Functional profiles revealed downregulation of carbohydrate and nucleotide metabolism pathways in fine-textured soils, suggesting potential constraints on carbon turnover and microbial energy flow under neonicotinoid stress. These contrasting responses may be partly explained by inherent structural differences between the soils. The Vertisol (clay) is characterised by shrink–swell behaviour due to its high-activity clays, resulting in a more dynamic and compactible soil structure that can influence pore connectivity and chemical-microbe contact, even under constant moisture. In contrast, the Luvisol soils (loamy sand and sandy loam) have more rigid and stable pore structures, which may moderate exposure effects and support greater microbial resilience. These structural differences likely shaped microbial community stability and network organisation, which in turn may have contributed to the stronger functional suppression observed in the fine-textured Vertisol.

These experimental insights were synthesised into a fate–effect–function conceptual model linking neonicotinoid persistence, microbial exposure, taxonomic restructuring, and functional consequences. The model provides a mechanistic framework for interpreting neonicotinoid–microbe interactions and their implications for biogeochemical cycling, biodegradation potential, and soil ecosystem services.

The findings of this thesis demonstrate that neonicotinoid effects on soil microbial communities are dynamic, compound-specific, and mediated by soil texture. By linking microbial structural and functional shifts to agroecosystem processes, this thesis advances the evidence base for neonicotinoid risk assessment and supports the development of management strategies aimed at safeguarding soil health and agricultural sustainability.

List of abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
16s rRNA	16S ribosomal ribonucleic acid
%IncMSE	Percent Increase in Mean Square Error
ACE	Abundance-based Coverage Estimator
AIC	Akaike Information Criterion
a.i.	Active ingredient
ANOVA	Analysis of Variance
ASV	Amplicon Sequence Variants
BH	Benzene Hexachloride
BIC	Bayesian Information Criterion
CAP	Constrained Analysis of Principal Coordinates
CID	Compound Identification
DADA2	Divisive Amplicon Denoising Algorithm 2
dbRDA	Distance-Based Redundancy Analysis
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
EC	Electrical Conductivity
ECEC	Effective Cation Exchange Capacity
ECs	Enzyme Commission numbers
ESP	Exchangeable Sodium Percentage
Faith's PD	Faith's Phylogenetic Diversity
gDNA	Genomic DNA
GLMs	Generalised Linear Models
HPLC	High-Performance Liquid Chromatography
IncNodePurity	Increase in Node Purity
IndVal	Indicator Value
IRAC	Insecticide Resistance Action Committee
ITS	Internal Transcribed Spacer
IUPAC	International Union of Pure and Applied Chemistry
IUSS	International Union of Soil Sciences
KOs	KEGG Orthology identifiers
K_{ow}	n-octanol/water partition coefficient
LDA	Linear Discriminant Analysis
LEfSe	Linear discriminant analysis Effect Size

LMMs	Linear Mixed-effects Models
MPD	Mean Pairwise Distance
NCBI	National Center for Biotechnology Information
NMDS	Non-metric Multidimensional Scaling
*n*AChR	Nicotinic acetylcholine receptor
NNR	Number Needed to Read
OM	Organic Matter
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
PICRUSt2	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
SRA	Sequence Read Archive
UniFrac	Unique Fraction metric

Table of Contents

List of Tables

List of Figures

Chapter 1 General introduction

1.1 Introduction

As the global population increases and the demand for food intensifies, agriculture faces mounting pressure to ensure food security (Beltran-Peña et al., 2020). Among the key challenges is the effective management of agricultural pests, which can significantly reduce crop yields (Dent & Binks, 2020; Fernández et al., 2022; Sharma & Ortiz, 2000). To minimise these losses, pesticides have become an essential component of modern crop protection. Pesticides comprise a diverse group of substances designed to prevent, destroy, repel or control harmful organisms and include insecticides, herbicides, fungicides, rodenticides and nematicides (Garud et al., 2024; Hassaan & El Nemr, 2020). Depending on their intended application, pesticides may also be classified according to their chemical composition, mode of action or mode of entry (Parra-Arroyo et al., 2022; Sulaiman et al., 2019). Among these groups, insecticides represent one of the most extensively used classes because insect pests remain a major constraint to agricultural productivity worldwide (Abubakar et al., 2020; Sharma et al., 2019).

The rapid evolution of pesticide usage mainly started with the Second World War, with the introduction of several synthetic chemical pesticides such as DDT, BHC, aldrin, dieldrin, endrin, etc (Damalas, 2009). Between the 1940s and 1970s, synthetic organics largely replaced botanical and inorganic insecticides, with organochlorines, organophosphates, carbamates, and pyrethroids becoming dominant (Tomizawa & Casida, 2003). Following the Green Revolution, the demand for chemical inputs, such as fertilisers and insecticides, increased substantially to support high-yielding crop varieties. Since the early 1990s, the use of insecticides in agriculture has begun to rise rapidly (FAO, 2024).

Organochlorines, due to their broad-spectrum efficacy, were widely adopted within a short time (Brain & Anderson, 2019; Kaushik & Kaushik, 2007) but later proven to be a potential threat to humans, amphibians, and the environment in terms of ecotoxicity, lethality, genotoxicity, and cytotoxicity (Blus, 2002; Jamil et al., 2005). Their high environmental persistence led to bans on organochlorine insecticides in many developed countries during the 1970s (Karasali & Maragou, 2016; Saeed et al., 2017). In this context, organophosphates were being used effectively as alternatives to organochlorines due to their comparatively rapid degradation rate (Cave et al., 2012). However, some organophosphates were found to be toxic at varying degrees to humans, social insects, and wildlife populations (Karasali & Maragou, 2016). They are also considered as environmental pollutants as they can

accumulate in plants, soil, and water and eventually enter the human food chain (Dar et al., 2020; Özkara et al., 2016). Consequently, carbamates and pyrethroids were introduced to the global insecticide market as replacements for organochlorine and organophosphate insecticides. Over time, target insect populations developed resistance to these insecticide groups, leading to a gradual loss of effectiveness (Tomizawa & Casida, 2003, 2005). In response, neonicotinoid insecticides were first introduced in the 1980s and subsequently became widely adopted throughout the late 20th century, representing a major shift in pest management (Anderson et al., 2015). They act as nicotinic acetylcholine receptor (nAChR) agonists within Subgroup 4A of the IRAC classification (IRAC, 2022; Jeschke & Nauen, 2008). Their efficacy, coupled with their systemic nature, enabled application via seed treatments or foliar sprays, and facilitated their rapid integration into agricultural practices worldwide (Buchholz & Nauen, 2002; Kundoo et al., 2018). The replacement of organophosphates, carbamates, phenyl-pyrazoles, and pyrethroids with neonicotinoids has been attributed to the combination of their physicochemical properties, systemic nature, low mammalian toxicity, and versatility in application (Elbert et al., 2008; Jeschke & Nauen, 2008).

However, environmental concerns have been raised with respect to the wide use of neonicotinoids due to their persistence, off-site transport and off-target effects. Some studies have shown that only around 5% of the neonicotinoid dose applied is absorbed by the plant, while the remainder disperses into the adjacent environment, entering soil, air, and water (Hladik & Kolpin, 2016; Wood & Goulson, 2017). Foremost among these concerns was the widespread decline of bee populations, both honeybees (*Apis mellifera*) and native wild bees (*Bombus* sp.) (Cutler et al., 2014; Woodcock et al., 2016), crucial for pollinating a substantial portion of our food crops. The harmful effects of neonicotinoids became a cause for concern when honey bee populations first declined in some US states during 2006-2007 (Gross, 2013; Oldroyd, 2007). Further research demonstrated that these insecticides inhibited development, sensory function, colony growth, and foraging efficiency of bee populations (Jacob et al., 2019; Muth & Leonard, 2019; Siviter et al., 2018).

Beyond pollinators, the non-target impacts of neonicotinoid insecticides encompass a wide array of unintended consequences, spanning terrestrial and aquatic ecosystems. In terrestrial ecosystems, neonicotinoid exposure can disrupt the behaviour and reproductive capabilities of predatory insects, such as ladybugs (*Coleomegilla* sp., *Harmonia* sp., *Hippodamia* sp., etc) and parasitoid wasps (*Microplitis* sp., *Nasonia* sp., etc) (Pisa et al., 2015; Stapel et al., 2000; Tappert et al., 2017). Reduced populations of these natural enemies can lead to increased pest pressure, potentially resulting in greater pesticide use—a classic example of unintended consequences in pest management strategies (Harsimran Kaur & Harsh, 2014; Sánchez-

Bayo, 2021). Aquatic invertebrates, such as mayflies (*Caenis* sp., *Cloeon* sp., etc) and dragonflies (*Ischnura* sp., *Orthetrum* sp., etc), are also highly susceptible to neonicotinoids, leading to cascading effects on aquatic food webs (Barmentlo et al., 2021; Roessink et al., 2013; Van den Brink et al., 2016). Neonicotinoid persistence and accumulation in soil is also of concern (van der Sluijs et al., 2015) because soil-dwelling invertebrates, such as earthworms (*Allolobophora* sp., *Eisenia* sp., *Lumbricus* sp., etc) and ground beetles (*Harpalus* sp., *Poecilus* sp., *Pterostihus* sp., etc) can be adversely affected (Gasparic et al., 2022; Ge et al., 2018; van Loon et al., 2022) potentially disrupting nutrient cycling and soil health (Gunstone et al., 2021). In essence, the non-target impacts of neonicotinoids in the surrounding ecosystem have emerged as a pressing ecological concern. Overall, the unintended impacts of neonicotinoids extend across trophic levels and ecosystems, raising concerns about the long-term sustainability of their use in modern agriculture.

1.2 Neonicotinoids in the soil ecosystem

1.2.1 Pathways to soil

Following application, neonicotinoid compounds enter the soil environment through several primary routes, including seed coating, soil drenching, foliar spray drift, irrigation return flows and plant residue deposition (Bonmatin et al., 2015; Pisa et al., 2015). Among these pathways, seed treatment is the most common, with less than 20% of the active ingredient typically taken up by the developing plant, depending on crop characteristics and environmental conditions (Sur & Stork, 2003). In fact, more than 90% of the applied compound can enter the environment, with the soil as a major sink for these chemicals (Goulson, 2013). Soil-applied formulations such as granules or drenches introduce the active compounds directly into the rhizosphere and are commonly used in high-value crops and horticultural systems due to their systemic activity and long-lasting protection (Alsafran et al., 2022; Sánchez-Bayo, 2014; Zhang et al., 2017). In addition to direct soil application, residues may enter the soil through processes such as foliar spray runoff and dust drift from treated seeds during planting (Girolami et al., 2013; Morrison et al., 2023). Dust generated during mechanical planting can be highly contaminated with neonicotinoids, and once released into the air, it can settle on nearby soils and non-target vegetation, contributing to off-site soil contamination (Krupke et al., 2012; Wintermantel et al., 2020). Collectively, these diverse entry routes highlight the multiple exposure pathways by which neonicotinoids can accumulate in agricultural soils.

1.2.2 Fate and transport

Once introduced into the soil, neonicotinoids are subject to a range of physicochemical and biological processes that influence their distribution, persistence, and mobility. Key factors governing their fate include the compound's solubility, sorption affinity, degradation rate, and interactions with soil properties such as texture, organic matter content, pH and microbial activity (Pietrzak et al., 2020). Due to their high-water solubility and variable sorption to soil particles, neonicotinoids are considered potentially mobile in soil environments. Their solubility makes them prone to leaching, which can lead to the contamination of deeper soil layers or groundwater, potentially affecting non-target ecosystems (Zhang et al., 2024c). Sorption behaviour is strongly influenced by both the chemical nature of the compound and soil properties (Zhang et al., 2018). Even neonicotinoids with similar molecular size and structure can exhibit distinct sorption–desorption kinetics depending on functional groups. For example, thiacloprid shows faster and stronger sorption than acetamiprid, likely due to its sulphur-containing thiazolidine ring, which affects its solubility, lipophilicity, and affinity for soil constituents (Modrić et al., 2023).

Neonicotinoids in soil also undergo various degradation processes. Photodegradation occurs when these compounds are exposed to sunlight, particularly UV radiation. While photodegradation is typically slower than other degradation processes, UVB radiation plays a significant role in the photodegradation of neonicotinoids, such as clothianidin and thiamethoxam, with degradation rates under UVB being much higher than those under UVA or visible light (Li et al., 2018b). Chemical degradation of neonicotinoids is influenced by environmental factors such as soil pH, temperature, and the presence of soil organic carbon and cation exchange capacity, with processes like nitrate reduction and hydrolysis contributing to their breakdown (Zhang et al., 2018).

1.2.3 Microbial interactions

The delicate balance of Earth's ecosystems relies on a multitude of intricate relationships and processes that unfold both visibly and imperceptibly. Within this subterranean world, a diverse and dynamic community of microorganisms plays a fundamental role in shaping soil health and ecosystem functioning (Ortiz & Sansinenea, 2022). As the cornerstone of agriculture and terrestrial ecosystems, the intricate web of interactions within the soil microbiome is both complex and essential. Yet, this delicate balance is under threat from agrochemicals, such as neonicotinoids and their potential effects on soil health and ecosystem sustainability (Aloo et al., 2021; Daisley et al., 2022). Soil fertility is a combination of its physical, chemical, and biological properties, of which the last is the least understood. Soils are enriched with

microorganisms, which play a pivotal role in improving fertility and nutrient cycling. One gram of healthy soil may comprise of about 100 million of bacteria, >0.15 mg of fungi, about 100,000 protozoa and other types of microorganisms which are involved in the breakdown of organic matter (Kalia & Gosal, 2011). Soil microorganisms are involved in not only organic matter breakdown but also in releasing nutrients from organic matter, fixing atmospheric nitrogen, increasing phosphorus availability, controlling pathogens, and improving soil structure, and are thus a key determinant of soil fertility (Parizadeh et al., 2021). Hence, a greater understanding of the fate of pesticides is required, primarily because the fact that it regulates the exposure to and thus, the effect on target and non-target organisms (Chaplain, 2011).

Neonicotinoids are readily dissolved in water with log K_{ow} values ranging from −0.66 to 1.26, making them potential to transport off-site (Hladik & Kolpin, 2016; Tomizawa & Casida, 2005). Thus, when neonicotinoids are applied to soil, they can leach into the upper 15–20 cm, where concentrations are often highest, increasing the likelihood of microbial exposure (Philipp et al., 2025; Zhang et al., 2021b). At the same time, the co-location of active microbial taxa within this layer can facilitate biodegradation and transformation of neonicotinoids by selective microbial taxa (Wu et al., 2021; Zhang et al., 2021b).

The impact of neonicotinoids on soil microorganisms is complex and multifaceted. Alterations in microbial community composition and abundance can occur following exposure to these insecticides (Wu et al., 2021; Yu et al., 2020). Certain microbial populations may decline, while others become more dominant, disrupting the delicate balance of soil biodiversity (Garg et al., 2021). These shifts in microbial communities can have cascading effects on soil functions, potentially leading to changes in nutrient availability, organic matter decomposition rates, and even soil structure (Lo, 2010; Tripathi et al., 2020).

Furthermore, neonicotinoids have been found to affect the enzymatic activity of soil microorganisms (Castillo-Díaz et al., 2017; Wang et al., 2014). Enzymes play a crucial role in breaking down organic matter and releasing nutrients that plants require for growth (Dotaniya et al., 2019; Wallenstein & Burns, 2011). Changes in enzyme activity can hinder nutrient cycling processes, potentially leading to nutrient imbalances in the soil and impacting plant health (Dai et al., 2021; Hui et al., 2023; Zhang et al., 2015b). Additionally, the ecological repercussions extend beyond the soil itself. Soil microorganisms are integral to the broader ecosystem, and their health is intimately tied to the well-being of plants, insects, and other organisms higher up the food chain (Bach et al., 2020).

The intricate interplay between neonicotinoids and soil microorganisms is a topic of growing concern in the realm of agricultural sustainability and environmental health. While neonicotinoids were initially designed to target specific pests, their unintended consequences

on soil microbial communities and the broader ecosystem cannot be ignored. Recognising the potential far-reaching impact of these insecticides on the soil microbiome underscores the importance of further research, sustainable agricultural practices, and a holistic approach to pest management that considers the delicate balance of life beneath our feet.

1.3 Immediate and persistent ecological effects in soil ecosystem

The ecological implications of neonicotinoids in soils cannot be fully understood without considering temporal dynamics. Their impacts on soil microorganisms, which drive key biogeochemical cycles, can differ markedly depending on whether exposure is acute and transient or chronic and persistent. Short-term effects are often associated with immediate physiological and metabolic disruptions, while long-term consequences involve deeper restructuring of microbial community composition and functionality. Distinguishing between these temporal dimensions is essential for evaluating how neonicotinoids influence soil health and ecosystem sustainability.

1.3.1 Short-term ecological effects

Short-term impacts emerge within days of application and are best captured by quantitative changes in metabolic activity and enzyme kinetics, followed by transient adjustments in community structure. In controlled soil assays, microcalorimetry and enzyme profiling showed that brief exposure to imidacloprid and acetamiprid at experimental concentrations of 10, 20, 40 and 80 mg kg^{-1} soil suppressed dehydrogenase (≈ 29.6 %), phosphomonoesterase (≈ 40.4 %), and urease (≈ 23–30 %), indicating an immediate constraint on microbial respiration and N–P turnover (Wang et al., 2014). Imidacloprid application at the recommended field rate (25 g a.i. ha^{-1}), as well as 2-, 5- and 10-fold higher application rates, has also been shown to depress a broader set of enzymatic activities and microbial biomass, such as β-glucosidase, fluorescein diacetate hydrolase, acid phosphatase, and urease in rice soils, again highlighting immediate metabolic interference (Mahapatra et al., 2017). These findings were corroborated by a field study in a maize rhizosphere, where imidacloprid seed dressing at 5 mL kg^{-1} reduced culturable bacteria, actinomycetes, fungi, and varying urease and invertase activities (Wu et al., 2016). Beyond metabolism, thiamethoxam triggers transient microbial community shifts. Initial community responses to thiamethoxam were evident by day 7 and were dose-modulated. Following thiamethoxam inputs at the recommended field application rate (1.8 mg kg^{-1} soil) and two elevated concentrations (18 and 180 mg kg^{-1} soil), bacterial diversity and composition shifted early and then trended back toward pre-exposure states as residues

declined; the amplitude and persistence of deviation depended strongly on soil type and exposure intensity (Wu et al., 2021). Evidence from these investigations indicates that short-term microbial responses occur across both field-relevant and experimentally elevated neonicotinoid exposures. This short-term phase is characterised by metabolic inhibition and temporary reconfiguration of community structure, prior to any longer-term accumulation or legacy restructuring.

1.3.2 Long-term ecological effects

Long-term effects arise because only a small fraction of seed-applied neonicotinoids is taken up by crops, leaving residues to persist in agricultural soils across seasons (Goulson, 2013). Field monitoring in corn–canola systems subjected to repeated clothianidin seed treatments detected mean soil residues of approximately 7.0 and 5.7 ng g^{-1}, respectively, indicating persistent, low-level exposure for soil biota without evidence of progressive accumulation under typical agricultural use (de Perre et al., 2015). Typical environmental half-lives for neonicotinoids span months and can exceed a year in cool or dry soils, favouring accumulation and sustained microbial contact (Bonmatin et al., 2015). Thiamethoxam further contributes to long-term pressure by metabolically converting to the more persistent clothianidin in soils (Kurwadkar & Evans, 2016). During a 56-day soil incubation, imidacloprid applied at the recommended field rate of 1 mg kg^{-1} soil and at a ten-fold elevated rate of 10 mg kg^{-1} soil, microbial communities exhibited directional changes in composition, with sensitive taxa declining and tolerant groups increasing (Cycoń et al., 2013). Using the same application rates, Cycoń and Piotrowska-Seget (2015a) demonstrated that, these structural adjustments were accompanied by functional narrowing and reduced redundancy in soil processes following chronic neonicotinoid inputs. Under the same exposure regime, long-term exposure can also shift the composition of nitrifying guilds (ammonia-oxidising archaea/bacteria), indicating potential legacy effects on nitrification capacity (Cycoń & Piotrowska-Seget, 2015b). Microbial transformation adds a further long-term dimension: bacteria can cleave the thiazolylmethyl–nitroguanidine linkage in clothianidin, yielding thiazole- and guanidine-derived products that persist in soils (Parte & Kharat, 2019). White-rot fungi such as *Phanerochaete sordida* also convert clothianidin to urea-type metabolites under laboratory conditions, demonstrating fungal contributions to residue turnover (Mori et al., 2017). Importantly, several transformation products retain equal or greater toxicity than their parents for selected endpoints, so biodegradation does not automatically equate to detoxification over long timescales (Shen et al., 2022).

1.4 Overview of key neonicotinoids

According to IRAC (2022), there are seven active ingredients of neonicotinoids commercially available in the global market. Among them, imidacloprid, thiamethoxam, and clothianidin are the most commonly used in modern crop production and are frequently reported in agricultural soils following repeated seed treatment applications (Sánchez-Bayo & Hyne, 2014). While these compounds share a common neurotoxic mode of action in insects, they differ in their chemical structures, which in turn underpins differences in solubility, persistence, and transformation pathways in soil. Their chemical structures are shown in Figure 1–1, and selected physicochemical properties are summarised in Table 1–1.

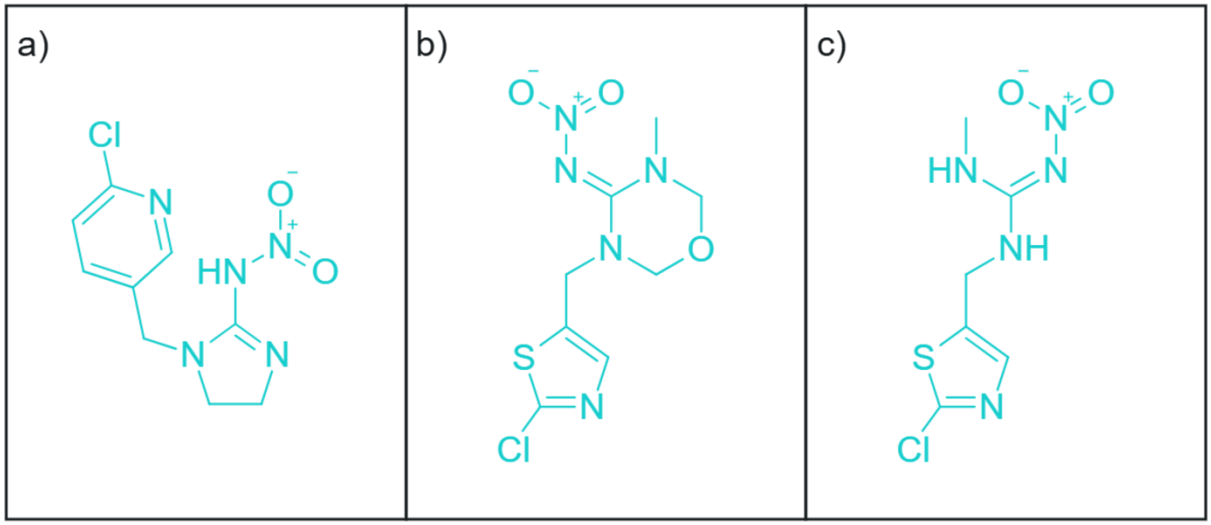

Figure 1–1: Chemical structures of the three neonicotinoid insecticides used: (a) imidacloprid, (b) thiamethoxam, and (c) clothianidin. Structures were reproduced using the Marvin web-based chemical structure editor (ChemAxon, 2025).

1.4.1 Imidacloprid

Imidacloprid (IUPAC: 1-[(6-chloro-3-pyridyl) methyl]-N-nitroimidazolidin-2-ylideneamine), a chloronicotinyl neonicotinoid (Figure 1–1a), was the first of its class to be commercialised for agricultural use and remains one of the most widely applied globally (Kitsiou et al., 2009; Talcott, 2013). Its persistence in soils varies widely with management and environmental conditions. Degradation tends to be slower in non-agricultural soils compared to cropped soils, and organic matter amendments can further influence persistence (Roberts & Hutson, 1999; Rouchaud et al., 1994). Imidacloprid degrades into a suite of metabolites, including 6-chloronicotinic acid, hydroxynicotinic acid, cyclic ureas, and several nitro- and nitroso-derivatives (Gervais et al., 2010). Sorption generally increases with soil organic matter (Liu et

al., 2006), but can decrease at higher concentrations or in soils rich in dissolved organic carbon, conditions that enhance its potential for leaching (Flores-Céspedes et al., 2002).

1.4.2 Thiamethoxam

Thiamethoxam (IUPAC: 3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene(nitro)amine), a second-generation thianicotinyl neonicotinoid (Figure 1–1b), is distinguished by its high water solubility and mobility in soils (Maienfisch et al., 2001). In soils, thiamethoxam displays high mobility due to its low sorption affinity, which raises the risk of leaching to deeper soil layers and groundwater (Zhou et al., 2025a). It is generally stable under acidic conditions but hydrolyses more rapidly in alkaline soils (Karmakar et al., 2009). A defining characteristic of thiamethoxam is its metabolic transformation to clothianidin, which is more persistent and frequently detected as the dominant residue following field applications (Jeschke & Nauen, 2005).

1.4.3 Clothianidin

Clothianidin (IUPAC: 1-(2-chlorothiazol-5-ylmethyl)-3-methyl-2-nitroguanidine), also a second-generation neonicotinoid of the nitroguanidine subclass (Figure 1–1c), is characterised by strong binding affinity to soil particles and pronounced persistence, with residues frequently detected long after application (de Perre et al., 2015; Goulson, 2013). In soils, clothianidin adsorption is strongly influenced by organic carbon and pH, with desorption often incomplete, and it generally degrades by first-order kinetics at rates that vary with soil type and moisture (Li et al., 2018c).

Table 1–1: General characteristics of imidacloprid, thiamethoxam, and clothianidin, compiled from PubChem compound entries (CIDs: 86287518, 5821911, 86287519) (National Center for Biotechnology Information, 2022)

Property	Imidacloprid	Thiamethoxam	Clothianidin
Molecular weight (g/mol)	255.66	291.72	249.68
Solubility (H_2O) (mg/L)	6.10×10^2 at 20°C	4.10×10^3 at 25°C	3.27×10^2 at 20°C
Octanol-water partition coefficient (log K_{ow})	0.57 at 21°C	−0.13 at 25°C	0.70 at 25°C
Vapor pressure (Pa)	9.0×10^{-10}	6.6×10^{-9}	1.3×10^{-10}

1.5 Research objectives and methodological approaches

The central objective of this thesis is to investigate how neonicotinoid seed treatments affect soil microbial communities and to evaluate the implications of these changes for soil health and agricultural sustainability. While the ecological risks of neonicotinoids have been extensively studied in pollinators and other aboveground organisms, their impacts on belowground ecosystems remain poorly understood. This knowledge gap is particularly concerning given the pivotal role of soil microorganisms in nutrient cycling, organic matter decomposition, and ecosystem resilience.

To address this gap, three commonly used neonicotinoids, imidacloprid, thiamethoxam, and clothianidin, were selected as model compounds due to their contrasting physicochemical properties, widespread agricultural application, and persistence in soils. The novelty of this research lies in its dual emphasis on bacterial and fungal communities, its integration of contrasting soil textures, and its use of multi-layered analyses combining diversity metrics, indicator species, co-occurrence networks, functional prediction, and correlations with soil properties.

The methodological framework integrates both synthesis and experimentation. A systematic review of the literature (Chapter 2) provides a global overview of the extent and consistency of neonicotinoid effects on soil microorganisms and highlights major gaps that require targeted study. Building on this foundation, a series of microcosm experiments were conducted to evaluate microbial responses at two temporal scales.

- Short-term experiments (Chapters 4 and 6) examined bacterial and fungal community dynamics during the first days to weeks of exposure. These chapters assessed changes in taxonomic composition, diversity indices, and network structures, while testing the hypothesis that distinct neonicotinoids induce unique temporal trajectories of microbial change.
- Extended experiments across soil textures (Chapters 5 and 7) evaluated microbial resilience and adaptation under prolonged exposure. By linking microbial shifts with soil physicochemical properties, these studies addressed how environmental context mediates pesticide–microbe interactions.

Finally, the thesis integrates experimental insights into a conceptual synthesis (Chapter 8), which links the environmental fate of neonicotinoids with microbial responses and soil ecosystem functions. The thesis concludes with Chapter 9, which synthesises findings across

chapters, reflects on limitations, and discusses implications for agricultural sustainability and pesticide regulation.

This framework collectively addresses five interlinked research questions:

1) To what extent do neonicotinoids affect soil microbial communities across existing studies?
2) How does this evidence inform understanding of the broader ecological impacts of neonicotinoid use?
3) How do different neonicotinoids influence microbial community composition, diversity, and functions over time?
4) How do soil texture and physicochemical properties mediate microbial community responses and patterns of adaptation under neonicotinoid exposure?
5) What are the implications of neonicotinoid-induced changes in soil microbial communities for soil health and agricultural sustainability?

Cognisant of these research questions, the research presented in this thesis is organised into four distinct experimental chapters, each characterised by its own specific objectives, methodologies, findings, and subsequent discussions.

1.6 Thesis structure

This thesis is presented in the format of a “Standard Thesis” (Figure 1–2). The integration of these chapters forms a cohesive body of work that advances understanding of neonicotinoid impacts on soil microbial communities. While each chapter is self-contained and focused on specific aspects of the research, together they provide a comprehensive exploration of the overarching research questions.

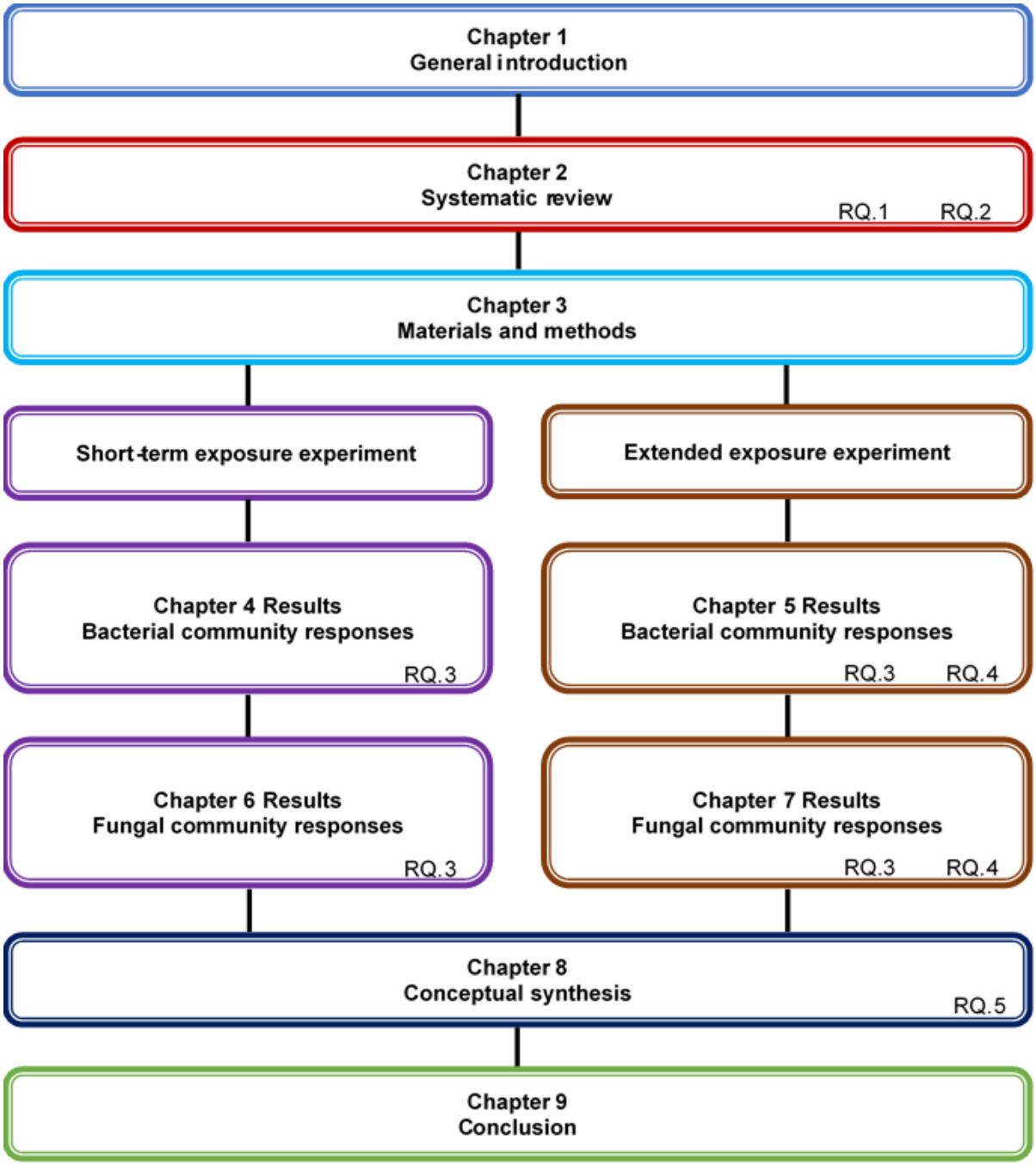

Figure 1–2: Structure of the thesis, showing the progression from a systematic review (Chapter 2) to experimental investigations of bacterial (Chapters 4–5) and fungal (Chapters 6–7) community responses under short- and extended exposures, culminating in a conceptual synthesis (Chapter 8) and overall conclusion (Chapter 9)

The thesis is organised as follows:

Chapter 1. General introduction provides an overview of the research context, outlines the research questions, and sets the stage for the subsequent chapters.

Chapter 2 synthesises existing knowledge on neonicotinoid effects on soil microbial communities, identifies inconsistencies, and highlights knowledge gaps.

Chapter 3 provides a comprehensive materials and methods that consolidates the core experimental procedures used throughout the research.

Chapter 4 examines the rapid effects of distinct neonicotinoid seed treatments on soil bacterial communities, focusing on diversity, composition, and interaction patterns.

Chapter 5 investigates bacterial community shifts under extended exposure to neonicotinoids across soils of contrasting textures, linking community dynamics with soil properties.

Chapter 6 assesses how soil fungal communities respond to different neonicotinoid seed treatments, with attention to temporal changes in diversity and taxonomic composition.

Chapter 7 analyses taxonomic and functional shifts in fungal communities under prolonged exposure, evaluating how soil texture mediates resilience and adaptation.

Chapter 8 develops a conceptual synthesis that links the environmental fate of neonicotinoids with microbial responses and soil ecosystem functions.

Chapter 9 summarises findings across all chapters, outlines ecological and regulatory implications, acknowledges limitations, and proposes directions for future research.

Chapter 2 Changes in soil microbial communities after exposure to neonicotinoids: A systematic review

2.1 Overview[1]

This chapter constitutes a foundational element of the thesis by establishing evidence base on neonicotinoid impacts on soil microbial communities. It addressed two central research questions. Firstly, it critically assessed the extent and consistency of evidence regarding the effects of neonicotinoid insecticides on soil microbial communities across a diverse range of studies. Despite the fundamental role of soil microbes in ecosystem processes, there were comparatively fewer studies investigating neonicotinoid impacts on these communities at the time of publication. By systematically reviewing and synthesising available literature, this chapter not only quantified the collective body of knowledge on the subject but also identified patterns, gaps, and inconsistencies within the available research. Secondly, it explored how this knowledge contributed to a more comprehensive understanding of the ecological impacts associated with the use of neonicotinoids. This systematic review was crucial because it served as the groundwork, providing the thesis with a robust scientific foundation. It not only elucidated the ecological consequences of neonicotinoid exposure on soil microbial communities but also underscored the need for further experimentation to address the identified gaps and variations within existing studies. By critically evaluating the available evidence, the chapter refined subsequent research questions, highlighted the importance of targeted empirical work, and established an evidence-based framework for investigating neonicotinoid–microbe interactions.

[1] This chapter has been published as:
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2.2 Systematic review

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MINI REVIEW

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Changes in soil microbial communities after exposure to neonicotinoids: A systematic review

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Abstract

Neonicotinoids are a group of nicotine-related chemicals widely used as insecticides in agriculture. Several studies have shown measurable quantities of neonicotinoids in the environment but little is known regarding their impact on soil microbial populations. The purpose of this systematic review was to clarify the effects of neonicotinoids on soil microbiology and to highlight any knowledge gaps. A formal systematic review was performed following PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analyses) guidelines using keywords in PubMed, SCOPUS and Web of Science. This resulted in 29 peer-reviewed articles, whose findings diverged widely because of variable methodologies. Field-based studies were few (28%). Imidacloprid was the most widely used (66%) and soil microbial communities were most sensitive to it. Spray formulations were used in 83% of the studies and seed treatments in the rest. Diversity indices were the most frequently reported soil microbial parameter (62%). About 45% of the studies found that neonicotinoids had adverse impacts on soil microbial community structure, composition, diversity, functioning, enzymatic activity and nitrogen transformation. Interactions with soil physicochemical properties were poorly addressed in all studies. The need for more research, particularly field-based research on the effects of neonicotinoids on soil microorganisms was highlighted by this review.

INTRODUCTION

In agriculture, crop quality and yield depend to a large extent on crop protection. Crop protection is defined as management practices that protect crops from weeds, pests and pathogens. Annually about 26%–40% of global potential crop yield may be lost due to weeds, pests and diseases (OECD/FAO, 2012) and therefore the losses could certainly be twofold without crop protection. Thus, to secure good quality and quantity crop production, farmers use pesticides from both natural and synthetic sources. Neonicotinoids are nicotine-moiety-based systemic insecticides (Kasiotis & Machera, 2015) that have been widely used globally since the early 1990s (Goulson, 2013). Structurally, neonicotinoids are hydroheterocyclic guanidines/ amidines conjugated with an aromatic heterocyclic group, electron-withdrawing group and elastic bond (Buszewski et al., 2019). They function as nicotinic acetylcholine receptor agonists by targeting the sodium/ potassium ionophore of an insect's central nervous system and disrupting cholinergic neurotransmission (Jeschke et al., 2011; Moffat et al., 2016; Seifert, 2014). Neonicotinoids are popular over other insecticides because of their ease of application, toxicity to a wide range of insects, high potency for controlling insects and low acute toxicity to mammalians (Thompson et al., 2020). Based on the primary site of action, there are seven active ingredients of neonicotinoids commercially available in the global pesticide market viz.

dinotefuran, clothianidin, thiacloprid, thiamethoxam, acetamiprid, nitenpyram and imidacloprid (IRAC, 2022).

Although neonicotinoids are intended to be taken up by the plant, a greater portion of the applied insecticide failed to reach the target plant tissue (Sur & Stork, 2003). Only about 5% of the applied active ingredients of neonicotinoids are taken up by the target plant and remaining majority dispersing to the surrounding environment in soil, air and water (Wood & Goulson, 2017). Neonicotinoids have a limited propensity for volatilization, which means during spray treatments, they are most likely to exist in a gaseous form for a short period of time (Bonmatin et al., 2015). These compounds are transported in the soil environment primarily by surface runoff, leaching and plant uptake (Borsuah et al., 2020; Pietrzak et al., 2020). It had been reported by Chrétien et al. (2017) that surface runoff was a significant transportation mode that contributed about 53% of the observed total losses of neonicotinoids. After entering the soil, neonicotinoids may undergo a number of processes: leaching through the soil profile, sorption, dissipation and degradation (Anderson et al., 2015; Dankyi et al., 2018; Pietrzak et al., 2020). The leaching behaviour of neonicotinoids is predominantly determined by sorption and desorption processes. Sorption affinities are generally influenced by soil organic carbon, soil texture and soil temperature (Anderson et al., 2015; Zhang et al., 2018). Degradation is also a crucial factor in determining how pesticides behave in soils. Neonicotinoids can be degraded abiotically viz. physical, photochemical and chemical degradation, biodegradation (plants and microorganisms), or through enzymatic activity by producing different metabolites (Zhang et al., 2018). Due to low volatility and high water solubility nature, neonicotinoids may leach into the surface and groundwater, raising safety concerns (Bonmatin et al., 2015).

To gain approval for use in agricultural systems, pesticides should be used in such a way that their residues do not accumulate in the surrounding environment with a minimal impact on non-target organisms (Lo, 2010). Many queries have been raised in recent decades concerning the possible effect of neonicotinoids on non-target species. Some studies revealed that neonicotinoids had some detrimental impacts on agriculturally useful species, such as earthworms and insect pollinators, notably honeybees (James et al., 2016; Pisa et al., 2015). A pesticide's fate in soil depends on physicochemical properties like solubility, adsorption, persistence, volatility, etc. The physicochemical properties of neonicotinoids like octanol/water partition coefficient (log K_{ow}) and dissociation constant (pKa) enable them to be systemic in nature, and hence, facilitate their pathway into plant tissues and their movement to all parts of plants (Simon-delso et al., 2015). A wide range of log K_{ow} values (−0.55 to 1.26) and long half-lives (DT_{50} value 3–545 days) also makes neonicotinoids potential to transport offsite (Hladik & Kolpin, 2016). So, being highly soluble in water, neonicotinoids can easily move downward and persist in the soil from days to years depending on formulations, soil texture, organic matter content, management practices and climatic conditions (Bonmatin et al., 2015). The downward movement usually occurs from the topsoil, which is considered as the region of greater microbial activity and the residues of neonicotinoids may pose a threat to soil microorganisms.

The term 'soil microbes' or 'soil microorganisms' refers to the diverse community of microorganisms and is broadly defined as a group of microscopic life forms that include bacteria, archaea, viruses and eukaryotes such as fungi (Flombaum et al., 2022). In agriculture, soil microorganisms are important in biogeochemical cycling, hence alteration in microbial populations eventually affects soil fertility and crop production. Microbial communities are analysed using a variety of methods, of which, microbial activity and abundance are well-known indices of soil quality (Lori et al., 2017). Neonicotinoids may potentially affect soil microbial populations in terms of diversity and abundance (Yu et al., 2020) and biochemical functioning (Cycoń & Piotrowska-Seget, 2015b; Mahapatra et al., 2017).

Individual study outcomes are likely to change due to experimental characteristics such as neonicotinoid active ingredients, treatment rates, soil type, or even the environmental circumstances in which the experiments are conducted (Bonmatin et al., 2015). Research on the effects of neonicotinoids on soil microbes is progressing, but a systematic assessment of neonicotinoid impacts on microbial activities and population in soils has not been previously reported. This systematic review aims to provide an understanding of neonicotinoids' impacts on soil microbiology and thus identify the disparities in the body of knowledge. The objective of this multi-approach systematic review was to analyse all available publications that investigated the changes in soil microbial communities after applying neonicotinoid insecticides.

METHODS

Literature exploration and appraisal

Literature searching strategy

We accessed the available literatures on the impacts of neonicotinoid insecticides on soil microbial communities included in SCOPUS, Web of Science and PubMed databases following PRISMA guidelines[1] (Page et al., 2021). The PRISMA flow diagram was prepared

[1]PRISMA stands for Preferred Reporting Items for Systematic Reviews and Meta-Analyses. It is an evidence-based minimum set of items for reporting in systematic reviews and meta-analyses which consists of a 27-item checklist and a 4-phase flow diagram. PRISMA primarily focuses on the reporting of reviews evaluating the effects of interventions, but can also be used as a basis for reporting systematic reviews with objectives other than evaluating interventions (e.g., evaluating aetiology, prevalence, diagnosis or prognosis).

by accessing an online tool (https://estech.shinyapps.io/prisma_flowdiagram/). The literature search was conducted by using the keywords 'neonicotinoids AND soil AND microorganisms', 'imidacloprid OR clothianidin OR thiamethoxam OR acetamiprid OR thiacloprid OR dinotefuran OR nitenpyram AND soil AND microbial AND communities', 'neonicotinoids AND soil AND bacterial AND communities' and 'neonicotinoids AND soil AND fungal AND communities' restricted to the publication date between 2000 and 2022. The search results were collated and a reference list was made for screening the titles and abstracts.

Screening of studies

Preliminarily identified articles were screened by reading the full texts for further assessment. Only papers that met the following criteria were included in this review:

1. The research must be a published original research paper in a peer-reviewed journal and available in English;
2. Applied neonicotinoid to the soil as formulation or seed treatment;
3. Provided soil microbial community-related data;
4. Applied appropriate controls as well as replication.

Extraction of data

Articles were selected based on the above-mentioned eligibility criteria. For each study, the following data were collected: title of the study, name of the authors, journal name, year of publication, type of neonicotinoids with exposure modality, study design, physicochemical properties, microbiological parameters and geographical location of sampling sites. Soil physicochemical properties included soil pH (in water), organic carbon (g/100 g), soil texture along with clay, silt and sand concentrations (g/100 g), and soil depth (m). The following formula by Lierop (1981) was used to convert pH values in $CaCl_2$ to pH in H_2O.

$Y = 0.53 + 0.98X$; X = pH values in $CaCl_2$, Y = pH values in H_2O. Where organic carbon values were reported as organic matter, it was converted using the following formula (Pluske et al., 2022):

$$\text{Organic matter} = 1.72 \times \text{Organic carbon}$$

Soil textural classes were classified using USDA Soil Texture Calculator (USDA, 2022) based on percent sand, silt and clay for those studies which did not mention the textural class of soil. In addition, the locations of the experimental sites of the selected studies were also extracted to see the geographical distribution of microbial studies of neonicotinoids. To visualise different parameters of this systematic review, data were plotted in R software version 4.2.1.

RESULTS

Analysis of search strategy

A total of 267 articles were found using keywords in SCOPUS, Web of Science and PubMed databases, and 5 articles were found by searching citations. After discarding repeated articles, 97 potential papers were identified for screening. Among these, 73 articles did not meet the inclusion requirements, hence they were excluded from the subsequent analyses. In general, this was due to experimentation on the degradation of neonicotinoids; or the absence of soil microbial experimentation. A sub-set of 29 articles (Table 1) with 119 sampling points was then established following the methodology. The number of articles in each phase of the article selection process is depicted in the PRISMA flow diagram (Figure 1). Afterward, the sensitivity, precision and number needed to read (NNR) of the search strategy of this systematic review were calculated by the formula shown below (Lee et al., 2012; Lefebvre et al., 2022).

$$\text{Sensitivity} = (\text{no. of relevant studies identified} \div \text{total no. of relevant reports in the resource}) \times 100$$

$$\text{Precision} = (\text{no. of relevant studies identified} \div \text{total no. of reports identified}) \times 100$$

$$\text{Number needed to read (NNR)} = 1/\text{Precision}$$

The sensitivity and precision of the search strategy were 28% and 11% respectively indicating low sensitivity and precision and as a result, reflecting low NNR (9.09).

Experimental design and geographical distribution

Out of 29 selected studies, 4 were pot experiments, 8 were field experiments and the remaining 17 were laboratory experiments. Seven different neonicotinoids (dinotefuran, clothianidin, thiacloprid, thiamethoxam, acetamiprid, imidacloprid and paichongding) were tested, of which imidacloprid was used in 19 studies, thiamethoxam in 5 studies, paichongding in 4 studies, clothianidin in 2 studies, dinotefuran in 2 studies, acetamiprid in 1 study and

TABLE 1 Summary of studies investigating neonicotinoids exposure to soil microbial population.

Author/study	Experiment	Exposure modality	Type of neonicotinoid	Country of study
Castillo Diaz et al., 2017	Field experiment	Spray application	Imidacloprid	Spain
Garg et al., 2021	Field experiment	Spray application	Imidacloprid	Not reported
Li et al., 2018	Field experiment	Seed treatment	Imidacloprid, Clothianidin	China
Mahapatra et al., 2017	Pot experiment	Spray application	Imidacloprid	India
Parizadeh et al., 2021	Field experiment	Seed treatment	Thiamethoxam	Canada
Wang et al., 2014	Laboratory experiment	Spray application	Imidacloprid, Acetamiprid	China
Wu et al., 2021	Laboratory experiment	Spray application	Thiamethoxam	China
Yu et al., 2020	Laboratory experiment	Spray application	Thiamethoxam, Dinotefuran	China
Zhang et al., 2018	Laboratory experiment	Spray application	Imidacloprid, Clothianidin, Thiacloprid	China
Cycoń & Piotrowska-Seget, 2015a	Field experiment	Spray application	Imidacloprid	Poland
Cai et al., 2016b	Laboratory experiment	Spray application	Paichongding	China
Cycoń & Piotrowska-Seget, 2015b	Field experiment	Spray application	Imidacloprid	Poland
Zhang et al., 2015	Laboratory experiment	Spray application	Imidacloprid	China
Astaykina et al., 2020	Laboratory experiment	Spray application	Imidacloprid	Russia
Cycoń et al., 2013	Field experiment	Spray application	Imidacloprid	Poland
Filimon et al., 2015	Field experiment	Spray application	Thiamethoxam	Romania
Ingram et al., 2005	Laboratory experiment	Spray application	Imidacloprid	Not reported
Streletskii et al., 2022	Laboratory experiment	Spray application	Imidacloprid	Not reported
Xiao et al., 2017	Laboratory experiment	Spray application	Imidacloprid	China
Zhang et al., 2021	Laboratory experiment	Spray application	Thiamethoxam	China
Yamaguchi et al., 2021	Laboratory experiment	Spray application	Dinotefuran	Japan
Cai et al., 2015	Laboratory experiment	Spray application	Paichongding	China
Cai et al., 2016a	Laboratory experiment	Spray application	Paichongding	China
Zhu et al., 2018	Laboratory experiment	Spray application	Paichongding	China
Deborah et al., 2013	Laboratory experiment	Spray application	Imidacloprid	India
Deborah & Madhuri, 2013	Laboratory experiment	Spray application	Imidacloprid	India
Singh & Singh, 2005a	Field experiment	Seed treatment	Imidacloprid	India
Singh & Singh, 2005b	Field experiment	Seed treatment	Imidacloprid	India
Singh & Singh, 2005c	Field experiment	Seed treatment	Imidacloprid	India

thiacloprid was used in 1 study as a treatment for microbial studies. No study was found that used nitenpyram. Among all the studied neonicotinoids, paichongding is a cis-nitromethylene type. About 83% of the selected studies (24 out of 29 articles) applied neonicotinoids as a spray and the remaining

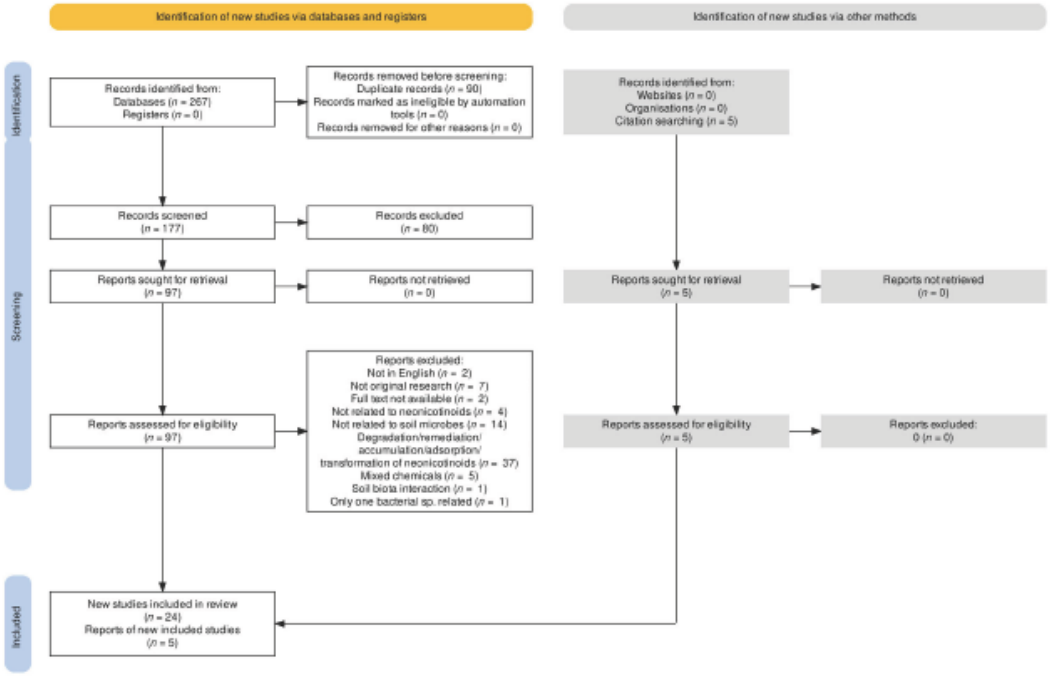

FIGURE 1 PRISMA (preferred reporting items for systematic review and meta-analyses) flow diagram.

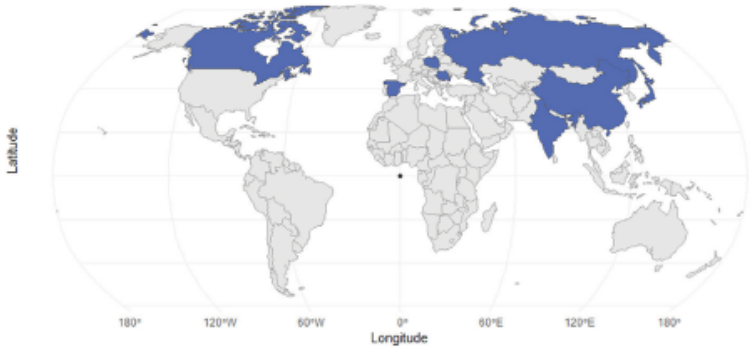

FIGURE 2 Geographical distribution of studies on neonicotinoid effects on soil microbial dynamics. Geographical reporting was at the country level.

17% of the studies (5 out of 29 articles) used neonicotinoids as a seed treatment.

In terms of the geographical location of neonicotinoid studies on soil microbes, the majority, 26 studies named the general locality of the sample sites of which, only 10 studies reported coordinate data and 3 studies did not specify study region or coordinates. We mapped all to acquire a global scale of relevant studies at the country level (Figure 2). The geographical distribution of studies of neonicotinoids on soil microbial communities reflected that all of the studies were conducted in the northern hemisphere with no work on neonicotinoids' impact on soil microbes found in South America, Africa and Oceania.

Soil physicochemical features

Soils used in all selected studies were collected from the field. For soil physicochemical factors, soil pH,

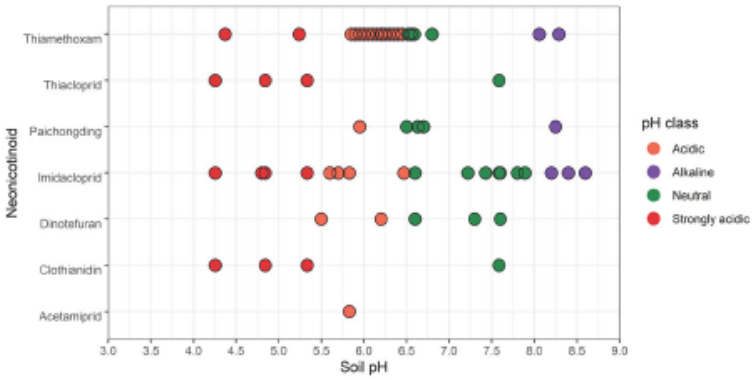

FIGURE 3 pH range of soils in selected studies. strongly acidic (pH <5.5); orange, acidic (pH 5.5–6.4); green, neutral (pH 6.5–7.9); and purple, alkaline (pH 8.0–9.2).

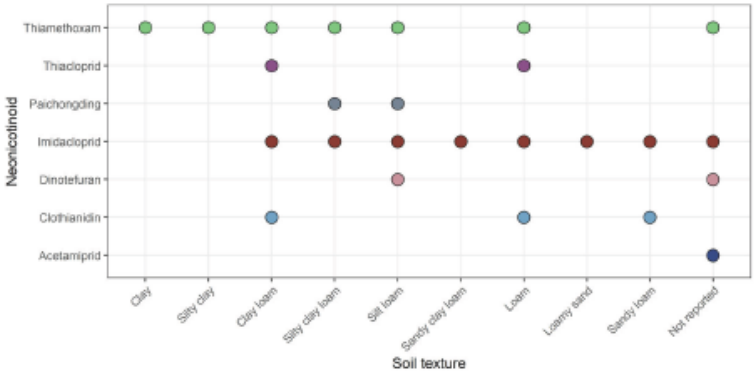

FIGURE 4 Soil textures in the selected studies on neonicotinoids. Each colour indicates a different type of neonicotinoid. Studies that did not mention the textural class were classified using the USDA Texture Calculator.

organic carbon concentration, soil texture, clay, silt and sand concentrations, and soil depth data were extracted to visualise the extent of edaphic variation in the selected microbial studies. Soil pH was reported in 28 studies and 26 studies reported organic carbon concentration. 18 studies reported the clay, silt and sand concentration while 7 studies reported the textural classes of the soil without clay, silt and sand. Samples of soil in the selected studies were taken from the top 0.12–0.20 m of the rhizosphere. No pattern of changes in microbial populations with soil organic carbon was discussed in the selected articles. The experimented soils of the selected studies exhibited a wide range of pH (Figure 3). About 59% were acidic (pH 5.5–6.4), 23% were neutral (pH 6.5–7.9), 10% were strongly acidic (pH <5.5) and 6% were alkaline (pH 8.0–9.2). No strongly alkaline (pH > 9.2) soils were part of the selected data set. Likewise, a wide range of textural classes was present in the selected papers (Figure 4). About 37% of the studied samples were clay while the least was loamy sand (3%).

Microbial parameters

We observed that 16 different microbial parameters were assessed by the various authors when determining the impacts of neonicotinoids on soil microbial populations. Diversity indices were reported most frequently (18 studies) followed by relative abundance (14 studies), enzymatic activity (11 studies), community structure (10 studies) and community composition (6 studies) (Figure 5). Diversity indices included abundance-based coverage estimators (ACE), shannon index, simpson index and chao1. Relative abundance was measured as the percent composition of a microorganism of a particular kind relative to the total number of microorganisms in the area. In

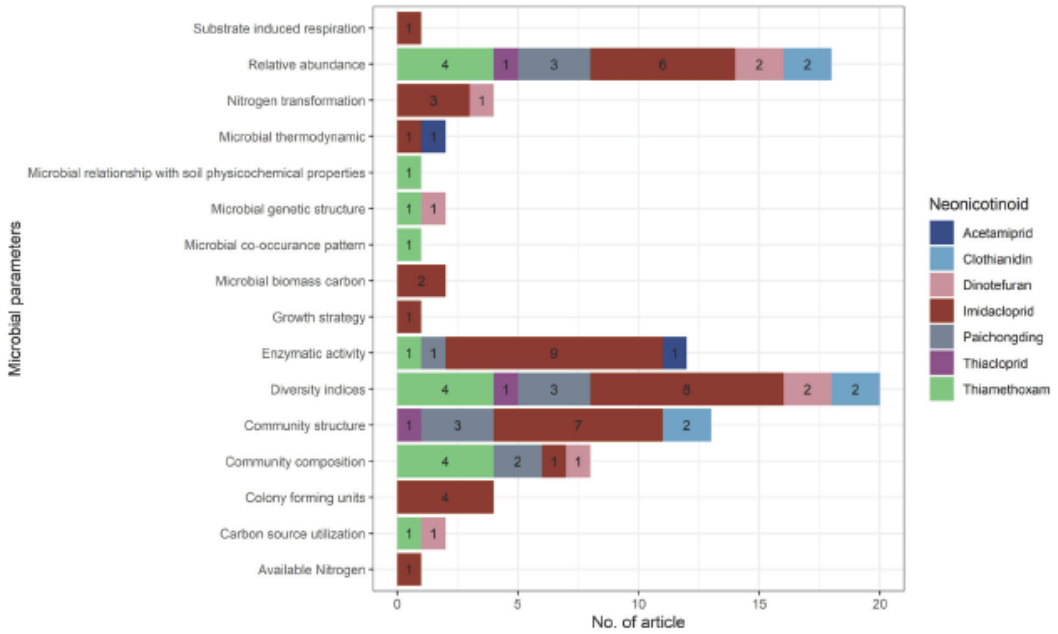

FIGURE 5 Microbial parameters of selected studies after exposure to neonicotinoids. Each colour indicates a different type of neonicotinoid and each number within horizontal bars is the number of articles for an individual neonicotinoid.

terms of enzymatic activity, dehydrogenase, urease, β-glycosidase, fluorescein diacetate hydrolase, acid phosphatase, phosphomonoesterase, protease, catalase, phosphatase, amylase and arginine deaminase activity were assessed in the selected studies. Community structure was monitored by measuring the genetic variability and community composition was measured by analysing which species were present and the abundance of their members.

Neonicotinoid studies that reported no adverse effect on the soil microbial population

Out of 29 selected studies, only five (17%) found no or very limited significant adverse effects on the relative abundance and diversity of microbial populations, some of which are responsible for the biodegradation of neonicotinoids from the neonicotinoids in question (imidacloprid, clothianidin and thiamethoxam) (Garg et al., 2021; Li et al., 2018; Parizadeh et al., 2021; Singh & Singh, 2005a; Zhang et al., 2015). PLFA, PCR-DGGE and CLPP approaches used by Cycoń et al. (2013) revealed that imidacloprid applied at field rate had a negligible impact on the community structure and biodiversity of native soil microorganisms. Wu et al. (2021) reported that thiamethoxam application had no significant effects on some soil metabolic processes such as the carbon metabolism (tricarboxylic acid cycle, pentose phosphate and glycolysis), nitrogen cycle and sulphur metabolism. Some studies reported that imidacloprid stimulates plant available nitrogen, nitrification, ammonification and nitrogen fixation processes in soil (Astaykina et al., 2020; Cycoń & Piotrowska-Seget, 2015a; Singh & Singh, 2005c; Zhang et al., 2018), while dinotefuran promotes ammonia oxidation (Yamaguchi et al., 2021). In terms of enzymatic activity, some studies found that imidacloprid had no adverse effect on microbial phosphatase, protease and urease activity in soil (Deborah & Madhuri, 2013; Ingram et al., 2005). Some studies reported that amylase, dehydrogenase, phosphomonoesterase and arginine deaminase activity was increased at field rate application of imidacloprid and acetamiprid (Singh & Singh, 2005c, b; Deborah et al., 2013; Wang et al., 2014). Cai et al. (2016b) reported that dehydrogenase, protease and catalase activities were all stimulated differently by paichongding. In low-saline soils, the soil bacterial diversity marginally decreased with increasing imidacloprid concentration, however, it might somewhat boost the soil bacterial diversity in moderately saline soils (Zhang et al., 2015).

Neonicotinoid exposure studies that reported adverse effects on soil microbial populations

Thirteen studies (45%) discussed the adverse effect of neonicotinoids (clothianidin, dinotefuran, imidacloprid, paichongding, thiacloprid and thiamethoxam) on soil microbial relative abundance, diversity, activity, community composition and community structure. Imidacloprid changed the relative abundance of some eukaryotic genera named *Apiotrichum*, *Gamsia*, *Humicola*, *Kitasatospora*, *Solicoccozyma* and prokaryotic genera named *Sphingomonas*, *Streptomyces* and *Terrabacter* (Streletskii et al., 2022). In comparison to bacteria, low relative abundances of the archaea genera *Crenarchaeota* and *Euryarchaeota* were found in loam and clay loam soil treated with imidacloprid, thiacloprid and clothianidin (Zhang et al., 2018). Parizadeh et al. (2021) reported that the relative abundance of various potentially beneficial soil bacteria, notably the rhizobacteria that encourage plant development, decreased after neonicotinoid seed treatment. Cai et al. (2016b) found that the actinomycic population and the cultivable fungal population significantly decreased at the beginning and after 60 days of the experiment respectively. In the study of Mahapatra et al. (2017), imidacloprid was applied at 4 different doses (recommended dose, double, 5 times and 10 times the recommended dose) and the results showed that imidacloprid treatment resulted in a decrease in the overall soil microbial biomass carbon and higher concentration of imidacloprid decreased bacterial, actinomycetes and fungal population. The reduction in bacterial and fungal population was 3 and 6.3% less, respectively, in the 10 times of the recommended dose treatment compared to control. Imidacloprid resulted in a reduction in the total of selected PLFA (phospholipid fatty acid) concentrations when compared to the controls, demonstrating an inhibitory action on soil bacteria and fungus (Xiao et al., 2017). Cycoń et al. (2013) reported that imidacloprid applied at a higher rate significantly altered the composition of the microbial community and reduced the biomass of the total, bacterial and fungal PLFAs as well as lower their metabolic activity. Imidacloprid sensitivity had been found in the Ammonia-Oxidizing Archaeal community in some nitrifying and N_2-fixing bacteria, potentially posing a threat to nitrogen cycles in soil (Cycoń & Piotrowska-Seget, 2015a, 2015b). Regardless of soil type, nitrite oxidation and *Methylotenera* spp. richness was suppressed when dinotefuran was used in high concentrations (Yamaguchi et al., 2021). In anaerobic soil, paichongding had been found to inhibit bacterial diversity as well as growth (Cai et al., 2015) and the soil bacterial community composition varied at both phylum and genus levels (Cai et al., 2015; Cai et al., 2016a). Paichongding also caused significant differences in eukaryal population before and after application and it could cause some phyla and genera to grow or shrink in anaerobic soils (Zhu et al., 2018). Five studies (17%) reported that acetamiprid, imidacloprid, paichongding and thiamethoxam inhibited soil enzymatic activities (cellulase, urease, dehydrogenase, catalase, phosphatase and phosphomonoesterase) ultimately hampering biogeochemical function of soil such as ammonification, nitrification and denitrification (Cai et al., 2016a; Castillo Diaz et al., 2017; Deborah et al., 2013; Filimon et al., 2015; Wang et al., 2014). Out of 24 studies, only one study (3%) reported that thiamethoxam application reduced soil bacterial co-occurrence patterns, which also depend on soil pH and cation exchange capacity (Zhang et al., 2021).

DISCUSSION

Despite the fact that a lot of time and effort has gone into researching and debating neonicotinoids' environmental effects, rigorous assessments of their soil ecotoxicology are rare. To our knowledge, this is the first systematic review to explore soil microbial health of exposure to neonicotinoids in the peer-reviewed literature. To address the possible changes in soil microbial communities after exposure to neonicotinoids, this systematic review was performed using the PRISMA guidelines, an approach commonly used in agro-environmental research (Koutsos et al., 2019; Moher et al., 2010; Vásquez-Dean et al., 2020).

Sensitivity (28%), precision (11%) and number of needed to read (9) metrics were calculated to evaluate the search strategy. The goal of the systematic review search strategy was to be as broad as feasible in order to include as much relevant research as possible in the review. A search strategy that strikes an equilibrium between high sensitivity and high precision is known as an optimum search (Petitti, 2000). Designing a search strategy always starts with a study of the key ideas and the keywords that will be used to describe each notion (Salvador-Oliván et al., 2019). Higher sensitivity eventually reduces the precision percentage and ultimately retrieves non-relevant studies in return (Dieste & Griman, 2007; Lefebvre et al., 2022). For instance, lower sensitivity and precision indicated that the search strategy extracted more relevant studies with higher accuracy but the reference standard only comprised a small number of papers, which might impair the accuracy of the performance evaluation (Dieste et al., 2009; Li et al., 2019). The NNR (number of needed to read) of the search strategy reflects that nine articles were read to find a relevant study to be included in this systematic review indicating substantially lower screening time.

An insight into the possible changes in soil microbial activity after applying neonicotinoid insecticides was provided by this approach, and through the analysis of

the selected articles knowledge gaps were identified. A scarcity of research articles related to the topic was a major limitation of this review. A total of 272 articles were found using the keywords in PubMed SCOPUS and Web of Science databases, and citation searching, while changing only the keyword 'neonicotinoid' to 'glyphosate' resulted in 1115 articles. From the view of the geographical distribution of 29 studies identified through this systematic approach, there was no study in the southern hemisphere of the world. According to Bass et al. (2015) Latin America, Asia and North America account for 75% of the world's neonicotinoid usage, with Europe making about 11% of overall sales. But from geographical distribution, it had been reflected that no microbial studies on neonicotinoid were conducted in Latin America. On February 28, 2018, the European Food Safety Authority (EFSA) website posted the conclusions of its risk assessment in relation to affecting bees and other environmental threats for the active ingredients imidacloprid, thiamethoxam and clothianidin, therefore, the recommendations to entirely outlaw the outdoor usage of these three active ingredients were maintained by the Commission services and endorsed by a major of Member States in the Regulatory Committee on April 27, 2018 (EFSA, 2018). On the other hand, a majority portion of the world still does not have sufficient evidence to assess the impact of neonicotinoids on soil biology and ecology. Different regions of the world have different climates- thus resulting in distinct soil development patterns. These facts also highlighted the need for soil microbiological research on neonicotinoids in order to have a comprehensive understanding of the physicochemical and microbiological features of soil after exposure to neonicotinoids across the world.

The reported data in the selected studies often differed among themselves. Most were carried out in the laboratory, with few occurring in the field and all had variable exposure modalities, thus, true meta-analysis was difficult to perform. On the other hand, studies on all commercially available neonicotinoids were scarcer which indicates more diverse group of neonicotinoids should be tested on soil microbial communities. For a better understanding of neonicotinoids-soil microorganism complexities, a more seamless transition from laboratory to field application is required because soils provide a wide range of crucial ecosystems that have components that might impact pesticide effects (de Santo et al., 2019; Kördel & Römbke, 2001; Parnell et al., 2016). Pesticide toxicity on soil microbes relies on soil characteristics, pesticides' physical and chemical behaviour, concentrations and mode of application (Kalia & Gosal, 2011). Foliar application and seed treatment of pesticides react differently in soil. According to Moorman (1989) foliar-sprayed pesticides concentrated on the soil surface initially but seed treatment created higher concentration of pesticides near the seed. In this systematic review, fewer numbers of microbial studies were found where the mode of application was seed treatment despite that globally, neonicotinoids are widely applied as seed treatment (Mourtzinis et al., 2019).

Important physicochemical drivers of soil microbial productivity were poorly controlled in the selected studies. Only one study reported the relation between microbial co-occurrence pattern and soil physicochemical properties and found that edaphic variables significantly influenced the response of soil bacterial networks to neonicotinoids (Zhang et al., 2021). Soil physicochemical properties are also strongly related to soil microbial activity and diversity (Buyer et al., 2010; Fierer et al., 2012) as well as the fate and behaviour of neonicotinoids in soil (Aseperi et al., 2020).

We also found that research on the effects of neonicotinoids on soil microorganisms has produced conflicting results. Some studies did not find any adverse effects of neonicotinoids on microorganisms (Garg et al., 2021; Li et al., 2018; Parizadeh et al., 2021; Singh & Singh, 2005a; Zhang et al., 2015), while some found significant changes in the microbial community in terms of relative abundance (Garg et al., 2021; Parizadeh et al., 2021; Streletskii et al., 2022; Wu et al., 2021; Zhang et al., 2018), diversity and structure (Cai et al., 2015; Wu et al., 2021; Yamaguchi et al., 2021; Yu et al., 2020; Zhang et al., 2015; Zhang et al., 2018; Zhang et al., 2021) and enzymatic activity (Cai et al., 2016a; Castillo Diaz et al., 2017; Deborah et al., 2013; Filimon et al., 2015; Wang et al., 2014). Such contradictory results may be because some authors investigated only eukaryotes, some only prokaryotes, and others a combination of the two groups. In our systematic review, we found that 13 studies had been conducted on prokaryotes, one on eukaryotes and five on both prokaryotes and eukaryotes. Recent studies have also challenged the notion that neonicotinoids primarily impact eukaryotic soil microbes (Wang et al., 2023) because they share some cellular features with insects (Lemelin & Fine, 2013) such as eukaryotic cell structure (Cooper, 2000), chitin in cell wall/exoskeleton (Numata & Kaplan, 2011) and cytoplasmic streaming (Goldstein & van de Meent, 2015).

Although neonicotinoids as group of target invertebrates (i.e., insects) and most soil microbes do not possess the specific target proteins for neonicotinoids, they can still be indirectly affected by these insecticides. Any pesticide can interact with enzymes in soil microorganisms, leading to unintended effects on their physiology and metabolism (Das et al., 2005; Floch et al., 2011) that had also been observed in neonicotinoids (Cycoń & Piotrowska-Seget, 2015a). When any change or disturbance occurs in the surrounding environment of microbial communities, they modify their pattern of habitat by increasing their abundance, or become more robust to the disturbance over time (Fierer et al., 2010;

Redford & Fierer, 2009) but, concurrently, there may have been a commensurate decline in functional microbial diversity (Tripathi et al., 2020). Long-term inconsistent and excessive pesticides usage disrupt the stability of microbial population (Lupwayi et al., 2009) hence making biogeochemical nutrient cyclings like nitrogen cycle and organic matter mineralisation sensitive to pesticide (Jat et al., 2021; Sim et al., 2022; Xu et al., 2001). On the other hand, the soil nutrient status was poorly addressed within the selected studies on neonicotinoids, and the findings may well reflect changes in soil nutrient conditions rather than their impacts on soil microbial populations.

Concerns have also been raised about the adverse effects of neonicotinoids on non-target soil fauna. Neonicotinoid seed treatment reduced earthworm growth, activity, behaviour and burrowing capacity (Capowiez et al., 2005; Capowiez et al., 2006; Ge et al., 2018) and soil treated with neonicotinoids at the concentration of 1 ppm or higher can pose mortality risk to earthworms (Pisa et al., 2015). Neonicotinoid exposure in soil resulted in a large increase in adult DNA damage as well as a significant reduction in earthworm reproduction that occurred concurrently with neonicotinoid bioaccumulation (Chevillot et al., 2017). Yu et al. (2021) reported that the relationships between Collembola, fungi and bacteria that regulate the mineralisation of maize and soil organic carbon are changed by dinotefuran. Clothianidin had been found to reduce the population of earthworms, springtails (de Lima e Silva et al., 2020) and oribatid mites (Ritchie et al., 2019).

Wang et al. (2023) investigated the responses of eukaryotic soil protozoa to imidacloprid exposure and found evidence of altered key differentially expressed genes involved in cell division, cytochrome P450, morphogenesis and phagocytosis pathways.

In summary, our findings suggest that although the effects of neonicotinoids on soil microbial life, in general, remain unclear, some detrimental effects on soil microbial and enzymatic activity were reported by a number of authors.

In this systematic review, we identified the following research gaps:

a. Most research on neonicotinoids and soil microorganisms had been conducted in laboratory settings or controlled environments with very few field studies. Consequently, more field-scale studies are needed to understand how neonicotinoids influence soil microbial dynamics under real-world agricultural conditions.
b. The existing literature is focused on foliar spray application while the effects of seed treatments remain understudied. Thus, there is a need for more spray versus seed treatment comparison experiments.
c. The relationship between specific physicochemical properties of soil and the observed changes in microbial habitat caused by neonicotinoids remains relatively unclear. Determining the key soil properties that are most influenced by neonicotinoid exposure and how they mediate microbial responses is crucial for targeted conservation and management strategies.
d. A knowledge gap exists with respect to the impact of neonicotinoids on nutrient availability, and consequently, modify microbial community dynamics.
e. While the primary neonicotinoid compounds had been extensively studied, there is a lack of comprehensive research on the formation and fate of their metabolites in various environmental settings. Understanding the transformation pathways and persistence of these metabolites is essential for assessing their potential impacts on ecosystems and non-target organisms.
f. The lack of comprehensive studies on the effects of neonicotinoids on soil eukaryotic microbes hinders our ability to fully assess the broader impacts of these chemicals on soil biodiversity and ecosystem services. Furthermore, it may limit the development of targeted and sustainable pest management strategies that consider the interactions between neonicotinoids and diverse soil microbial communities. To gain a comprehensive understanding of the impacts of neonicotinoids on eukaryotic microbes, more targeted studies are warranted to investigate the mechanisms of toxicity, the potential for resistance or adaptation and the ecological consequences of such effects on soil microbial communities.

CONCLUSION

The current information on the microbial communities in relation to the exposure of neonicotinoids was compiled and visualised by this systematic review through an integrative approach. Due to diverse methodologies with different research outputs, a comprehensive meta-analysis was difficult to perform. The severity of neonicotinoid toxicity on soil microbes was determined by the dose and duration of exposure. Imidacloprid was the mostly used (66%) neonicotinoid class in soil microbial studies and the soil microbial population was sensitive to it as exposure to unfavourable physiological states could have detrimental effects on their survival and growth, leading to a decrease in their abundance eventually affecting metabolic processes. Laboratory studies dominated over field experiments for the studies of microbial parameters in neonicotinoid-exposed soil. Even though heterogeneity was high, 45% of the selected studies found significant negative impacts on soil microbial community structure, composition,

functioning, diversity, enzymatic activity and nitrogen transformation after neonicotinoid exposure. The scarcity of studies highlighted the need for further research on this topic.

AUTHOR CONTRIBUTIONS

Sharmin Akter: Conceptualization (equal); methodology (lead); writing – original draft (lead). **Nilantha R. Hulugalle:** Supervision (supporting); writing – review and editing (lead). **Julia Jasonsmith:** Conceptualization (equal); supervision (supporting); writing – review and editing (supporting). **Craig L. Strong:** Conceptualization (equal); supervision (lead); writing – review and editing (supporting).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Chapter 3 Materials and methods

3.1 Overview of experimental design

Two controlled microcosm experiments were conducted to investigate bacterial and fungal community responses to neonicotinoid seed treatments in soil over short and extended exposure periods. The short-term experiment examined the effects of imidacloprid, thiamethoxam and clothianidin in a single soil type across six sampling times over ten days. The extended experiment assessed imidacloprid exposure in three contrasting soils differing in texture across five sampling time points over 28 days. All experiments were performed under standardised incubation conditions to minimise external variability.

3.2 Soil collection, characterisation and preparation

3.2.1 Field sampling

Surface soil (A horizon: ~0.2m) was collected from minimally disturbed, shaded pastures. Three different soils were used across the experiments. Two soils were sourced from Canberra (35.28°S, 149.13°E), Australia and comprised kaolinitic clay. These soils were weakly aggregated, with most aggregates measuring <5 mm in diameter. They were collected from shaded pastures and were characterised by long-term mulching, high macroinvertebrate and earthworm activity. The third soil was collected from Narrabri (30.33°S, 149.78°E), Australia and was dominated by smectite clay. The Canberra and Narrabri sites have Köppen–Geiger climate classifications of temperate oceanic (Cfb) and hot semi-arid (BSh), respectively (Beck et al., 2018). All collection sites had no known history of neonicotinoid application. Samples were initially evaluated for textural class by field-texturing before being processed in the laboratory.

3.2.2 Laboratory processing

All samples were air-dried in the laboratory for one week. As the Canberra soils were weakly aggregated, most aggregates loosened during sieving. In contrast, due to the strong aggregation of the Narrabri soil, larger aggregates were mechanically ground using a jaw crusher. All soils were then homogenised to create composite samples and sieved through a ≤ 2 mm mesh to remove dried leaves, roots, and other debris.

3.2.3 Particle size and physicochemical analyses

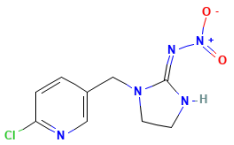
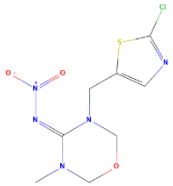
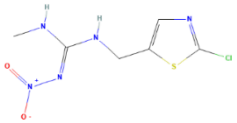
Following sieving, particle size was determined using the modified hydrometer method (Carter & Gregorich, 2007). Final textural classes were assigned as loamy sand, sandy, and clay using USDA soil textural triangle (Soil Survey Division Staff, 1993). The loamy sand and sandy loam soils originated from Canberra and were classified as red Luvisols, while the clay soil from the Narrabri site was classified as a Vertisol (IUSS Working Group WRB, 2015). Composite samples from each soil were analysed for thirteen key physicochemical properties. These were pH, electrical conductivity (EC), organic matter (OM), total carbon (C), total nitrogen (N), carbon-to-nitrogen ratio (C:N), available phosphorus (P), exchangeable calcium (Ca^{2+}), exchangeable magnesium (Mg^{2+}), exchangeable potassium (K^{+}), effective cation exchange capacity (ECEC), exchangeable sodium percentage (ESP), and the calcium-to-magnesium ratio (Ca:Mg). Detailed descriptions of the analytical procedures are provided in the appendix in Table A. 1.

3.3 Neonicotinoid treatments and seed material

3.3.1 Active ingredients

Analytical standards (≥ 98.0 % HPLC area purity) of three neonicotinoid chemicals were purchased from Sigma-Aldrich Pty Ltd (A Subsidiary of Merck), Australia, with these being imidacloprid ($C_9H_{10}ClN_5O_2$), thiamethoxam ($C_8H_{10}ClN_5O_3S$), and clothianidin ($C_6H_8ClN_5O_2S$). The characteristics of the neonicotinoids are listed in Table 3–1. Chemicals were stored according to the manufacturer's recommendations (Sigma-Aldrich, 2022a, 2022b, 2023).

Table 3–1: Characteristics of the neonicotinoids used in this study.

Product name	Chemical formula	Structure (Adapted from PubChem)	Molecular weight (g/mol)	Purity (HPLC area %)	Melting point
Imidacloprid PESTANAL®, analytical standard	$C_9H_{10}ClN_5O_2$		255.66	≥ 98.0	141 – 146°C
Thiamethoxam PESTANAL®, analytical standard	$C_8H_{10}ClN_5O_3S$		291.71	≥ 98.0	137 – 143°C
Clothianidin PESTANAL®, analytical standard	$C_6H_8ClN_5O_2S$		249.68	≥ 98.0	171 – 176°C

3.3.2 Seed material and treatment protocol

Wheat seeds (*Triticum aestivum* L.) were used for all experiments and purchased locally from Canberra, Australia, with no specific cultivar information available. For the short-term experiment, seeds were treated with analytical-grade active ingredients to simulate the application of three commercial neonicotinoid formulations at commercial application rates: 120 mL of 60% imidacloprid (Bayer CropScience Australia, 2023) and clothianidin (Bayer CropScience, 2023), and 120 mL of 35% thiamethoxam (Syngenta Australia, 2023). These application rates correspond to 72 g active ingredient (a.i.) per 100 kg of seed for imidacloprid and clothianidin (60% a.i.) and 42 g a.i. per 100 kg of seed for thiamethoxam (35% a.i.).

To replicate these rates for 20 g of seed used per treatment, the following amounts of analytical-grade compounds were used: 14.4 mg of imidacloprid, 14.4 mg of clothianidin, and 8.4 mg of thiamethoxam. Each compound was weighed into a sterile 50 mL specimen jar inside a certified fume cupboard. 5 mL of Milli-Q water was added to dissolve the compound. After complete dissolution, 20 g of wheat seeds were added to the solution. The contents were gently agitated to ensure uniform aqueous coating. The treated seeds were left in sealed jars at room temperature for 1 hour to allow adequate absorption. Unlike commercial formulations that often include polymers, dyes, or other additives, only the active ingredient dissolved in Milli-Q water was used in this study. This simplified treatment avoided potential confounding effects from auxiliary components and allowed assessment of the direct microbial response to neonicotinoids alone. Seeds were used immediately after this treatment period without drying or storage. Control seeds were handled using the same procedure but without the addition of insecticides.

For the extended experiment, wheat seeds were devitalised by heat treatment at 100°C for one hour to prevent germination. This procedure was confirmed to be effective in a preliminary trial and then applied to all seeds used in the experiment to ensure uniform pesticide–soil interactions. Seeds were treated with imidacloprid at the same commercial application rate following the same procedural workflow described above for the short-term experiment, and untreated seeds served as controls. All seed treatments were carried out under appropriate laboratory safety protocols, including the use of gloves, lab coat, face shield, safety goggles, and respiratory protection. All containers and tools were either sterilised between treatments or disposed of according to institutional biosafety guidelines.

3.4 Microcosm setup, incubation and sampling

3.4.1 Pot preparation

Polypropylene pots (30 mL volume) were used in both microcosm experiments. Two 1.5 mm diameter holes were drilled in the sides of each pot to facilitate future sampling, which were sealed with adhesive tape until the time of sampling. Forty-gram samples of soil were dispensed into each pot and compacted for uniformity using a penetrometer set to a pressure of approximately 294 kPa.

3.4.2 Seed planting and moisture control

Each pot received two wheat seeds, either treated with neonicotinoids at commercially relevant rates or left untreated as controls. Seeds were planted at a depth of ~1 cm below the soil surface. The soil was uniformly moistened from the soil surface to a gravimetric moisture content of 32 g/100 g using deionised water (Milli-Q) to provide sufficient moisture for biological processes without causing waterlogging, ensuring uniform conditions across all pots. No additional watering was applied during the experiment. No fertiliser was added to prevent nutrient-driven community shifts unrelated to the experimental treatment.

3.4.3 Incubation conditions

After watering, the pots were covered with lids and placed in a dark environment at a constant room temperature of 22°C to maintain stable conditions. Keeping pots sealed minimised evaporation and contamination, while darkness reduced photodegradation of neonicotinoids and plant-mediated microbial variation.

3.5 Experimental design

Pots were arranged in a fully randomised design in both experiments to minimise positional effects and to ensure that all treatment combinations were equally represented across sampling days.

3.5.1 Short-term experiment

Four treatment conditions were established (control, imidacloprid, thiamethoxam, and clothianidin). Since time was a key experimental variable, soil sub-samples were collected at six specific intervals—day 1 (before treatment), 2 (24 hours after treatment), 3, 5, 7, and 10—to observe temporal changes in bacterial communities. To minimise variability, soil sub-samples were collected each day beginning at 9:30 a.m., following a consistent sampling order

across treatments. Each treatment and control group included five replications for each sampling day, resulting in a total of 120 pots.

3.5.2 Extended experiment

Three soils with contrasting textures (loamy sand, sandy loam, and clay) were used to evaluate the modifying influence of edaphic properties on microbial responses. Two treatment conditions were applied: imidacloprid-treated seeds and untreated controls. Soil sub-samples were collected at five intervals (days 3, 7, 14, 21, and 28) with sampling initiated at the same time each day. Triplicate samples were included in each treatment and control group at each sampling time, resulting in a total 90 pots.

3.6 Soil sub-sampling

On each sampling day, designated pots from each treatment group were withdrawn from the experiment for soil sub-sampling. A clean micro spatula was pushed horizontally through the full diameter of the pot via the side hole positioned immediately below the seed placement depth to collect a soil core (~0.25 g). To prevent contamination, disposable nitrile gloves were used for each sample, and the spatula was sterilised between collections. Collected soil samples from the microcosms were immediately processed for subsequent DNA extraction to preserve microbial integrity and minimise any potential changes in the microbial community. After pot preparation, the entire experiment was carried out inside a fume cupboard to ensure safety and minimise contamination.

3.7 DNA extraction and quantification

Genomic DNA (gDNA) was extracted on the same day of sub-sampling using DNeasy® PowerSoil® Pro Kit (QIAGEN, Germany) following the manufacturer's protocol (Wu, 2022). Subsequently, the concentrations of gDNA were determined using a Qubit 4™ Fluorometer with the dsDNA BR Assay KitTM for Qubit. The quality of the gDNA was assessed employing NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), after which the extracted gDNA samples were securely stored at −20˚C, awaiting downstream analysis.

3.8 Next-generation sequencing

Amplicon libraries were prepared using a two-step PCR workflow. The first-step PCR used locus-specific primers modified with Illumina overhang adapter sequences, and the second-step PCR attached unique dual indexes and full Illumina adapters. Paired-end targeted amplicon sequencing was performed on the MiSeq v3 platform (Illumina Inc., San Diego, CA,

USA) with a read length of 2 × 300 bp, and all sequencing was conducted at the Ramaciotti Centre for Genomics (UNSW, Australia). During demultiplexing, Illumina adapters and dual indexes were trimmed from all sequences.

3.8.1 Bacterial 16S rRNA gene amplification

The V1 – V3 regions of the 16S rRNA gene were amplified using the primer pair 27f – 519r. The first-step PCR used the 27f forward primer sequence, which consisted of the Illumina forward overhang sequence (5' – TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG – 3') and the forward primer (27f) (5' – AGAGTTTGATCMTGGCTCAG – 3') (Lane, 1991). The 519r reverse primer sequence comprised the Illumina reverse overhang sequence (5' – GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG – 3') and the reverse primer (519r) (5' – GWATTACCGCGGCKGCTG – 3') (Lane et al., 1985).

3.8.2 Fungal internal transcribed spacer (ITS) amplification

The ITS1 region of the fungal rDNA was amplified using the primer pair ITS1f – ITS2 (White et al., 1990). The first-step PCR used the ITS1f forward primer sequence, which consisted of the Illumina forward overhang sequence (5′ – TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG – 3′) and the forward primer (ITS1f) (5′ – CTTGGTCATTTAGAGGAAGTAA – 3′). The ITS2 reverse primer sequence comprised the Illumina reverse overhang sequence (5′ – GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG – 3′) and the reverse primer (5′ – GCTGCGTTCTTCATCGATGC – 3′).

3.9 Bioinformatics workflow

3.9.1 Sequence processing

All sequence processing and downstream analyses were conducted in R Studio v4.4.2 (R Core Team, 2024), unless otherwise specified. The initial metagenomic data consisted of paired-end FASTQ files generated by Illumina sequencing. These files had been demultiplexed by sample, with barcodes, Illumina adapters, and unique dual indices already trimmed during the demultiplexing process. Non-biological nucleotides (primers) were removed from the demultiplexed FASTQ files using the Cutadapt tool (v2.10) prior to import into R for further processing (Martin, 2011).

3.9.2 Amplicon data processing

The paired-end demultiplexed sequences were processed using the DADA2 workflow for quality filtering, denoising, read merging, chimera removal and amplicon sequence variant (ASV) inference (Callahan et al., 2016). Taxonomy was assigned using either SILVA 138.1 database for bacteria (McLaren & Callahan, 2021) or UNITE v10.0 database for fungi (Abarenkov et al., 2024). Locus-specific parameters, truncation settings, and filtering thresholds are described in the relevant experimental chapters. ASV tables and associated taxonomic assignment were imported into R to create phyloseq objects (phyloseq v1.48.0; McMurdie & Holmes, 2013). Representative sequences were added as DNAStringSet objects (Pagès et al., 2024). A sequence-derived phylogenetic tree was generated from ASV representative sequences using `DECIPHER` for alignment (Wright, 2024), `phangorn` for tree inference (Schliep, 2011). The data were filtered to retain only bacterial sequences for 16S datasets or fungal sequences for ITS datasets, with chloroplast and mitochondrial ASVs removed. Samples containing zero counts across all ASVs were excluded to avoid artefactual bias in downstream analyses. The resulting phyloseq objects were used for statistical analyses. Rarefaction or additional read-count normalisation was not applied because rarefaction discards valid sequence information and reduces statistical power (McMurdie & Holmes, 2014). Instead, analyses were performed using the complete ASV dataset with relative abundance, phylogenetically informed distance matrics and model-based approaches.

3.10 Functional prediction

Functional profiles were inferred using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) (Douglas et al., 2020). ASVs were placed into a reference phylogeny using `place_seqs.py` tool, and hidden-state prediction was applied to estimate marker gene copy numbers and enzyme annotations based on phylogenetic placement. Enzyme Commission (EC) numbers and, where applicable, KEGG orthologs (KOs) were inferred from marker gene abundances. Metabolic pathway abundances were predicted using the `pathway_pipeline.py` tool, with all pathway annotations derived from the MetaCyc reference database embedded within the PICRUSt2 workflow. Analyses were executed in a dedicated Conda environment configured according to the installation and execution recommendations provided for PICRUSt2. Resulting pathway profiles were used for downstream comparative analyses, with the specific statistical models applied as described in the relevant chapter-level methods. PICRUSt2 was selected because functional metagenomics was not sequenced directly, allowing inference of functional potential from amplicon data.

3.11 Statistical analyses and community profiling

All statistical analyses were conducted in the R environment using packages appropriate for ecological community data to evaluate microbial community diversity, composition, structure, and functional profiles across experimental variables. Depending on the experimental design and data distribution, appropriate parametric or non-parametric models were applied, including generalised linear models, linear mixed-effects models, permutation-based tests, and pairwise post-hoc comparisons. Richness, evenness, and phylogenetic diversity metrics were calculated with significance assessed using established model diagnostics. Community dissimilarity was quantified using taxonomic or phylogenetic distance matrices and visualised with ordination methods, and permutational multivariate analysis of variance (PERMANOVA) was used to assess experimental effects. Differential abundance analyses were applied to identify microbial taxa and predicted functional pathways associated with specific treatment conditions. Statistical frameworks based on linear discriminant analysis (LDA) and count-based modelling approaches were used depending on data type and experimental design. Where relevant, correlation-based approaches, indicator taxa analysis, and co-occurrence network construction were applied to explore ecological associations. Specific statistical frameworks, parameter values, thresholds, and diversity metrics vary between experimental chapters and are detailed within the respective chapter-level methods sections.

3.12 Data availability

Raw demultiplexed sequencing reads generated in this thesis have been deposited in the NCBI Sequence Read Archive (SRA) under experiment-specific BioProject accession numbers. These datasets will be made publicly accessible upon publication of the corresponding manuscripts. Accession identifiers are provided in the data availability statements within each chapter.

Chapter 4 Bacterial resilience and vulnerability to neonicotinoid seed treatments in soil: Short-term community responses

4.1 Overview[2]

This chapter investigated the short-term responses of soil bacterial communities to imidacloprid, thiamethoxam and clothianidin seed treatments applied to wheat under controlled microcosm conditions. Soil samples were collected over a 10-day period to examine temporal changes in bacterial diversity, community composition and taxonomic abundance using 16S rRNA gene amplicon sequencing. The results showed that bacterial communities responded differently to the three neonicotinoids despite their similar mode of action. While overall diversity remained relatively stable, transient changes in diversity occurred at specific sampling times. Community structure was influenced primarily by sampling time, whereas differential abundance analyses identified compound-specific responses among individual bacterial taxa. These findings indicated that short-term exposure to neonicotinoid seed treatments elicited selective rather than widespread changes in soil bacterial communities. This chapter provides the first experimental assessment of bacterial responses presented in this thesis and forms the basis for the subsequent investigation of longer-term effects of neonicotinoid exposure on soil microbial communities.

[2] This chapter has been published as:
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4.2 Published paper

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Bacterial Resilience and Vulnerability to Neonicotinoid Seed Treatments in Soil: Short-Term Community Responses

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ABSTRACT

We explored the short-term impacts of three neonicotinoids (imidacloprid, thiamethoxam, and clothianidin) on soil bacterial community composition and diversity in wheat-planted microcosms. Neonicotinoids were applied as seed treatments, and soil samples were collected over 10 days. Overall alpha diversity metrics showed no significant treatment- or time-dependent shifts; however, post hoc analyses revealed transient, treatment-specific responses at individual sampling time points. Thiamethoxam and clothianidin significantly increased diversity and evenness at early time points, while imidacloprid reduced diversity by Day 10. Clothianidin was also associated with a short-term increase in estimated species richness. Actinobacteriota and Proteobacteria dominated across treatments, with Firmicutes increasing and Bacteroidota declining with time. The minor phylum Methylomirabilota exhibited a significant treatment effect. Sampling day and day-treatment interaction significantly influenced community structure. *Mesorhizobium* enriched under all neonicotinoids. Imidacloprid enhanced *Massilia* and suppressed Solirubrobacterales and Chloroflexia. Thiamethoxam enriched *Gaiella*, *Solirubrobacter*, and *Massilia* but suppressed *Nitrospira*. Clothianidin enriched *Solirubrobacter* and *Lysobacter* but suppressed *Methyloceanibacter* and *Nitrospira*. Haliangiaceae were positively correlated with sampling days, while Flavobacteriaceae and Microscillaceae were negatively correlated. Yersiniaceae and Solirubrobacteraceae were negatively correlated with imidacloprid and Mycobacteriaceae with thiamethoxam. These findings highlight the need for longer-term and functional investigations into neonicotinoid impacts on soil microbial communities and ecosystem health.

1 | Introduction

Within the surface millimetre of the soil lies a diverse microscopic community that collectively functions to produce the complex web of interactions described as the soil ecosystem (Manda et al. 2023). This microscopic community or soil microbiome is a highly dynamic assemblage of bacteria, fungi, protozoa, archaea, and other microorganisms. The quality and health of soil are often characterised by the functional capacity of these microbes (Potts et al. 2023; Hou et al. 2020). They are collectively responsible for maintaining soil fertility and ecosystem resilience and drive key soil processes such as nutrient cycling, organic matter decomposition, and soil structure maintenance (Uzoh et al. 2021; Kästner and Miltner 2018; Dini-Andreote and Van Elsas 2019). Among the soil microorganisms, bacteria are one of the most dominant, with their biomass often ranging from 100 to 10,000 times greater than that of other key microbial groups in the soil microbiome (Fierer 2017). Bacteria perform critical roles in several biochemical processes such as carbon mineralisation, nitrogen transformation and sulfur reduction and oxidation, with these processes fundamental to broader ecosystem health (Basu et al. 2021), organic matter decomposition, soil fertility, and nutrient availability (Yadav et al. 2021). Additionally, bacteria

promote plant growth via biogeochemical processes such as fixing atmospheric nitrogen, solubilising phosphate through the secretion of organic acids, and releasing soluble potassium from otherwise insoluble rocks and silicate materials (Alori et al. 2017; Ribeiro et al. 2020; Raza et al. 2023; Sindhu et al. 2014). However, these crucial processes are susceptible to disturbances through human activities, particularly the widespread use of chemical inputs in modern farming practices (Yang et al. 2021; Martyniuk et al. 2019).

Modern agriculture practices impose a range of obvious and subtle stresses that can lead to instability and changes in the composition of soil microbial communities (Ni et al. 2025; Gupta et al. 2022). One such stress has been the introduction and rapid increase in the use of neonicotinoid insecticides, which are highly effective in protecting crops from a broad range of insect pests (Thompson et al. 2020; Thany 2023) and are used at different phases of cultivation and during post-harvest storage (Liu et al. 2010; Orikpete et al. 2025). Neonicotinoids are a class of systemic insecticides characterised by nitroguanidine (—N—NO_2) or cyanoamidine (=N—CN) functional groups, which confer high affinity for insect nicotinic acetylcholine receptors (nAChRs) (Buszewski et al. 2019). They are widely applied for the control of sap-feeding pests such as aphids, leafhoppers, whiteflies, and thrips, as well as several soil-dwelling coleopteran larvae (Jeschke and Nauen 2005; Rawal et al. 2025). They are especially controversial because their adverse effects on organisms at the bottom of the food chain are considered to have been inadequately studied (Bakker et al. 2020; Overton et al. 2021; Umina et al. 2019; Bonmatin et al. 2021). They can be applied in several ways—sprayed onto leaves, drenched into the soil, applied to seeds as treatment, or used as granules over various crops (Briceño et al. 2024). Their longevity in soil, high solubility in water, and weak adsorption properties enable neonicotinoids to persist and travel through soil and water systems (Bonmatin et al. 2015; Lamers et al. 2011; Li et al. 2018; Zhang et al. 2023).

Neonicotinoids' potential toxicity to non-target soil microorganisms has been highlighted by several studies. Castillo-Díaz et al. (2017) observed immediate significant reductions in bacterial abundance and shifts in community structure following imidacloprid application. Similarly, Wu et al. (2021) further noted that thiamethoxam had a short-term impact on soil bacterial abundance, leading to a reduction in microbial diversity and changes in bacterial community structure. Li et al. (2018) reported that higher concentrations of clothianidin suppressed bacterial species richness, though overall community diversity was unaffected.

Neonicotinoids not only affect bacterial diversity and abundance but also disrupt key bacterial-driven processes, particularly nutrient cycling and community interactions. Imidacloprid inhibited nitrifying bacteria, reducing nitrification rates and stimulating ammonification, while also suppressing critical processes like nitrogen transformation and carbon mineralisation (Mahapatra et al. 2017; Cycoń and Piotrowska-Seget 2015). Yamaguchi et al. (2021) reported that dinotefuran promoted ammonia oxidation but suppressed nitrite oxidation, further disrupting nitrogen cycling.

In addition to changes in community structure and specific processes, neonicotinoids also induce broader metabolic and functional shifts within bacterial communities. Yu et al. (2020) found that dinotefuran and thiamethoxam altered carbon metabolism in bacterial communities and changed the genetic profiles of specific bacterial taxa, potentially leading to shifts in soil microbial community function. Neonicotinoid seed treatments selectively affected bacterial gene expression, particularly genes related to regulatory functions, metabolic activities, DNA repair and most notably, heat shock proteins, with these effects varying across and between growing seasons (Parizadeh et al. 2023). These metabolic changes reflected how neonicotinoid exposure can reshape bacterial communities, potentially leading to long-term disruptions in soil ecosystems.

In Australian wheat (*Triticum aestivum* L.) production, neonicotinoids play a key role in pest management (Overton et al. 2021; Umina et al. 2019). Neonicotinoid seed treatments provide effective protection for young wheat plants against aphids (Labrie et al. 2020; Miao et al. 2014), but their use within the soil raises concerns regarding their persistence and potential ecological impacts on soil microbial communities (Wettstein et al. 2016; Zheng et al. 2022). This study investigated the impact of three neonicotinoids, applied as seed treatments in wheat, on soil bacterial communities. Imidacloprid, thiamethoxam, and clothianidin were selected because they are among the most widely used neonicotinoid seed treatments in cereal production and represent closely related yet chemically distinct compounds with differing soil persistence and degradation profiles (Thompson et al. 2020). Seed treatment was selected to reflect common agricultural practice and realistic exposure pathways, where microbial communities are exposed to active ingredients via root exudates, seed leachates, and rhizosphere interactions. We hypothesised that (i) neonicotinoid seed treatment would alter the soil bacterial community composition and structure, leading to changes in diversity and taxa interactions, and (ii) these changes would vary over time, with each neonicotinoid causing distinct temporal patterns in community structure and taxa abundance shifts. To test these hypotheses, an experiment was conducted for 10 days in small pots under controlled laboratory conditions. Neonicotinoids were applied as a seed treatment to wheat seeds in separate microcosms. Soils associated with treated seeds were sampled to capture the immediate and temporal dynamics of bacterial responses. The short duration of the experiment was intentional, aimed not at chemical risk assessment but at capturing early-stage microbial responses to neonicotinoid seed treatments.

2 | Materials and Methods

2.1 | Chemical and Seed Material

Analytical standards (≥ 98.0% HPLC area purity) of three neonicotinoid chemicals were purchased from Sigma-Aldrich Pty Ltd. (A subsidiary of Merck), Australia, with these being imidacloprid ($C_9H_{10}ClN_5O_2$), thiamethoxam ($C_8H_{10}ClN_5O_3S$), and clothianidin ($C_6H_8ClN_5O_2S$). Wheat seeds were purchased locally in Canberra, Australia with no specific cultivar information available.

2.2 | Soil Sampling and Preparation

Five kilograms of the A horizon from a Red Luvisol (Iuss Working Group Wrb 2015) soil with no pesticide application history was collected and transported back to the laboratory for analysis. The sampling location has a long history of being well-mulched and shaded with minimal disturbance. The A horizon was ~0.2 m deep, dominated by <10 mm aggregates and showed a high incidence of macroinvertebrate & earthworm activity.

The collected soil sample was carefully homogenised to create a composite soil sample. To ensure uniformity, the homogenised soil was sieved (≤2 mm) to remove roots, stones and earthworms. The soil had a sandy loam texture (70 g/100 g sand (0.05–2 mm), 14 g/100 g silt (0.02–0.05 mm), and 16 g/100 g clay (< 0.02 mm)). Its pH was 6.7, electrical conductivity ($EC_{1:5}$) 0.41 dS/m, total organic matter 8.4 g/100 g, total carbon 4.8 g/100 g, total nitrogen 0.43 g/100 g, C/N 11.0, Bray-1 extractable phosphorus 232 mg/kg, exchangeable Ca 18 cmol(+)/kg, exchangeable Mg 3.5 cmol(+)/kg, exchangeable K 1.4 cmol(+)/kg, exchangeable Na < 0.07 cmol(+)/kg, effective CEC 23 cmol(+)/kg, exchangeable sodium percentage (ESP) 0.16 and Ca/Mg 5.1. All methods used to analyse the soil are summarised in Table S1.

2.3 | Seed Treatment and Experimental Setup

Wheat seeds were treated with analytical-grade active ingredients to simulate the application of three commercial neonicotinoid formulations at field-relevant rates. The target application rate for each compound was based on the recommended commercial dose of 120 mL of formulated product per 100 kg of seed, using products with the following active ingredient (a.i.) concentrations: 60% for imidacloprid (Bayer Cropscience Australia 2023), 60% for clothianidin (Bayer Cropscience 2023) and 35% for thiamethoxam (Syngenta Australia 2023). This corresponds to active ingredient application rates of 72 g a.i. per 100 kg seed for imidacloprid and clothianidin, and 42 g a.i. per 100 kg of seed for thiamethoxam. To replicate these rates for 20 g of seed used per treatment, the following amounts of analytical-grade (99.7% purity) compounds were used: 14.4 mg of imidacloprid, 14.4 mg of clothianidin, and 8.4 mg of thiamethoxam. Each compound was weighed into a sterile 50 mL specimen jar inside a certified fume cupboard. 5 mL of Milli-Q water was added to dissolve the compound, followed by the addition of 20 g of wheat seeds. The contents were gently agitated to ensure uniform aqueous coating. The treated seeds were left in sealed jars at room temperature for 1 h to allow adequate absorption. Seeds were used immediately after this treatment period without drying or storage.

Control seeds were handled using the same procedure but without the addition of insecticides. All treatments were carried out under appropriate laboratory safety protocols, including the use of gloves, lab coat, face shield, safety goggles and respiratory protection. All containers and tools were either sterilised between treatments or disposed of according to institutional biosafety guidelines.

We used 30 mL pots with lids, each containing 40 g of soil. Two small holes were made in the sides of each pot prior to filling them with soil and were temporarily sealed with adhesive tape. To standardise the starting conditions, we compacted the soil in each pot with a penetrometer set at a pressure of approximately 29,400 Pa. Soil properties that could potentially influence bacterial populations, such as temperature, pH, moisture, texture, and electrical conductivity (Akter et al. 2023) were identical in all pots.

Two treated or control seeds were planted in each pot. The soil was moistened to a gravimetric moisture content of 32 g/100 g using Milli-Q water (deionised water) to maintain consistent moisture conditions across all pots. After watering, the pots were capped with lids and incubated in the dark at room temperature (22°C). No fertiliser was added.

Since time was a key experimental variable, soil samples were collected at six specific intervals: day 1 (pretreatment baseline, equivalent to time zero), 2 (24 h after treatment), 3, 5, 7, and 10, to observe temporal changes in bacterial communities. To minimise variability, soil samples were collected each day beginning at 9:30 AM, following a consistent sampling order across treatments. Each treatment and control group included five replications for each sampling day, resulting in a total of 120 pots arranged in a randomised complete block design.

On each sampling day, designated pots from each treatment group were withdrawn from the experiment for soil sampling. A clean micro spatula was pushed horizontally through the full diameter of the pot via the side hole to collect a soil core (~0.25 g). To prevent contamination, disposable nitrile gloves were used for each sample, and the spatula was sterilised between collections. Collected soil samples from the microcosms were immediately processed for subsequent DNA extraction to preserve microbial integrity and minimise any potential changes in the microbial community. After pot preparation, the entire experiment was carried out inside a fume cupboard to ensure safety and minimise contamination.

2.4 | Extraction of Genomic DNA and Next-Generation Amplicon Sequencing

Genomic DNA (gDNA) was extracted from the soil samples using the DNeasy PowerSoil Pro Kit (QIAGEN, Germany) on the same day of soil sample collection, following the manufacturer's protocol (Wu 2022). Subsequently, the concentrations of gDNA were determined using a Qubit 4 Fluorometer with the dsDNA BR Assay Kit for Qubit. The quality of the gDNA was assessed employing the NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), after which the extracted gDNA was securely stored at −20°C, awaiting downstream analysis.

The extracted gDNA samples were sequenced by the Ramaciotti Centre for Genomics (UNSW, Australia), targeting the 16S rRNA gene. The primer pairs 27f–519r for the V1–V3 regions of the 16S rRNA gene were used to amplify these regions (Lane et al. 1985; Lane 1991). Pair-end targeted amplicon sequencing was performed on the MiSeq v3 platform using a two-step PCR

workflow (Illumina Inc., San Diego, CA, United States) with a read length of 2×300 bp. Detailed primer sequences are provided in Table S2.

2.5 | Bioinformatics

The initial metagenomic data consisted of paired-end FASTQ files generated by Illumina sequencing. These files had been demultiplexed by sample, with barcodes, Illumina adapters, and unique dual indices already trimmed during the de-multiplexing process. Non-biological nucleotides (primers) were removed from the demultiplexed FASTQ files using Cutadapt tool (v2.10) (Martin 2011) prior to processing. The sequencing data were processed following the version 1.8 DADA2 workflow, using the 'dada2' package (v1.32) (Callahan et al. 2016). The quality of control was performed using 'plotQualityProfile'. Sequences were truncated to 275bp for forward reads and 245bp for reverse reads, with a maximum of 2 and 5 expected errors, respectively, based on the observed quality score distribution (QS > 20). Primer removal was verified using 'vcountPattern' using the Biostrings package (v2.72.0; Pagès et al. 2024). The remaining sequences were dereplicated, and error rates were estimated using the 'learnErrors' function with 'nbases = 1e8', leveraging a large dataset to improve error model accuracy. Sample inference was conducted with 'pool = "pseudo"', which allows pseudo-pooling across samples to improve sensitivity for rare variants. Paired-end reads were merged with a minimum overlap of 10 bases. Chimeric sequences were identified and removed using the 'removeBimeraDenovo' function with the 'consensus' method to detect chimeras across all samples. The 'allowOneOff = TRUE' parameter was used to permit slight mismatches in percent sequences. Additionally, 'minFoldParentOverAbundance = 2' was applied to ensure that percent sequences were at least twice as abundant as potential chimeras, providing a conservative yet reliable approach to chimera removal. These steps produced the final unique amplicon sequence variant (ASVs) table. Finally, for taxonomic classification, the 'assignTaxonomy' function in DADA2 was used with the SILVA 138.1 database (Mclaren and Callahan 2021), adding species-level annotation using the 'addSpecies' function. All analyses were conducted within v4.4.2 of R Studio (R Core Team 2024).

2.6 | Data Processing

The ASV tables and taxonomic data generated from the DADA2 pipeline were processed in R Studio to create phyloseq objects using the phyloseq package (v1.48.0; Mcmurdie and Holmes 2013). Representative sequences were added as DNAStringSet objects to the phyloseq object for further integration. A placeholder phylogenetic tree was generated using the 'rtree' function in the ape package (v5.8; Paradis and Schliep 2019) and incorporated into the phyloseq objects, ensuring the tree tip labels corresponded to the ASV names. The data were subsequently filtered to retain only bacterial sequences, excluding chloroplast and mitochondrial ASVs. Additionally, samples with zero counts across all ASVs were then removed to prevent empty samples from impacting subsequent analyses. The resulting phyloseq objects were then used for downstream statistical analysis.

Sequencing depths across samples ranged from 391 to 11,565 reads (Table S3). Rarefaction curves (observed ASVs versus sequencing depth) were generated to assess sequencing depth adequacy and comparability across samples (Figure S1). Sequencing depth did not differ significantly among treatment groups (Kruskal–Wallis test, $\chi^2 = 1.003$, df = 3, $p = 0.8$), supporting balanced comparison across treatments. Because this study aimed to compare bacterial community patterns across treatments and sampling days rather than to exhaustively characterise total soil bacterial diversity, downstream analyses were conducted without rarefaction.

2.7 | Statistical Analysis

The impact of imidacloprid, thiamethoxam, and clothianidin on bacterial community composition and diversity was assessed by calculating alpha diversity using the Shannon, Simpson (1-D), Chao1 and ACE indices. These indices were used to measure community richness and evenness across different treatment groups over time. Before conducting the main analysis, Shapiro–Wilk tests were used to assess normality, and Levene's tests were employed to check for homogeneity of variances. While some combinations of Treatment and Day showed normality ($p > 0.05$), others exhibited significant deviations from normality ($p < 0.05$). Despite these deviations, Generalised Linear Models (GLMs) without random effects were fitted using a Gamma distribution with a log link function in the 'glmmTMB' package (v1.1.10; Brooks et al. 2017). Day 1 (pre-treatment) was used as the baseline within each treatment, while the control group served as the baseline across treatments. Model fit was evaluated by examining residuals, checking for overdispersion, and comparing models using AIC (Akaike Information Criterion) and BIC (Bayesian Information Criterion) values. Post hoc comparisons were performed using estimated marginal means in the 'emmeans' package (v1.10.6; Lenth 2024) to explore significant differences within treatments over time and between treatments on the same day.

Relative abundance data were aggregated at the phylum level to explore broad taxonomic shifts across treatments. Log-transformed data were tested for normality using the Shapiro–Wilk test. Phyla with normally distributed data (Shapiro–Wilk $p \geq 0.05$) were analysed using a two-way ANOVA to assess the main effects of day and treatment, while those with non-normal distributions (Shapiro–Wilk $p < 0.05$) were analysed using the non-parametric Kruskal-Wallis test. Due to low variability and the presence of many zero values, we focused on the main effects rather than interactions to enhance the usefulness and interpretability of results. This analysis was performed using the packages 'phyloseq' (v1.48.0; Mcmurdie and Holmes 2013), 'dplyr' (v1.1.4; Wickham et al. 2023), 'car' (v3.1.3; Fox and Weisberg 2019), and 'ggplot2' (v3.5.1; Wickham 2016).

Beta diversity was assessed using Bray–Curtis dissimilarity calculated at the ASV level computed with the 'phyloseq' package (v1.48.0; Mcmurdie and Holmes 2013), and the PERMANOVA test was applied using the 'adonis2' function from the 'vegan' package (v2.6.6.1; Oksanen et al. 2024) to evaluate the impact of treatment, time, and their interaction on microbial community structure.

TABLE 1 | Results of Type II Wald chi-square analysis of variance (ANOVA) from generalised linear models (GLMs) assessing the effects of treatment, sampling day, and their interaction on bacterial alpha diversity indices.

	Treatment group			Sampling day			Treatment group × sampling day		
Diversity index	χ^2	df	p	χ^2	df	p	χ^2	df	p
Shannon	1.5	3	0.6	3.1	5	0.6	15.2	15	0.4
Simpson	3.1		0.3	4.8		0.4	20.9		0.1
Chao1	0.3		0.9	4.9		0.4	18.8		0.2
ACE	0.3		0.9	4.6		0.4	19.0		0.2

To identify key taxa that were differentially abundant between treatment groups, pairwise differential abundance analysis was performed using the Linear Discriminant Analysis Effect Size (LEfSe) method with the function 'run_lefse' from the 'microbiomeMarker' package (v1.10.0; Cao et al. 2022) to identify taxa significantly enriched between control and treatment groups based on Linear Discriminant Analysis (LDA) scores. Taxa with LDA scores ≥ 3.0 and $p \leq 0.05$ were considered discriminative biomarkers.

Following the identification of differentially abundant taxa, we investigated how neonicotinoid treatments influenced bacterial interactions and family-level abundances over time. Spearman's rank correlation was used to explore the relationships between bacterial families and experimental factors such as treatment and sampling day. This analysis was conducted using the 'microViz' package (v0.12.5; Barnett et al. 2021).

Alpha diversity indices were visualised using estimated marginal means (EMM) line plots to compare microbial diversity across treatments and sampling days. Relative abundance bar plots were used to present bacterial composition at the phylum level. Principal Coordinates Analysis (PCoA) plots based on Bray-Curtis distance matrices were used to illustrate shifts in bacterial community structure. LDA score plots from LEfSe analysis were used to visualise differentially abundant taxa between treated and control groups. A correlation heatmap, coupled with a dendrogram, displayed Spearman's rank correlations between bacterial families and experimental factors, providing insights into the relationships among taxa and their responses to neonicotinoid treatments.

3 | Results

3.1 | Community Composition and Diversity Patterns

Alpha diversity metrics showed limited overall variation across neonicotinoid treatments and sampling days (Table 1). The generalised linear model showed that significant differences in bacterial diversity occurred across treatments and time; it detected no significant main effects of Treatment or Day, and no significant Treatment × Day interactions for Shannon, Simpson, Chao1, or ACE indices. Notwithstanding the absence of significant global effects, post hoc pairwise comparisons indicated a small number of transient, day-specific differences relative to the control within individual sampling days (Figures S2–S3). Shannon diversity, a metric reflecting both the richness and evenness of microbial communities (Figure S2a), revealed that thiamethoxam and clothianidin significantly increased microbial diversity compared to the control. Specifically, thiamethoxam resulted in a significant increase in evenness on day 2 ($p = 0.04$), while clothianidin similarly increased diversity on day 3 ($p = 0.05$). In contrast, imidacloprid showed a significant decrease in Shannon diversity compared to both day 1 and the control on day 10 ($p = 0.01$).

The Simpson index, a measure of community dominance (Figure S2b), revealed consistent trends. Thiamethoxam and imidacloprid both significantly increased Simpson's D on Days 2 and 3, respectively ($p = 0.03$), suggesting reduced evenness at early stages. However, by Day 10, Simpson's D significantly decreased under imidacloprid ($p = 0.01$), indicating a subsequent loss of evenness. This suggests that while imidacloprid initially promoted a more even community distribution, dominance by a few taxa became stronger again by Day 10. Clothianidin increased estimated species richness, as indicated by higher Chao1 (Figure S3a) and ACE indices (Figure S3b) on Day 3 compared to the control ($p = 0.03$).

3.2 | Taxonomic Distribution Shifts

The bacterial community in the Red Luvisol soil investigated for this research was comprised of 24 different phyla, of which Actinobacteriota consistently dominated across all treatments (Figure 1). Across all treatments, the five most abundant phyla, with a relative abundance exceeding 5%, were Actinobacteriota (32%–45%), Proteobacteria (27%–33%), Firmicutes (7%–14%), Bacteroidota (6%–11%), and Myxococcota (4%–5%).

The analysis of bacterial community composition at the phylum level revealed stable patterns for dominant phyla and selective changes among minor phyla (Tables S4 and S5). Actinobacteriota and Proteobacteria, the most abundant phyla, showed no significant effects of treatment or sampling day ($p > 0.05$). Firmicutes significantly increased over time ($p = 0.02$), with the highest relative abundance observed under imidacloprid on Day 10, though treatment effects were non-significant. Bacteroidota significantly decreased over time ($p = 0.01$) without treatment effects.

Among the minor phyla, Methylomirabilota exhibited a significant treatment effect ($p = 0.001$), while Desulfobacterota and

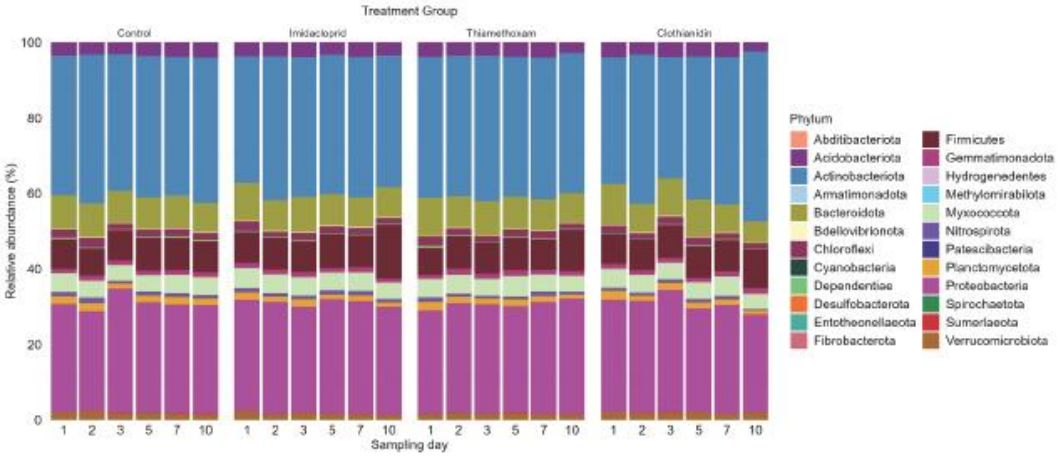

FIGURE 1 | Phylum-level taxonomic distribution and temporal variation in bacterial relative abundance (%) across different treatments.

Chloroflexi showed low relative abundances with slight, non-significant decreasing trends over time ($p > 0.05$). Other phyla, Planctomycetota, Abditibacteriota and Verrucomicrobiota, remained stable across treatments and sampling days, with no significant changes detected.

3.3 | Community Structural Dynamics

Sampling days significantly influenced the structure of bacterial communities ($R^2 = 0.05$, $p = 0.01$, Table S6). Moreover, the interaction between treatment and sampling days was also significant ($R^2 = 0.14$, $p = 0.004$), indicating that the effect of treatment varied over time. Treatment alone did not significantly affect community structure ($p > 0.05$), suggesting that temporal dynamics and their interaction with treatments were the primary drivers of changes in bacterial community structure.

The PCoA plots were used to visualise community structural patterns associated with sampling days and their interaction with different neonicotinoids. The first two principal coordinates explained 15.01% of the total variation in the community data, with PC1 accounting for 11.42% and PC2 3.59% of the variation (Figure 2).

Despite the statistical significance, the ellipses for different treatments overlapped substantially, and no distinct clustering patterns were observed. The overlapping ordination space suggests that while temporal changes and their interaction with treatments significantly influenced community structure, the effects were subtle.

3.4 | Differential Abundance and Key Taxonomic Indicators

The pairwise LEfSe identified significant shifts in the bacterial community structure when comparing control and neonicotinoid-treated groups. The LDA score plot (Figure 3) illustrated bacterial taxa that were either enhanced or suppressed in relative abundance by the neonicotinoids relative to control across different taxonomic levels. Several taxa showed an enhanced response under imidacloprid, including *Massilia* sp. within Oxalobacteraceae, Micromonosporaceae, and *Mesorhizobium* sp. within the Rhizobiaceae family, with LDA scores ranging from 3.14 to 3.43. Conversely, a broader set of taxa exhibited a suppressed response, including Myxococcaceae, the *Pir4* lineage (Pirellulaceae), *RB41* (Pyrinomonadaceae), Chitinophagaceae, Blastocatellia, Thermomicrobiales, and Solirubrobacterales (LDA = −3.03 to −4.02).

Similarly, thiamethoxam was associated with pronounced bacterial community responses relative to the control. Several taxa exhibited enhanced responses, including members of Gaiellales–Gaiellaceae–*Gaiella* lineage, as well as the families Oxalobacteraceae and Rhizobiaceae, and affiliated genera such as *Solirubrobacter*, *Marmoricola*, *Massilia*, and *Mesorhizobium*, with LDA scores ranging from 3.05 to 3.74. In contrast, thiamethoxam was associated with suppressed responses across multiple lineages, including 67–14 (Solirubrobacterales), Planctomycetes, the Nitrospirota lineage (Nitrospiria–Nitrospirales–Nitrospiraceae–*Nitrospira*), the *Pir4 lineage* (genus-level taxon within Pirellulaceae), the Pedosphaerales–Pedosphaeraceae lineage, and *Aquicella* (LDA = −3.00 to −4.05).

For clothianidin, enhanced responses were observed for several taxa, including the Propionibacteriales–Nocardioidaceae–Nocardioides lineage, *Solirubrobacter*, Intrasporangiaceae, Rhizobiaceae, Micromonosporales–Micromonosporaceae, *Mesorhizobium* and *Lysobacter* (LDA = 3.01–3.93). Conversely, suppressed responses were detected across multiple bacterial groups, including Chloroflexia, Planctomycetes, *Methyloceanibacter*, the Nitrospirota lineage (Nitrospiria–Nitrospirales–Nitrospiraceae–*Nitrospira*), the Diplorickettsiales–Diplorickettsiaceae lineage, the *Pir4 lineage* (Pirellulaceae), and Blastocatellia (LDA = −3.18 to −3.48).

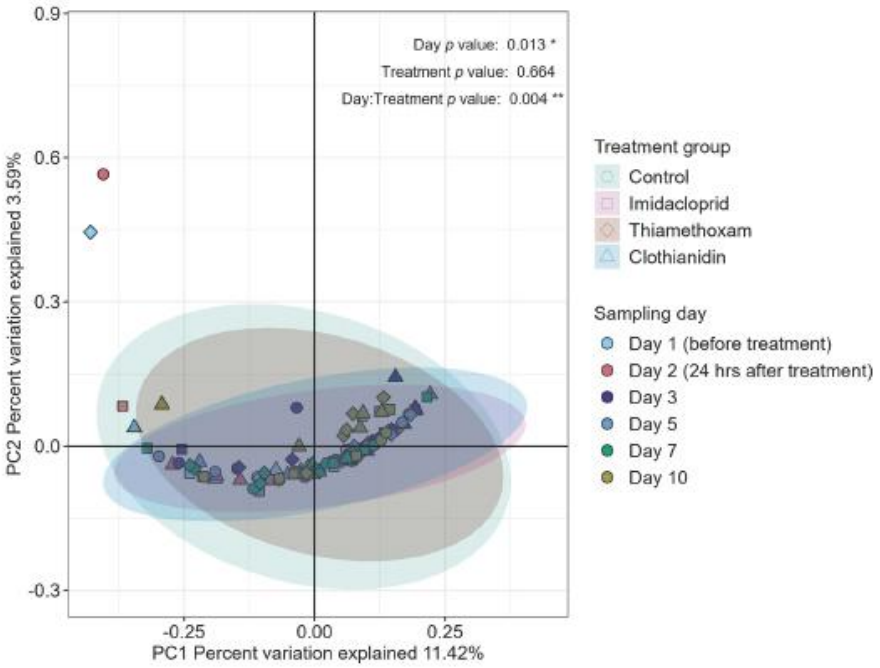

FIGURE 2 | Principal Coordinate Analysis (PCoA) plot of bacterial community structure based on Bray–Curtis dissimilarities at the ASV level. Shapes represented different neonicotinoid treatments, and colours indicated sampling days. Significance levels were set as '***' $p \leq 0.001$, '**' $p \leq 0.01$, '*' $p \leq 0.05$.

3.5 | Correlation Analysis and Taxa Interaction Patterns

The bacterial correlation heatmap (Figure 4) illustrated how different bacterial families responded to neonicotinoid treatments over time. Several families showed statistically significant correlations with sampling days and treatments; however, correlation strengths were generally weak ($|\rho| < 0.6$), indicating small effect sizes. Representative scatter plots are shown in Figure S4.

67–14 had a positive correlation with days ($\rho = 0.21$, $p = 0.02$), followed by Haliangiaceae ($\rho = 0.21$, $p = 0.02$), Gaiellaceae ($\rho = 0.24$, $p = 0.01$), Weeksellaceae ($\rho = 0.25$, $p = 0.01$), Diplorickettsiaceae ($\rho = 0.25$, $p = 0.01$), Nocardioidaceae ($\rho = 0.27$, $p = 0.003$), Paenibacillaceae ($\rho = 0.27$, $p = 0.003$), and Erwiniaceae ($\rho = 0.29$, $p = 0.001$). Whereas Solirubrobacteraceae ($\rho = 0.20$, $p = 0.03$) and Rhizobiaceae ($\rho = 0.25$, $p = 0.01$) had a positive correlation with treatments. Conversely, a total of 13 microbial families exhibited significant negative correlations with sampling days, indicating that these families decreased in relative abundance over time. Flavobacteriaceae exhibited the strongest negative correlation with sampling days ($\rho = -0.56$, $p < 0.001$). Similarly, Microscillaceae ($\rho = -0.44$, $p < 0.001$) and Sandaracinaceae ($\rho = -0.41$, $p < 0.001$) showed significant negative correlations, indicating a consistent decrease in their abundance over time. Other families such as Pirellulaceae ($\rho = -0.36$, $p < 0.001$), BIrii41 (an uncultured family within Polyangiales; $\rho = -0.34$, $p < 0.001$), and JG30-KF-CM45 (an uncultured family within Thermomicrobiales; $\rho = -0.29$, $p = 0.001$) also exhibited negative correlations with sampling days. In addition to the temporal effects, Nitrospiraceae ($\rho = -0.27$, $p = 0.003$) showed a significant negative correlation with treatments, indicating that this family decreased in relative abundance under the applied treatments.

Treatment-specific analyses revealed that Yersiniaceae ($\rho = -0.21$, $p = 0.02$), 67–14 ($\rho = -0.20$, $p = 0.03$), and Solirubrobacteraceae ($\rho = -0.19$, $p = 0.03$) were significantly negatively correlated with imidacloprid. Mycobacteriaceae ($\rho = -0.19$, $p = 0.04$) was negatively correlated with thiamethoxam treatment, whereas SC-I-84 (Burkholderiales) exhibited a positive correlation with clothianidin ($\rho = 0.21$, $p = 0.02$).

4 | Discussion

4.1 | Shifts in Diversity Patterns of Bacterial Community Across Neonicotinoid Treatments

The observed shifts in bacterial diversity across different neonicotinoid treatments provide further insights into how these chemicals influence soil microbial communities over time. However, the lack of extensive literature specifically addressing neonicotinoid effects applied as seed treatments on microbial communities makes it difficult to directly compare these findings. This highlights the novelty of our study, as it contributes new insights into the effect of neonicotinoids on soil bacterial communities. By revealing differential impacts across diversity metrics, our research fills a critical gap in understanding

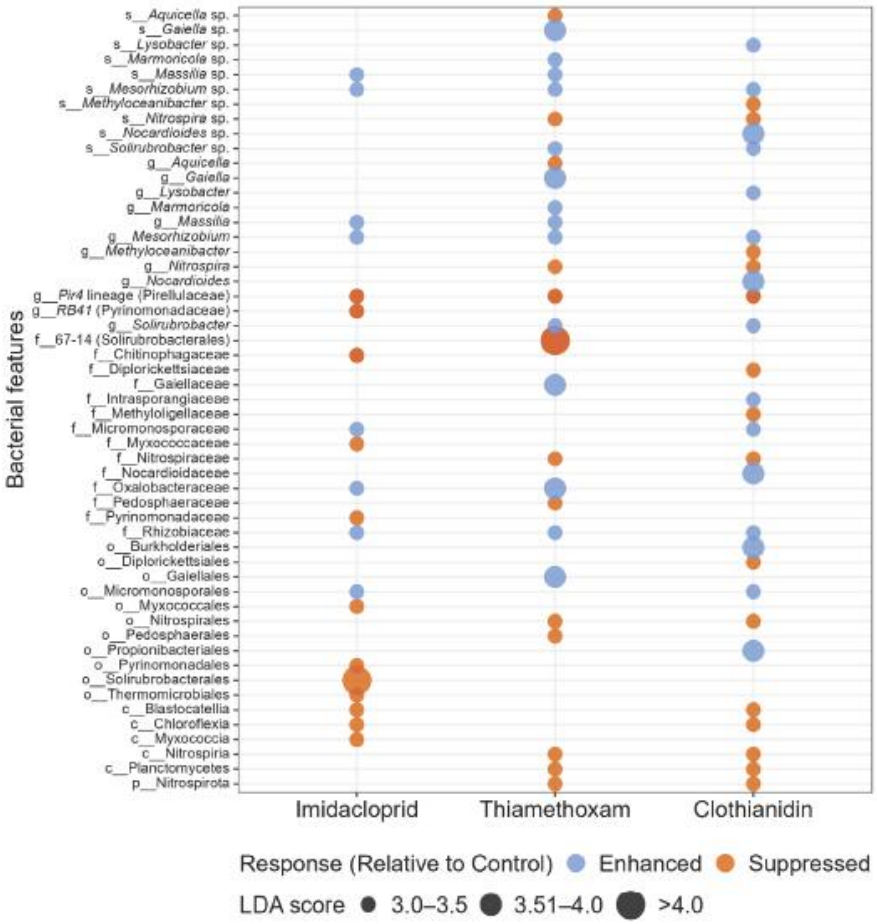

FIGURE 3 | Differential bacterial taxa enriched or suppressed under neonicotinoid treatments relative to the control, identified using LEfSe (LDA $\geq 3.0, p \leq 0.05$). Point size reflects the magnitude of the LDA effect size.

the ecological consequences of these widely used insecticides. Similar studies have shown that the response of microbial communities can vary depending on the diversity metric used (Liu et al. 2023), suggesting that richness and evenness are differentially sensitive to different chemical stresses (Herath et al. 2017; Meena et al. 2020).

The initial increase in microbial diversity observed in the Shannon and Simpson indices under thiamethoxam and clothianidin treatments may reflect the early-stage responses of the soil microbial communities to pesticide exposure. This phenomenon has been observed in other studies, where microbial communities demonstrated an increase in diversity shortly after pesticide exposure, likely as a result of microbial adaptation and the exploitation of newly available ecological niches (Pertile et al. 2021). During this initial phase, certain microorganisms may thrive by utilising the pesticide as a source of energy, leading to a temporary rise in diversity (Gangola et al. 2023, 2021). One possible mechanism behind this early rise in diversity is the activation of resistance mechanisms in microbial populations. Thiamethoxam and clothianidin may activate efflux pumps and increase bacterial cell membrane permeability, promoting horizontal gene transfer and enabling the rapid spread of resistant strains, thereby temporarily boosting microbial diversity (Qiu et al. 2022). However, this increase in diversity is not necessarily sustained. Over time, the pesticide treatments increasingly favour certain resistant strains, causing the gradual decline of less resistant taxa. This is consistent with the concept of 'selective pressure', where only those microorganisms capable of resisting the pesticide survive, reducing the overall diversity within the community (Hawkins et al. 2019; Qiu et al. 2022). As a result, we observed a decrease in diversity over time, particularly under the imidacloprid treatment. This decline in diversity, visible in the Simpson index, suggests that over time, the microbial community becomes more dominated by a few pesticide-resistant taxa, while the rest of the community is suppressed, a pattern often associated with neonicotinoid exposure. This is in line with findings showing that higher doses of imidacloprid significantly reduced bacterial evenness and richness over time, likely due to the displacement of sensitive species by resistant or

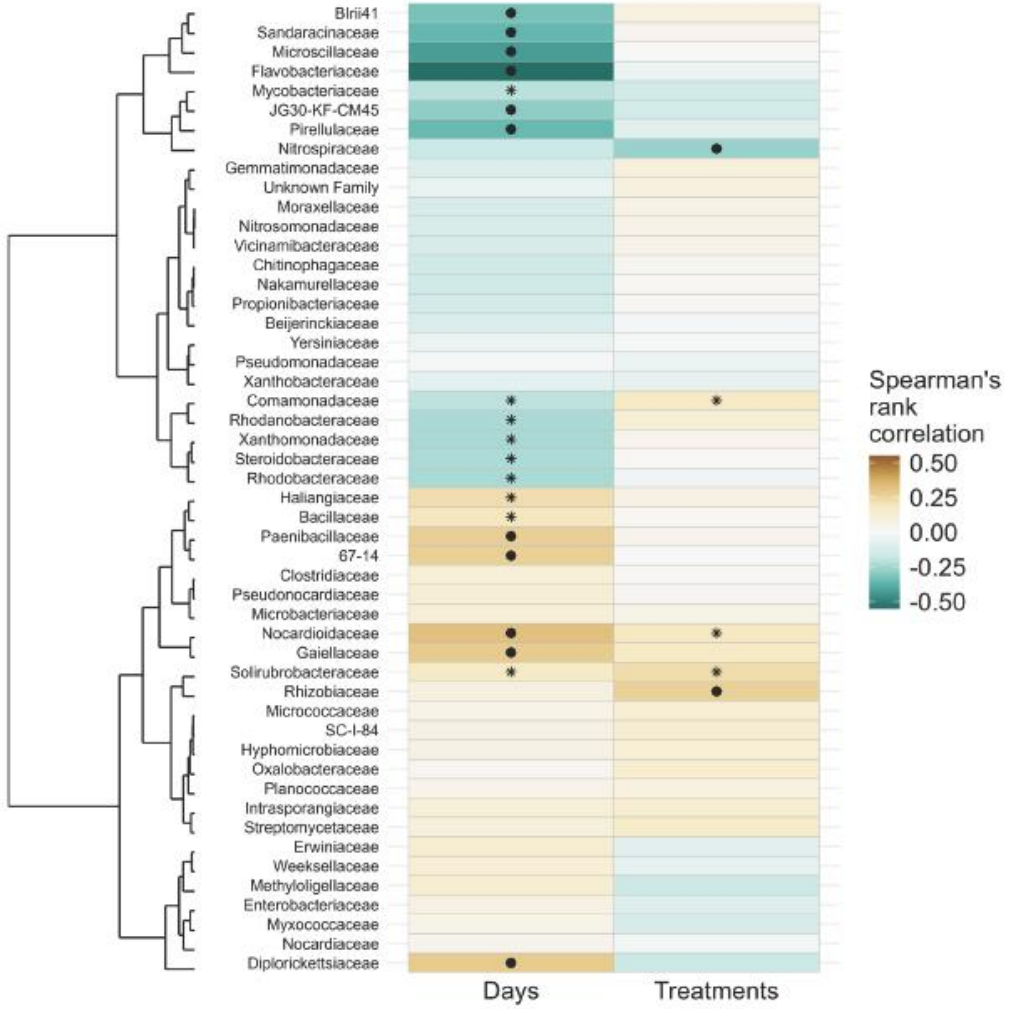

FIGURE 4 | Correlation heatmap and dendrogram showing bacterial family-level correlations with days and treatments. Significant positive and negative correlations are marked by asterisks ($p < 0.05$, unadjusted), and FDR-adjusted correlations are highlighted with filled circles (FDR-corrected $p < 0.05$).

imidacloprid-degrading taxa, with both time and dosage playing key roles in shaping the community (Cycoń et al. 2013).

Clothianidin's impact on species richness, as indicated by the ACE and Chao1 indices on day three, suggests that this compound may promote a broader range of bacterial taxa in the early stages of exposure. The temporary increase in richness may reflect an initial proliferation of opportunistic taxa before community structure begins to stabilise. Although direct studies on clothianidin's effect are limited, similar patterns of increased bacterial richness have been observed with other chemicals. For example, a mixture of acetochlor and atrazine applied to surface soil has been shown to significantly increase ACE and Chao1 indices in deeper soil layers (0.2–0.4 m), suggesting that certain chemical compounds can enhance bacterial richness under specific conditions (Chen et al. 2021). Given the short-term nature of this study, it is possible that longer exposure times may reveal further changes, such as potential recovery or prolonged impacts. Zhang et al. (2019) reported similar patterns with the organochlorine pesticide 1,3-Dichloropropene, applied as a pre-plant fumigant, where the initial decrease in bacterial richness was followed by recovery at later stages. These differential findings of imidacloprid, thiamethoxam, and clothianidin on bacterial diversity underscore the complex interactions between neonicotinoids and soil bacterial communities. Significant differences in bacterial diversity indices across treatments reflect the variability in microbial responses, influenced by factors such as environmental conditions, the reactivity of different neonicotinoids, exposure duration, and the specific composition and resilience of microbial taxa. While short-term increases in

diversity were observed, the long-term effects of pesticide exposure could lead to a reduction in bacterial diversity, potentially compromising soil health. Although shifts in bacterial diversity occurred with treatments and time, these changes were not uniform. This is consistent with the mode of action of neonicotinoids: they are not classified as broad-spectrum microbial toxins, and their primary toxicity is directed towards insect nervous systems via nicotinic acetylcholine receptors (Jeschke et al. 2011). Nevertheless, neonicotinoids can influence microbial communities indirectly by altering rhizosphere conditions, modifying root exudation patterns, or exerting selective pressure on sensitive taxa (Cycoń and Piotrowska-Seget 2015). Therefore, a consistent decline in microbial diversity is not necessarily expected. Instead, the observed temporal and compound-specific shifts may reflect community restructuring, short-term functional responses, or the emergence of tolerant taxa. These transient changes, even in the absence of clear directional trends, may have important ecological implications, particularly if they affect keystone taxa or microbial functional capacity (Allison and Martiny 2008).

4.2 | Phylum-Level Taxonomic Distribution in Response to Neonicotinoids

The bacterial community composition across different neonicotinoid treatments revealed both stable and shifting community dynamics. While the dominant phyla, such as Actinobacteriota and Proteobacteria, exhibited minimal responses to imidacloprid, thiamethoxam, and clothianidin exposure, notable changes were observed among minor phyla over time. These observations suggest that, although some groups of bacteria remain resilient to pesticide stress, others may undergo selective shifts depending on their tolerance to the chemicals and the dynamic soil environment. The stability of Actinobacteriota and Proteobacteria in the presence of neonicotinoids aligns with findings from previous studies, which indicate that these phyla are often dominant in soil and show strong resilience to neonicotinoid as well as other pesticide applications (Li et al. 2018; Galic et al. 2024). This resilience may be attributed to their metabolic diversity and ecological role in soil biogeochemical processes, where they contribute to biodegrade complex organic compounds, including pesticides, which helps maintain their abundance despite exposure to neonicotinoids (Bhende and Dafale 2023; Mawang et al. 2021; Ma et al. 2022; Wang et al. 2023).

Conversely, the rise in Firmicutes under imidacloprid may be attributed to this phylum's spore-forming ability, which supports survival in chemically stressed environments (Beskrovnaya et al. 2021; Filippidou et al. 2016). Comparable increases in Firmicutes abundance have been noted in soils treated with other neonicotinoids, such as thiamethoxam (Wu et al. 2021) and Paichongding (Cai et al. 2016) as well as with non-neonicotinoid chemicals like dithiocarbamates (Feld et al. 2015) and ethanol-free chloroform (Domínguez-Mendoza et al. 2014). In contrast, Bacteroidota exhibited a significant decrease in relative abundance over time, a trend that suggests susceptibility to neonicotinoid exposure. This aligns with previous studies that reported a decline in Bacteroidota abundance under paichongding stress conditions, indicating that this phylum may be more vulnerable to the effects of neonicotinoids (Cai et al. 2016). Another study reported that imidacloprid changed the abundance of Bacteroidota, with differences occurring between early and later sampling points (Streletskii et al. 2023). Such declines suggest that their metabolic functions could be disrupted by pesticide stress, leading to a decrease in their abundance. Minor phylum Methylomirabilota exhibited significant treatment effects, suggesting that some less-abundant microbial groups are more sensitive to neonicotinoid exposure. This aligns with previous research, where fumigation treatment of chloropicrin in soils led to a reduction in the abundance of Methylomirabilota over time (Tekeu et al. 2023). While the specific mechanisms remain unclear, it is possible that the decrease in Methylomirabilota in our study could be related to the stress induced by neonicotinoids, thus disrupting microbial community stability.

4.3 | Community Structure Responses

This study demonstrates that the interaction between sampling day and neonicotinoid treatment significantly influences bacterial community structure in the soil. These findings suggest that microbial responses to neonicotinoids develop progressively over time, rather than as immediate reactions to treatment alone. Such time-dependent shifts align with previous research, where temporal variation, alongside factors like habitat or host environment, serves as a critical driver of bacterial community composition (Parizadeh et al. 2021). Similarly, studies on chloropicrin and metam sodium reported that sustained exposure leads to community shifts, supporting the role of prolonged chemical presence in altering microbial dynamics (Sennett et al. 2022). This underscores the importance of temporal dynamics in shaping microbial community structure, especially under prolonged chemical stress, as microbial communities in terrestrial ecosystems can exhibit diverse temporal changes, from immediate responses to seasonal variations (Schmidt et al. 2007; Bardgett et al. 2005).

The PCoA analysis showed that temporal and treatment interactions led to subtle structural changes in bacterial communities, without distinct clustering patterns. This lack of separation may reflect the functional redundancy often seen in microbial communities, where diverse taxa can fulfil similar roles, stabilising function despite taxonomic shifts (Allison and Martiny 2008). Additionally, the complexity of soil microhabitats likely introduces spatial variability, potentially masking distinct clustering in ordination space (Fierer and Jackson 2006). Such subtle responses align with studies indicating that microbial adaptation to stressors, including chemical exposure, tends to accumulate over time rather than producing immediate changes (Shade et al. 2012). Furthermore, the partial variance explained by PCoA suggests that other environmental factors, not captured here, might also influence community dynamics (Ramette 2007). These findings highlight that while the interaction of treatment with three different neonicotinoids and time significantly influences bacterial communities, the resulting shifts are complex and gradual. This suggests that each neonicotinoid may contribute uniquely to changes in soil microbial dynamics, with potential implications for soil ecosystem stability under prolonged chemical exposure.

4.4 | Taxonomic Indicators of Bacterial Community Response to Neonicotinoids

The differential abundance analysis highlights specific bacterial taxa that respond uniquely to each neonicotinoid treatment, indicating selective effects on bacterial community composition with implications for multiple biogeochemical cycles. Taxa that are important within key biogeochemical cycles benefited from soil exposure to neonicotinoids. All three neonicotinoids led to the enhancement of specific bacterial taxa. *Mesorhizobium*, a genus known for nitrogen fixation (Laranjo et al. 2014; Colombi et al. 2023), was enhanced under all treatments, indicating that neonicotinoids may stimulate nitrogen cycling, which is beneficial for soil fertility and plant growth. Additionally, *Mesorhizobium*'s production of 1-aminocyclopropane-1-carboxylic acid deaminase can help alleviate plant stress by limiting ethylene formation, a hormone that is typically produced in response to stressors such as drought, salinity, and heavy metals (Wanjofu et al. 2022). This enzymatic activity may support plant growth under various environmental stress conditions. Imidacloprid also enriched the Micromonosporaceae family, which is involved in the breakdown of complex organic compounds such as cellulose, chitin, lignin and pectin (Trujillo et al. 2014). These bacteria are also stress-tolerant, capable of nitrogen fixation and decomposition of carbon compounds, all of which are important for maintaining soil health (Skidmore et al. 2022). Thiamethoxam also enhanced *Massilia* sp., *Gaiella* sp. and *Solirubrobacter* sp., whereas clothianidin enhanced the latter one. The bacterial genus *Massilia* exhibits significant ecological importance through its adaptability across diverse ecosystems, from extreme environments like deserts and glaciers to polluted soils and airborne communities, where it aids in pollutant bioremediation, enhances plant resilience in phytoremediation, and serves as a potential bioindicator of environmental stress (Amirhosseini et al. 2025). *Gaiella* sp. promote the biodegradation of organic pollutants by using intermediate metabolites and is involved in phosphate absorption and organic matter decomposition (Gu et al. 2023; Wei et al. 2024). Similarly, *Solirubrobacter* sp. plays a crucial role in soil organic matter assimilation and biogeochemical cycling and contributes significantly to soil fertility and nutrient availability (Jiang et al. 2023; Jara-Servin et al. 2024). Clothianidin also enriched *Lysobacter*, a predatory Gammaproteobacterium that employs T4SS-mediated contact-dependent killing of gram-negative soil bacteria (Shen et al. 2021). This enrichment reveals pesticide-specific selection for microbial predators possessing lytic enzymes and antibiotics that suppress soilborne pathogens while maintaining nutrient cycling, demonstrating functional resilience under neonicotinoid stress.

While some key taxa benefited by soil exposure to neonicotinoids, the suppression of other bacterial taxa is concerning as they too play critical roles in soil health and ecosystem functions. Imidacloprid and clothianidin suppressed Chloroflexia, which is involved in degradation of complex carbohydrates through diverse CAZymes and contributes to carbon cycling (Freches and Fradinho 2024; Zheng et al. 2021). The reduction of Chloroflexia could impair plant-derived carbohydrate decomposition and slow down carbon cycling in soil (Dobrzyński et al. 2026). The suppression of *Nitrospira* sp. by both thiamethoxam and clothianidin is particularly troubling because these taxa are involved in key nitrogen processes. The suppression of *Nitrospira* sp., responsible for nitrification, further points to potential disruptions in the nitrogen cycle by impairing nitrite oxidation to nitrate, potentially leading to reduced nutrient mineralisation for plants (Takahashi et al. 2020; Meng et al. 2023). Clothianidin also suppressed *Methyloceanibacter* sp., a flexible methylotroph that scavenges C_1 compounds, participates in methane-linked food webs via cross-feeding and couples C_1 oxidation to nitrogen transformations, especially in moist, redox-dynamic soil environments (Howland et al. 2025; Rasmussen et al. 2024; Vekeman et al. 2016). This could elevate methane emissions, hinder N-cycling, and disrupt microbiome stability, underscoring pesticide risks to soil C/N homeostasis (Spokas et al. 2007; Itoh et al. 2014).

4.5 | Correlation and Taxa Interaction Patterns

The correlation analysis reveals distinct short-term responses of bacterial families to neonicotinoid treatments, indicating that neonicotinoids influence microbial dynamics in a treatment-specific and time-sensitive manner. The positive correlations of 67–14 (*Solirubrobacterales*), *Haliangiaceae, Gaiellaceae, and Weeksellaceae* with sampling days may indicate an initial resilience or adaptation, allowing these families to maintain or slightly increase in abundance under early neonicotinoid exposure. This aligns with findings from other studies showing that certain bacterial taxa can exhibit resilience to pesticide stress, potentially due to metabolic flexibility or adaptive mechanisms (Shahid et al. 2023; Jiao et al. 2019). In contrast, Flavobacteriaceae and Microscillaceae exhibited negative correlations with days, suggesting short-term sensitivity to neonicotinoid stress or competitive interactions within the microbial community. This variability in bacterial resilience highlights the influence of different chemical treatments, with certain microbial groups being more sensitive to specific pesticides, which can affect their roles in maintaining ecosystem stability (Meena et al. 2020).

Beyond the general trends that were observed, each treatment had unique effects on specific bacterial families. The significant negative correlation of Yersiniaceae with imidacloprid suggests that this family may be sensitive to neonicotinoid exposure. Despite its pathogenic significance (Seabaugh and Anderson 2024), there is a lack of evidence regarding the ecological importance of Yersiniaceae in soil ecosystems. Similarly, the negative correlation of 67–14 with imidacloprid indicates its susceptibility to neonicotinoid exposure. Given its positive correlation with microbial biomass carbon and specific surface area, the suppression of 67–14 may reflect disruptions in key soil processes, such as carbon cycling and the maintenance of soil structure, which are essential for soil health and microbial community stability (Company et al. 2022). The negative correlation of Solirubrobacteraceae with imidacloprid aligns with previous research where this family was significantly depleted by chloropicrin applied as a soil fumigant (Zhan et al. 2021). As a family associated with the rhizosphere, Solirubrobacteraceae is likely involved in plant nutrition and soil health (Jiang et al. 2023). While its specific functional roles are unclear, it is known to possess broad ecological capabilities, thriving in both aquatic and terrestrial environments, including disturbed

soils (Evdokimova et al. 2024). Similarly, Mycobacteriaceae, recognised for its role in the degradation of hydrocarbons and pollutants (Conte et al. 2021; Alidoosti et al. 2024), was negatively correlated with thiamethoxam in our study, suggesting its sensitivity to neonicotinoid exposure. This finding supports previous studies that demonstrated that a non-ionic surfactant hindered the proliferation of Mycobacteriaceae, affecting its ability to degrade polycyclic aromatic hydrocarbons (Lladó et al. 2015). Thus, Mycobacteriaceae may also be sensitive to neonicotinoids, whose presence in soil may reduce their efficacy in biodegradation processes. In contrast, clothianidin was positively correlated with SC-I-84 (Burkholderiales). This aligns with findings from previous studies, which suggest that pollutants such as Ni, Cu, Pb, and organic contaminants favour the growth of SC-I-84 (Wang et al. 2024).

We recommend future research focus on long-term studies to assess cumulative neonicotinoid effects on microbial community composition, particularly in relation to soil ecosystem functions. Comparative analyses of individual neonicotinoids could further clarify how each compound differentially impacts soil microbial composition, diversity, and function. Additionally, incorporating environmental parameters such as soil pH, moisture, organic matter, and texture could provide insights into the interactions between neonicotinoids and natural soil conditions, enhancing the understanding of microbial stability and resilience under varying field conditions. Mechanistic studies examining metabolic pathways and adaptation responses in neonicotinoid-affected taxa are also essential for advancing knowledge of how prolonged chemical exposure influences soil microbial networks, functional resilience, and specific ecological roles. A more functional perspective could reveal the broader implications of microbial shifts, shedding light on how these communities sustain or alter critical soil processes over extended exposure periods.

5 | Conclusion

Our results showed that neonicotinoid seed treatments altered microbial diversity, taxonomic composition, and community structure during short-term exposure. Each neonicotinoid induced unique shifts in bacterial diversity and taxa interactions, with evidence of both resilience and sensitivity among bacterial taxa. Initial increases in diversity under thiamethoxam and clothianidin were followed by declines, particularly under imidacloprid, suggesting progressive selective pressure during continued exposure. Although dominant phyla remained relatively stable, less abundant taxa showed sensitivity, and community changes over time suggest that continued exposure favours stress-tolerant populations. The enrichment of genera *Mesorhizobium*, *Massilia*, *Gaiella*, *Solirubrobacter*, and *Lysobacter* suggests adaptive or stress-tolerant capacities within certain taxa, whereas the suppression of Chloroflexia, *Nitrospira*, and *Methyloceanibacter* points to potential vulnerabilities in carbon and nitrogen cycling pathways. Given the limited body of literature on neonicotinoid seed treatments, this research provides insights into early soil microbial responses. Further long-term and functional investigations are needed to elucidate metabolic pathways, functional adaptations, and resilience mechanisms under repeated and cumulative neonicotinoid exposure across soils with differing chemical and physical properties.

Author Contributions

Sharmin Akter: conceptualisation, methodology, investigation, laboratory analysis, data analysis, data curation, visualisation, writing – original draft. **Julia Jasonsmith:** supervision, method validation, writing – review and editing. **Nilantha R. Hulugalle:** supervision, method validation, writing – review and editing. **Craig L. Strong:** supervision, method validation, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The raw sequencing data produced in this study have been deposited in the NCBI Sequence Read Archive under accession number PRJNA1251256. Other datasets relevant to the findings of this article are provided within the main text and the accompanying Supporting Information files.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Summary of physicochemical properties of the soil samples analysed in this study. **Table S2:**. Primer sequences used for amplification of the 16S rRNA gene (V1–V3 region). **Table S3:**. Sequencing depths by treatments. **Table S4:**. Bacterial relative abundance (%) at the phylum level across different treatments over time. **Table S5:**. Statistical test of different bacterial phyla across different treatments and time, with effect direction assessed using median and negative significance highlighted by the red circle and positive significance highlighted by the green circle. **Table S6:**. PERMANOVA results showing the influence of different neonicotinoid treatments, sampling day, and their interaction on soil bacterial community structure measured by Bray–Curtis dissimilarity matrix. **Figure S1:**. Rarefaction curves showing sequencing depth for each sample. **Figure S2:**. Alpha diversity indices for bacteria, measured by (a) Shannon index, (b) Simpson index. Points represent estimated marginal means (EMMs) ± 95% confidence intervals for each treatment group (Control, Imidacloprid, Thiamethoxam, and Clothianidin). Asterisks indicate significant differences among treatments within each day based on Generalised Linear Model analysis ('**' ≤ 0.01, '*' ≤ 0.05). **Figure S3:**. Alpha diversity indices for bacteria, evaluated through (a) Chaol index, (b) ACE index. Points represent estimated marginal means (EMMs) ±95% confidence intervals for each treatment group (Control, Imidacloprid, Thiamethoxam and Clothianidin). Asterisks indicate significant differences among treatments within each day based on Generalised Linear Model analysis ('*' ≤ 0.05). **Figure S4:**. Scatter plot showing CLR-transformed relative abundance of bacterial families that showed significant Spearman correlations ($p \leq 0.05$) with sampling day and/or neonicotinoid treatments.

Chapter 5 Texture and neonicotinoid exposure shape bacterial assemblages and functions in agricultural soils: Responses over prolonged exposure

5.1 Overview[3]

This chapter investigates how soil texture influences bacterial community responses to prolonged exposure to the neonicotinoid insecticide imidacloprid. Building on the short-term experiment presented in the previous chapter, this study examines whether bacterial responses become more pronounced or differ following extended exposure and whether these responses vary among contrasting agricultural soils. Three contrasting soil textures were evaluated to determine how inherent soil properties mediate changes in bacterial diversity, community composition, ecological interactions and predicted functional potential. By integrating taxonomic, ecological network and functional prediction approaches, this chapter provides a comprehensive assessment of the mechanisms through which soil characteristics influence microbial responses to neonicotinoid stress. The findings contribute to a broader understanding of how soil physicochemical properties shape the ecological consequences of neonicotinoid exposure and help explain why pesticide impacts cannot be generalised across different soil environments.

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Texture and Neonicotinoid Exposure Shape Bacterial Assemblages and Functions in Agricultural Soils: Responses Over Prolonged Exposure

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ABSTRACT

The physical and chemical properties of soil fundamentally shape its microbial communities. In a controlled 28-day microcosm experiment, we assessed bacterial community responses to imidacloprid in three soils with differing textures and classifications: a loamy sand (11 g/100 g clay; red Luvisol), a sandy loam (16 g/100 g clay; red Luvisol) and a clay soil (56 g/100 g clay, Vertisol). Analyses included 16S rRNA gene amplicon sequencing, indicator species analysis, co-occurrence network analysis and PICRUSt2-based functional prediction. Imidacloprid exposure elicited soil-specific shifts in bacterial community structure, primarily altering evenness rather than richness; however, overall diversity patterns were more strongly governed by soil texture and sampling time. Indicator species analysis identified distinct sensitive and tolerant taxa in each soil texture, with a core set of taxa remaining largely unchanged. Co-occurrence network analysis showed decreased network complexity and increased modularity under imidacloprid, particularly in loamy sand and clay soils, suggesting altered bacterial interaction patterns. Predicted functional profiles showed upregulation of stress-response pathways and downregulation of energy/nutrient metabolism pathways, implying a community-level shift toward stress adaptation. Although taxonomic richness remained relatively stable, these reorganisations of community interactions and functional potential suggest changes in bacterial resilience and biogeochemical cycling, which may have implications for long-term soil health.

1 | Introduction

Soil is a complex, heterogeneous medium typically comprised of inorganic mineral particles, organic material, water and air, which together support diverse microbial life critical for agricultural productivity (Buscot 2005). The relative proportions of sand-, silt- and clay-sized particles dictate a soil's texture. This texture is a fundamental determinant of the soil's physical properties, including moisture, porosity, strength and nutrient availability (Fernandez-Illescas et al. 2001; Zhu et al. 2021; Lynch et al. 2021). Furthermore, soil texture also governs microbial access to resources and habitat stability through its effects on pore connectivity and substrate availability (Patel et al. 2021; Kravchenko and Guber 2017). Fine-textured soils rich in clay provide higher water retention but can limit gas exchange and create microenvironments with reduced oxygen levels (Ben-Noah and Friedman 2016; Al-Kaisi et al. 2017). These conditions often select for anaerobic or facultative microbial populations, influencing community composition (Tecon and Or 2017). In addition, swelling clays are prone to shrinkage and cracking during drying cycles, which can alter pore connectivity and create preferential flow paths (Qi et al. 2022; Luo et al. 2023). These structural changes may also lead to localised variation in soil physical and chemical properties, influencing microbial habitat stability (Weisskopf et al. 2010; Hullugalle et al. 2001). In contrast,

sandy soils with larger pores and lower water-holding capacity, promote aerobic microbial activity but can expose microbes to environmental stressors such as desiccation (Yang and van Elsas 2018). The distinct physical and chemical environments shaped by soil texture thus create a mosaic of niches, promoting microbial diversity and functional specialisation (Seaton et al. 2020; Naveed et al. 2016; Carson et al. 2010). Texture also dictates how chemicals move through soils and interact with microbial populations. Clays typically exhibit high nutrient retention due to increased surface area adsorption and cation exchange capacity (Wijayawardena et al. 2016; Kumari and Mohan 2021; Sigmund et al. 2022), affecting soil fertility and contaminant mobility (Sigmund et al. 2022). Silty soils provide a similar but reduced, long-lasting supply of nutrients to microbial communities through slower solute diffusion due to smaller particles and denser pore structure (Matichenkov et al. 2020). In contrast, sandy soils exhibit increased leaching, which results in more transient availability of both nutrients and contaminants (Nunan et al. 2017).

Imidacloprid is the most widely used neonicotinoid insecticide and has been extensively studied in the context of soil microbial responses (Akter et al. 2023; Wang et al. 2023). It has low volatility and bioaccumulation potential, with a propensity to leach through the soil profile due to its weak sorption properties (Fouad and Abdel-Raheem 2024; Zhang et al. 2018). The mobility of imidacloprid is particularly enhanced in soils with low organic matter, where reduced adsorption increases leaching potential (Bernardino et al. 2021). Its persistence in soil is highly variable, ranging from 40 days to over 2 years depending on physicochemical properties (Broznić and Milin 2013; Sarkar et al. 2001). Bonmatin et al. (2015) detected imidacloprid in 100% of soils sown with treated seeds in the same year and in 97% of soils one to 2 years after application, with accumulation observed in consecutively treated plots. Prolonged exposure to imidacloprid can lead to shifts in microbial diversity (Cycoń and Piotrowska-Seget 2015b; Castillo-Díaz et al. 2017), affecting critical microbial functions, including nutrient cycling, nitrogen fixation, and organic matter decomposition (Cycoń and Piotrowska-Seget 2015a). These disruptions in microbial processes can ultimately influence soil fertility and plant growth, and therefore, sustainable agriculture and ecosystem stability. Understanding the impacts of imidacloprid on soil microbiota, specifically bacteria, across different soil textures over time will provide greater clarity on the broader environmental consequences of neonicotinoids. In this context, soil physicochemical properties play an important role, as they influence the mobility, sorption and persistence of chemicals in soils (Du et al. 2022; Korz and Muñoz 2025), and are likely to affect bacterial responses to neonicotinoids across different soil textures.

In this study we aimed to (i) identify bacterial taxa and community features (indicator taxa, co-occurrence patterns and predicted functional pathways) associated with imidacloprid exposure across three soil textures, thereby examining how bacterial responses vary among soils, including temporal variation in diversity and community structure, and (ii) identify the relationships between bacterial diversity and key soil properties including pH, electrical conductivity (EC), organic matter (OM), total carbon (C), total nitrogen (N), available phosphorus (P), exchangeable calcium (Ca), exchangeable magnesium (Mg), exchangeable potassium (K), effective cation exchange capacity (ECEC), exchangeable sodium percentage (ESP) and Ca:Mg ratio.

We hypothesised that bacterial responses to imidacloprid would vary across soil textures over time due to differences in physicochemical properties influencing imidacloprid availability and microbial habitat conditions, and that these responses would be reflected in both community structure and functional potentials.

2 | Materials and Methods

2.1 | Neonicotinoid and Seed Material

Analytical-grade standard of imidacloprid ($C_9H_{10}ClN_5O_2$; ≥98.0% purity, HPLC) was procured from Sigma–Aldrich Pty Ltd., Australia and stored according to the manufacturer's recommendations (Sigma–Aldrich 2022). Wheat seeds (*Triticum aestivum* L.) were purchased locally from Canberra, Australia and stored in a cool, dry laboratory environment until use.

2.2 | Soil Sampling and Preparation

Three soil samples with varying textures were collected from undisturbed, shaded pastures in Canberra (35.28° S, 149.13° E) and Narrabri (30.33° S, 149.78° E), Australia, which have Köppen–Geiger climate classifications of temperate oceanic (Cfb) and hot semi-arid (BSh), respectively (Beck et al. 2018). All collection sites had no history of pesticide application. Soil samples were collected from the surface (A horizon: ~0.2 m), and initial field hand texturing provided a quick textural assessment to ascertain suitability. The samples were then transported to the Fenner School of Environment and Society laboratory at the Australian National University, Australia, for detailed analysis.

All samples were air-dried on the laboratory bench for 1 week. As the Canberra soils were weakly aggregated, most aggregates loosened during sieving. In contrast, due to the strong aggregation of the Narrabri high-clay soil, larger aggregates were mechanically ground using a jaw crusher. Each soil type was then homogenised to create composite samples and sieved through a ≤2 mm mesh to remove dried leaves, roots and other debris. Particle size was determined using the modified hydrometer method (Carter and Gregorich 2007) and their textures were determined to be loamy sand, sandy loam and clay (Soil Science Division Staff 2017). The loamy sand and sandy loam soils originated from Canberra and were classified as red Luvisols, while the clay soil from the Narrabri site was classified as a Vertisol (IUSS Working Group WRB 2015).

Thirteen soil physicochemical properties were analysed from composite samples of each soil. These included pH, EC, OM, total C, total N, C:N ratio, available P, exchangeable Ca, exchangeable Mg, exchangeable K, ECEC, ESP and Ca:Mg ratio. The analytical methods used for these measurements are provided in Table S1. A summary of the soil physicochemical and textural properties is presented in Table 2.

2.3 | Experimental Design

Wheat seeds were oven-dried at 100°C for 1 h to prevent germination and associated uptake of imidacloprid by emerging seedlings, thereby maintaining consistent distribution of the insecticide within the soil across pots. Germination tests were performed prior to the trial to confirm the efficacy of heat sterilisation.

Forty-gram samples of soil were dispensed into 30 mL polypropylene pots and compacted for uniformity using a penetrometer set to a pressure of ~294 kPa. Before adding the soil, two 1.5 mm diameter holes were drilled in the sides of the pot to allow targeted sampling of soil in close proximity to the treated seeds at the designated sampling time. The holes were sealed with adhesive tape until sampling.

Wheat seeds were treated with imidacloprid at commercial application rates of 120 mL of 60% active ingredient per 100 kg seeds (Bayer CropScience Australia 2023). Two imidacloprid-treated seeds were planted in each pot, and initial soil moisture was adjusted to 32 g/100 g on a gravimetric basis using deionised water to provide sufficient moisture for biological processes without causing waterlogging, ensuring uniform conditions across all pots. Moisture was not re-adjusted during incubation. Following watering, the pots were covered and held in darkness at a constant room temperature of 22°C to maintain stable conditions.

Arranged in a randomised complete block design, each soil type had 15 pots that contained neonicotinoid-treated seeds and 15 pots with non-neonicotinoid seeds. Soil sub-samples were collected at five intervals (Days 3, 7, 14, 21 and 28) with sampling initiated at the same time each day. Control and imidacloprid-treated soils were sampled concurrently at each time point, allowing treatment effects to be distinguished from temporal changes during incubation. Triplicate samples were included in each treatment and control group at each sampling time.

On each sampling day, six pots per soil type assigned to that time point were removed from the experiment, and ~0.25 g of soil was withdrawn from each pot using a mini spatula through the pre-drilled holes in the pot walls. Soil samples were immediately processed for DNA extraction. Each pot was sampled once at its assigned time point.

2.4 | DNA Extraction and Targeted Amplicon Sequencing

Genomic DNA (gDNA) was extracted from soil samples using the DNeasy PowerSoil Pro Kit, following the manufacturer's protocol (Wu 2022). DNA concentrations were determined using a Qubit 4 Fluorometer (Thermo Fisher Scientific), and quality was assessed with a NanoDrop Lite Spectrophotometer. The V1–V3 regions of the 16S rRNA gene were amplified using primers 27f (Lane 1991) and 519r (Lane et al. 1985) and sequenced at the Ramaciotti Centre for Genomics (UNSW, Australia) using the Illumina MiSeq platform with 2×300 base pair read lengths.

2.5 | Bioinformatics Analyses

Illumina-derived paired-end FASTQ files were analysed following prior demultiplexing and removal of barcodes, adapters and indices. Primers were removed using Cutadapt (v2.10) (Martin 2011). The paired-end demultiplexed data were processed with the DADA2 workflow (Callahan et al. 2016), including quality filtering based on truncation lengths and expected error thresholds. Reads were truncated to 275 base pairs for forward and 235 base pairs for reverse with an expected error rate of 2 and 5, respectively. Pseudo-pooling ('pool = "pseudo"') was used for sequence inference after dereplication and error estimation. Reads were merged with a minimum 10 bp overlap followed by removal of chimaeras. A final amplicon sequence variant (ASV) table was obtained, and taxonomy was assigned using version 138.1 of SILVA (McLaren and Callahan 2021).

Across samples, sequencing depths ranged from 1274 to 12,110 reads (Table S2). Observed ASV richness across sequencing depth is illustrated using rarefaction curves in Figure S1. All analyses were performed in R Studio v4.4.2 (R Core Team 2024). The sequence data have been uploaded to the Sequence Read Archive of NCBI (PRJNA1251260).

2.6 | Functional Prediction

Functional pathway abundance was predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) pipeline (Douglas et al. 2020). First, study sequences were placed into a reference tree using the 'place_seqs.py' tool to assign sequences to a phylogenetic tree. Hidden-state predictions for 16S rRNA gene copy numbers were made using the 'hsp.py' tool. Functional predictions for EC numbers and KEGG orthologs (KOs) were obtained based on marker gene abundances.

Pathway abundances were inferred using the 'pathway_pipeline.py' function, which integrates the predicted EC numbers and KOs into bacterial pathway profiles. All pathway predictions were based on the MetaCyc reference database embedded within the PICRUSt2 pipeline. The resulting pathway abundance data were then used for downstream analysis.

2.7 | Data Processing and Statistical Analyses

The non-chimeric sequence table and taxonomy table from the DADA2 pipeline were exported along with metadata to create a phyloseq object using v1.48 phyloseq package (McMurdie and Holmes 2013). Filtering steps retained bacterial taxa while removing chloroplast and mitochondrial sequences, along with samples containing no ASVs. A sequence-derived phylogenetic tree was generated from ASV representative sequences using 'DECIPHER' for alignment (Wright 2024) and 'phangorn' for tree inference (Schliep 2011) and merged with the object, which was then saved for downstream analysis.

To assess alpha diversity, the Chao1 richness estimator and Pielou's Evenness index were calculated, while phylogenetic

diversity was evaluated using Faith's Phylogenetic Diversity (Faith's PD) and Mean Pairwise Distance (MPD). Each diversity measure was modelled using linear mixed-effects models (LMMs), with treatment, soil texture and time as fixed effects and replicate as a random effect. Analysis of Deviance was performed to assess the significance of these factors, and Dunnett's test was used for pairwise comparisons between control and imidacloprid treatments across different soils and sampling days. Indicator Species Analysis was performed using the 'multipatt' function from the indicspecies package (Cáceres and Legendre 2009) to identify taxa that were significantly associated with combinations of soil-treatment categories. The results were filtered to retain taxa with an IndVal greater than 0.7 and p values ≤ 0.05, indicating strong indicator species for specific conditions. Ecological Distance-Based Redundancy Analysis (dbRDA) was performed to explore the bacterial community composition using the Weighted UniFrac distance matrix, which is based on relative abundances and accounts for differences in sequencing depth. Permutational multivariate analysis of variance (PERMANOVA) was applied to evaluate the statistical significance of the main effects (treatment, soil and day) and their interactions (Anderson 2017). The relationship between bacterial communities and soil properties was explored by correlating the Shannon diversity index with various soil properties. A random forest model was applied to assess the importance of soil properties in explaining the variation in Shannon diversity. To control for potential confounding factors, a Partial Mantel test was performed to evaluate the influence of soil properties on bacterial community structure, while accounting for treatment, soil type and sampling day as covariates. The co-occurrence network analysis was conducted by aggregating the taxa at the Order level using Spearman's rank correlation. Only correlations meeting thresholds of p values ≤ 0.05 and $|\text{rho}| \geq 0.8$ were considered significant. Network parameters, including transitivity, average path length, modularity and network diameter, were calculated for each treatment and soil type. Differential abundance analysis (DAA) of predicted functional pathway abundance was performed using the 'edgeR' method, with significance determined based on Benjamini–Hochberg (BH) false discovery rate (FDR)-adjusted p values. All figures were generated in R using 'ggplot2' (Wickham 2016), 'ggridges' (Wilke 2024), 'ggvenn' (Yan 2023), 'igraph' (Csárdi et al. 2025; Csárdi and Nepusz 2006) and 'vegan' (Oksanen et al. 2024) along with related packages.

3 | Results

3.1 | Diversity Profiles in Different Soil and Imidacloprid

Analysis of deviance revealed a significant effect of soil on Chao1 richness (Table S3; Figure 1a), indicating that bacterial richness varied across different soil textures. However, Dunnett's test did not detect significant differences between the control and imidacloprid-treated samples in relation to any soil texture. Contrastingly, Pielou's evenness was significantly influenced by time (Table S4; Figure 1b), suggesting temporal shifts in community distribution. A treatment effect was detected only in clay soil on Day 21, where evenness was reduced under imidacloprid compared with the control (estimate = −0.08, $p = 0.003$). No other treatment-related differences were observed across soils or time points. Phylogenetic diversity metrics (Faith's PD and MPD) showed limited and inconsistent responses across soils and time, with no clear overall treatment effects. A significant increase in MPD was observed in sandy loam soil on Day 21, indicating a localised shift in phylogenetic structure (Tables S5–S6; Figure S2b).

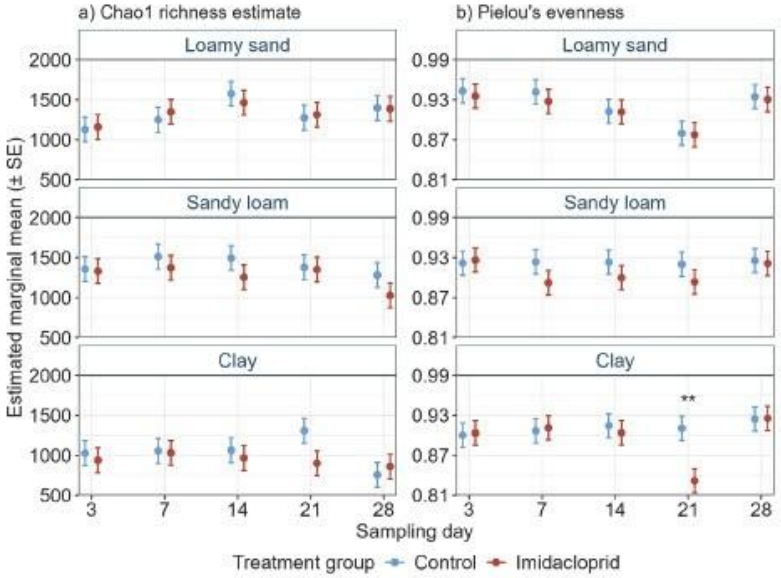

FIGURE 1 | Changes in bacterial alpha diversity across soil textures and treatments over time. (a) Chao1 richness and (b) Pielou's evenness. Points represent estimated marginal means ± SE ($n = 3$).

3.2 | Changes in Bacterial Community Composition and Structure

3.2.1 | Indicator Species Analysis

A total of 1668 bacterial ASVs were identified through indicator species analysis (IndVal > 0.7, $p \leq 0.05$; Figure 2). Across soils, most ASVs showed no significant association with either treatment, and many were shared between control and imidacloprid treatments, while only a small proportion were uniquely associated with either group. This pattern varied among soil textures. In loamy sand, 10 ASVs (0.6%) were uniquely associated with the control and 20 (1.2%) with imidacloprid, while in sandy loam, 28 ASVs (1.7%) were unique to the control and 13 (0.8%) to imidacloprid. The strongest imbalance occurred in clay soil, where 20 ASVs (1.2%) were uniquely to the control and only one ASV (0.1%) was uniquely associated with imidacloprid. Most ASVs in clay were either non-indicators (66.8%) or shared between treatments (32.0%). This pronounced asymmetry indicates that bacterial taxa in clay soil were more strongly associated with the control than with imidacloprid treatment, with very few taxa showing consistent association with the treatment condition. The taxonomic distribution of these indicator species analyses is shown in Table S7.

3.2.2 | dbRDA Analysis of Community Structure

Analysis based on dbRDA scores using PERMANOVA revealed a dominant effect of soil texture on bacterial community structure ($R^2 = 0.623$, $p = 0.001$), with additional contributions from the soil×sampling day interaction ($R^2 = 0.082$, $p = 0.001$) and sampling day ($R^2 = 0.055$, $p = 0.001$), while the treatment effect was small ($R^2 = 0.008$, $p = 0.05$). The dbRDA plot (Figure 3) visually supported these findings, showing distinct clustering of bacterial communities by soil texture along the constrained axes. Loamy sand samples were positioned at lower CAP2 values, while sandy loam samples clustered at higher CAP2 values. Clay soils formed a distinct cluster in the positive region of CAP1, with slightly positive CAP2 values, separating them from the other soil types. CAP1 and CAP2 together explained 78.9% of the constrained variation (CAP1: 59.4%, CAP2: 19.5%), corresponding to 48.1% and 15.8% of the total variation, respectively, with additional variation captured by higher-order axes. Within each soil type, control and imidacloprid-treated samples largely overlapped, consistent with the small effect of treatment on community structure.

3.3 | Bacterial Community-Soil Properties Interaction

Soil physicochemical properties varied across the three soil textures and are summarised in Table 1.

According to the Random Forest model, 16.2% of the variance in Shannon diversity was explained by soil properties, with a mean squared residual of 0.109. Among the 13 soil properties tested, Mg ranked highest, followed by Ca, pH and ECEC, while OM, total N, ESP, available P and Ca:Mg consistently ranked as the most important predictors based on both %IncMSE and IncNodePurity metrics (Figure 4). These properties contributed relatively more to model performance and decision tree structure, indicating that bacterial diversity was associated with a suite of interrelated soil properties rather than a single dominant predictor. In contrast, K, EC and the C:N ratio showed negligible importance, suggesting limited predictive value for Shannon diversity in this system.

Partial Mantel analysis confirmed a significant relationship between soil physico-chemical properties and bacterial community structure after controlling for treatment, soil type and sampling day ($r = 0.36$, $p = 0.001$). Vector fitting onto the NMDS ordination (Figure 5) revealed that multiple soil physicochemical properties were differentially associated with bacterial community ($p \leq 0.032$). Strong gradients along NMDS1 were defined by ESP, Mg, pH, OM, C and Ca:Mg ($R^2 \geq 0.87$), while N and P were moderately associated with NMDS2 ($R^2 = 0.61, 0.50$, respectively). In contrast, C:N showed a weak association ($R^2 = 0.08$). These results indicate that bacterial community variation aligned with multiple, correlated soil physicochemical gradients.

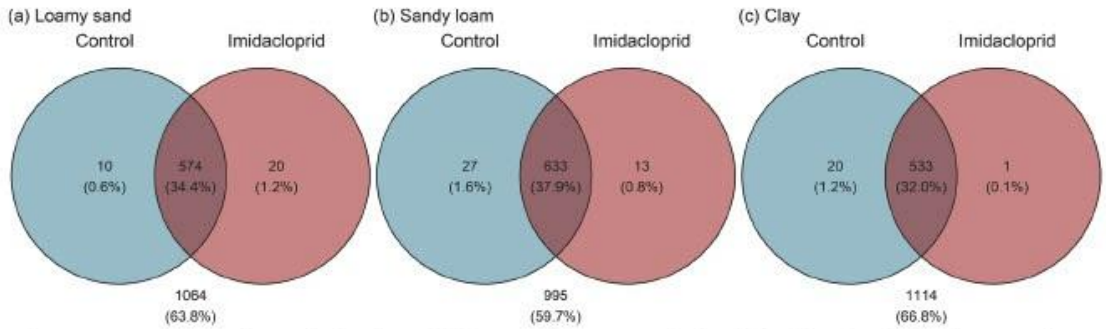

FIGURE 2 | Venn diagrams showing the distribution of indicator taxa between control and imidacloprid at ASV, order, family, genus, and species level across three soil-texture: (a) Loamy sand, (b) Sandy loam and (c) Clay (IndVal > 0.7; p value ≤ 0.05). The blue regions represent taxa unique to the control treatment, the red regions represent taxa unique to the imidacloprid treatment, and the overlapping areas indicate taxa that are significant for both treatments.

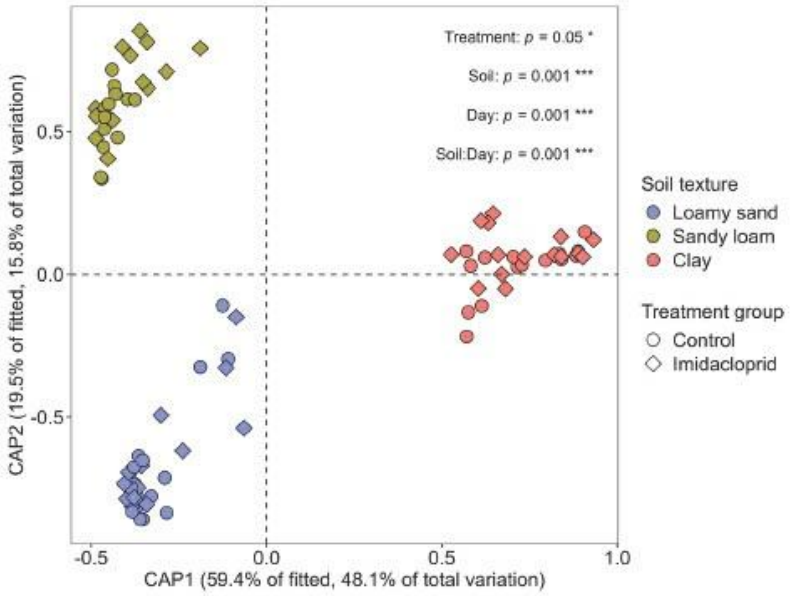

FIGURE 3 | Distance-based redundancy analysis (dbRDA) plot illustrating bacterial community structure using Weighted UniFrac distances. Colours correspond to different soil textures, while shapes represent the treatment groups. Significance levels are denoted as ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$.

3.4 | Co-Occurrence Network Analysis

Bacterial association patterns differed significantly across soil types and treatments. The topological characteristics of the networks were calculated and are summarised in Table 2.

In loamy sand soil, imidacloprid treatment was associated with a decrease in the number of edges (196 vs. 321 in control) and an increase in modularity (0.47 vs. 0.37), suggesting a less connected and more modular network structure. In sandy loam soil, imidacloprid treatment corresponded to an increase in edges (273 vs. 223 in control) and modularity (0.43 vs. 0.32 in control), suggesting a more connected and more modular network structure. In clay soil, the network under imidacloprid had fewer edges (57 vs. 94 in control) and higher modularity (0.63 vs. 0.44 in control), suggesting a less connected but more modular structure. The network plots for each soil type and treatment are presented in Figure 6, illustrating the network structure and providing a visual representation of the associations between taxa. These visual patterns are consistent with the numerical trends in Table 2, particularly the reduced connectivity and increased modularity, indicative of a more fragmented network structure observed in the clay soil under imidacloprid. The taxon names corresponding to the network nodes for each soil are provided in Tables S8–S10.

3.5 | Functional Pathway Abundance

The PICRUSt2-predicted functional pathway analysis identified a total of 427 pathways across the different soil textures. In loamy sand soil, 12 pathways exhibited significant changes ($p_{adj} \leq 0.05$) under neonicotinoid seed treatment (Figure 7). Key upregulated pathways included enterobacterial common antigen biosynthesis and superpathway of L-arginine, putrescine and 4-aminobutanoate degradation, consistent with stress-related functions. Conversely, lactose and galactose degradation I were significantly decreased, indicating shifts in predicted carbohydrate metabolism.

In sandy loam soil, 36 pathways showed significant changes ($p_{adj} \leq 0.05$) under imidacloprid treatment (Figure 8). Prominent upregulations included the superpathway of L-arginine, putrescine and 4-aminobutanoate degradation and the superpathway of L-tryptophan biosynthesis, while the superpathway of both taurine degradation and of pyrimidine ribonucleosides degradation was downregulated, indicating shifts in amino acid and nucleotide metabolism.

In clay-textured soil, 29 pathways exhibited significant changes ($p_{adj} \leq 0.05$) under imidacloprid treatment (Figure 9). Notable upregulation was observed in the superpathway of $(Kdo)_2$-lipid A biosynthesis and enterobacterial common antigen biosynthesis, consistent with stress-related functions. Several pathways, including adenosine nucleotides degradation IV and sucrose degradation II, were downregulated, indicating changes in predicted energy metabolism.

Overall, across soil types, pathway shifts consistently reflected increased stress-related functions alongside reductions in carbohydrate, nucleotide and energy metabolism, with more extensive changes in sandy loam and clay soils.

TABLE 1 | Physicochemical properties of the three soils used in the experiment.

Properties	Loamy sand (Red Luvisol)	Sandy loam (Red Luvisol)	Clay (Vertisol)
pH (1:5 H_2O)	6.33	6.71	8.74
EC (dS/m) (1:5 H_2O)	0.069	0.406	0.286
OM (g/100 g)	5.7	8.4	2.8
Total C (g/100 g)	3.3	5.1	1.7
Total N (g/100 g)	0.19	0.43	0.13
C:N ratio	17	11	12
Available P (mg P/kg soil)	35	232	20
Exchangeable Ca ($cmol_+$/kg)	6.3	18	25
Exchangeable Mg ($cmol_+$/kg)	1.9	3.5	15
Exchangeable K ($cmol_+$/kg)	0.44	1.4	1.3
ECEC	8.8	23	44
ESP	1.2	0.16	7.4
Ca:Mg ratio	3.4	5.1	1.7
Sand (g/100 g)	82.08	70.03	28.51
Silt (g/100 g)	6.79	13.94	15.24
Clay (g/100 g)	11.14	16.03	56.25

4 | Discussion

4.1 | Bacterial Community Dynamics

A reduction in bacterial community evenness was observed only in clay soil on Day 21 (Figure 1b), consistent with previous findings from Parizadeh et al. (2021), who reported significant reductions in bacterial evenness and richness in clay and clay loam soils exposed to thiamethoxam seed treatment. However, in our study, this pattern was not consistently observed across soils or time points. The reduction in evenness may be related to the higher adsorption capacity of clay soils, which may prolong localised microbial exposure to imidacloprid (Muema et al. 2016; Kızılkaya et al. 2004; Masini and Abate 2021; Dankyi et al. 2018). The clay-textured soil used in this study was a Vertisol, characterised by smectitic minerals and shrink-swell behaviour, properties that may further influence the retention and spatial distribution of imidacloprid within the soil matrix, potentially contributing to the observed bacterial responses (Muchaonyerwa et al. 2006; Niaz et al. 2023), although these processes were not directly measured in the present study.

Phylogenetic diversity showed limited and soil-specific responses. An increase in MPD was observed only in sandy loam soil on Day 21, indicating a localised shift in phylogenetic structure (Table S6; Figure S2b). This may reflect reduced competitive dominance or stress-induced suppression of dominant lineages, allowing the coexistence of phylogenetically diverse, stress-tolerant taxa (Webb et al. 2002) or increased influence of stochastic processes, such as random colonisation or ecological drift under chemical disturbance (Pérez-Valera et al. 2015; Chase and Myers 2011). Ecological filtering may also contribute to such patterns depending on trait distribution (Pavoine and Bonsall 2011). However, this increase was not observed at other sampling times or in other soil types.

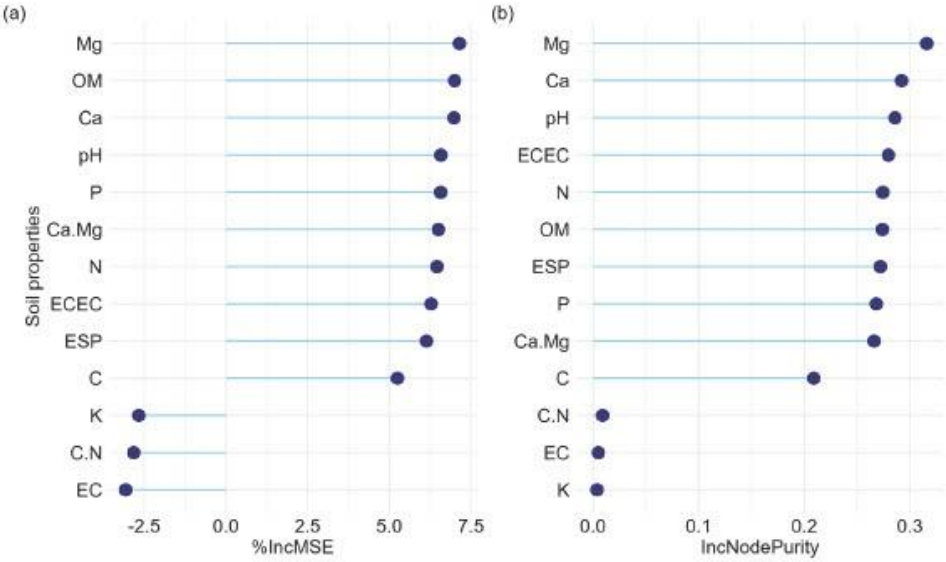

FIGURE 4 | Importance of soil physicochemical properties in predicting bacterial alpha diversity (Shannon index) based on random forest analysis. (a) Shows variable importance ranked by the percent increase in mean squared error (%IncMSE), and (b) shows importance based on increase in node purity (IncNodePurity).

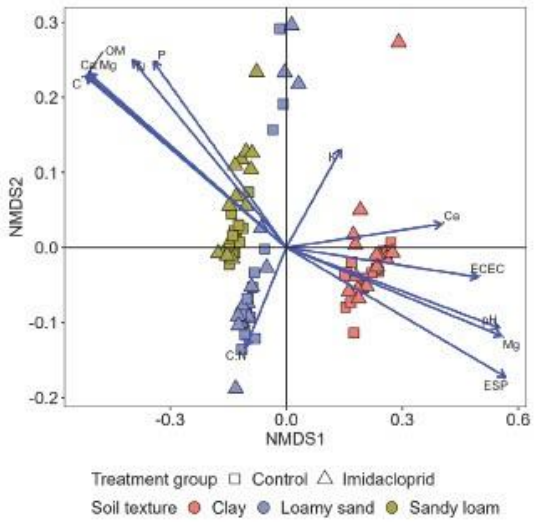

FIGURE 5 | Non-metric multidimensional scaling (NMDS) ordination of bacterial community structure based on weighted UniFrac distances. Points represent individual samples, coloured by soil texture and shaped by treatment group. Vectors indicate fitted correlations of soil physicochemical properties with community structure; arrow direction shows the gradient, and length reflects the strength of association.

Indicator species analysis showed that most taxa were shared between the control and imidacloprid treatments, indicating broad overlap in bacterial communities during the 28-day exposure (Figure 2). Taxa unique to each treatment may represent differential associations under untreated versus neonicotinoid-exposed conditions. Across all three soils, several taxa were strongly associated with control treatments, notably *Pseudomonas*, *Solirubrobacter*, *Ensifer*, *Marmoricola*, *Rhizobium*, *Microlunatus* and *Chryseolinea*. These taxa are known to play critical roles in nitrogen cycling, phosphorus mobilisation and organic matter degradation, suggesting potential shifts in taxa associated with nutrient-related functions under imidacloprid exposure (Khoshru et al. 2025; Jara-Servin et al. 2024; Fox et al. 2007; Potera 2007). This finding aligns with reports of reduced abundance of beneficial rhizosphere bacteria under neonicotinoid exposure (Parizadeh et al. 2021; Gorshkov et al. 2024). These patterns may suggest potential impacts on symbiotic nitrogen fixation and nutrient cycling, as N_2-fixing bacteria are particularly sensitive to imidacloprid (Cycoń and Piotrowska-Seget 2015a). In contrast, several genera were uniquely associated with imidacloprid-treated soils, including *Mycobacterium*, *Flavobacterium*, *Nakamurella*, *Noviherbaspirillum*, *Rhodococcus* and *Pseudarthrobacter*. These taxa are ecologically equipped to tolerate or metabolise toxic organic compounds like neonicotinoids (Ramírez et al. 2023; Alvarez et al. 2017). In clay soil, the strongest imbalance between control- and treatment-associated taxa was observed, with few taxa uniquely associated with imidacloprid, suggesting a less consistent microbial response to treatment.

The inherent differences among the loamy sand, sandy loam, and clay soil communities along with successional changes

TABLE 2 | Summary of topological characteristics of co-occurrence networks across soil textures and treatments.

Soils	Treatments	Nodes	Edges	transitivities	Average path lengths	Modularities	Graph densities	Network diameters	Average degrees	Clustering coefficients
Loamy sand	Control	63	321	0.57	3.04	0.39	0.16	8	10.19	0.65
	Imidacloprid	52	195	0.52	2.28	0.46	0.15	6	7.50	0.61
Sandy loam	Control	53	223	0.46	2.54	0.31	0.16	6	8.42	0.52
	Imidacloprid	54	268	0.49	2.70	0.43	0.19	8	9.93	0.51
Clay	Control	35	94	0.53	1.98	0.44	0.16	4	5.37	0.68
	Imidacloprid	35	57	0.32	3.68	0.63	0.10	9	3.26	0.37

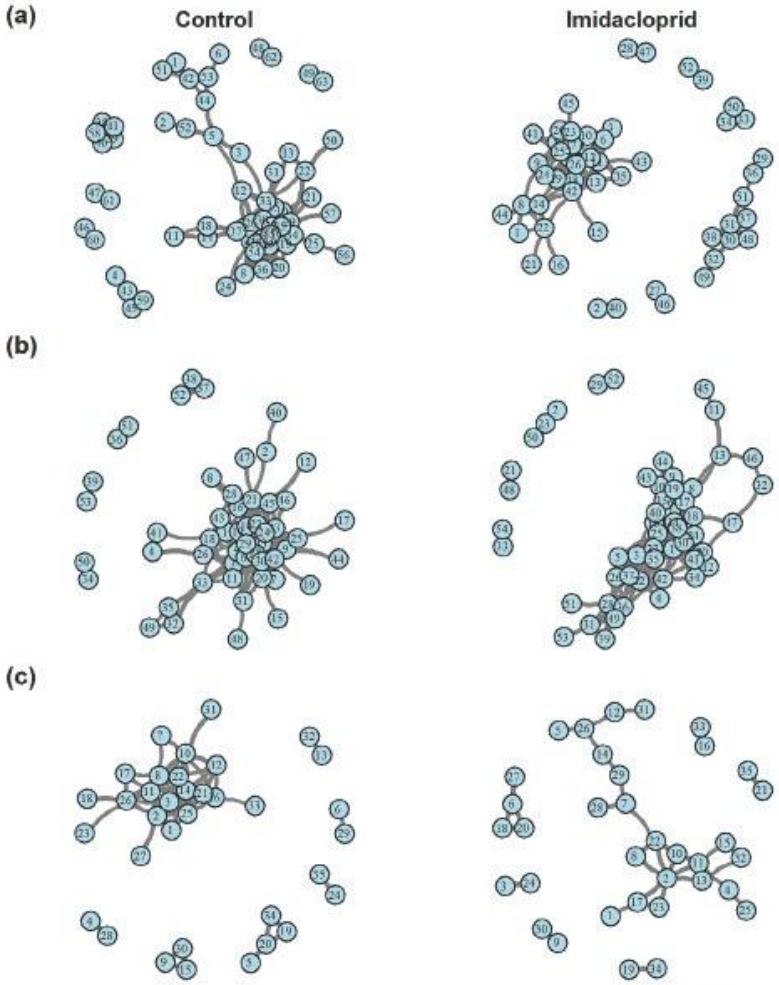

FIGURE 6 | Co-occurrence networks for bacterial taxa at the order level in different soil types and treatments. (a) Loamy sand, (b) Sandy loam and (c) Clay soil under control and imidacloprid treatments. Nodes represent microbial taxa, and edges represent significant interactions between taxa ($|rho| \geq 0.8$, $p \leq 0.05$).

over the 28-day period accounted for substantially more variation in microbial profiles than the presence or absence of the pesticide (Figure 3). This result underscores that the baseline edaphic properties and temporal dynamics of the microcosms outweighed the treatment effect on the microbiome. Such findings are consistent with broader ecological patterns, where soil physico-chemical properties, particularly texture, exert strong selective pressure on microbial communities, leading to distinct assemblages in sands versus clays, regardless of experimental treatments (Liu et al. 2024). This emphasises the critical role of soil texture in structuring bacterial communities, consistent with previous research that has demonstrated the importance of soil characteristics in paichongding-treated soils (Cai et al. 2016). Similarly, a study applying thiamethoxam in two soil types, silt loam and silt clay, found that both soil texture and time significantly shaped microbial community structure, with pesticide effects varying across soil types and over time (Wu et al. 2021). Soil texture may also influence imidacloprid behaviour through sorption–desorption processes that differ between sandy and clay-rich soils, which may contribute to the muted treatment effects observed across soils (Krohn and Hellpointner 2002). In finer-textured soils (e.g., our vertisol soil), greater sorption of imidacloprid to soil particles, particularly clay surfaces, has been reported (Fouad and Abdel-Raheem 2024), with organic matter acting as an important sorptive medium for imidacloprid (Didović et al. 2022).

These findings further highlighted that inherent soil properties strongly shape microbial diversity and community structure (Table 1; Figures 4 and 5). High OM and total carbon C create abundant energy sources and microhabitats, often supporting greater bacterial diversity (Tian et al. 2021; Bastida et al. 2021). Likewise, richer N and P availability can promote diverse microbial growth, as soil bacterial diversity and composition are largely driven

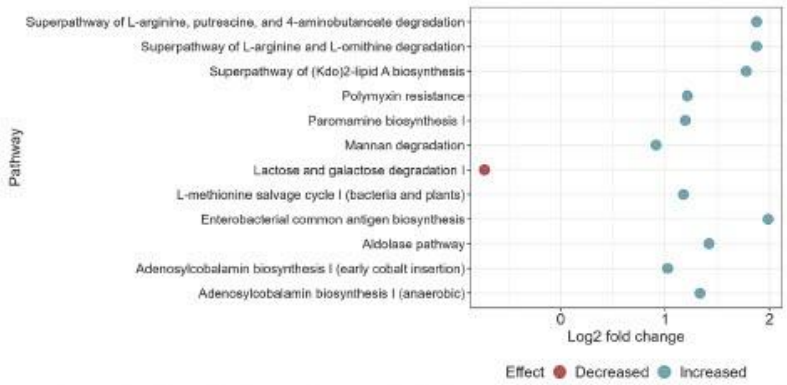

FIGURE 7 | Changes in functional pathway abundance in loamy sand soil. The *x*-axis shows the log2 fold change (log2FC) in abundance, while the *y*-axis represents significant ($p_{adj} \leq 0.05$) individual pathways. Blue points indicate pathways predicted to be more abundant than the control, whereas red points indicate pathways predicted to be less abundant.

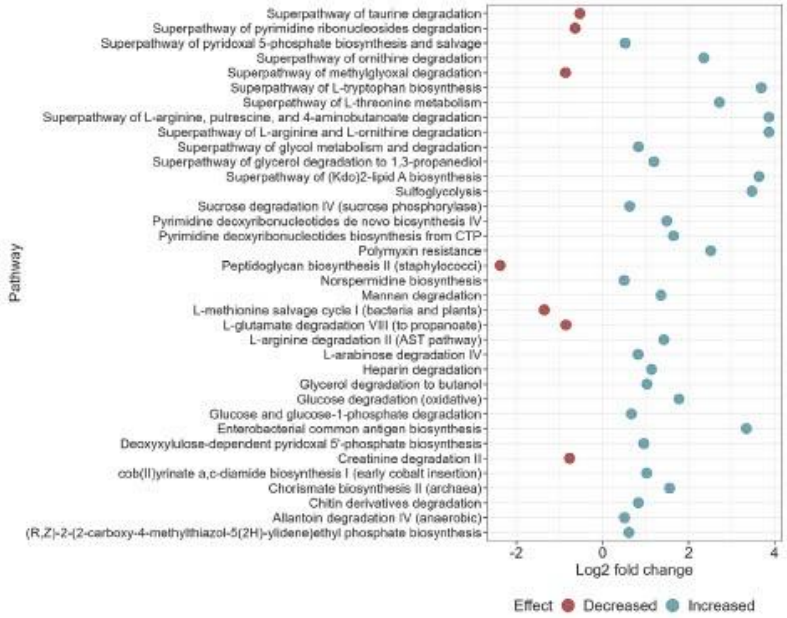

FIGURE 8 | Changes in functional pathway abundance in sandy loam soil. The *x*-axis shows the log2 fold change (log2FC) in abundance, while the *y*-axis represents significant ($p_{adj} \leq 0.05$) individual pathways. Blue points indicate pathways predicted to be more abundant than the control, whereas red points indicate pathways predicted to be less abundant.

by total C, N and P stoichiometry (Zhang et al. 2023; Delgado-Baquerizo et al. 2017). The association of sandy loam soils with nutrient-related soil properties suggests that nutrient-enriched soils select for microbial communities adapted to exploit these resources. Consistent with these patterns, clay soils with elevated Ca, Mg, pH, EC and ESP harboured distinct communities tolerant of high base cations (Tang et al. 2019; Kaiser et al. 2016; Zhang et al. 2024). The strong soil physicochemical predictors identified likely reflect underlying environmental gradients associated with substrate availability and habitat heterogeneity, explaining the observed differences in bacterial communities across soil textures.

4.2 | Bacterial Community Network Structure

Consistent with previous studies, correlation-based patterns may reflect co-oscillation of taxa in response to shared environmental conditions rather than direct relationships, and therefore caution is needed in their interpretation (de Vries et al. 2018; Freilich et al. 2018). Imidacloprid exposure induced significant changes in the microbial co-occurrence network across different soil types. Loamy sand and clay soils showed pronounced network fragmentation, with imidacloprid greatly reducing the number of edges, signifying fewer microbial co-occurrences,

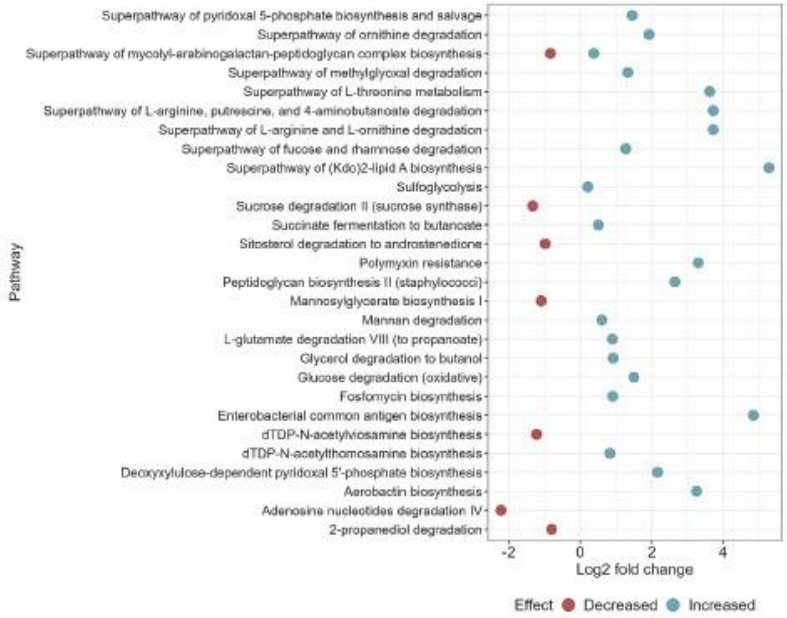

FIGURE 9 | Changes in functional pathway abundance in clay-textured soil. The *x*-axis shows the log2 fold change (log2FC) in abundance, while the *y*-axis represents significant ($p_{adj} \leq 0.05$) individual pathways. Blue points indicate pathways predicted to be more abundant than the control, whereas red points indicate pathways predicted to be less abundant.

and increasing modularity, leading to more compartmentalised subnetworks. Such fragmentation suggests that many associations were disrupted, isolating microbes into smaller modules, as observed in similar findings where thiamethoxam was applied in clay soil (Zhang, Zhang, et al. 2021). In the clay-textured soil, lower microbial connectivity under imidacloprid may also be influenced by the inherently discontinuous macropore networks of Vertisols, which are known to limit air diffusion and isolate microbial habitats in swelling clays (Ruan et al. 2021). In sandy loam textured soil, by contrast, imidacloprid increased the number of edges alongside higher modularity, hinting that association patterns were reorganised, even as the network became partitioned into modules. This could reflect selection for stress-tolerant microbes that associate strongly with each other, thereby compensating for the loss of sensitive species (Li et al. 2022). These network shifts align with broader patterns of microbial responses to agrochemicals, which have been associated with disrupted symbioses and loss of keystone taxa that maintain community structure, which can impair ecosystem functions reliant on microbial interactions (Lv et al. 2025; Meyer et al. 2024; Zhang, Song, et al. 2021).

4.3 | Functional Pathway Changes Across Soils

The functional profiles presented in this study therefore reflect inferred metabolic potential rather than direct measurement of bacterial activity. Under imidacloprid exposure, each soil type showed a distinct yet convergent shift in microbial functional profiles consistent with stress adaptation. In all soil textures analysed, pathways linked to stress resilience and catabolism of amino compounds were enriched, whereas those for carbohydrate and nucleotide utilisation were repressed. For example, the significant upregulation of polyamine degradation (the L-arginine/putrescine/4-aminobutanoate superpathway) suggests activation of a general stress response, consistent with altered polyamine metabolism of microbes under adverse condition (Niu et al. 2024) and previous evidence that polyamine catabolism contributes to bacterial stress responses (Schneider et al. 2013). Likewise, increased biosynthesis of gram-negative cell envelope components (e.g., enterobacterial common antigen and Kdo_2-lipid A) is consistent with potential cell envelope fortification under chemical stress, in line with observations for difenoconazole pesticide pressure, which induced membrane-associated protective functions (Feng et al. 2025). Conversely, the downregulation of pathways for complex sugar breakdown (e.g., lactose/galactose, sucrose) and nucleotide metabolism indicated metabolic reorganisation under imidacloprid exposure. These patterns aligned with reports that pesticide-exposed soil communities prioritised stress-defence and detoxification mechanisms such as contaminant degradation enzymes at the expense of growth-oriented metabolism (Senabio et al. 2024). While the specific pathways affected varied across loamy sand, sandy loam and clay, the overall functional response was one of community-level stress adaptation and metabolic reorganisation in the presence of imidacloprid. Ecologically, these functional shifts have profound implications for soil ecosystem processes. Future studies incorporating measurements of soil enzyme activities could further clarify how these predicted functional shifts translate into changes in soil biogeochemical processes, while direct quantification of imidacloprid residues would help to better characterise exposure dynamics and strengthen

interpretation of bacterial responses across contrasting soil textures.

5 | Conclusion

Imidacloprid exposure across loamy sand, sandy loam and clay textured soils induced soil-texture specific shifts in bacterial communities. Alpha diversity showed a context-dependent response to imidacloprid, with a reduction in community evenness observed only in clay soil at a single time point, while richness remained unaffected. Soil texture was the primary driver of community composition, with time contributing marginally. Indicator species analysis revealed that many bacterial taxa were shared between control and imidacloprid treatments, suggesting a degree of community stability, while certain taxa unique to each soil texture emerged as particularly sensitive or tolerant. Co-occurrence network analysis further demonstrated that imidacloprid was associated with changes in bacterial association patterns, resulting in less connected and more modular networks, characterised by reduced complexity and increased modularity, particularly in clay and loamy sand soils. In parallel, functional pathway prediction indicated a community-wide stress response, with upregulation of stress-related pathways, such as amino acid degradation and membrane biosynthesis, and concurrent downregulation of pathways involved in energy and nutrient metabolism, including carbohydrate and nucleotide degradation. Taken together, these findings illustrate that while overall taxonomic richness remained relatively stable, imidacloprid exposure substantially reorganised bacterial interactions and functional capacities under the applied conditions, a reorganisation that may diminish soil microbial resilience, decelerate nutrient cycling and ultimately impair long-term soil health. We emphasise, however, that the effects of imidacloprid exposure on soil bacterial communities were dependent on soil texture, which exerted a stronger influence on diversity, structure and function.

Author Contributions

Nilantha R. Hulugalle: conceptualization, methodology, supervision, writing – review and editing. **Sharmin Akter:** conceptualization, methodology, data curation, investigation, validation, formal analysis, visualization, project administration, writing – original draft, writing – review and editing. **Craig L. Strong:** conceptualization, methodology, supervision, project administration, writing – review and editing. **Julia Jasonsmith:** conceptualization, methodology, supervision, writing – review and editing. **James O. Latimer:** resources, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All raw sequencing data have been submitted to the NCBI Sequence Read Archive under the accession number PRJNA1251260. Supporting datasets and materials necessary to interpret the findings are included in the main manuscript and Supporting Information.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Analytical methods used to determine soil physicochemical properties. **Table S2:** Summary of sequencing depth across treatment, soil, and sampling day groups. **Table S3:** Analysis of deviance results for Chao1 richness estimate. **Table S4:** Analysis of deviance results for Pielou's evenness. **Table S5:** Analysis of deviance results for Faith's phylogenetic diversity (Faith's PD). **Table S6:** Analysis of deviance results for Mean pairwise distance (MPD). **Table S7:** Indicator taxa at the Order level across control and imidacloprid treatments in different soil types. Orders present in both treatments may represent distinct indicator ASVs differing at lower taxonomic ranks. **Table S8:** Taxon names corresponding to the nodes of the co-occurrence network for loamy sand soil, showing the treatments (control and imidacloprid). **Table S9:** Taxon names corresponding to the nodes of the co-occurrence network for sandy loam soil, showing the treatments (control and imidacloprid). **Table S10:** Taxon names corresponding to the nodes of the co-occurrence network for clay soil, showing the treatments (control and imidacloprid). **Figure S1:** Rarefaction curve showing sequencing depth per sample. **Figure S2:** Changes in phylogenetic diversity of bacterial communities across soil textures and treatments over time. (a) Faith's phylogenetic diversity (PD) and (b) mean pairwise distance (MPD). Points represent estimated marginal means ± SE ($n = 3$).

Chapter 6 Fungal responses to neonicotinoid seed treatments in soil: Temporal shifts in community diversity and composition

Abstract[4]

Neonicotinoid seed treatments are widely used in agriculture, yet their effects on soil fungal communities remain underexplored. This study investigates how three commonly applied neonicotinoids- imidacloprid, thiamethoxam, and clothianidin alter fungal community diversity, composition, and temporal dynamics in soil microcosms. Amplicon sequencing of fungal ITS regions was performed on soil samples collected across multiple time points following neonicotinoid exposure. While overall diversity indices remained largely stable across treatments, post-hoc comparisons identified a transient reduction in fungal diversity under clothianidin exposure on day 2. Beta diversity analyses revealed significant effects of sampling day and a treatment-by-time interaction, indicating temporally variable responses. While dominant phyla such as Ascomycota and Basidiomycota remained relatively stable, several less abundant phyla, including Mortierellomycota, Rozellomycota and Olpidiomycota declined significantly over time. Functional guild analysis further revealed a clothianidin-driven increase in saprotroph abundance and a decrease in symbiotroph abundance. Differential abundance analysis revealed compound-specific responses. Imidacloprid was associated exclusively with suppressed fungal biomarkers, whereas thiamethoxam induced both enriched and depleted taxa. Clothianidin produced the strongest compositional response, with the greatest number of discriminatory fungal biomarkers, and was accompanied by increased saprotroph abundance and reduced symbiotroph abundance. These findings underscore the importance of including fungal community responses in ecological risk assessments and highlight the need for long-term, functionally oriented studies in agroecosystem contexts.

Keywords: Insecticide, soil fungi, ITS amplicon sequencing, fungal diversity, temporal dynamics

[4] This chapter has been submitted to *Microbial ecology* (June 2026; currently under review).

6.1 Introduction

Fungi are indispensable components of terrestrial ecosystems. Within soil, they perform essential ecological roles. Through the secretion of extracellular enzymes, they can degrade a broad spectrum of organic matter, facilitating decomposition and regulating soil carbon and nutrient balances (Burke et al., 2011; Zhao et al., 2021). This functional versatility underpins nutrient cycling, carbon sequestration, and plant health. Mycorrhizal fungi, in particular, form mutualistic associations with plant roots, enhancing uptake of nutrients like phosphorus and nitrogen, while many other fungi act as biocontrol agents against pathogens (Calderon & Dangi, 2024; Mishra et al., 2024; Weng et al., 2022). Soil fungi also encompass plant pathogenic taxa, reflecting the wide range of ecological functions performed by fungal communities (Frąc et al., 2018; Marín & Kohout, 2021; Rillig, 2026). Thus, fungal diversity and activity are central to maintaining soil fertility and ecosystem resilience. Beyond nutrient cycling, soil fungi also play a crucial role in detoxification and structural enhancement of soils. They assist in the breakdown of complex pollutants, including heavy metals and xenobiotics, through assimilatory and dissimilatory reactions, thereby restoring soil health in chemically degraded environments (Akhtar & Mannan, 2020; Wang et al., 2024b). Arbuscular mycorrhizal fungi contribute significantly to soil aggregation through the secretion of glomalin-related soil proteins, a group of glycoprotein reported to stabilise soil particles and enhance porosity, water retention and resistance to erosion (Agnihotri et al., 2022; Li et al., 2022b; Wu et al., 2014). These fungi also enrich the hyphosphere microbiome, fostering symbiotic networks that amplify nutrient flux and biotic interactions vital for sustainable agriculture (Zhang et al., 2024b).

Despite their ecological importance, soil fungal communities are increasingly exposed to anthropogenic pressures, including agrochemical use. Among modern pesticides, neonicotinoids are synthetic analogues of nicotine that act systemically and target insect nicotinic acetylcholine receptors, making them widely used as seed treatments to protect crops from early-season pests (Moffat et al., 2016; Roy et al., 2019). While their effectiveness in pest control is well established, their unintended impacts on non-target soil microbiota, particularly fungi, remain an area of growing concern.

Neonicotinoids are known to persist in soils and can be absorbed by plant roots (Wood & Goulson, 2017), migrating into the rhizosphere, the biologically active zone influenced by root exudates and rich in microbial interactions (Ji et al., 2025). Their systemic nature allows for continuous release from coated seeds into the surrounding soil, thereby exposing microbial communities over extended periods (Chagnon et al., 2015). Fungal taxa in this zone are

particularly susceptible due to their intimate association with plant roots and critical functional roles, including nutrient exchange, structural stabilisation and signalling functions vital to plant-microbe interactions (Sattiraju et al., 2023). Fungal communities, due to their physiological and ecological distinctiveness, may exhibit responses divergent from bacteria when exposed to chemical stress. For instance, *Phanerochaete chrysosporium*, a white-rot fungus, can degrade imidacloprid while also stimulating other microbial degraders, potentially altering community composition (Shang et al., 2023). Yet, not all fungi possess such detoxification capacities; many may undergo suppressed growth, disrupted sporulation, or a loss of competitive fitness, thereby altering soil functional potential (Malla et al., 2022).

Neonicotinoid exposure can modify soil microbial communities. These changes include reductions in microbial biomass carbon, disruptions in enzyme activity, and shifts in microbial populations (Mahapatra et al., 2017). Neonicotinoid seed treatments can reduce microbial alpha diversity and affect beneficial taxa during active crop growth phases (Parizadeh et al., 2021). Neonicotinoids can also alter microbial-mediated carbon and nitrogen cycling pathways (Yu et al., 2020) and reduce the abundance of keystone taxa (Briceño et al., 2024). A growing body of evidence also suggests that neonicotinoids negatively impact network complexity, although the emphasis has largely been on bacterial communities (Zhang et al., 2021b).

However, most studies rely on spiking of soils with neonicotinoids, often at concentrations exceeding field-realistic levels, and moreover, fungal responses to neonicotinoids remain significantly underrepresented and underexplored (Akter et al., 2023). The use of unrealistic exposure levels and limited fungal focus reduces the ecological relevance of existing findings and complicates efforts to perform accurate environmental risk assessments. Therefore, the current study seeks to address this critical knowledge gap by characterising the temporal dynamics of soil fungal communities following exposure to commercially formulated neonicotinoid seed treatments applied at field-realistic rates.

The main objective of this 10-day microcosm study was to investigate how neonicotinoid seed treatments affect soil fungal communities, specifically by: (i) assessing changes in community composition, structure, and diversity, and (ii) examining how these changes vary over time.

6.2 Materials and methods

Procedures relating to seed treatment, microcosm preparation, incubation, sequencing, and community-data processing are detailed in Chapter 3. The methods outlined below describe the components specific to the short-term fungal microcosm experiment.

6.2.1 Experimental variables

Four treatment conditions were applied using seeds coated with imidacloprid, thiamethoxam, or clothianidin, along with untreated controls. Soil sub-samples were collected on days 1 (pre-treatment), 2, 3, 5, 7, and 10 to track short-term shifts in fungal communities with five replicate pots sampled per treatment at each timepoint.

6.2.2 Bioinformatics

Sequences were processed using DADA2 ITS pipeline. Forward and reverse reads were trimmed by removing 50 and 80 nucleotides, respectively from the 3′ ends to eliminate terminal low-quality bases. Reads were then filtered using a maximum expected error threshold of 2 for forward and 3 for reverse reads. Paired-end reads were merged using a minimum overlap of 12 base pairs. Chimeric sequences were identified using a consensus-based approach that permitted one mismatch during detection and required parental sequences to be at least twice as abundant as potential chimeras, ensuring stringent removal of artefactual variants. Error correction was performed using pseudo-pooling.

6.2.3 Statistical analysis

Alpha diversity indices (Shannon, Simpson, Chao1, ACE) were computed to assess within-sample richness and evenness. Normality and homogeneity of variance were tested using the Shapiro-Wilk (Shapiro & Wilk, 1965) and Levene's tests (Levene, 1960), respectively. A linear mixed-effect model was used to assess the effect of treatments and sampling day (Gałecki & Burzykowski, 2013). To evaluate the significance of the fixed effect, an Analysis of Variance (ANOVA) was conducted (Fox & Weisberg, 2018), followed by Tukey-adjusted post-hoc pairwise comparisons of estimated marginal means to identify significant differences (Lenth, 2024). Beta diversity was estimated using Bray–Curtis dissimilarities (Bray & Curtis, 1957), visualised via Principal Coordinates Analysis (PCoA), and statistically evaluated with PERMANOVA (Anderson, 2017). Fungal ecological guilds were assigned using FUNGuild retaining only highly probable and probable classifications (Nguyen et al., 2016). Relative abundances of saprotrophs, symbiotrophs, and pathotrophs were calculated for each sample, with taxa assigned to multiple trophic modes partitioned equally among guilds. Treatment effects on guild relative abundance were assessed using beta regression with treatment and sampling day included as fixed effects (Cribari-Neto & Zeileis, 2010). Differentially abundant taxa were identified through linear discriminant analysis (Liu, 2013).

6.3 Results

6.3.1 Sequencing summary

Sequencing depths across samples ranged from 80 to 75046 reads (median = 36978). A summary table and barplot of sequencing depths are provided in Appendix D (Table D. 1, Figure D. 1). The sample with exceptionally low sequencing depth was excluded from subsequent analysis.

6.3.2 Alpha diversity patterns

The ANOVA results for Shannon entropy indicated no significant effects of treatment, sampling day, or their interaction (Table D. 2). In contrast, Simpson's dominance index, Chao1 richness and the ACE estimator showed a significant effect of sampling day (Table D. 2). Post-hoc contrasts revealed significant declines at Day 10 in Simpson's index (P = 0.03), Chao1 richness (P = 0.03), and ACE estimator (P = 0.03) under imidacloprid relative to Day 1. Similarly, under thiamethoxam, Chao1 richness (P = 0.02) and the ACE estimator (P = 0.02) were significantly lower at Day 10 compared to Day 1 (Table D. 3, Figure D. 2).

6.3.3 Multivariate patterns of fungal community structure

Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarities revealed substantial overlap among fungal communities across sampling days and treatments, indicating the absence of strong visual clustering. The first two principal coordinates explained 30.94% and 9.31% of the total variation in community composition, respectively (Figure 1–1).

Despite the visual similarity, PERMANOVA results demonstrated a significant effect of sampling day (R^2 = 0.10, P = 0.001) and a significant interaction between treatment and sampling day (R^2 = 0.15, P = 0.014), while the effect of treatment alone was not significant (P > 0.05). These findings suggest that although compositional differences were not visually distinct, fungal community structure was modestly shaped by temporal dynamics and their interaction with neonicotinoid exposure.

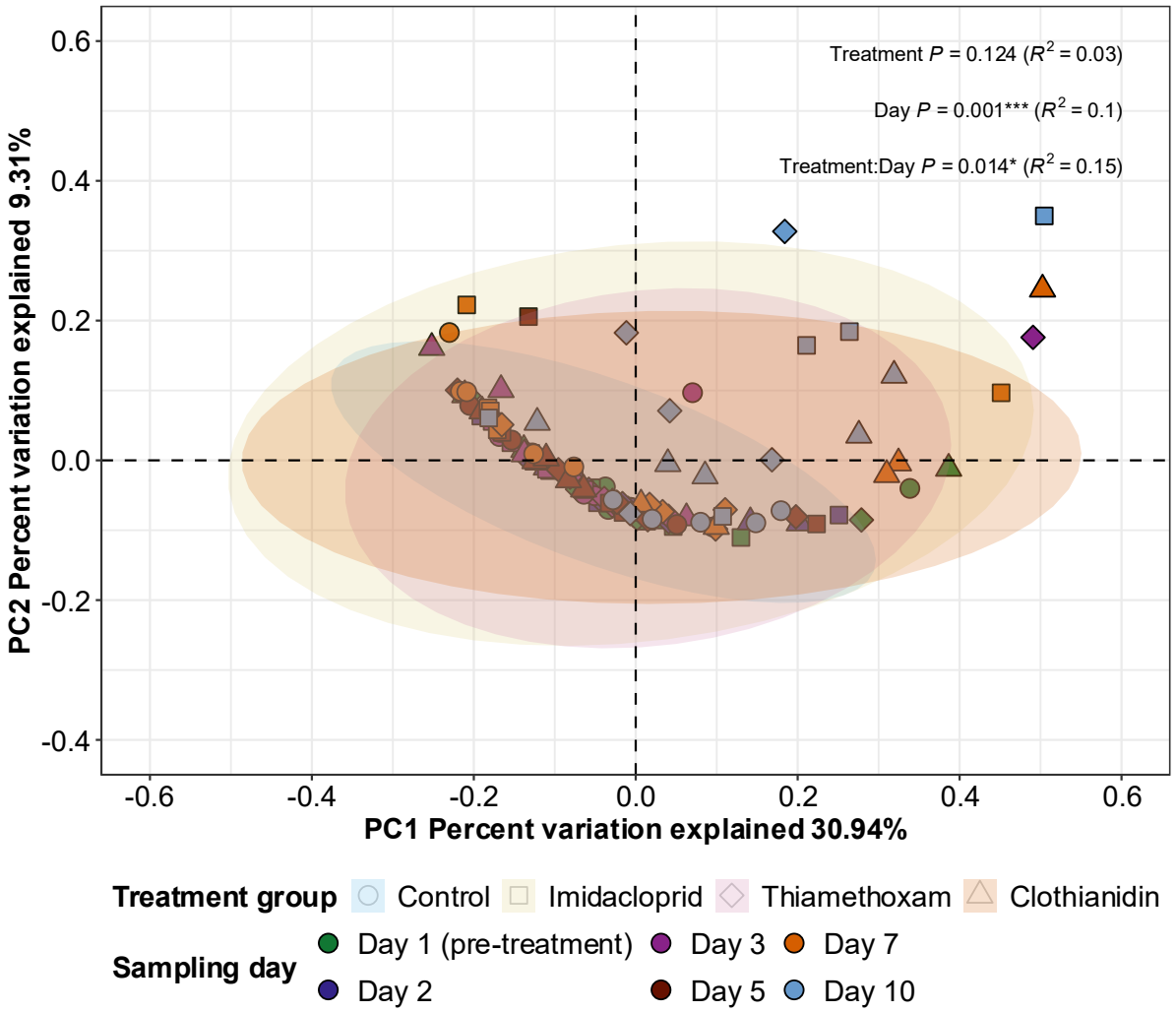

Figure 6–1: Principal Coordinates Analysis of fungal community structure based on Bray–Curtis dissimilarities. Each point represents an individual soil sample, with colours indicating sampling day and shapes representing neonicotinoid treatment. Ellipses denote 95% confidence intervals under a multivariate normal distribution

6.3.4 Relative abundance patterns

Analysis of relative abundance at the phylum level revealed that the fungal community was predominantly composed of Ascomycota (55–72%) and Basidiomycota (12–20%), which remained relatively stable across all sampling days and treatments (Figure 6–2a). Mortierellomycota, accounting for 9–16% of the community, showed a significant decline over time (P = 0.04, ANOVA), reflecting a temporal trend. Mortierellomycota, accounting for 9–16% of the community, showed a significant day effect (P = 0.01, ANOVA), with generally lower relative abundance at the end of the experiment. Rozellomycota and Olpidiomycota also varied significantly across sampling days (P = 0.03 an P = 0.05 respectively), although

Olpidiomycota occurred at very low relative abundance. No significant treatment effects were identified for any of these phyla. Several low-abundance phyla, including Zoopagomycota, Mucoromycota, and Blastocladiomycota, maintained relative abundances below 0.1% and did not exhibit statistically significant variation across treatments or time.

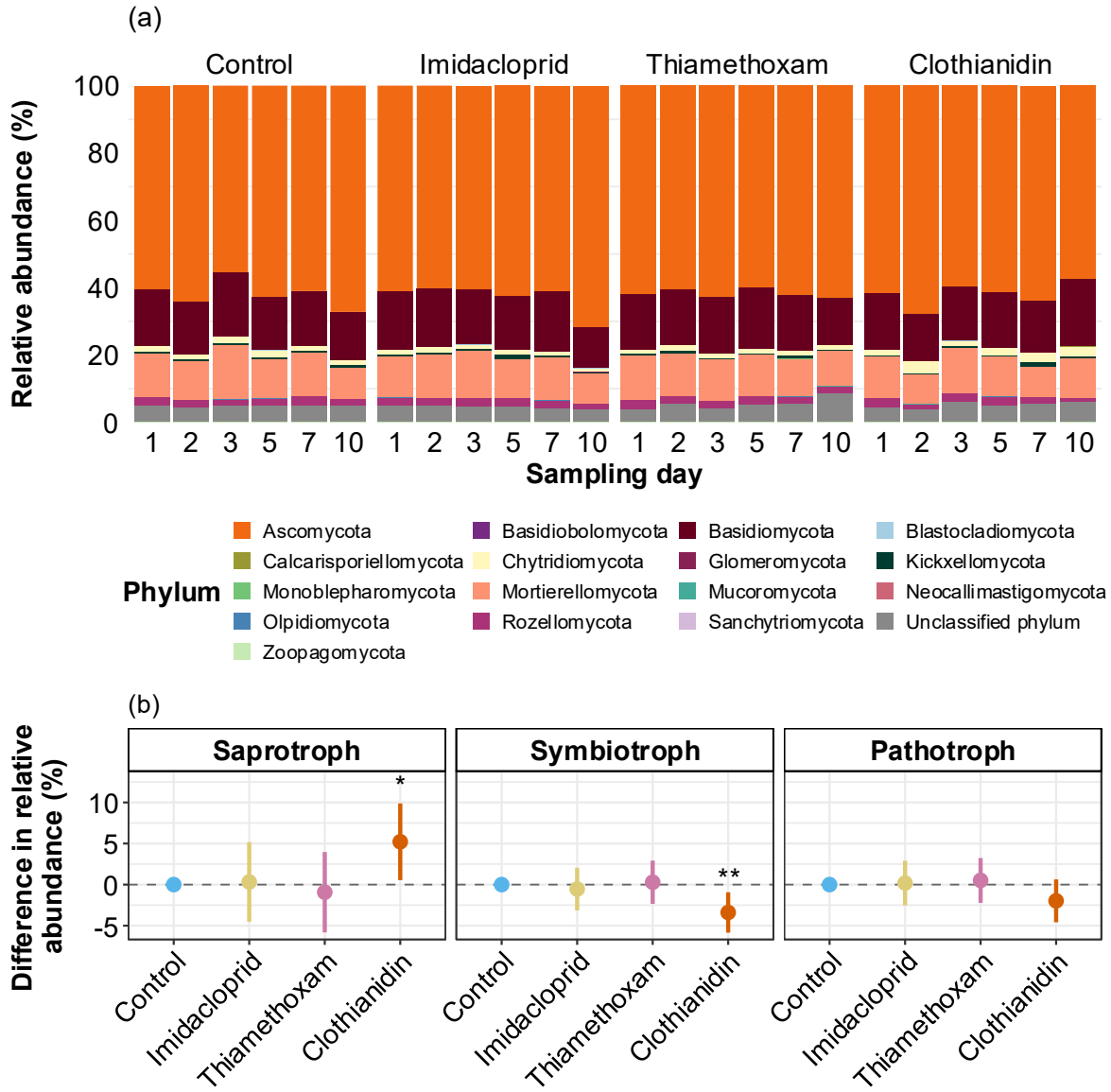

Figure 6–2: Taxonomic and functional responses of fungal communities to neonicotinoid seed treatments. (a) Phylum-level community composition normalised to relative abundance and averaged across quintuplicate soil microcosms for each treatment and sampling day. (b) Response-scale contrasts in fungal trophic guild relative abundance estimated from beta-regression models. Points represent percentage-point differences in relative abundance relative to the control treatment. The dashed line indicates no difference from the control. Asterisks indicate significant contrasts relative to the control treatment ('*' $P < 0.05$, '' $P < 0.01$).**

Functional guild analysis showed that saprotrophs were the dominant guild, accounting for 74.8% of assigned reads, whereas pathotrophs and symbiotrophs represented 12.7% and 12.5% of the community, respectively. While no treatment effects were detected at the phylum level, guild composition responded to clothianidin exposure (Figure 6–2b). After accounting for sampling day, clothianidin increased saprotroph abundance by 5.2 percentage points relative to the control ($P = 0.02$) and reduced symbiotroph abundance by 3.4 percentage points ($P = 0.003$). In contrast, no significant effects of imidacloprid or thiamethoxam were detected, and pathotroph abundance remained unchanged across treatments.

6.3.5 Discriminative fungal taxa identified by LEfSe analysis

Linear discriminant analysis effect size (LEfSe) identified several fungal taxa that responded significantly to neonicotinoid treatments, highlighting both enrichment and suppression patterns across taxonomic levels. Clothianidin produced the strongest taxonomic response, with 38 discriminatory fungal features identified, followed by imidacloprid with 19 features and thiamethoxam with 8 features (Figure 6–3).

Under imidacloprid exposure, all discriminatory features exhibited suppressed responses relative to the control. The strongest suppressed biomarkers included *Linnemannia gamsii*, Glomerellales, Plectosphaerellaceae, Sordariales, Helotiales and Chytridiomycota (LDA = −3.64 to −3.92). Thiamethoxam induced comparatively fewer responses, with enhanced biomarkers including *Terfezia* sp. and *Fusicolla* sp. (LDA = 3.07–3.47), whereas *Apiotrichum*, *Peziza*, *Humicola nigrescens* and Sordariomycetes were suppressed (LDA = −3.01 to −3.96). Clothianidin elicited both enhanced and suppressed responses, with Agaricomycetes, Microascales and Microascaceae exhibiting positive responses (LDA = 3.91–4.30), while Pleosporales, Pleosporaceae, Glomerellales, Plectosphaerellaceae, *Lophiotrema* and *Acrostalagmus* were among the most strongly suppressed biomarkers (LDA = −3.78 to −4.30).

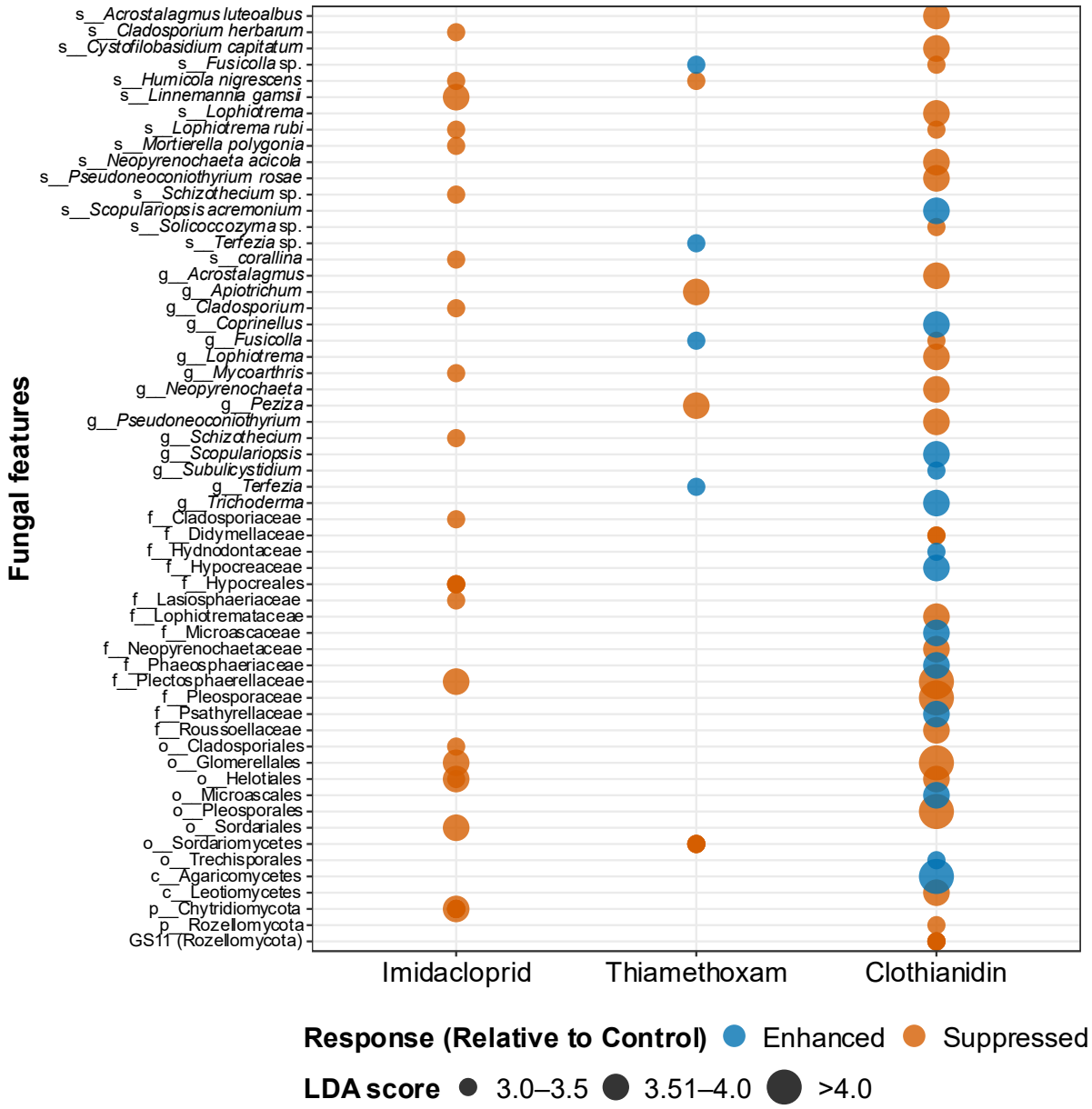

Figure 6–3: Differentially abundant fungal taxa identified through pairwise LEfSe analysis comparing each neonicotinoid treatment with the control.

6.4 Discussion

Neonicotinoid impacts on soil fungal communities are an emerging concern yet remain poorly understood compared to bacterial responses. Our findings demonstrate that exposure to three widely used neonicotinoids (imidacloprid, thiamethoxam, and clothianidin) can alter fungal community structure and composition, with effects shaped by temporal dynamics. The observed patterns suggest that while fungal communities exhibit some resilience in overall diversity, pesticide-induced compositional shifts may have significant ecological implications

The transient reduction in Shannon, Simpson, and ACE diversity indices under clothianidin exposure suggests a short-term disturbance to fungal community composition. Such short-term diversity losses, likely due to the acute sensitivity of certain taxa or inhibitory effects on fungal growth and sporulation, are commonly observed following chemical perturbation (Celar & Kos, 2016) and are consistent with previous reports of short-term microbial diversity disturbances after imidacloprid application (Li et al., 2018a). As the observed response was restricted to the early phase of the experiment, it is interpreted as a transient effect rather than evidence of a persistent shift in fungal diversity. The subsequent stabilisation of diversity over time indicates potential community resilience, likely mediated by functional redundancy and compensatory growth of tolerant taxa (Biggs et al., 2020; Branco et al., 2022). This resilience may be partly attributed to overlapping ecological roles among fungal taxa, where the decline of sensitive decomposers or root-associated fungi is buffered by other functionally similar organisms capable of sustaining key soil processes.

Soil fungal communities are known to exhibit pronounced temporal dynamics, driven by seasonal variation, plant phenology, and environmental fluctuations (Bowd et al., 2023; Fox et al., 2022; Hannula et al., 2019). In line with these patterns, we observed a significant effect of sampling day on fungal community, confirming that temporal variability is a key factor shaping community structure in our system. However, the explained variation was modest (R^2 = 0.10), which is typical in soil microbial studies, where temporal turnover often explains only a small fraction of community variance compared to more dominant spatial or environmental factors (Shade et al., 2013). A continental-scale analysis reported large seasonal changes in fungal communities, yet commonly measured covariates could explain only a limited portion of this turnover (Averill et al., 2019). Despite the modest explained temporal variation, the significant interaction between sampling day and neonicotinoid treatment indicates that fungal community responses to neonicotinoid exposure differed across time rather than being static, a pattern consistent with previous observations in fungal responses to ecosystem disturbances (Parladé et al., 2019).

Beyond overall community structure, examining phylum-level responses provides a deeper understanding of how neonicotinoid exposure and temporal dynamics may differentially affect key fungal groups and their ecological roles. At the phylum level, the dominant groups (Ascomycota and Basidiomycota) remained relatively stable across treatments and time, a finding consistent with prior studies reporting the broad taxonomic resilience of major fungal phyla to chemical disturbance (Astaykina et al., 2020; Li et al., 2018a). However, significant temporal declines in Mortierellomycota, Rozellomycota, and Olpidiomycota suggest that less abundant, opportunistic groups are more sensitive to environmental fluctuations (Lin et al.,

2021). These shifts could have notable functional implications for soil ecosystems. Members of the Mortierellomycota phylum play key ecological roles in soils, including the enhancement of plant growth via the synthesis of phytohormones like indoleacetic acid and gibberellins, as well as facilitating phosphorus uptake through both nutrient exchange and the secretion of organic acids that mobilise inorganic phosphorus (Sang et al., 2022; Zhu et al., 2022). Similarly, Rozellomycota, which are predominantly parasites of chytrids, algae, and oomycetes, contribute to decomposition processes through the extracellular secretion of degradative enzymes, enabling nutrient absorption via diffusion (Gleason et al., 2012; Zhou et al., 2025b). The observed declines in these phyla suggest that neonicotinoid exposure could have cascading effects on soil nutrient dynamics, potentially impacting plant health and productivity. These effects are likely magnified when the impacted taxa play structurally or metabolically distinct roles within the broader fungal community.

Differential abundance analysis revealed that neonicotinoids exerted selective effects on specific fungal taxa, with several Ascomycota lineages repeatedly identified as discriminatory biomarkers. Under imidacloprid and clothianidin exposure, members of Glomerellales, Plectosphaerellaceae, Sordariales and Pleosporales were among the taxa most consistently suppressed. Similar groups have previously been reported from pesticide-impacted and contaminated environments, suggesting that fungal responses to chemical disturbance are highly taxon-dependent and may reflect differences in physiological tolerance or ecological strategy (Guo et al., 2023; Lemmel et al., 2021; Sliti et al., 2024; Zheng et al., 2025). In contrast, clothianidin enriched members of Agaricomycetes and Microascales, while thiamethoxam promoted taxa such as Terfezia and Fusicolla. The enrichment of Fusicolla may indicate shifts in root-associated fungal assemblages or differential responses of fungal taxa to pesticide-induced stress, consistent with previous observations that seed treatments can influence fungal colonisation patterns in plant roots (Nettles et al., 2016). The coexistence of enriched and suppressed biomarkers is consistent with environmental filtering processes, whereby disturbance favours organisms possessing traits that confer greater tolerance to stress while reducing the abundance of more sensitive community members (Briceño et al., 2024; Millar & Bennett, 2016; Treseder et al., 2021)

While this study reveals neonicotinoid-driven shifts in fungal community composition, it does not directly quantify pesticide residues, soil enzyme activities, or functional shifts at the gene or metabolite level. Moreover, the temporal scope may not capture legacy or delayed responses, particularly for slower-growing fungal guilds. Future studies employing metagenomics, metatranscriptomics, or stable isotope tracing could provide deeper insight into how these compositional changes translate into altered ecosystem processes such as

nutrient cycling, carbon sequestration, or plant–microbe interactions. Long-term monitoring across agricultural regimes would also help contextualise short-term microbial responses within broader agroecosystem resilience frameworks.

Additionally, integrating environmental metadata such as soil physicochemical parameters and crop phenology would further clarify the context-dependence of fungal responses. While this study focused on taxonomic shifts, coupling these data with bioindicators of soil functionality, such as extracellular enzyme activity or mycorrhizal colonisation rates, could more directly link compositional change to ecosystem function. Such integrative approaches are essential for translating microbial observations into actionable insights for sustainable agriculture and pesticide regulation. Expanding this work across multiple soil types, crop systems, and climatic zones will also be critical to determine the generality of neonicotinoid impacts. Given the differential sensitivity observed across fungal taxa and temporal points, mechanistic experiments that assess fungal physiological traits under pesticide exposure could further resolve the drivers of community shifts and resilience. Ultimately, these efforts will improve our capacity to predict, mitigate, and manage pesticide impacts on belowground biodiversity and ecosystem functioning.

6.5 Conclusion

Neonicotinoid seed treatments can modulate soil fungal communities in taxon-specific and temporally dynamic ways. While overall diversity remained largely stable, dominant phyla including Ascomycota and Basidiomycota remained relatively stable in abundance, whereas the functionally important phylum Mortierellomycota, and the less abundant phyla Rozellomycota, and Olpidiomycota declined over time independently of treatment. Differential abundance analyses revealed distinct patterns of fungal enrichment and suppression among the three neonicotinoids, with clothianidin producing the strongest compositional response and the greatest number of discriminatory fungal biomarkers. Functional guild analysis further demonstrated that clothianidin altered the relative abundance of saprotrophic and symbiotrophic fungi, suggesting that ecological responses may not always be evident from broad taxonomic patterns alone. These findings indicate that neonicotinoids, even in the absence of broad community collapse, can reshape fungal assemblages in ecologically meaningful ways. This underscores the need to include fungal responses in pesticide risk assessments and to adopt integrative approaches that link microbial composition to ecosystem function. Future research should prioritise long-term, functionally explicit investigations to better understand how these compositional shifts influence agroecosystem sustainability.

6.6 Availability of data and material

All raw sequencing data have been submitted to the NCBI Sequence Read Archive under the accession number PRJNA1256647. Supporting datasets and materials necessary to interpret the findings are included in the main chapter and associated appendix.

Chapter 7 Soil texture shapes fungal community responses to neonicotinoid stress: Taxonomic and functional shifts over extended exposure

Abstract[5]

Neonicotinoids can persist in soil and disrupt non-target microbial communities, yet fungal responses remain poorly understood. We investigated how imidacloprid exposure interacts with soil texture to shape fungal diversity, community organisation and predicted metabolic potential. Microcosm experiment was conducted over 28 days using three contrasting soils: loamy sand (Red Luvisol; 11 g/100 g clay), sandy loam (Red Luvisol; 16 g/100 g clay) and clay (Vertisol; 56 g/100 g clay). Imidacloprid was imposed via wheat (*Triticum aestivum*) seeds, treated at a commercial application rate. Fungal communities were profiled using ITS amplicon sequencing with complementary ecological and PICRUSt2-based functional analyses. Soil physicochemical properties were strongly associated with fungal community composition, with pH, exchangeable Ca^{2+}, Mg^{2+}, effective cation exchange capacity, exchangeable sodium percentage and organic matter emerging as the strongest predictors. Loamy sand exhibited significant reductions in fungal diversity together with increased network connectivity and limited predicted functional changes. Sandy loam showed no significant changes in fungal diversity and comparatively few predicted functional shifts, which may reflect the higher organic matter content of this soil. Clay soils exhibited reduced network cohesion, fewer indicator taxa and the broadest suppression of predicted metabolic pathways, indicating the strongest community-level response to imidacloprid. These findings demonstrate that soil texture and physicochemical properties strongly mediate fungal responses to neonicotinoid exposure and should be explicitly considered when assessing ecological risks across agricultural landscapes.

Keywords: Imidacloprid, seed treatment, red luvisol, vertisol, co-occurrence network, functional prediction

[5] This chapter has been submitted to *Biology and Fertility of Soils* (July 2026, currently under review).

7.1 Introduction

Neonicotinoid insecticides, a novel class of neuroactive compounds modelled after nicotine, have become central to pest management in modern agriculture (Mandal et al., 2025). These compounds share a nicotinoid scaffold with a characteristic nitro-substituted pharmacophore that confers potent agonistic activity at insect nicotinic acetylcholine receptors (Kimura-Kuroda et al., 2012). Since their introduction in the early 1990s, they have been widely adopted due to their high efficacy against a broad range of insect pests, systemic movement within plant tissues, and perceived environmental safety relative to older chemistries (García-Hernández et al., 2025). By the 2000s, they were in widespread use across more than 120 countries (Zhao et al., 2020), with imidacloprid remaining the most extensively applied compound in this class (Casida, 2018).

A key innovation driving neonicotinoids' success was their systemic activity and versatility in application. These chemicals can be applied as foliar sprays, soil drenches or granules, but are most commonly applied as seed coatings that provide the germinating crop with internal insecticidal protection (Wood & Goulson, 2017). Nonetheless, seed treatment was initially viewed as an environmentally preferable delivery method because it minimises off-target drift compared to spraying, and early toxicological profiles suggested low non-target impacts (Mörtl et al., 2020). However, decades of intensive use have revealed unintended environmental persistence and non-target effects, prompting re-evaluation of their ecological safety. Notably, neonicotinoids exhibit high water solubility and low volatility, with soil degradation half-lives ranging from days to years, enhancing their potential to persist and redistribute across environmental compartments beyond the initial application zone (Hladik et al., 2018). Repeated applications can thus result in residual build-up in agricultural soils (Wu et al., 2020). These residues have become nearly ubiquitous in intensively farmed landscapes, raising concerns about chronic exposure in non-target organisms (Humann-Guilleminot et al., 2019).

Indeed, the same insecticidal specificity that made neonicotinoids so effective has also manifested in harm to beneficial insects such as the western honey bee (*Apis mellifera*) and the buff-tailed bumblebee (*Bombus terrestris*) (Camp & Lehmann, 2021; Williams et al., 2015), prompting restrictions on several major neonicotinoids in the European Union and other regions (Yan et al., 2024).

More recently, research focus has shifted toward the less visible but ecologically vital consequences of neonicotinoid persistence, particularly their influence on soil microbial communities and biogeochemical functions. A substantial portion of applied neonicotinoid inevitably remains in the soil matrix, where it may interact with the soil microbiome (Li et al.,

2025). Once in the soil, the ecological impact of these compounds is shaped not only by their chemical properties but also by soil physical characteristics, particularly texture, which governs fundamental soil characteristics such as pore structure, water retention, and surface adsorption capacity (Upadhyay & Raghubanshi, 2020). Texture can strongly influence microbial habitat conditions and the fate of xenobiotics in soil environments. Coarse-textured soils typically allow for faster infiltration and chemical leaching, potentially exposing deeper microbial communities, whereas fine-textured soils exhibit higher surface area and cation exchange capacity, enhancing pesticide retention and altering exposure duration (Rajakaruna & Boyd, 2008; Xia et al., 2020). These physicochemical differences may also shape microbial diversity and resilience. For example, fungal community structure has been observed to vary significantly across soil textures, even within similar cropping systems, with loam and clay soils supporting distinct functional and taxonomic assemblages (Wang et al., 2021b). Despite these insights, little is known about how soil texture modulates microbial community responses to neonicotinoid exposure, a critical knowledge gap that may explain inconsistent findings in pesticide–microbiome studies.

In situ and laboratory experiments increasingly indicate that neonicotinoid residues can disrupt soil microbial communities and functions (Yu et al., 2020). Observed effects include reductions in microbial biomass and enzymatic activity, shifts in community composition, and impaired nutrient cycling (Castillo-Díaz et al., 2017; Cycoń & Piotrowska-Seget, 2015a). However, this emerging body of work has largely centred on bacterial communities commonly assessing bacterial abundance and 16S rRNA gene profiles, often alongside soil enzyme activities, while comparatively little is known about soil fungi. Fungal communities represent a major component of soil biodiversity and drive key ecosystem processes (Treseder & Lennon, 2015), yet their responses to neonicotinoid contamination remain understudied. Existing studies indicate that fungal communities may exhibit distinct responses compared to bacteria; for example, imidacloprid exposure has been shown to significantly alter bacterial diversity, whereas fungal community composition appeared largely unchanged under identical conditions (Zhang et al., 2015a). These patterns raise the possibility that fungal communities may respond differently than bacteria to neonicotinoid exposure, but comprehensive data are lacking. This knowledge gap is increasingly recognised as an important frontier, since understanding fungal responses is crucial for a full accounting of neonicotinoids' ecological impacts on soil health.

To address these gaps, this study investigated how imidacloprid exposure in three soils varying in texture influenced fungal communities. We characterised shifts in fungal diversity, taxonomic structure, indicator taxa, and predicted ecological functions in response to pesticide

exposure across these soils. Additionally, we examined how these community patterns correlated with a set of key soil properties such as pH, electrical conductivity (EC), organic matter (OM), total carbon (C), total nitrogen (N), carbon-to-nitrogen ratio (C:N), available phosphorus (P), calcium (Ca), magnesium (Mg), potassium (K), effective cation exchange capacity (ECEC), exchangeable sodium percentage (ESP), and the calcium-to-magnesium ratio (Ca:Mg). These insights provide a fungal-specific perspective on how soil texture influences neonicotinoid–microbe interactions at the community level.

7.2 Materials and methods

General experimental procedures, sequencing workflows, and community-data processing are outlined in Chapter 3. The methods presented below describe components unique to the extended fungal microcosm experiment.

7.2.1 Experimental variables

Fungal communities were assessed across three soil textures (loamy sand, sandy loam, clay) and two exposure groups (imidacloprid-treated vs. control) over 28 days. Soil sub-samples were collected on days 3, 7, 14, 21, and 28, with three replicates per soil–treatment combination at each sampling point.

7.2.2 Bioinformatics

Sequences were processed through quality filtering, denoising, and ASV inference followed the DADA2 ITS workflow. Forward and reverse reads were trimmed from the 3′ ends by 50 and 80 bases, respectively, to remove low-quality tails, and filtered using expected error thresholds of 2 (forward) and 3 (reverse). Reads were merged with a minimum overlap of 12 bp. Chimera removal was performed using a consensus method allowing one mismatch and requiring parent sequences to be twice as abundant as potential chimeras. Pseudo-pooling was used for improved error correction.

7.2.3 Statistical analyses

Fungal asymptotic richness was calculated using the Chao1 estimator and relative evenness using Pielou’s index, while evolutionary breadth and relatedness were calculated using Faith’s Phylogenetic Diversity (PD) and Mean Pairwise Distance (MPD). All indices were analysed using linear mixed-effects models, specifying exposure group, soil texture, and time as fixed effects, and including replicate as a random effect to account for repeated measurements within the experimental design (Bates et al., 2015). This modelling framework represents a

three-way factorial ANOVA, enabling interpretation of both main effects and interactions. Post-hoc comparisons were conducted using Dunnett's test.

Community structure was examined using distance-based redundancy analysis (dbRDA) on Weighted UniFrac distance matrices (Legendre & Anderson, 1999; Lozupone et al., 2007). PERMANOVA was performed using the same distance matrix to evaluate the individual and interactive effects of exposure group, soil texture, and time points, with significance assessed using 999 permutations (Anderson, 2017). Associations between Shannon diversity and soil physicochemical properties were evaluated using Pearson correlation, random forest modelling with variable importance quantified by %IncMSE and IncNodePurity, and partial Mantel tests to control for potential confounding variables. Indicator taxa were determined by assessing the strength and fidelity of associations between ASVs and specific exposure group–soil texture combinations (Dufrêne & Legendre, 1997). Taxa with indicator values exceeding 0.7 and statistical significance at $P \leq 0.05$ were considered characteristic of their respective groups. Predictive functional profiling was performed using PICRUSt2 (Douglas et al., 2020), a standalone workflow executed in its native conda environment, generating EC numbers and MetaCyc pathways per sample. Differential abundance analysis of the predicted pathways was performed using edgeR, stratified by soil texture (Robinson et al., 2010).

7.3 Results

7.3.1 Sequencing summary

Sequencing depths across samples ranged from 1249 to 78220 reads (median = 42132.5). A summary table and barplot of sequencing depths are provided in Appendix E (Table E. 1, Figure E. 1)

7.3.2 Fungal community diversity, structure, and environmental associations

The effects of exposure group, soil texture, time, and their interactions on fungal diversity indices were evaluated using Type II Wald χ^2 tests (Table E. 2). Significant main effects and interactions varied among indices, with soil texture and time consistently influencing richness (Chao1), evenness (Pielou's), and phylogenetic diversity (Faith's PD, MPD). Post-hoc contrasts showed that Chao1 richness was significantly lower in imidacloprid-treated than control loamy sand on day 14 (estimate = −251.2, $P = 0.01$) and day 28 (estimate = −267.4, $P = 0.003$). Similarly, Pielou's evenness was lower in imidacloprid-treated loamy sand on day 14 (estimate = −0.2, $P = 0.02$), while Faith's PD was reduced on days 14 (estimate = −18.5,

P = 0.01) and 28 (estimate = −18.7, P = 0.007). In contrast, MPD was significantly higher under imidacloprid than the control in clay (Figure E. 2b) on day 3 (estimate = 0.02, P = 0.04) and day 7 (estimate = 0.03, P = 0.01). No significant treatment-control differences were detected in sandy loam soil for any diversity metric.

dbRDA analysis showed that fungal community structure was significantly influenced by soil texture, time, and their interaction (P = 0.001). PERMANOVA indicated that soil texture explained the greatest proportion of variation in fungal community structure (R^2 = 0.44), followed by sampling time (R^2 = 0.19) and their interaction (R^2 = 0.10). Neither exposure group nor its interactions with soil texture or sampling time significantly affected community structure (P > 0.05). The dbRDA ordination (Figure 7–1) showed clear clustering according to soil texture, whereas substantial overlap between control and imidacloprid samples was observed within each soil type.

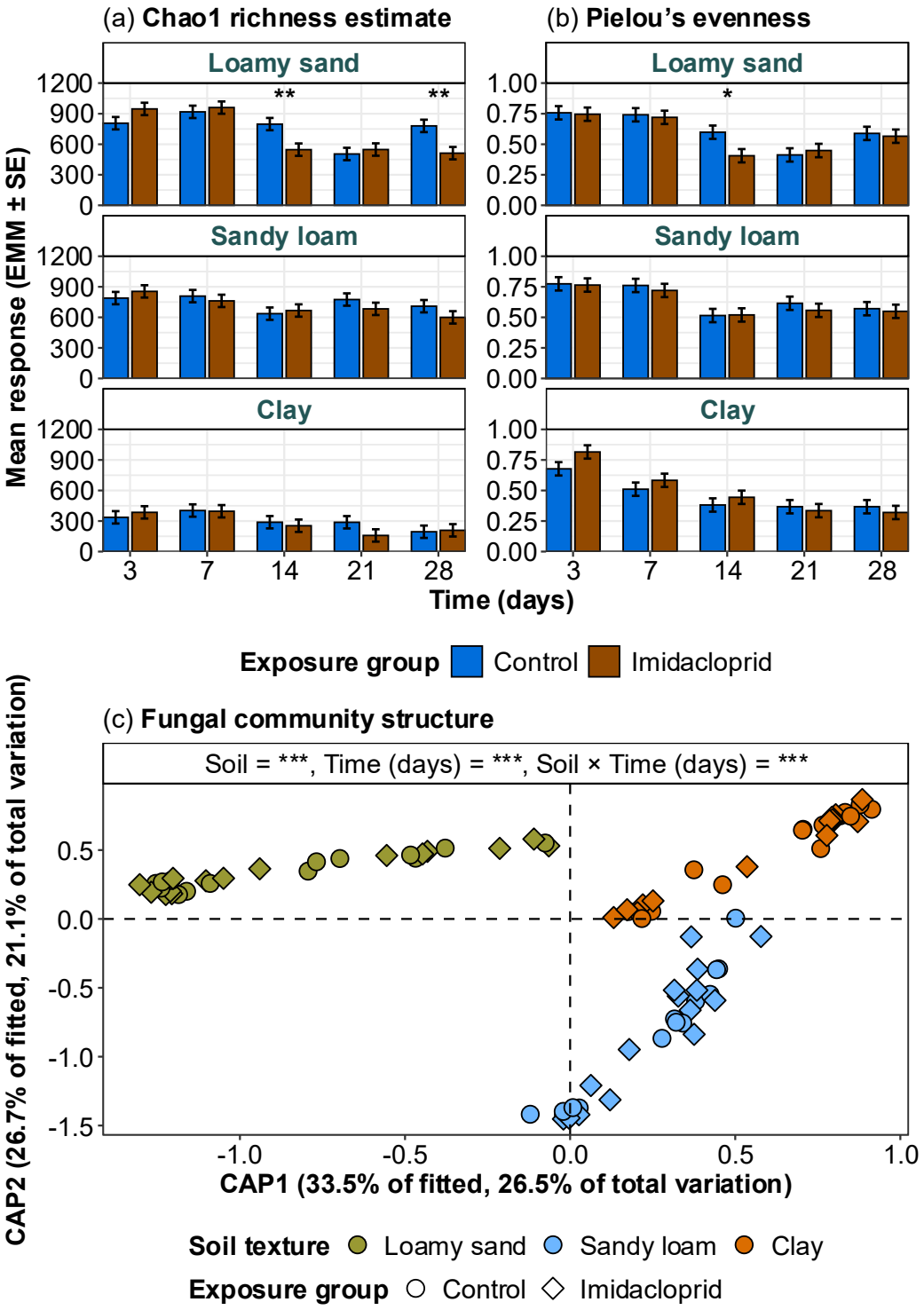

Figure 7–1: Responses of fungal richness, evenness and community structure across three soil textures under imidacloprid exposure. (a) Chao1 richness estimate and (b) Pielou's evenness are presented as estimated marginal means (EMM ± SE). (c) Distance-based redundancy analysis (dbRDA) ordination based on Weighted UniFrac distances. Statistical significance of the main effects and interactions is reported in the panel. Significance levels are indicated as '*' $P \leq 0.05$, '' $P \leq 0.01$, and '***' $P \leq 0.001$.**

Soil physicochemical characteristics varied across the three textures considered in this study (Table A. 1) and were associated with differences in fungal community composition. Clay soil was strongly alkaline (pH 8.74) with elevated exchangeable Ca^{2+} (25 $cmol_+kg^{-1}$), Mg^{2+} (15 $cmol_+kg^{-1}$), and Na^+ (3.2 $cmol_+kg^{-1}$), resulting in the highest effective cation exchange capacity (44 $cmol_+kg^{-1}$). In contrast, loamy sand sol was slightly acidic (pH 6.33) with lower ECEC (8.8 $cmol_+kg^{-1}$), low Ca^{2+} and Mg^{2+}. Sandy loam had higher organic matter (8.4%), greater total C and N contents (4.8% and 0.43%, respectively), and the highest available P (232 mg P kg^{-1} soil), indicating comparatively greater nutrient availability than the other soils. Multiple complementary analyses consistently identified soil physicochemical properties as major correlates of fungal community composition.

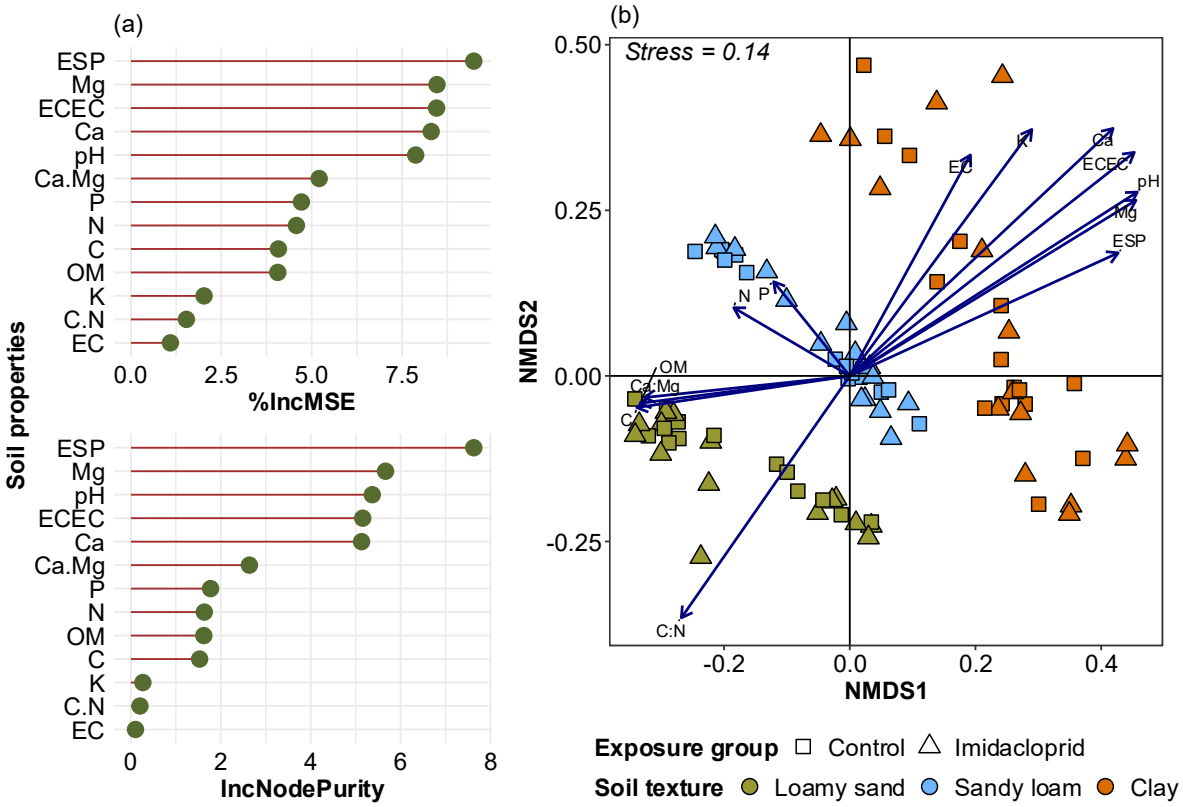

Figure 7–2: Edaphic controls on fungal community structure across soils. (a) Variable-importance ranks for measured soil properties from random-forest models relating edaphic factors to fungal community variation. Two complementary importance metrics Percentage Increase in Mean Squared Error (%IncMSE) and Increase in Node Purity (IncNodePurity) are show, with larger values indicating stronger predictors. (b) Non-metric multidimensional scaling (NMDS, Bray–Curtis) of fungal communities. Blue arrows are envfit vectors for soil properties; arrow length indicates the strength of correlation with the ordination, and direction indicates increasing values.

Random Forest analysis showed that soil properties collectively explained 22.3% of the variation in fungal Shannon diversity, with ESP, Mg^{2+}, ECEC, Ca^{2+}, and soil pH emerging as the most influential predictors across both %IncMSE and IncNodePurity metrics (Figure 7–2a). Ordination-based vector fitting revealed distinct soil-associated gradients in fungal community structure (Figure 7–2b, Table E. 3), with all measured soil properties significantly correlated with community structure (FDR-adjusted $P = 0.001$), and ECEC ($R^2 = 0.29$), Ca^{2+} ($R^2 = 0.24$), pH ($R^2 = 0.23$), Mg^{2+} ($R^2 = 0.22$) and ESP ($R^2 = 0.20$) showing the strongest associations.

7.3.3 Co-occurrence patterns and indicator taxa

Across soils, imidacloprid altered fungal co-occurrence network topology, although the magnitude of these changes differed among soil textures (Table 7–1, Figure E. 3). In loamy sand, the imidacloprid network exhibited greater connectivity than the control network, with increases in the number of nodes, edges, average degree and graph density, accompanied by shorter average path length and lower modularity, indicating a more integrated network structure. Sandy loam showed a similar pattern, with higher connectivity and reduced modularity under imidacloprid. In contrast, clay soil displayed a less cohesive network following imidacloprid exposure. Although more nodes were present, network density, average degree, transitivity and clustering coefficient decreased, while average path length and network diameter increased, indicating reduced local clustering and weaker overall network cohesion. Permutation analysis indicated that these changes were statistically significant only in clay soil, where transitivity and clustering coefficient decreased and average path length increased under imidacloprid ($P < 0.05$).

Indicator species analysis identified distinct fungal taxa associated with each soil texture-exposure group combination (Figure 7–3). Across all soil textures, the control treatment contained more unique indicator species than the imidacloprid treatment. Loamy sand had four unique indicator species under the control compared with one under imidacloprid, whereas sandy loam contained seven and three unique indicator species, respectively. In clay, four unique indicator species were associated with the control and three with imidacloprid. Most unique indicator species occurred at low relative abundances (< 0.07%), although *Fusarium equiseti* (control, sandy loam) and *Dactylonectria macrodidyma* (imidacloprid, clay) were comparatively more abundant than the remaining indicator species. Indicator values ranged from 0.70 to 0.94, with the strongest associations observed for *Thelonectria nodosa* in imidacloprid-treated sandy loam and *Dactylonectria macrodidyma* in imidacloprid-treated clay.

Table 7–1: Key network topology metrics for fungal co-occurrence networks across soil textures and exposure groups. Different superscript letters within a soil texture indicate significant differences between exposure groups based on permutation tests ($P < 0.05$).

Soil	Treatment	Node	Edge	Transitivity	Average path length	Modularity	Graph density	Network diameter	Average degree	Clustering coefficient
Loamy sand	Control	69	190	0.62	2.55	0.36	0.08	7	5.51	0.72
	Imidacloprid	75	448	0.60	2.00	0.23	0.16	5	11.95	0.72
Sandy loam	Control	47	73	0.48	2.87	0.60	0.07	6	3.11	0.65
	Imidacloprid	46	121	0.51	2.43	0.33	0.12	6	5.26	0.62
Clay	Control	35	35	**0.89[a]**	**1.27[a]**	0.83	0.06	3	2.00	0.89
	Imidacloprid	45	42	**0.35[b]**	**2.93[b]**	0.73	0.04	6	1.87	0.47

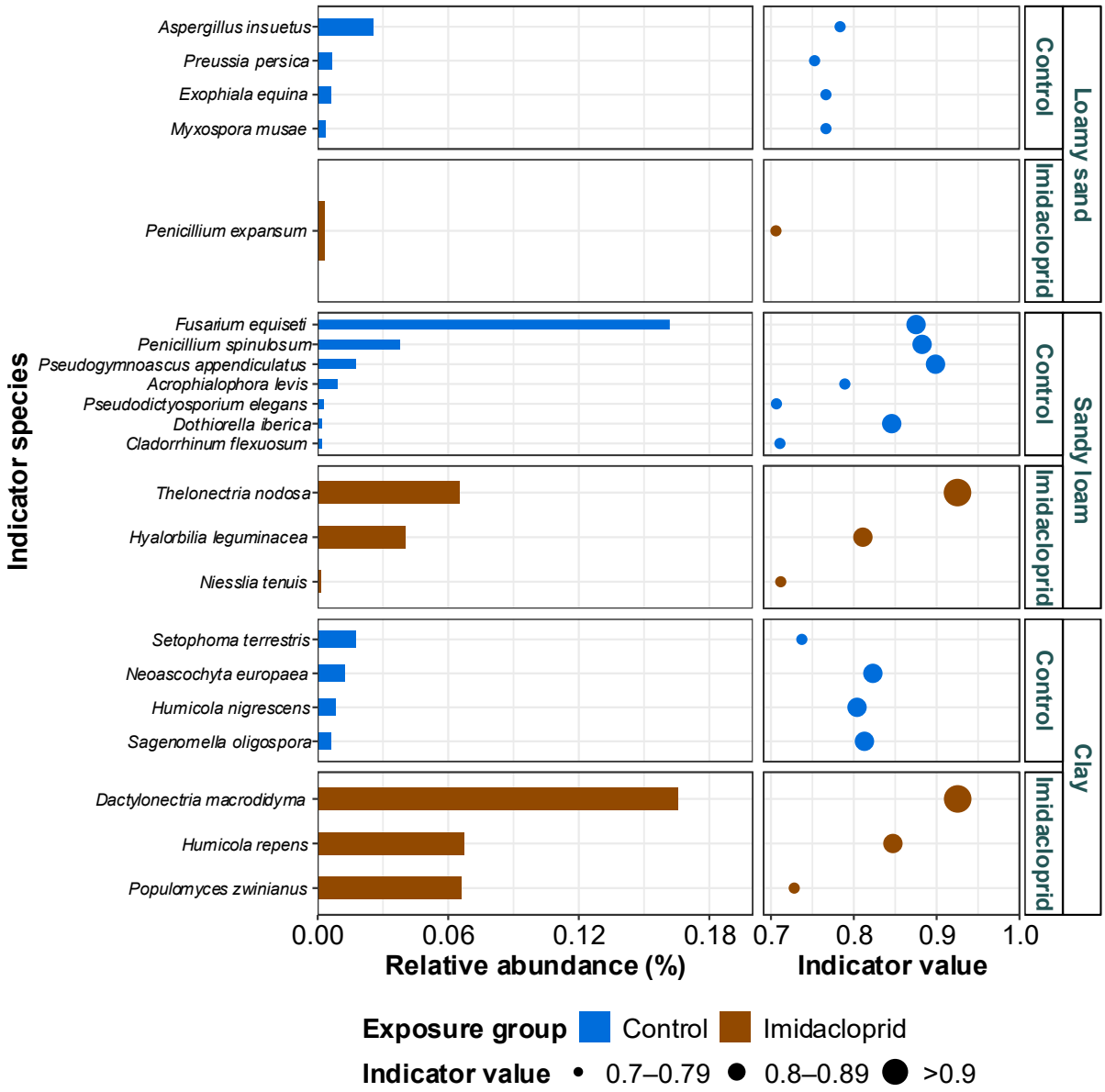

Figure 7–3: Species-level fungal indicator taxa uniquely associated with individual soil texture-exposure group combinations. The left panel shows the mean relative abundance (%) of each indicator species across all samples, and the right panel shows the corresponding indicator value (IndVal). Only species-level indicator taxa exclusively associated with a single soil texture-exposure group combination with IndVal > 0.70 and *P* ≤ 0.05 are shown.

7.3.4 Predicted functional shifts

PICRUSt2-predicted MetaCyc pathway analysis identified 77 predicted metabolic pathways across the three soil textures, of which only a subset differed significantly between imidacloprid-treated and control soils (Figure 7–4). The magnitude and direction of functional

responses varied markedly among soil textures. In loamy sand, only two biosynthetic pathways were significantly upregulated, specifically L-methionine biosynthesis III and the superpathway of pyrimidine ribonucleosides salvage, both related to amino acid and nucleotide metabolism (Figure 7–4a). In sandy loam, functional responses were modest, with upregulation of sucrose biosynthesis I and III, changes in amino acid degradation including L-leucine and L-tryptophan degradation, and downregulation of pyrimidine nucleotide biosynthesis pathways (Figure 7–4b). Clay soil showed the strongest functional response to imidacloprid (Figure 7–4c), with L-arginine biosynthesis I (via L-ornithine) as the only significantly upregulated pathway. In contrast, numerous pathways were significantly downregulated, including those associated with carbohydrate metabolism (sucrose degradation III, sucrose biosynthesis I and III, glycogen biosynthesis I, glycogen degradation I, starch degradation V, and glucose and glucose-1-phosphate degradation), fatty acid biosynthesis (stearate and gondoate biosynthesis), purine nucleotide biosynthesis (5-aminoimidazole ribonucleotide biosynthesis I and II and the superpathway of 5-aminoimidazole ribonucleotide biosynthesis), and exopolysaccharide precursor biosynthesis (colanic acid building blocks biosynthesis).

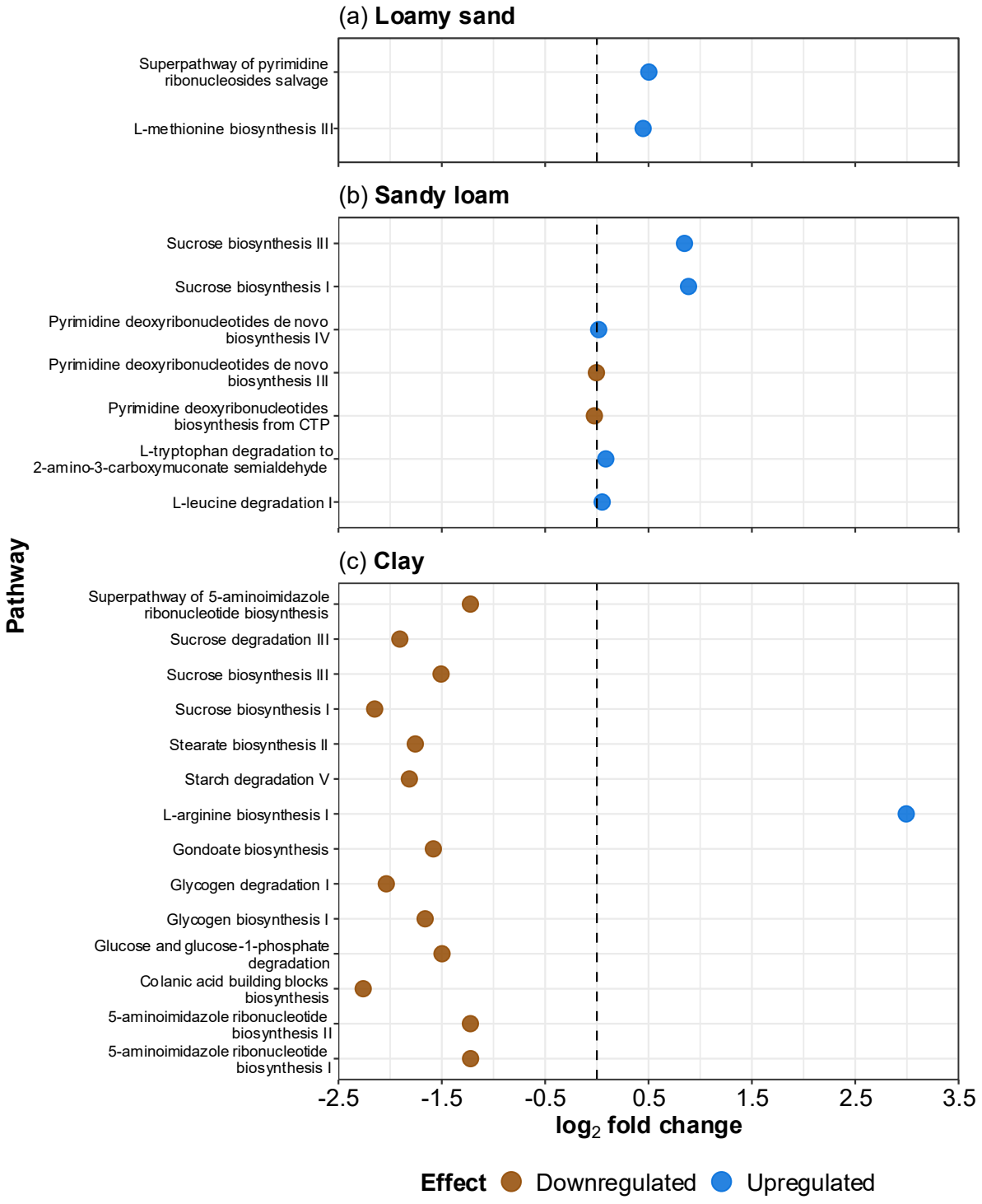

Figure 7–4: Texture-dependent shifts in predicted fungal pathways under imidacloprid. Differentially abundant MetaCyc pathways predicted with PICRUSt2 are shown as log_2 fold change of imidacloprid relative to control. Panels are fit within each soil, and only pathways with FDR < 0.05 are plotted.

7.4 Discussion

This study showed that the consequences of imidacloprid exposure for soil fungi are conditional on soil texture and chemistry but were not uniform across soils. In sandy loam, no significant changes were detected in richness, evenness, or phylogenetic diversity, suggesting that fungal communities in this texture were resilient to imidacloprid exposure. Such stability is likely linked to the physicochemical properties of sandy loam (Table A. 1), including the highest organic matter content among the soils in this study, good aeration, and balanced pore structure, which together can enhance sorption, leaching, and microbial degradation of imidacloprid, thereby reducing its persistence and bioavailability (Anhalt et al., 2008). In contrast, loamy sand exhibited significant reductions in Chao1 richness, Pielou's evenness, and Faith's phylogenetic diversity at the later sampling times (days 14 and 28; Figure 7–1, Figure E. 2). These concurrent declines indicate that prolonged imidacloprid exposure reduced fungal richness, community evenness, and phylogenetic diversity, suggesting a progressive contraction of both taxonomic and evolutionary diversity in loamy sand (Matsuoka et al., 2019; Riedo et al., 2025). Clay soil displayed a different response. Richness, evenness, and Faith's PD remained largely unchanged, whereas MPD increased during the early stages of exposure (days 3 and 7), indicating shifts in the phylogenetic structure of the community despite relatively stable overall diversity. These contrasting responses suggest that soil texture influences not only the magnitude but also the nature of fungal community responses to imidacloprid (Köninger et al., 2026), while also supporting the role of environmental filtering in shaping fungal community assembly across contrasting soil environments (Kivlin et al., 2014).

These diversity responses occurred against a background in which fungal community composition was structured primarily by soil texture and sampling time, while imidacloprid exerted soil-dependent rather than consistent effects across soils. Ordination revealed clear clustering according to soil texture, with clay communities associated with gradients in pH, ESP, Mg^{2+}, Ca^{2+} and ECEC, whereas loamy sand and sandy loam occupied the opposing region of the ordination. These edaphic gradients match global syntheses in which pH and base-cation regimes are first-order determinants of fungal biogeography and turnover (Bahram et al., 2018; Tedersoo et al., 2014) and likely interact with texture-dependent pesticide fate to determine realised exposure. Such interactions help explain why imidacloprid effects were strongest in loamy sand and clay, but negligible in sandy loam, whose physicochemical properties may buffer communities against pesticide disturbance.

Consistent with the dominant influence of soil texture on fungal community assembly, imidacloprid was associated with soil-specific changes in fungal co-occurrence networks, indicating that pesticide exposure altered potential ecological interactions in a texture-dependent manner. Although the magnitude of network responses varied among soils, only clay exhibited statistically significant changes in network topology, whereas loamy sand and sandy loam showed trends towards greater connectivity following imidacloprid exposure. Both Luvisol soils (loamy sand and sandy loam) displayed a tendency towards more connected fungal networks following imidacloprid exposure, characterised by increases in edge number, graph density and average degree together with reduced modularity. Although these changes were not statistically significant, they suggest that fungal associations became more integrated under pesticide exposure. Similar increases in network connectivity under environmental disturbance have been interpreted as community reorganisation, whereby interactions become concentrated among the remaining taxa despite reductions in network compartmentalisation (Price et al., 2021). In sandy loam, the comparatively high organic matter content may further have reduced the bioavailability of imidacloprid through enhanced sorption and microbial degradation, thereby buffering fungal associations against more pronounced network disruption (Liu et al., 2006). Smectitic clay minerals possess high cation-exchange capacity and pronounced shrink-swell behaviour, properties that enhance pesticide retention and modify pore connectivity, potentially prolonging pesticide-microbe interactions (Bühmann & Schoeman, 1995; Cattle & Field, 2013; Chittamart et al., 2010; Jablonowski et al., 2012). By comparison, the kaolinitic mineralogy of the Luvisols provides fewer sorption sites and maintains more stable pore networks, which may reduce prolonged pesticide exposure and limit disruption of fungal associations (Jozefaciuk et al., 2021; Polati et al., 2006; Xiao et al., 2024).

Indicator species analysis complemented the network analysis by showing a consistent reduction in the number of unique indicator taxa under imidacloprid across all soil textures. Because indicator taxa are defined by their high fidelity and specificity to particular environmental groups (Cáceres & Legendre, 2009), , this reduction suggests that fewer fungal taxa remained strongly associated with specific soil-exposure combinations following imidacloprid exposure. Most unique indicator taxa occurred at low relative abundances (Figure 7–3b), indicating that the reduction primarily affected the rarer components of the fungal community. Although rare taxa contribute little to overall community abundance, they are increasingly recognised as important reservoirs of taxonomic diversity and specialised ecological functions, contributing to community resilience under environmental change (Jousset et al., 2017). Consequently, the consistent loss of unique indicator taxa across all soil textures suggests that imidacloprid may reduce the ecological distinctiveness of fungal

communities by altering the taxa most closely associated with particular soil-exposure environments.

Predicted functional profiles further suggested that the observed changes in fungal community composition and network organisation were accompanied by shifts in potential metabolic capacity that differed among soil textures. Functional responses were relatively limited in the two Luvisol soils but became substantially more pronounced in the clay Vertisol, indicating that soil properties influenced not only fungal community structure but also the predicted metabolic responses to imidacloprid. Although these pathways were inferred from taxonomic composition rather than directly measured, the contrasting functional profiles were consistent with the soil-dependent responses observed in diversity and co-occurrence analyses. In loamy sand, only two predicted biosynthetic pathways were significantly enriched, involving amino acid metabolism and nucleotide salvage. The limited number of altered pathways suggests that fungal communities maintained much of their predicted metabolic capacity despite reductions in diversity. Increased amino acid biosynthesis is consistent with cellular stress responses, as amino acid metabolism contributes to nitrogen regulation, cellular maintenance and oxidative stress protection (Chen & Dickman, 2005; López-Berges et al., 2010). Similarly, enhanced nucleotide salvage may represent a metabolically efficient strategy for maintaining nucleic acid turnover under environmental stress, avoiding the energetic costs of de novo nucleotide synthesis (Boer et al., 2009). In sandy loam, responses were modest, with sucrose biosynthesis pathways upregulated, nucleotide biosynthesis downregulated, and additional shifts in amino acid degradation. Increased sucrose metabolism may reflect reallocation of carbon into extracellular polysaccharides that strengthen soil aggregation and buffer cells through water retention, nutrient diffusion, biofilm formation, and detoxification processes (Costa et al., 2018; Kayoumu et al., 2025). In contrast, downregulation of nucleotide biosynthesis suggests a more conservative metabolic response. These relatively limited functional shifts are consistent with the minimal changes observed in fungal diversity and network topology, supporting the hypothesis that the higher organic matter content of sandy loam reduced imidacloprid bioavailability and moderated its effects on fungal communities (Zhang et al., 2018). Clay soils displayed the greatest predicted functional disruption, with widespread downregulation of pathways involved in carbohydrate metabolism, fatty acid biosynthesis, purine nucleotide biosynthesis and exopolysaccharide precursor production, while only L-arginine biosynthesis increased. These broad reductions suggest that prolonged imidacloprid exposure constrained multiple aspects of fungal metabolic potential. The high smectite content and cation-exchange capacity of Vertisols are known to promote stronger pesticide sorption and prolonged retention (Cox et al., 2001), increasing the likelihood of sustained microbial exposure. Under prolonged chemical stress, microorganisms commonly

reallocate resources away from growth-associated metabolism towards cellular maintenance and stress tolerance, consistent with reports of neonicotinoid-induced oxidative stress and metabolic disruption (Ralser et al., 2007; Xu et al., 2022). The broad suppression of predicted metabolic pathways in clay, together with reduced network cohesion and fewer unique indicator taxa, suggests that imidacloprid affected multiple dimensions of fungal community organisation, with potential implications for ecosystem functioning where functional redundancy is limited (Allison & Martiny, 2008).

Future work should validate these predicted pathways with shotgun metagenomics, metatranscriptomics or targeted enzyme assays. Quantifying imidacloprid residues, porewater concentrations, and sorption dynamics across contrasting soil textures would strengthen the mechanistic links between pesticide bioavailability and microbial responses. Experiments manipulating organic matter and clay mineralogy could disentangle the relative contributions of organic and mineral sorption, while recovery experiments following pesticide withdrawal would provide insight into fungal resilience and functional recovery.

7.5 Conclusion

This study demonstrates that the effects of imidacloprid on soil fungal communities are strongly mediated by soil texture and physicochemical properties rather than occurring uniformly across soils. Fungal responses ranged from minimal changes in sandy loam to pronounced alterations in diversity in loamy sand and community organisation and predicted metabolic potential in clay soils. These soil-dependent responses were reflected in fungal diversity, co-occurrence network topology, indicator taxa and predicted metabolic pathways, highlighting the close links between soil physicochemical conditions and microbial responses to neonicotinoid exposure. These findings show that the ecological consequences of neonicotinoid seed treatments cannot be generalised across soil types and should instead be interpreted within the context of soil texture and chemistry. Integrating taxonomic, ecological, and predicted functional perspectives, provides a more comprehensive framework for assessing pesticide impacts and strengthening ecological risk assessment across heterogeneous agricultural soils.

7.6 Availability of data and material

The raw sequencing data associated with this chapter have been deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA1256652. Additional supporting materials required to interpret the results are available within the chapter and its appendix.

Chapter 8 Conceptualising neonicotinoid–microbe interactions: Fate, effects and functional shifts

8.1 Introduction

The preceding chapters demonstrated that neonicotinoids can substantially alter bacterial and fungal communities, with the strength and direction of responses depending on compound type, soil texture, and exposure duration. These findings extend beyond taxonomic shifts to reveal changes in co-occurrence networks, predicted functions, and indicators of ecological stability. In this thesis, “stress” is used to describe the environmental conditions associated with neonicotinoid exposure, rather than a discrete disturbance event. Following Plante (2017), disturbance involves an unpredictable event that directly removes microbial biomass, whereas gradual changes in environmental conditions are considered environmental stress. Accordingly, the taxonomic, ecological and predicted functional changes observed throughout this thesis are interpreted as microbial community responses associated with the environmental stress imposed by neonicotinoid seed treatments. However, individual changes in diversity, community composition or predicted function are not, in isolation, considered definitive indicators of physiological stress, as similar patterns may also reflect microbial adaptation or altered ecological interactions (Griffiths & Philippot, 2013).

This chapter consolidates those results into a conceptual framework that explains how neonicotinoid exposure interacts with soil properties to reshape microbial diversity, interactions, and functions. The model highlights microorganisms as both sensors of pesticide stress and potential participants in transformation processes. By placing experimental findings within this framework, the chapter provides a broader ecological interpretation of neonicotinoid–microbe interactions and their consequences for soil functioning.

8.2 Framing the conceptual model

The systematic review (Chapter 2) showed that most published studies reported effects of neonicotinoids on soil microorganisms without accounting for soil properties such as pH, organic matter, cation exchange capacity, or texture. This omission limited ecological interpretation. The experiments presented in Chapters 4–7 addressed this gap by explicitly incorporating soil texture and examining microbial responses under controlled short- and prolonged exposures.

The conceptual model (Figure 8–1) brings these experimental findings together within a broader framework. It shows how neonicotinoids applied as seed treatments enter soil systems, where they may be transformed or persist, and then interact with microbial communities. The solid boxes and arrows represent processes that were demonstrated in this thesis, including microbial uptake, community and network-level responses, and functional changes. The dashed boxes and arrows indicate processes that were not measured directly but are hypothesised ecological consequences by the observed microbial responses. The model links these experimental results to the underlying processes that might control neonicotinoid fate and highlights how microbial responses such as changes in diversity, community structure, and function can influence soil ecosystem services like nutrient cycling and organic matter turnover.

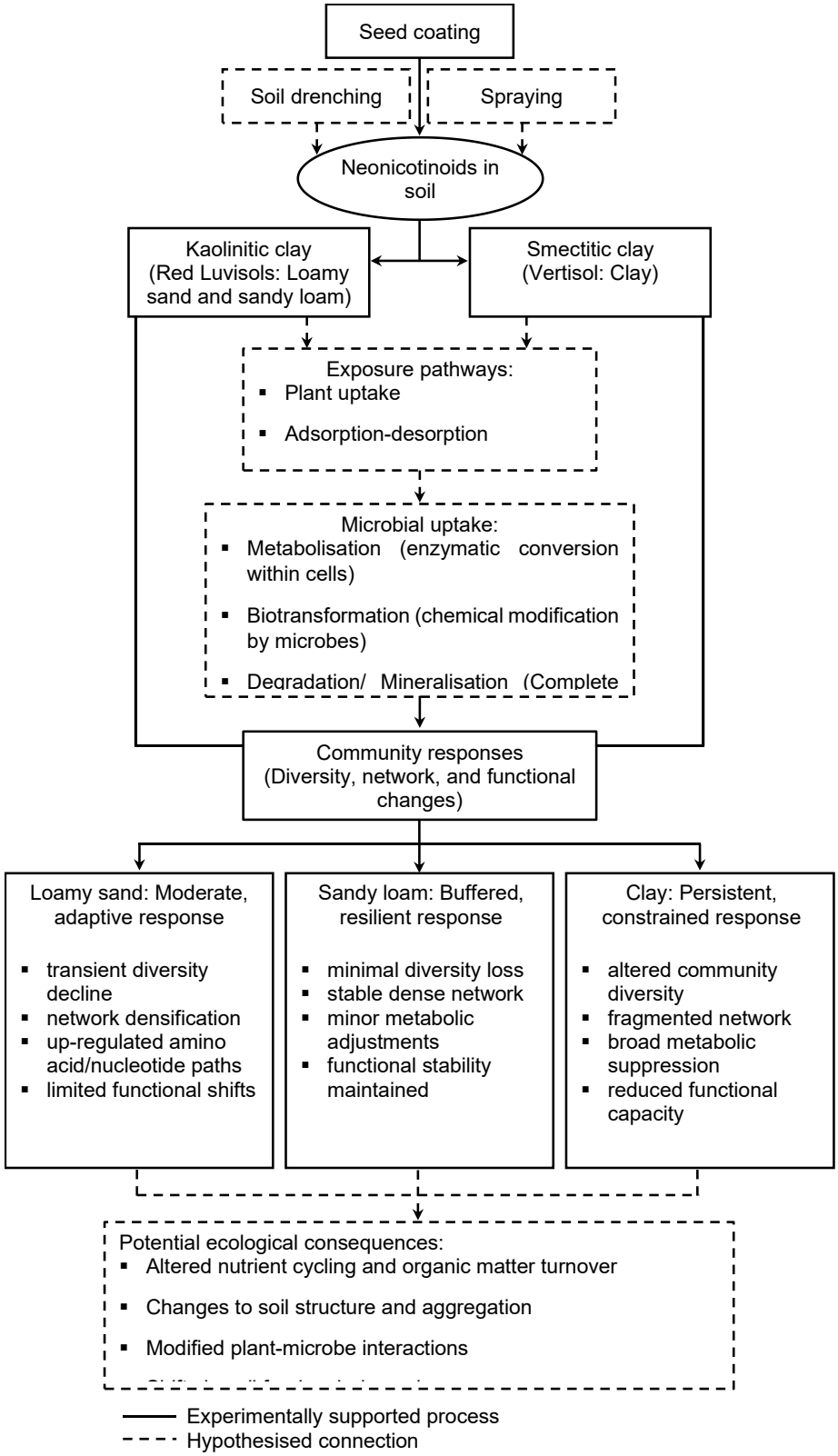

Figure 8–1 Conceptual model of neonicotinoid fate, microbial response, and functional consequences in soil ecosystems. The solid boxes and arrows represent experimentally supported processes demonstrated in this thesis, whereas dashed components depict hypothesised ecological consequences

8.3 Integrating experimental insights

Building on the conceptual model outlined in Figure 8–1, this section integrates findings from the bacterial and fungal experimental chapters to show how the observed microbial responses align with the proposed mechanisms of neonicotinoid fate and effect. This section synthesises findings from the bacterial and fungal experimental chapters, placing them within the conceptual framework of neonicotinoid fate, effects, and functional shifts. The combined results demonstrate that neonicotinoids reshape soil microbial communities in ways that reflect the model's solid-line processes, with outcomes strongly influenced by compound identity, soil texture, and time since exposure. These include measurable shifts in diversity, community composition, network organisation, and predicted functional capacity, all of which correspond to the stress-response and functional-change stages of the framework.

8.3.1 Bacterial and fungal responses in context

Across experiments, different components of bacterial diversity responded differently to neonicotinoid exposure. Richness showed limited and compound-specific responses, with increases observed under clothianidin, whereas prolonged imidacloprid exposure had little effect on bacterial richness. Evenness appeared more sensitive under particular soil conditions, with reductions detected only in clay soil following imidacloprid exposure. Phylogenetic diversity exhibited only localised responses, with changes in bacterial phylogenetic structure restricted to specific soil conditions.

The transient diversity responses observed in this study are consistent with patterns reported for microbial communities exposed to environmental stress. Exposure to environmental stress may temporarily favour fast-growing or rare microorganisms, leading to a short-lived increase in community diversity and functional potential before stabilising or reverting to pre-stress conditions (Griffiths & Philippot, 2013). Similar patterns have been observed across multiple stress contexts, including temperature perturbations (Allison & Martiny, 2008), community resilience studies (Shade et al., 2012), and broad-scale meta-analyses of microbiome stress responses (Rocca et al., 2019).

Taxonomic composition analyses confirmed that Actinobacteriota and Proteobacteria remained dominant across all treatments, whereas LEfSe analyses revealed selective shifts within these broader groups. Across the three neonicotinoids, taxa including *Mesorhizobium*, *Massilia*, members of the Rhizobiaceae and Micromonosporaceae, and, depending on the compound, *Solirubrobacter*, *Nocardioides* and *Lysobacter* were enriched, whereas lineages such as Nitrospira, Planctomycetes, Pir4 and Blastocatellia were consistently suppressed.

These contrasting responses indicate that neonicotinoids act as selective filters, favouring some bacterial taxa while reducing the relative abundance of others rather than producing uniform responses across bacterial communities.

Fungal communities responded to neonicotinoid exposure through changes in richness, evenness and phylogenetic diversity rather than consistent shifts across all components of diversity. Richness declined under imidacloprid in loamy sand but remained unchanged in sandy loam. Evenness also varied among soils, indicating that fungal diversity responses were not expressed uniformly across soil types. Phylogenetic diversity exhibited contrasting soil-specific responses, with clay soils showing changes in phylogenetic structure rather than declines in diversity.

These diversity responses were accompanied by taxon-specific shifts identified by LEfSe. Members of Glomerellales, Plectosphaerellaceae, Sordariales and Pleosporales were consistently suppressed under imidacloprid and clothianidin, whereas clothianidin enriched taxa such as Agaricomycetes and Microascales and thiamethoxam promoted *Fusicolla* and *Terfezia*. These contrasting responses are consistent with environmental filtering, whereby neonicotinoid exposure favours fungal taxa with greater tolerance while reducing more sensitive members of the community. At the functional guild level, however, this taxonomic reorganisation was reflected only under clothianidin, which increased saprotrophs and reduced symbiotrophs, whereas imidacloprid and thiamethoxam produced no detectable changes in predicted guild composition.

Exposure to pesticides and related chemical stressors in soil environments consistently leads to selective shifts in microbial communities, characterised by the enrichment of tolerant or metabolically versatile bacterial and fungal taxa alongside the decline of more sensitive lineages. This selective filtering shapes the community composition and function, rather than causing uniform suppression across all microbial groups (Ma et al., 2021; Steiner et al., 2024; Walder et al., 2022).

Within the conceptual model (Figure 8–1), this sequence represents the transition from an early adaptive phase to a selective-pressure phase, where microbial uptake and transformation processes give way to community restructuring and loss of functional redundancy.

8.3.2 Temporal dynamics

Across both bacterial and fungal communities, sampling time consistently influenced community composition and generally explained more variation than neonicotinoid treatment. In the short-term experiments, significant treatment × sampling day interactions indicated that

microbial responses to neonicotinoids varied over time despite the absence of significant treatment effects alone, suggesting that treatment effects were expressed only during specific stages of exposure. In contrast, the prolonged experiments demonstrated that soil texture became the dominant driver of microbial community composition, with sampling time and the soil × sampling time interaction explaining additional variation, whereas treatment effects were weak or not significant. These findings highlight the importance of considering temporal dynamics when evaluating neonicotinoid effects on soil microbial communities, as the magnitude and persistence of microbial responses are influenced by soil properties, including clay mineralogy and associated shrink-swell behaviour, which can influence pore structure, water availability and compound persistence, rather than texture alone (Griffiths et al., 2008; Jiao et al., 2019; Longepierre et al., 2021).

These temporal shifts reflect the process outlined in the conceptual model (Figure 8–1), where exposure pathways and microbial uptake occur dynamically over time. The early window of pronounced community change likely corresponds to the phase of highest neonicotinoid bioavailability (Simon-delso et al., 2015), when diffusion and adsorption–desorption processes favour greater exposure of microorganisms to the applied pesticide (Wauchope et al., 2002). During this period, transient increases in diversity may arise from short-term stimulation of tolerant or metabolically adaptable taxa (Wu et al., 2018). As degradation and sorption reduce chemical availability and cumulative stress effects emerge, diversity patterns stabilise or decline (de Souza et al., 2017; Ewere et al., 2024).

8.3.3 Community interactions and network stability

Co-occurrence network analysis revealed soil-specific rewiring of microbial associations under imidacloprid exposure. Although these networks do not demonstrate direct biological interactions, they provide insights into how taxa co-occur and how community organisation changes in response to environmental stress (Berry & Widder, 2014; Faust & Raes, 2012).

In bacterial communities, imidacloprid reduced network connectivity and increased modularity in loamy sand and clay, whereas sandy loam exhibited increased connectivity despite higher modularity. Ecologically, these contrasting patterns suggest that bacterial communities were reorganised differently among soil types. In loamy sand and clay, the more compartmentalised network structure indicates greater differentiation in the responses of bacterial taxa to imidacloprid, resulting in more distinct groups of co-occurring microorganisms. In contrast, the greater connectivity observed in sandy loam suggests that bacterial taxa continued to exhibit coordinated abundance patterns despite exposure, indicating community reorganisation without the widespread separation of co-occurring taxa observed in the other soils.

In fungi, the more integrated networks observed in loamy sand and sandy loam suggest that fungal taxa continued to occur in coordinated assemblages despite imidacloprid exposure, indicating comparatively less disruption to community organisation. In contrast, clay soil exhibited reduced connectivity, clustering and transitivity together with increased path length, indicating marked reorganisation of fungal association patterns. Ecologically, this suggests that fungal assemblages in clay were redistributed into different groups of co-occurring taxa, reflecting the greatest restructuring of fungal community organisation among the three soils.

The contrasting bacterial and fungal network responses observed here are consistent with previous studies demonstrating that bacteria and fungi exhibit distinct community trajectories under environmental disturbance (Sun et al., 2017) and differ in the way their co-occurrence networks respond to stress (de Vries et al., 2018; He et al., 2017).

These soil-specific changes in microbial association patterns correspond to the stress-response phase in the conceptual model (Figure 8–1), where chemical disturbance can reorganise microbial association networks, resulting in either more integrated or more fragmented community organisation depending on habitat and stress intensity (Wang et al., 2021a).

8.3.4 Functional implications

Functional prediction revealed consistent enrichment of stress-related and amino acid degradation pathways, such as the superpathway of L-arginine, putrescine, and 4-aminobutanoate degradation, across soils, suggesting community-level responses to xenobiotic stress. Conversely, carbohydrate metabolism pathways (e.g., lactose and galactose degradation, sucrose degradation) and nucleotide biosynthesis pathways were frequently downregulated, particularly in clay soils, suggesting potential constraints on microbial energy turnover and biosynthetic capacity. Fungal functional predictions corroborated these findings, showing reduced carbohydrate metabolism and fatty acid biosynthesis pathways in fine-textured soils, suggesting a reduction in decomposer functionality under prolonged exposure.

These predicted pathway shifts represent the functional dimension of the stress response within the conceptual model (Figure 8–1), reflecting a reallocation of metabolic potential from growth and biosynthesis toward degradation and stress mitigation. Such predicted shifts, if sustained may translate into potential ecological consequences by reducing energy turnover, organic matter decomposition, and nutrient cycling (Ni et al., 2025).

8.4 Microbial interfaces with neonicotinoid fate

This section represents the hypothesised interfaces presented by the dashed lines in the conceptual model (Figure 8–1), illustrating that microbial communities both influence and are influenced by neonicotinoid fate.

Once in the soil, neonicotinoids may be degraded through microbial and abiotic processes, or may accumulate and persist, prolonging exposure to soil organisms (Pang et al., 2020), while abiotic degradation occurs through hydrolysis, photolysis, and oxidation (Jia et al., 2023). Both processes generate transformation products which may be less toxic, equally toxic, or more bioactive than the parent compound (Shen et al., 2022).

Persistent residues and transformation products create a chronic exposure regime that exerts multiple effects on microbial communities. These include community composition shifts, reductions in diversity, and disruptions to microbial co-occurrence networks (Gangola et al., 2023; Zhang et al., 2021b). Such microbial impacts cascade into functional consequences, including impaired nutrient cycling, reduced biosynthetic capacity, greater susceptibility to soil-borne pathogens, and altered plant–microbe interactions (Cycoń & Piotrowska-Seget, 2015a; Wang et al., 2025; Wang et al., 2024c).

It is important to recognise that microorganisms are not merely passive recipients of contamination, but active participants in determining their persistence and transformation in soil. Their interactions with neonicotinoids can be grouped into three major interfaces: biodegradation, toxicological responses, and feedback effects on soil processes that influence pesticide fate. These interfaces are conceptual and derived from previous studies rather than direct experimental evidence. They are included to provide a mechanistic context for the observed microbial responses described in this thesis, showing how microbial processes can collectively influence neonicotinoid persistence and soil ecological functions.

8.4.1 Biodegradation pathways

A variety of bacteria and fungi are capable of metabolising neonicotinoids, using them as nitrogen or carbon sources and transforming parent compounds into hydroxylated, reduced, or demethylated products (Gautam & Dubey, 2023). A recent synthesis of studies highlights that degradation potential is widespread but heterogeneous across neonicotinoids and microbial taxa (Randhawa, 2024). Imidacloprid shows more variable outcomes, reflecting differences in microbial community composition and exposure history. *Bradyrhizobiaceae* strain SG-6C *and Bacillus alkalinitrilicus* from agricultural soils have been shown to remove > 90 % of applied imidacloprid (Sharma et al., 2014; Shettigar et al., 2012), while *Klebsiella*

pneumoniae and *Ochrobactrum* BCL-1 achieve more moderate (70–78 %) degradation (Hu et al., 2013; Phugare et al., 2013). Complementary fungal studies demonstrate similar trends, with *Aspergillus terreus* and *Phanerochaete chrysosporium* achieving ≈ 96–97 % degradation compared with ≈ 64 % by *Trametes versicolor* (Hu et al., 2022; Mohammed & Badawy, 2017; Weaver et al., 2023). Thiamethoxam generally exhibits high microbial degradability, with near-complete removal frequently reported under laboratory conditions. Several *Pseudoxanthomonas* and *Stenotrophomonas* strains, along with mixed soil consortia, achieve > 95 % degradation (Zhou et al., 2014), and *Phanerochaete chrysosporium* shows ≈ 98 % fungal removal efficiency (Chen et al., 2021a). Clothianidin remains one of the most recalcitrant neonicotinoids. *Ochrobactrum anthropi* and *Acinetobacter johnsonii* from glasshouse soils remove ≈ 79 % of clothianidin (Wang et al., 2019), whereas field-isolated *Pseudomonas* strains typically achieve only partial degradation, indicating slower breakdown under natural soil conditions (Parte & Kharat, 2019). Fungal contributions are comparatively modest, with *Phanerochaete sordida* showing ≈ 37 % removal, while co-culturing *Phlebia brevispora* with growth-promoting bacteria reduced clothianidin recovery by > 40 % compared with axenic cultures, indicating enhanced biodegradation under synergistic conditions (Harry-Asobara & Kamei, 2019; Mori et al., 2024).

8.4.2 Toxicological responses

While some microbes contribute to degradation, many suffer negative impacts from exposure to neonicotinoids. In saline and agricultural soils, imidacloprid application has been shown to reduce bacterial diversity and suppress enzyme activities (such as dehydrogenase, urease, acid phosphatase), with more pronounced effects at higher dosage and longer exposure (Mahapatra et al., 2017; Singh et al., 2024; Zhang et al., 2015a). Thiamethoxam exposure alters soil bacterial community composition and metabolic potential, particularly under high treatment conditions, and reduces microbial enzyme activities (including dehydrogenase and nitrate reductase) especially in rhizosphere and orchard soils (Shukla et al., 2024; Wu et al., 2021). Clothianidin's persistence in tropical soil systems (up to 90 days post-application) suggests prolonged exposure risks; given that extended residue presence generally correlates with adverse effects on non-target soil fauna and likely microbial community stress, its persistence increases the window for potential toxicity (Ramasubramanian, 2021).

8.4.3 Feedbacks on soil processes

Microbial activity indirectly modifies pesticide fate by altering soil physicochemical properties. Organic matter turnover can increase sorption sites or release bound residues, changing bioavailability (Fomsgaard & Kristensen, 1999; Reemtsma et al., 2003). Microbial respiration

and metabolite production can locally shift pH and redox conditions, which in turn influence the rates of hydrolysis and oxidation of neonicotinoids (Iqbal et al., 2025; Van Eerd et al., 2003). Additionally, the production of extracellular polymers and biofilms can affect pesticide mobility, either by promoting immobilisation or by enhancing transport through soil aggregates (Breitenbach et al., 2022; Kidinda et al., 2023).

8.5 Broader implications for soil health and agriculture

This section extends beyond the “Potential ecological consequences” illustrated in the model (Figure 8–1) to demonstrate how the conceptual framework developed in this study can inform broader soil-health assessment, agricultural management, and regulatory practices. The results of this thesis have direct implications for maintaining soil ecosystem services, agricultural productivity, and long-term sustainability. Because neonicotinoid effects vary with soil properties and exposure duration, risk assessments should explicitly incorporate spatial and temporal heterogeneity.

Current regulatory frameworks for pesticide evaluation, including those established by the Organisation for Economic Co-operation and Development (OECD, 2000a, 2000b) and adopted by Australian Pesticides and Veterinary Medicines Authority (APVMA, 2019), European Food Safety Authority (EFSA, 2014) and the United States Environmental Protection Agency (USEPA, 2012), rely on internationally harmonised tests that quantify carbon and nitrogen transformation as indicators of nutrient cycling. These OECD guidelines remain essential for benchmarking soil microbial function and ensuring comparability across jurisdictions. However, they focus on standardised short-term endpoints and cannot fully represent how soil-specific properties and microbial interactions influence pesticide behaviour and long-term ecological outcomes. Recent studies have expanded this perspective by identifying microbial endpoints that better reflect ecological disturbance. Swaine et al. (2025) showed through a global meta-analysis that ammonia-oxidising microorganisms (AOM), measured via *amoA* gene abundance, provide a consistent and sensitive indicator of pesticide-induced stress. Their work supports the integration of molecular microbial endpoints into ecotoxicological assessments.

The research presented in this thesis contributes to that broader perspective by identifying the structural and compositional attributes that underpin functional stability, including diversity, interaction strength, and texture-specific sensitivity. In this study, soils with higher cation-exchange capacity or elevated exchangeable-sodium percentage retained neonicotinoids for longer periods, likely prolonging microbial exposure and delaying community recovery. These results highlight that soil texture and chemistry strongly mediate pesticide persistence and

microbial stress responses, providing mechanistic insight that complements conventional functional assays.

Extending this evidence to landscape and cumulative-risk contexts, Mu et al. (2023) combined field monitoring and modelling to demonstrate that compounds such as imidacloprid, chlorpyrifos, and carbendazim pose chronic and mixture-driven hazards to soil organisms. Similarly, Lu et al. (2024) applied a risk-entropy framework to show that higher cumulative pesticide residue loads were associated with reduced bacterial diversity and consistent shifts in indicator taxa such as *Bacillus* and *Sphingomonas*. Together, these studies confirm that chemical persistence and mixture effects translate into measurable biological change, reinforcing the need to integrate community-level microbial indicators alongside established OECD endpoints.

From an agroecological perspective, the selective enrichment and suppression of key microbial taxa have consequences for nutrient cycling, plant–microbe interactions, and the soil food web. Suppression of nitrifiers and saprotrophic fungi under certain treatments could slow nitrogen turnover and organic matter decomposition, respectively, with potential knock-on effects on crop nutrient availability. Conversely, the enrichment of diazotrophic taxa such as *Mesorhizobium* under some neonicotinoid exposures may transiently enhance biological nitrogen fixation, though whether this translates into agronomic benefit remains uncertain. These results underline the need for long-term monitoring of soil functional indicators to detect shifts that could compromise fertility before yield losses become apparent.

The observed alterations in microbial network architecture also carry implications for ecosystem resilience. More fragmented and modular networks, as observed in clay soils under imidacloprid exposure, may be less capable of buffering disturbance, increasing the risk of regime shifts under repeated chemical inputs or climatic stress. In contrast, the comparatively more integrated network structure observed in sandy loam suggest greater maintenance of microbial community organisation under imidacloprid exposure. This comparatively buffered response may reflect the combined influence of soil properties, including higher organic matter, that can moderate microbial responses to pesticide exposure. This aligns with emerging evidence that microbiome stability is an important predictor of soil multifunctionality and should be considered when evaluating management practices.

Finally, the downregulation of core metabolic pathways involved in carbohydrate metabolism, nucleotide biosynthesis, and energy turnover raises concerns about the biogeochemical capacity of soils subjected to chronic neonicotinoid exposure. Reduced microbial processing of carbon substrates could alter soil carbon cycling, although the direction of longer-term effects on carbon sequestration remains uncertain. Reduced decomposition may limit

microbial transformation of organic matter into stable carbon, whereas reduced microbial respiration could also increase carbon retention. Thus, the implications of these findings extend beyond agronomy to encompass global soil-carbon management goals and the resilience of terrestrial ecosystems to anthropogenic stressors.

Sustainable agriculture depends on maintaining soil biological integrity while optimising productivity. As Robinson (2024) emphasised, aligning technological innovation with ecocentric soil stewardship is essential for achieving long-term agroecosystem resilience. Together, the conceptual framework developed in this study connects the environmental behaviour of neonicotinoids with microbial responses that influence soil function. It offers an integrated understanding of how chemical stress can affect microbial stability and ecological balance. By relating these patterns, the framework provides insight into how this knowledge can support more sustainable agricultural practices.

8.6 Conclusion

This chapter synthesised the experimental findings of this thesis into a conceptual model that explains how neonicotinoid behaviour and microbial ecology interact to shape soil processes. The model highlights microorganisms as both responders to and mediators of chemical stress, influencing neonicotinoid transformation and soil ecological processes.

It demonstrates that soil texture, compound chemistry, and exposure duration jointly determine the magnitude and direction of microbial responses. These factors shape community composition, network structure, and predicted functional capacity. The conceptual interfaces of biodegradation, toxicological responses, and feedback effects provide a mechanistic framework for interpreting the observed patterns and for anticipating similar outcomes across different soils and compounds.

By linking microbial mechanisms with ecological outcomes, the framework clarifies how neonicotinoid stress can influence nutrient cycling, organic matter turnover, and the stability of soil functions that underpin fertility and climate regulation. It provides a scientific foundation for incorporating microbial indicators into neonicotinoid-risk assessment and soil-health evaluation, strengthening the ecological dimension of sustainable agriculture.

Chapter 9 Conclusions and future directions

9.1 Executive summary

This thesis addressed five interlinked research questions designed to evaluate how neonicotinoid seed treatments influence soil microbial communities and the implications of these effects for soil health and agricultural sustainability. The research combined systematic synthesis, short-term multi-compound experiments, and prolonged texture-specific exposure trials to provide a comprehensive, multi-layered analysis.

RQ1: To what extent do neonicotinoids affect soil microbial communities across existing studies?

This was addressed in **Chapter 2**, which conducted a PRISMA-guided systematic review of 29 peer-reviewed articles from PubMed, SCOPUS, and Web of Science. The review found that imidacloprid was the most frequently studied compound (66%), with spray formulations dominating (83%), and only 28% of studies were conducted under field conditions. Diversity indices were the most commonly measured microbial endpoints (62%). Approximately 45% of studies reported adverse effects on microbial diversity, composition, functioning, enzymatic activity, or nitrogen transformations. However, results were highly heterogeneous, largely due to methodological variation, exposure design, and lack of standardisation across studies.

RQ2: How does this evidence inform understanding of the broader ecological impacts of neonicotinoid use?

Also addressed in **Chapter 2**, the review highlighted several critical gaps that constrain ecological interpretation: limited representation of fungal communities, underreporting of soil texture and physicochemical factors, and a scarcity of studies linking microbial shifts to biogeochemical processes. These findings provided the rationale for the experimental components of this thesis, which incorporated multiple neonicotinoids, both bacterial and fungal communities, texture-stratified microcosms, and analyses of diversity, network structure, and predicted function.

RQ3: How do different neonicotinoids influence microbial community composition, diversity, and functions over time?

This question was addressed in **Chapters 4**–**7**, which together examined temporal dynamics across both short-term (10 days) and prolonged (28 days) exposures. Chapters 4 and 6 investigated bacterial and fungal responses to imidacloprid, thiamethoxam, and clothianidin during the first 10 days of exposure and revealed compound-specific and time-dependent

patterns. Thiamethoxam and clothianidin significantly increased bacterial richness and evenness early in exposure, whereas imidacloprid caused a temporary reduction in Shannon diversity and evenness at later stages. Fungal communities were generally more stable but still exhibited significant temporal responses, including richness declines under clothianidin and shifts in phylogenetic diversity under imidacloprid. **Chapters 5** and **7** extended the investigation to a 28-day experiment with imidacloprid, capturing delayed responses and demonstrating that time remained an important driver of microbial change. Gradual adjustments in evenness, community structure, and co-occurrence network properties became more pronounced toward the later stages of exposure, indicating that microbial reorganisation continues well beyond the first 10 days. Together, these results show that neonicotinoid effects are dynamic over time, encompassing both immediate disruptions and slower, progressive adjustments in microbial communities.

RQ4: How do soil texture and physicochemical properties mediate microbial community responses and patterns of adaptation under neonicotinoid exposure?

Explored in **Chapters 5** and **7**, the 28-day imidacloprid study demonstrated strong texture effects. Clay soils exhibited reduced evenness, fragmented and modular co-occurrence networks, and downregulation of carbohydrate and nucleotide metabolism, whereas sandy loam maintained denser, more connected networks. Edaphic factors, including ESP, pH, Ca, Mg, OM, and total C, were significant predictors of community structure and diversity, confirming that soil properties modulate the magnitude and trajectory of neonicotinoid effects.

RQ5: What are the implications of neonicotinoid-induced changes in soil microbial communities for soil health and agricultural sustainability?

Synthesised in **Chapter 8**, a fate–effect–function conceptual model was developed linking neonicotinoid persistence, microbial exposure, and the resulting taxonomic and network-level, and predicted functional changes. The model highlighted that these effects may constrain carbon turnover, nutrient cycling, and neonicotinoid biodegradation potential, particularly in fine-textured soils, with potential consequences for long-term soil fertility, ecosystem resilience, and agricultural sustainability.

9.2 Key contributions

- Multi-compound, multi-phase design: Provided one of the few comparative studies examining three neonicotinoids under identical conditions for short-term effects and focusing on a single compound (imidacloprid) for texture-specific prolonged assessment.

- Integration of complementary analyses: Combined diversity indices, taxonomic composition, network topology, and functional predictions to characterise both structural and functional dimensions of microbial responses.
- Soil-specific mechanistic insight: Demonstrated that clay soils show stronger network fragmentation and functional suppression, highlighting the importance of edaphic context in determining ecological outcomes.
- Conceptual synthesis: Developed a fate–effect–function framework linking neonicotinoid persistence, microbial exposure, community restructuring, and functional outcomes, and explicitly extends these insights to agricultural sustainability and soil health. This synthesis highlighted how microbial shifts under pesticide stress can influence nutrient cycling, organic matter dynamics, and bioremediation capacity, providing a mechanistic basis for improving neonicotinoid risk assessments and guiding evidence-based policy and management decisions.

9.3 Limitations

9.3.1 Experimental design constraints

The work was conducted under controlled microcosm conditions to minimise variability and enable precise treatment comparisons. However, microcosms cannot fully reproduce field-scale heterogeneity in soil moisture, temperature fluctuations, plant–soil interactions, and multi-season pesticide carry-over. Consequently, the absolute magnitude of community shifts observed here may differ under field conditions, where microbial exposure is influenced by tillage, root exudates, and rainfall-driven redistribution of residues (Karas et al., 2018; Radolinski et al., 2019; Summerton et al., 2023; Sun et al., 2021).

9.3.2 Chemical exposure characterisation

Direct measurement of neonicotinoid residues and metabolites was not performed, so exposure was inferred from treatment application rates and durations rather than measured concentrations. Without residue kinetics, it is not possible to quantify sorption, transformation, or leaching losses, nor to confirm whether observed microbial recovery reflects true chemical dissipation or community adaptation under continued exposure (Wirsching et al., 2020). Moreover, potential formation of biologically active metabolites was not assessed, limiting mechanistic interpretation of observed effects.

9.3.3 Functional inference

Functional profiles in this thesis were inferred from 16S rRNA gene and ITS amplicon sequencing data using PICRUSt2, which enabled the identification of putative shifts in key metabolic pathways associated with neonicotinoid exposure. While this approach is widely accepted for deriving functional potential (Matchado et al., 2024), it does not measure actual gene expression or metabolic activity and may be influenced by gaps in reference genome representation. Confidence in functional conclusions is strengthened by direct validation via metagenomics, metatranscriptomics, metabolomics, or enzyme assays (Aguiar-Pulido et al., 2016).

9.3.4 Scope of microbial coverage

This thesis focused on bacteria and fungi, which represent major drivers of soil processes, but did not characterise archaea, protists, viruses, or soil fauna. These groups can significantly influence nutrient cycling and pesticide degradation, and their omission constrains interpretation of whole-microbiome responses (Astapati & Nath, 2023; Beaumelle et al., 2023).

9.4 Future research directions

The findings of this thesis open several promising avenues for further investigation that could enhance mechanistic understanding and improve ecological relevance. A critical next step is to pair chemical residue measurements with microbial analyses to link neonicotinoid persistence, transformation rates, and microbial community shifts more directly. This integration would help disentangle whether observed microbial changes reflect adaptation under continuous exposure, recovery following dissipation, or indirect alterations in soil nutrient dynamics.

Future studies should also explore a broader range of exposure scenarios, including multiple concentration levels and varying exposure durations, to capture potential dose–response relationships and thresholds of microbial disruption or resilience. Multifactorial designs incorporating co-occurring stressors, such as moisture fluctuations or nutrient imbalances, could reveal interactive effects that are highly relevant under field conditions.

To strengthen the functional interpretation of microbial shifts, direct process-level measurements (e.g., carbon mineralisation, nitrification rates, enzyme activities) and integrated multi-omics approaches should complement amplicon-based inference. This would allow for a more precise characterisation of the active metabolic pathways and ecosystem processes affected by neonicotinoid exposure.

Finally, long-term field-based studies are needed to evaluate microbial responses under realistic agronomic conditions, across multiple seasons and soil types. Such studies would capture resilience timescales and provide evidence essential for improving risk assessment frameworks and guiding sustainable soil management.

Together, these research directions will move the field toward a predictive understanding of pesticide–microbe interactions, enabling more accurate evaluation of their implications for soil health and agricultural productivity.

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Appendix A: Physicochemical properties of the soils used in this thesis

Table A. 1: Overview of the physicochemical properties of the soil samples analysed in this study.

Parameter	Unit	Method	Result			Method reference
Texture	–	USDA textural triangle	Loamy sand	Sandy loam	Clay	Soil Survey Division Staff (1993)
Clay	g/100 g	Hydrometer method	11.14	16.03	56.25	Carter and Gregorich (2007)
Silt		Hydrometer method	6.79	13.94	15.24	
Sand		Hydrometer method	82.07	70.03	28.51	
pH		1:5 soil:water suspension	6.33	6.71	7.74	Rayment and Lyons (2011)
Electrical conductivity (EC)	dS/m	1:5 soil:water extract	0.069	0.406	0.286	
Organic matter (OM)	g/100 g	Calculation: Total Carbon × 1.75	5.7	8.4	2.8	
Effective Cation exchange capacity (ECEC)	–	A sum of Ca, Mg, K, Na	8.8	23.0	44.0	
Exchangeable Ca	$cmol_+/kg$	Ammonium	6.3	18.0	25.0	
Exchangeable Mg		Acetate (1M	1.9	3.5	15.0	
Exchangeable K		NH_4OAc)	0.44	1.40	1.30	
Exchangeable Na			0.10	<0.065	3.20	
Calcium	%	Base saturation	72	78	56	
Magnesium		calculations -	21	15	33	
Potassium		Cation cmol+/kg	5.0	6.2	2.9	
Sodium - ESP		/ ECEC x 100	1.20	0.16	7.40	
Ca:Mg ratio	–	Calculation: Ca/ Mg (cmol+/kg)	3.4	5.1	1.7	
Phosphorus	mg P/kg	Fluoride-extractable P (Bray 1-P)	35	232	20	
Total Carbon	g/100 g	LECO TruMac Analyzer	3.3	4.8	1.6	LECO Corporation (2015)
Total Nitrogen		LECO TruMac Analyzer	0.19	0.43	0.13	
C:N ratio	–	Calculation: Total Carbon/ Total Nitrogen	17	11	12	

Appendix B: Supplementary materials for Chapter 4

Table B. 1: Summary statistics of sequencing depths across all samples.

Treatment	No. of samples	Mean reads (± SD)	Median reads	Minimum reads	Maximum reads
Control	30	5718 ± 2212	6132	391	9657
Imidacloprid	30	6313 ± 2301	6752	1881	11209
Thiamethoxam	30	6102 ± 1975	6626	728	9861
Clothianidin	30	6328 ± 2696	5572	2052	11565

Table B. 2: Bacterial relative abundance (%) at the phylum level across different treatments over time.

Treatment	Relative abundance (%)																							
	Control						Imidacloprid						Thiamethoxam						Clothianidin					
Day	1	2	3	5	7	10	1	2	3	5	7	10	1	2	3	5	7	10	1	2	3	5	7	10
Phylum																								
Abditibacteriota	0.00	0.01	0.00	0.02	0.01	0.02	0.02	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.03	0.00	0.01	0.00	0.02	0.01	0.01
Acidobacteriota	3.51	3.18	3.13	3.54	3.74	4.00	3.57	3.63	3.80	3.22	3.74	3.41	3.75	3.51	3.45	3.72	4.03	2.79	3.91	3.18	3.79	3.64	3.82	2.43
Actinobacteriota	36.92	39.34	36.13	37.49	36.82	38.49	33.55	38.16	37.07	36.92	37.35	34.95	37.42	37.15	38.55	37.05	37.57	37.14	33.55	39.48	32.22	37.93	39.02	44.91
Armatimonadota	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.01
Bacteroidota	8.98	9.05	8.47	8.41	8.61	7.70	10.05	8.10	9.29	8.37	7.80	7.97	10.10	8.34	9.16	8.60	8.19	7.74	10.95	7.56	9.93	9.81	7.42	5.66
Bdellovibrionota	0.14	0.18	0.19	0.16	0.24	0.22	0.21	0.16	0.21	0.19	0.24	0.22	0.16	0.18	0.11	0.18	0.15	0.21	0.15	0.26	0.25	0.22	0.19	0.15
Chloroflexi	2.14	2.44	1.57	1.86	1.82	1.76	2.46	1.54	2.00	1.63	1.66	1.49	2.35	1.83	1.35	1.77	1.92	1.48	1.74	1.50	1.91	2.06	1.53	1.27
Cyanobacteria	0.03	0.02	0.02	0.04	0.04	0.07	0.03	0.00	0.01	0.04	0.03	0.02	0.05	0.04	0.03	0.02	0.06	0.02	0.03	0.02	0.03	0.05	0.02	0.02
Dependentiae	0.26	0.21	0.11	0.23	0.38	0.27	0.20	0.15	0.22	0.21	0.25	0.25	0.51	0.16	0.20	0.27	0.19	0.19	0.23	0.11	0.18	0.14	0.23	0.14
Desulfobacterota	0.02	0.01	0.03	0.00	0.01	0.01	0.02	0.00	0.02	0.00	0.01	0.00	0.03	0.01	0.00	0.01	0.02	0.04	0.00	0.00	0.00	0.00	0.04	0.02
Entotheonellaeota	0.06	0.04	0.02	0.00	0.03	0.01	0.04	0.08	0.02	0.04	0.01	0.02	0.01	0.03	0.01	0.03	0.02	0.01	0.04	0.04	0.05	0.02	0.02	0.00
Fibrobacterota	0.07	0.08	0.07	0.04	0.13	0.10	0.06	0.03	0.06	0.09	0.05	0.06	0.06	0.09	0.01	0.04	0.02	0.04	0.08	0.11	0.07	0.16	0.09	0.02
Firmicutes	7.73	7.11	7.92	8.61	8.45	8.20	8.16	8.21	8.05	8.99	8.39	14.09	7.11	8.47	8.39	8.57	8.00	10.85	7.92	7.98	8.52	8.22	8.18	10.42
Gemmatimonadota	1.30	1.46	1.28	1.23	1.30	1.23	1.35	1.39	1.34	1.21	1.38	1.09	1.06	1.75	1.34	1.55	1.26	1.23	1.31	1.33	1.41	1.24	1.36	1.49
Hydrogenedentes	0.00	0.02	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.01	0.00	0.02	0.05	0.02	0.03	0.01	0.00	0.02	0.01	0.00
Methylomirabilota	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.05	0.01	0.01	0.02	0.02	0.01	0.03	0.03	0.02	0.02	0.00	0.00	0.00	0.02	0.02	0.00
Myxococcota	4.93	4.39	4.27	4.32	4.77	4.77	5.33	4.87	4.85	4.75	4.91	4.32	4.82	4.91	4.39	5.35	4.46	4.20	4.99	4.62	4.38	4.41	5.14	4.04
Nitrospirota	1.00	1.39	0.63	1.01	1.09	1.03	1.03	0.94	1.05	0.98	1.01	0.78	1.20	0.75	0.84	0.77	1.23	0.60	0.86	0.72	0.86	0.87	0.98	0.29
Patescibacteria	0.03	0.04	0.03	0.02	0.01	0.01	0.05	0.03	0.03	0.04	0.06	0.06	0.02	0.04	0.03	0.04	0.00	0.07	0.03	0.04	0.03	0.01	0.00	0.03
Planctomycetota	2.12	2.18	1.41	1.81	1.91	1.64	2.01	1.43	1.97	1.32	1.59	1.30	2.21	1.70	1.39	1.80	1.52	1.22	2.36	1.49	2.01	1.62	1.43	1.05
Proteobacteria	28.63	26.39	32.84	29.27	28.77	28.71	29.40	29.59	27.98	30.21	29.88	28.40	27.38	29.06	29.00	28.16	29.59	30.53	29.63	29.58	32.19	27.35	28.71	26.03
Spirochaetota	0.02	0.00	0.00	0.02	0.00	0.01	0.00	0.03	0.03	0.02	0.01	0.02	0.01	0.03	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.01	0.01	0.00
Sumerlaeota	0.01	0.00	0.01	0.01	0.03	0.01	0.01	0.01	0.02	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.01	0.00	0.01	0.01
Verrucomicrobiota	2.08	2.43	1.86	1.90	1.84	1.72	2.43	1.61	1.94	1.74	1.60	1.49	1.69	1.92	1.68	1.99	1.69	1.55	2.16	1.94	2.12	2.18	1.75	2.00

Table B. 3: Statistical test of different bacterial phyla across different treatments and time, with effect direction assessed using median and negative significance highlighted by the red circle and positive significance highlighted by the green circle.

Phylum	Test type	*P* value (Day)	*P* value (Treatment)
Abditibacteriota	Kruskal-Wallis	0.13	0.90
Acidobacteriota	Kruskal-Wallis	0.18	0.94
Actinobacteriota	Kruskal-Wallis	0.20	0.56
Armatimonadota	Kruskal-Wallis	0.10	0.94
Bacteroidota	Kruskal-Wallis	**0.01**	0.99
Bdellovibrionota	ANOVA	0.77	0.30
Chloroflexi	ANOVA	0.09	0.47
Cyanobacteria	ANOVA	0.51	0.17
Dependentiae	Kruskal-Wallis	0.19	0.22
Desulfobacterota	Kruskal-Wallis	0.16	0.60
Entotheonellaeota	ANOVA	0.11	0.62
Fibrobacterota	ANOVA	0.86	0.13
Firmicutes	Kruskal-Wallis	**0.02**	0.54
Gemmatimonadota	ANOVA	0.32	0.80
Hydrogenedentes	Kruskal-Wallis	0.56	0.21
Methylomirabilota	Kruskal-Wallis	0.92	**0.001**
Myxococcota	ANOVA	0.07	0.46
Nitrospirota	ANOVA	0.10	0.16
Patescibacteria	ANOVA	0.35	0.10
Planctomycetota	ANOVA	**0.01**	0.41
Proteobacteria	ANOVA	0.59	0.98
Spirochaetota	ANOVA	0.41	0.29
Sumerlaeota	Kruskal-Wallis	0.34	0.81
Verrucomicrobiota	ANOVA	0.11	0.10

Table B. 4: PERMANOVA results showing the influence of different neonicotinoid treatments, sampling day, and their interaction on soil bacterial community structure measured by Bray–Curtis dissimilarity matrix.

Source of variation	PERMANOVA				
	df	SS	R^2	F	PR (>F)
Day	5	1.14	0.05	1.20	0.013*
Treatment	3	0.54	0.02	0.95	0.664
Day:Treatment	15	3.26	0.14	1.14	0.004*
Residual	96	18.23	0.79	NA	NA
Total	119	23.17	1.00	NA	NA

*Signif. codes: '***' ≤ 0.001 '**' ≤ 0.01 '*' ≤ 0.05*

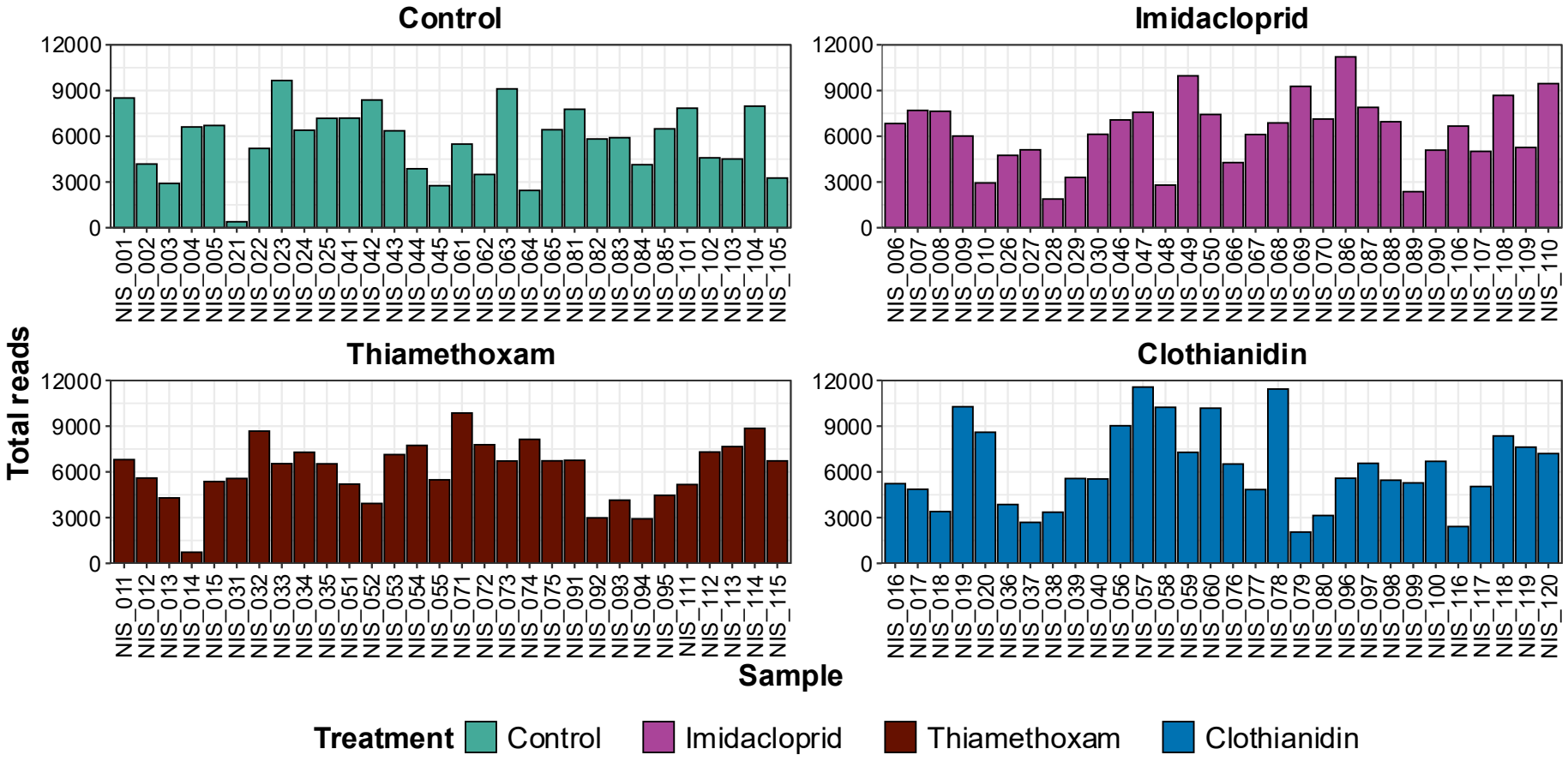

Figure B. 1: Barplot showing sequencing depth for each sample. Samples were not rarefied.

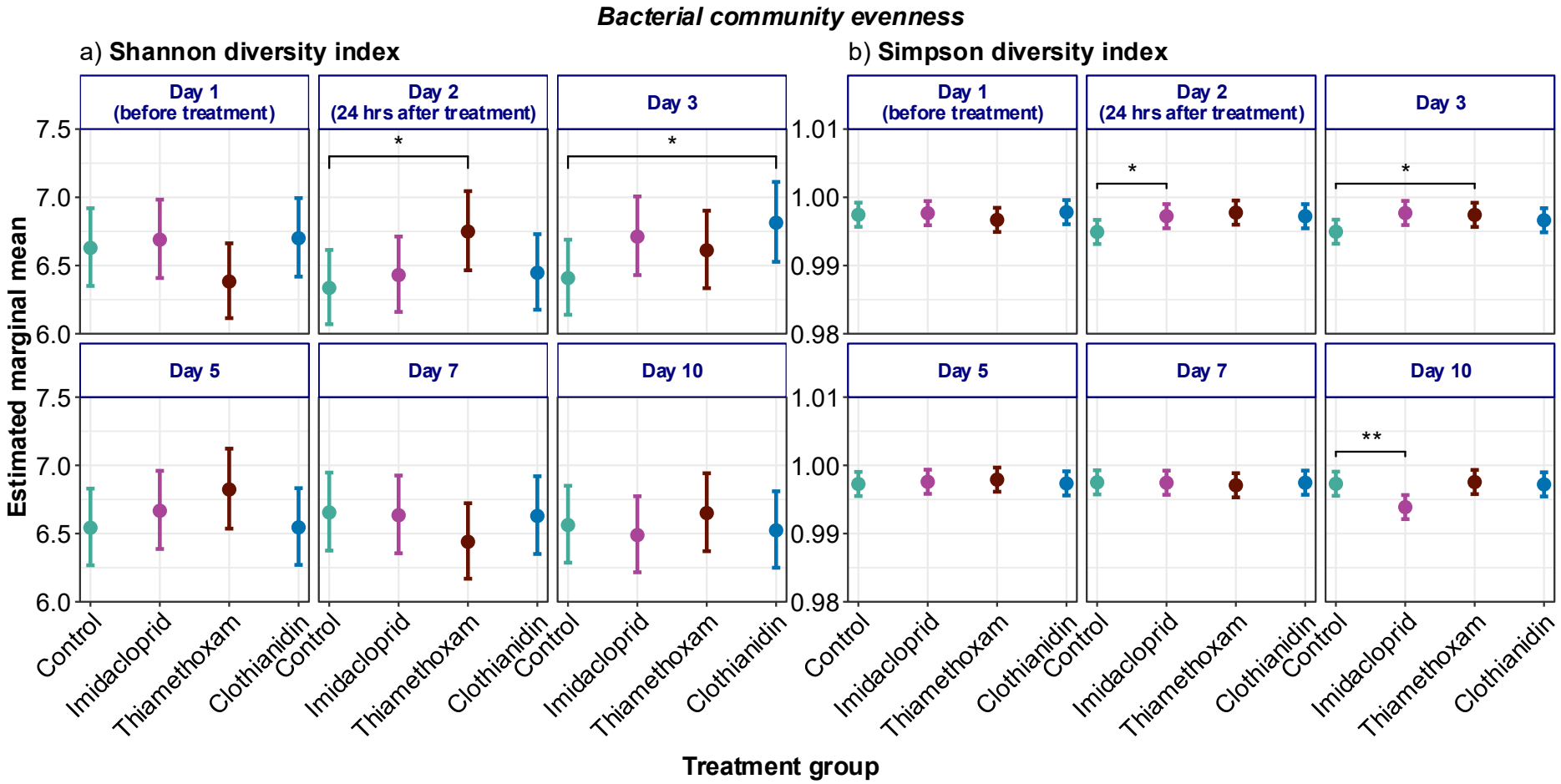

Figure B. 2: Alpha diversity indices for bacteria, measured by a) Shannon index, b) Simpson index. Points represent estimated marginal means (EMMs) ± 95% confidence intervals (n = 5) for each treatment group (Control, Imidacloprid, Thiamethoxam, and Clothianidin). Asterisks indicate significant differences among treatments within each day based on Generalised Linear Model analysis ('' $P \leq 0.01$, '*' $P \leq 0.05$).**

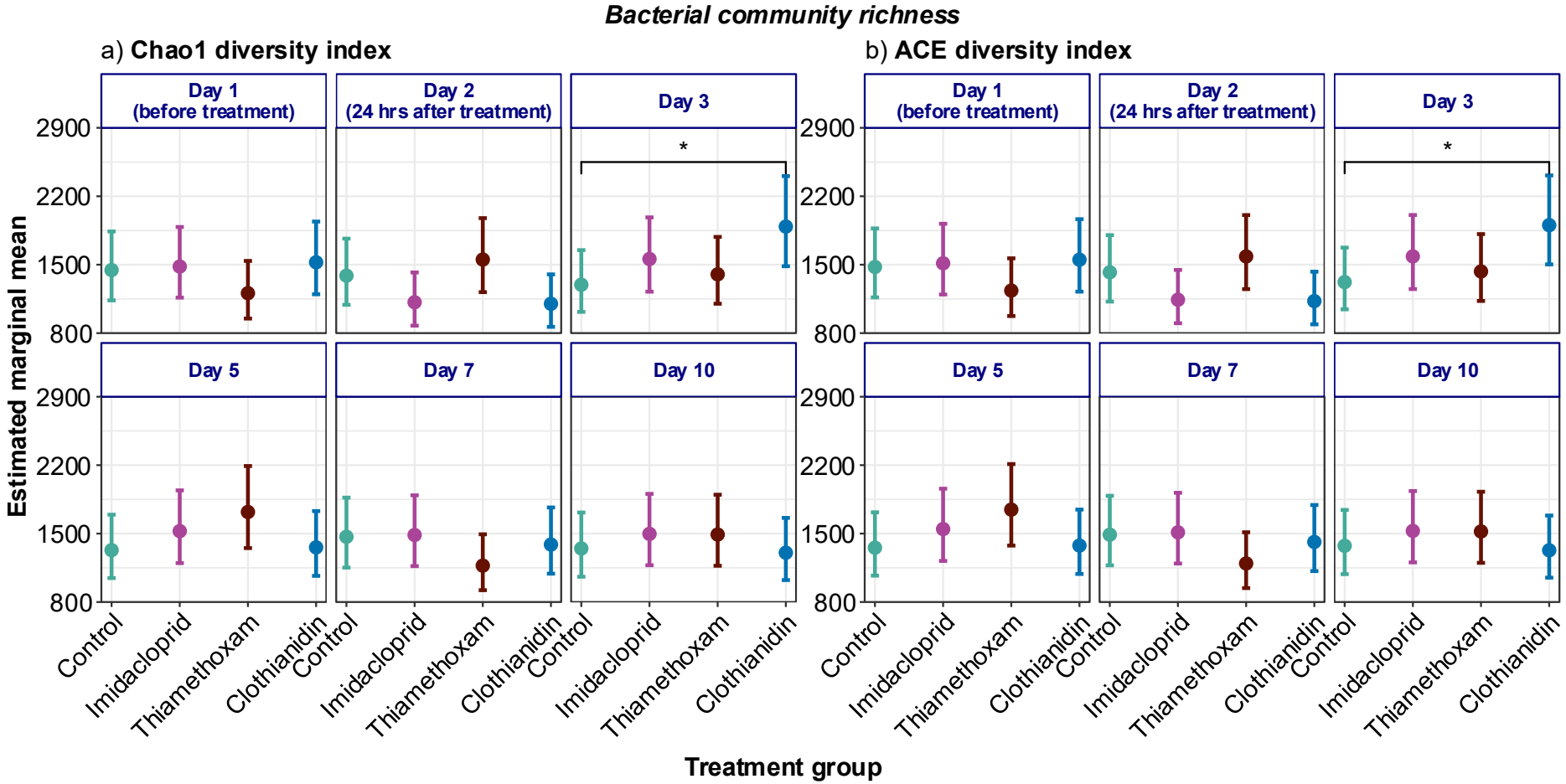

Figure B. 3: Alpha diversity indices for bacteria, evaluated through a) Chao1 index, b) ACE index. Points represent estimated marginal means (EMMs) ± 95% confidence intervals (n = 5) for each treatment group (Control, Imidacloprid, Thiamethoxam, and Clothianidin). Asterisks indicate significant differences among treatments within each day based on Generalised Linear Model analysis ('*' $P \leq 0.05$).

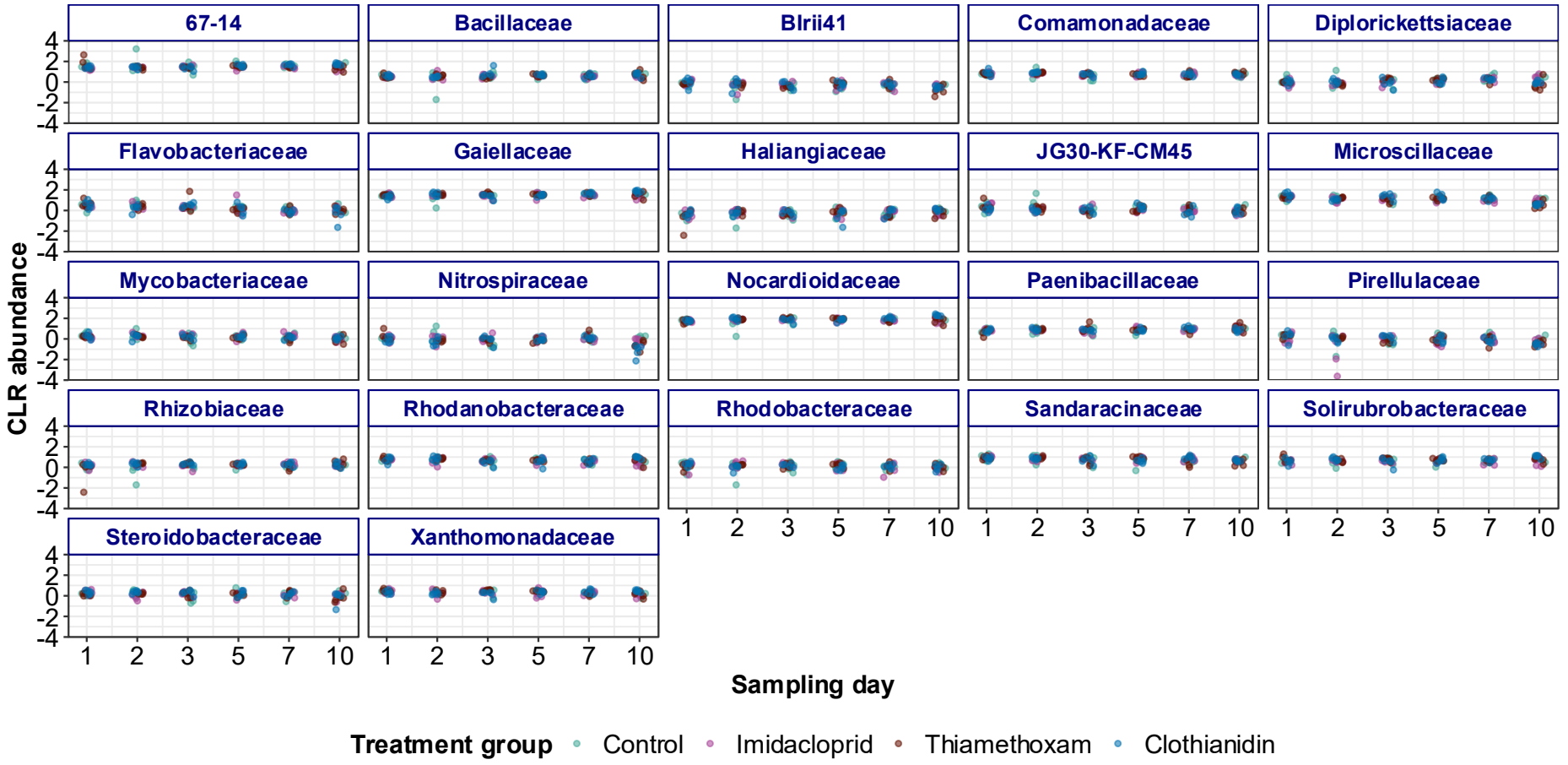

Figure B. 4: Scatter plot showing CLR-transformed relative abundance of bacterial families that showed significant Spearman correlations (P ≤ 0.05) with sampling day and/or neonicotinoid treatments.

Appendix C: Supplementary materials for Chapter 5

Table C. 1: Summary of sequencing depth across treatment, soil, and sampling day groups.

Treatment	Soil	Day	n	Mean	SD	Min	Max
Control	Loamy sand	3	3	5147	3246	2235	8646
		7		5608	2370	2874	7086
		14		8905	2746	6499	11896
		21		8113	2175	6053	10387
		28		7064	274	6752	7264
	Sandy loam	3	3	6969	2260	5360	9553
		7		8306	803	7662	9205
		14		8180	1328	7069	9651
		21		8190	4119	3469	11054
		28		6521	449	6011	6854
	Clay	3	3	7155	1657	6051	9060
		7		6942	2295	4461	8989
		14		6386	1324	5191	7810
		21		8962	1605	7480	10666
		28		3588	1350	2036	4484
Imidacloprid	Loamy sand	3	3	5716	4183	1274	9579
		7		6285	1249	4924	7380
		14		8417	3114	4933	10930
		21		8883	812	7963	9501
		28		6879	1234	5465	7740
	Sandy loam	3	3	6815	2867	5128	10125
		7		8494	3183	5559	11878
		14		7244	3832	2819	9480
		21		8480	3536	5047	12110
		28		4723	650	4304	5472
	Clay	3	3	6604	1849	4491	7925
		7		6701	2529	3802	8453
		14		6126	2782	3277	8836
		21		5704	1429	4331	7183
		28		4257	1336	2829	5476

Table C. 2: Analysis of deviance results for Chao1 richness estimate.

Index	Effect	Chi-square	Df	Pr (>Chisq)	Significance
Chao1	Treatment	1.85	1	0.17	
	Soil	32.24	2	9.97×10^{-8}	***
	Day	5.96	4	0.20	
	Treatment:Soil	1.21	2	0.55	
	Treatment:Day	0.89	4	0.93	
	Soil:Day	8.34	8	0.40	
	Treatment:Soil:Day	3.61	8	0.89	

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

Table C. 3: Analysis of deviance results for Pielou's evenness

Index	Effect	Chi-square	Df	Pr (>Chisq)	Significance
Pielou's evenness	Treatment	3.69	1	0.05	.
	Soil	4.21	2	0.12	
	Day	19.07	4	7.61×10^{-4}	***
	Treatment:Soil	0.54	2	0.76	
	Treatment:Day	3.66	4	0.45	
	Soil:Day	7.45	8	0.49	
	Treatment:Soil:Day	5.65	8	0.69	

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

Table C. 4: Analysis of deviance results for Faith's phylogenetic diversity (Faith's PD).

Index	Effect	Chi-square	Df	Pr (>Chisq)	Significance
Faith's PD	Treatment	0.11	1	0.74	
	Soil	44.97	2	1.72×10^{-10}	***
	Day	27.17	4	1.84×10^{-5}	***
	Treatment:Soil	1.60	2	0.45	
	Treatment:Day	2.58	4	0.63	
	Soil:Day	12.01	8	0.15	
	Treatment:Soil:Day	4.75	8	0.78	

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

Table C. 5: Analysis of deviance results for Mean pairwise distance (MPD).

Index	Effect	Chi-square	Df	Pr (>Chisq)	Significance
MPD	Treatment	3.74	1	0.05	.
	Soil	47.44	2	4.99×10^{-11}	***
	Day	10.47	4	0.03	*
	Treatment:Soil	1.42	2	0.491	
	Treatment:Day	8.76	4	0.07	.
	Soil:Day	7.92	8	0.44	
	Treatment:Soil:Day	6.48	8	0.59	

*Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

Table C. 6: Indicator taxa at the Order level across control and imidacloprid treatments in different soil types. Orders present in both treatments may represent distinct indicator ASVs differing at lower taxonomic ranks.

Soil texture	Control indicator taxa (Order)	Imidacloprid indicator taxa (Order)
Loamy sand	Rhizobiales, Pseudomonadales, Propionibacteriales, Cytophagales, Solirubrobacterales, Propionibacteriales, Vicinamibacterales	Polyangiales, Micrococcales, Corynebacteriales, Propionibacteriales, Rhizobiales, Flavobacteriales, Solirubrobacterales, Frankiales, Burkholderiales, Cytophagales, Corynebacteriales, Vicinamibacterales
Sandy loam	Rhizobiales, Gaiellales, Kineosporiales, Burkholderiales, Propionibacteriales, Solirubrobacterales, Cytophagales, Thermomicrobiales, Streptomycetales, Caulobacterales, Gemmatimonadales, Alicyclobacillales, Xanthomonadales, Corynebacteriales, Chitinophagales, Rhodobacterales, Micropepsales, Acetobacterales	Gammaproteobacteria Incertae Sedis, Burkholderiales, Cytophagales, Propionibacteriales, Streptomycetales, Pseudonocardiales, Acetobacterales, Rhizobiales, Pseudomonadales, Chitinophagales, Flavobacteriales
Clay	Streptomycetales, Gemmatimonadales, Solirubrobacterales, Burkholderiales, Pseudonocardiales, Frankiales, Gaiellales, Bacillales, Thermomicrobiales, Vicinamibacterales, Cytophagales, Propionibacteriales	Rhizobiales

Table C. 7: Taxon names corresponding to the nodes of the co-occurrence network for loamy sand soil, showing the treatments (control and imidacloprid).

Node number	Control Taxon (Order)	Node number	Imidacloprid Taxon (Order)
1	Azospirillales	1	Acetobacterales
2	Blastocatellales	2	Acholeplasmatales
3	Chitinophagales	3	Alicyclobacillales
4	Chthoniobacterales	4	Bacillales
5	Corynebacteriales	5	Corynebacteriales
6	Paenibacillales	6	Desulfotomaculales
7	Acetobacterales	7	Frankiales
8	Acidimicrobiales	8	Gemmatales
9	Acidobacteriales	9	Micromonosporales
10	Alicyclobacillales	10	Propionibacteriales
11	Babeliales	11	Solirubrobacterales
12	Bacillales	12	Streptomycetales
13	Diplorickettsiales	13	Acidobacteriales
14	Fibrobacterales	14	Bdellovibrionales
15	Frankiales	15	Bryobacterales
16	Gaiellales	16	Dongiales
17	Gammaproteobacteria Incertae Sedis	17	Gaiellales
18	Gemmatales	18	Gammaproteobacteria Incertae Sedis
19	Gemmatimonadales	19	Gemmatimonadales
20	Haliangiales	20	Kineosporiales
21	Kineosporiales	21	Nitrospirales
22	Micrococcales	22	Polyangiales
23	Micromonosporales	23	Pseudonocardiales
24	Micropepsales	24	Reyranellales
25	Nitrospirales	25	Steroidobacterales
26	Pedosphaerales	26	Thermoanaerobaculales
27	Pirellulales	27	Acidiferrobacterales
28	Polyangiales	28	Dethiobacterales
29	Propionibacteriales	29	Azospirillales
30	Reyranellales	30	Burkholderiales
31	Rhodobacterales	31	Chitinophagales
32	Solirubrobacterales	32	Cytophagales
33	Sphingomonadales	33	Flavobacteriales
34	Streptomycetales	34	Pseudomonadales
35	Streptosporangiales	35	Elsterales
36	Thermoanaerobaculales	36	Kallotenuales
37	Thermomicrobiales	37	Fibrobacterales
38	Kallotenuales	38	Myxococcales
39	Obscuribacterales	39	Oscillospirales
40	Rickettsiales	40	Thermincolales
41	Silvanigrellales	41	Micrococcales
42	Burkholderiales	42	Vicinamibacterales
43	Cytophagales	43	Sphingomonadales
44	Flavobacteriales	44	Haliangiales
45	Pseudomonadales	45	Streptosporangiales
46	Micavibrionales	46	Longimicrobiales

Node number	Control Taxon (Order)	Node number	Imidacloprid Taxon (Order)
47	Desulfitibacterales	47	Ectothiorhodospirales
48	Dethiobacterales	48	Verrucomicrobiales
49	Rokubacteriales	49	Saccharimonadales
50	Caldilineales	50	Rhodospirillales
51	Rhizobiales	51	Rhizobiales
52	Xanthomonadales	52	Veillonellales-Selenomonadales
53	Sphingobacteriales		
54	Pseudonocardiales		
55	Vicinamibacterales		
56	Tepidisphaerales		
57	Steroidobacterales		
58	Veillonellales-Selenomonadales		
59	Verrucomicrobiales		
60	Sumerlaeales		
61	Hydrogenedentiales		
62	Opitutales		
63	Thermincolales		

Table C. 8: Taxon names corresponding to the nodes of the co-occurrence network for sandy loam soil, showing the treatments (control and imidacloprid).

Node number	Control Taxon (Order)	Node number	Imidacloprid Taxon (Order)
1	Azospirillales	1	Acetobacterales
2	Blastocatellales	2	Alicyclobacillales
3	Chitinophagales	3	Bacillales
4	Chthoniobacterales	4	Caldilineales
5	Corynebacteriales	5	Caulobacterales
6	Rhizobiales	6	Corynebacteriales
7	Acetobacterales	7	Frankiales
8	Alicyclobacillales	8	Micromonosporales
9	Babeliales	9	Propionibacteriales
10	Bacillales	10	Solirubrobacterales
11	Bdellovibrionales	11	Bdellovibrionales
12	Diplorickettsiales	12	Dongiales
13	Frankiales	13	Gaiellales
14	Gaiellales	14	Gammaproteobacteria Incertae Sedis
15	Gammaproteobacteria Incertae Sedis	15	Gemmatimonadales
16	Gemmatimonadales	16	Nitrospirales
17	Haliangiales	17	Polyangiales
18	Micrococcales	18	Reyranellales
19	Micromonosporales	19	Steroidobacterales
20	Myxococcales	20	Chthoniobacterales
21	Nitrospirales	21	Lachnospirales
22	Pirellulales	22	Rhizobiales
23	Polyangiales	23	Fimbriimonadales
24	Propionibacteriales	24	Haliangiales
25	Pseudonocardiales	25	Micrococcales
26	Reyranellales	26	Rhodobacterales

Node number	Control Taxon (Order)	Node number	Imidacloprid Taxon (Order)
27	Rhodobacterales	27	Thermomicrobiales
28	Solirubrobacterales	28	Burkholderiales
29	Steroidobacterales	29	Chitinophagales
30	Streptosporangiales	30	Clostridiales
31	Caldilineales	31	Flavobacteriales
32	Anaerolineales	32	Paenibacillales
33	Burkholderiales	33	Pseudomonadales
34	Fimbriimonadales	34	Bacteriovoracales
35	Flavobacteriales	35	Rhodospirillales
36	Micavibrionales	36	Azospirillales
37	Acidiferrobacterales	37	Verrucomicrobiales
38	Kiloniellales	38	Pirellulales
39	Nitrococcales	39	Enterobacterales
40	Fibrobacterales	40	Vicinamibacterales
41	Paenibacillales	41	Planctomycetales
42	Vicinamibacterales	42	Xanthomonadales
43	Microtrichales	43	Streptomycetales
44	Gemmatales	44	Pseudonocardiales
45	Streptomycetales	45	Sphingomonadales
46	Thermomicrobiales	46	Microtrichales
47	Planctomycetales	47	Tepidisphaerales
48	Cytophagales	48	Myxococcales
49	Pseudomonadales	49	Sphingobacteriales
50	Isosphaerales	50	Oligoflexales
51	Spirochaetales	51	Cytophagales
52	Symbiobacteriales	52	Salinisphaerales
53	Vampirovibrionales	53	Rokubacteriales
		54	Rickettsiales

Table C. 9: Taxon names corresponding to the nodes of the co-occurrence network for clay soil, showing the treatments (control and imidacloprid).

Node number	Control Taxon (Order)	Node number	Imidacloprid Taxon (Order)
1	Azospirillales	1	Alicyclobacillales
2	Chitinophagales	2	Bacillales
3	Rhizobiales	3	Desulfotomaculales
4	Babeliales	4	Gemmatales
5	Bacillales	5	Micromonosporales
6	Frankiales	6	Obscuribacterales
7	Gaiellales	7	Propionibacteriales
8	Gemmatimonadales	8	Solirubrobacterales
9	Glycomycetales	9	Bryobacterales
10	Haliangiales	10	Gaiellales
11	Micrococcales	11	Gemmatimonadales
12	Nitrospirales	12	Nitrospirales
13	Pirellulales	13	Rubrobacterales
14	Propionibacteriales	14	Rhizobiales
15	Pyrinomonadales	15	Sphingomonadales
16	Reyranellales	16	Symbiobacteriales
17	Rhodobacterales	17	Burkholderiales

Node number	Control Taxon (Order)	Node number	Imidacloprid Taxon (Order)
18	Tepidisphaerales	18	Aneurinibacillales
19	Burkholderiales	19	Bacteriovoracales
20	Cytophagales	20	Glycomycetales
21	Thermomicrobiales	21	Ardenticatenales
22	Vicinamibacterales	22	Vicinamibacterales
23	Anaerolineales	23	Cytophagales
24	Isosphaerales	24	Fibrobacterales
25	Xanthomonadales	25	Rhodobacterales
26	Tistrellales	26	Steroidobacterales
27	Planctomycetales	27	Salinisphaerales
28	Bacteroidales	28	Pseudonocardiales
29	Micromonosporales	29	Xanthomonadales
30	Salinisphaerales	30	Diplorickettsiales
31	Polyangiales	31	Polyangiales
32	Solirubrobacterales	32	Thermomicrobiales
33	Steroidobacterales	33	Syntrophobacterales
34	Sphingobacteriales	34	Erysipelotrichales
35	Thermoactinomycetales	35	Pedosphaerales

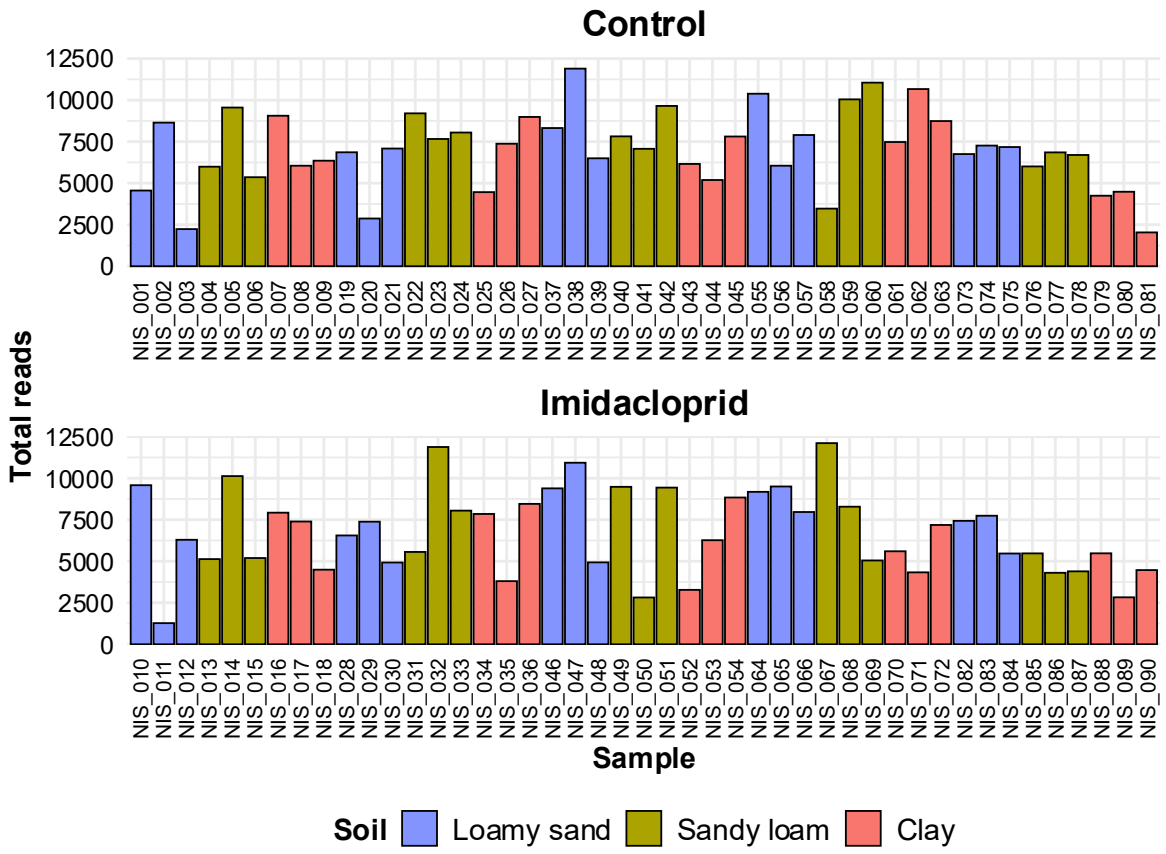

Figure C. 1: Barplot showing sequencing depth for each sample. Samples were not rarefied.

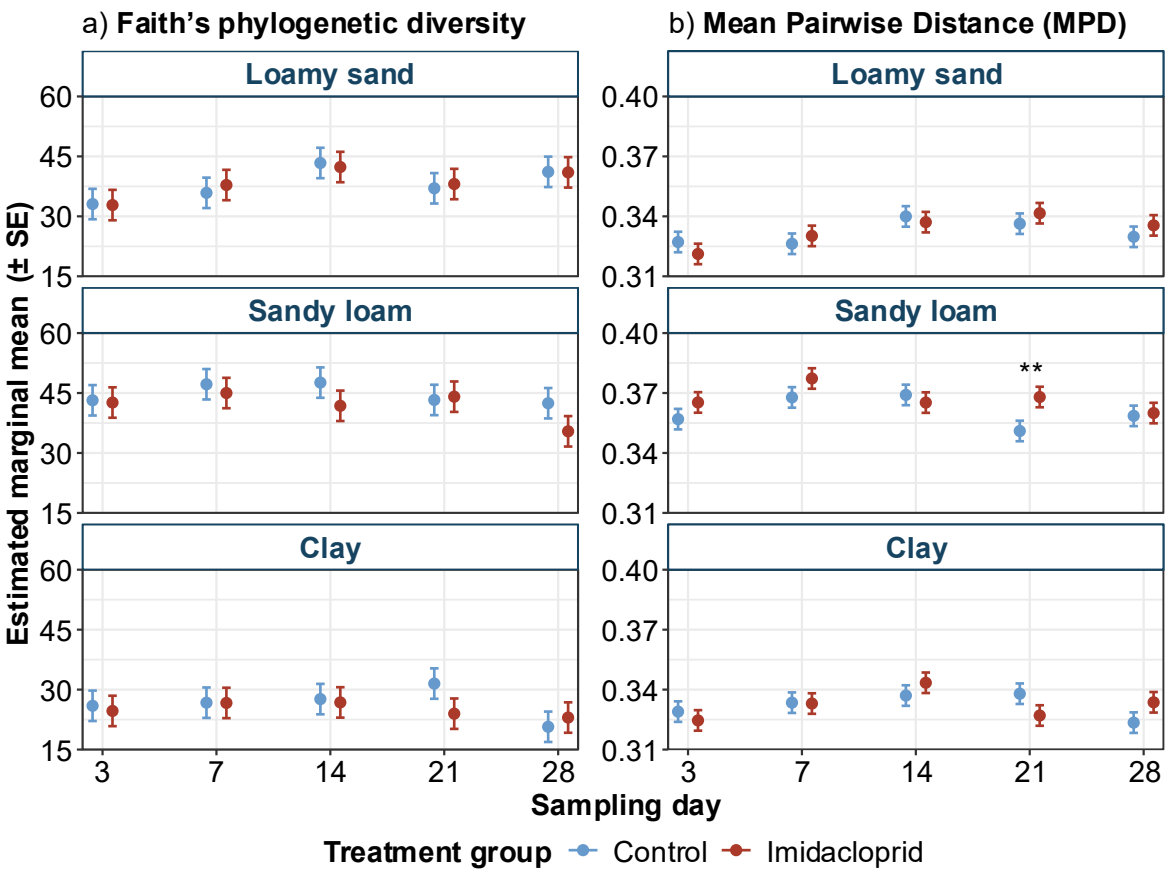

Figure C. 2: Changes in phylogenetic diversity of bacterial communities across soil textures and treatments over time. (a) Faith's phylogenetic diversity (PD) and (b) Mean Pairwise Distance (MPD). Points represent estimated marginal means ± SE (n = 3).

Appendix D: Supplementary materials for Chapter 6

Table D. 1: Summary statistics of sequencing depths across all samples.

Statistic	Value
Min.	80
1st Qu.	31727.7
Median	36978
Mean	38270.2
3rd Qu.	45893
Max.	75046

Table D. 2: Result of Type II Wald chi-square tests for linear mixed-effects models assessing the effects of treatment, sampling day and their interaction on fungal alpha diversity indices (Shannon entropy, Simpson's dominance index, Chao1 richness, and ACE estimator). Replicate was included as a random effect in each model. Significant effects are highlighted in bold

Factor	Response	χ^2	Df	*P* value
Shannon	Treatment	1.16	3	0.76
	Sampling day	4.02	5	0.55
	Treatment:Sampling day	17.29	15	0.30
Simpson	Treatment	2.29	3	0.51
	Sampling day	4.35	5	0.50
	Treatment:Sampling day	15.30	15	0.43
Chao1	Treatment	2.63	3	0.45
	Sampling day	14.30	5	**0.01**
	Treatment:Sampling day	21.14	15	0.13
ACE	Treatment	2.65	3	0.45
	Sampling day	14.56	5	**0.01**
	Treatment:Sampling day	21.14	15	0.13

Table D. 3: Estimated marginal means (± standard error) of fungal alpha diversity indices across treatments and sampling days, with corresponding 95% confidence intervals (lower–upper CL). Indices include Shannon entropy, Simpson's dominance index, Chao1 richness, and ACE estimator. Values were obtained from linear mixed-effects models with Treatment, Day, and their interaction as fixed effects, and Replicate as a random effect

Treatment	Contrast	Shannon	Simpson	Chao1	ACE
		Estimated difference			
Control	Day 2 – Day 1	−0.03	−0.001	11.4	8.9
	Day 3 – Day 1	−0.22	−0.013	−14.8	−18.4
	Day 5 – Day 1	0.05	0.002	3.3	0.9
	Day 7 – Day 1	−0.10	−0.001	−25.5	−30.7
	Day 10 – Day 1	−0.05	−0.006	−67.1	−72.6
Imidacloprid	Day 2 – Day 1	−0.05	−0.002	−39.7	−38.8
	Day 3 – Day 1	0.02	−0.002	−23.6	−22.6
	Day 5 – Day 1	−0.07	−0.002	−48.8	−49.6
	Day 7 – Day 1	−0.28)	−0.013	−106.4	−107.7
	Day 10 – Day 1	−0.42	**−0.023 (0.03)**	**−141.0 (0.03)**	**−143.6 (0.03)**
Thiamethoxam	Day 2 – Day 1	0.20	0.005	19.9	17.9
	Day 3 – Day 1	0.09	0.001	−25.3	−26.1
	Day 5 – Day 1	0.17	0.006	25.4	23.2
	Day 7 – Day 1	0.17	0.005	−12.9	−14.2
	Day 10 – Day 1	-0.27	−0.014	**−147.5 (0.02)**	**−149.4 (0.02)**
Clothianidin	Day 2 – Day 1	0.15	0.003	−18.5	−18.7
	Day 3 – Day 1	−0.02	−0.0001	11.8	12.9
	Day 5 – Day 1	0.09	0.001	36.3	36.6
	Day 7 – Day 1	0.32	0.007	21.6	20.1
	Day 10 – Day 1	0.14	−0.006	−46.0	−47.4

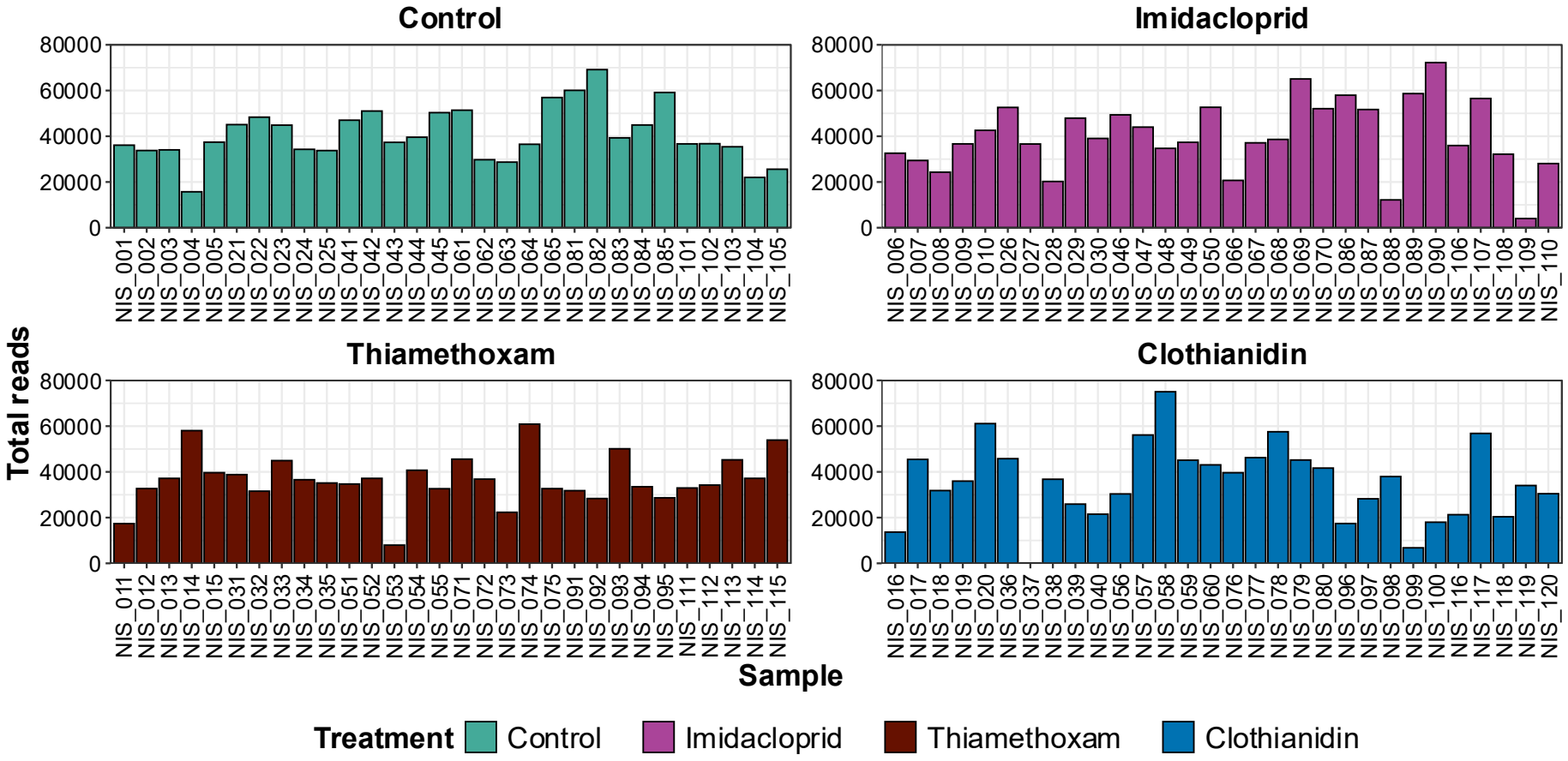

Figure D. 1: Barplot showing sequencing depth for each sample. Samples were not rarefied.

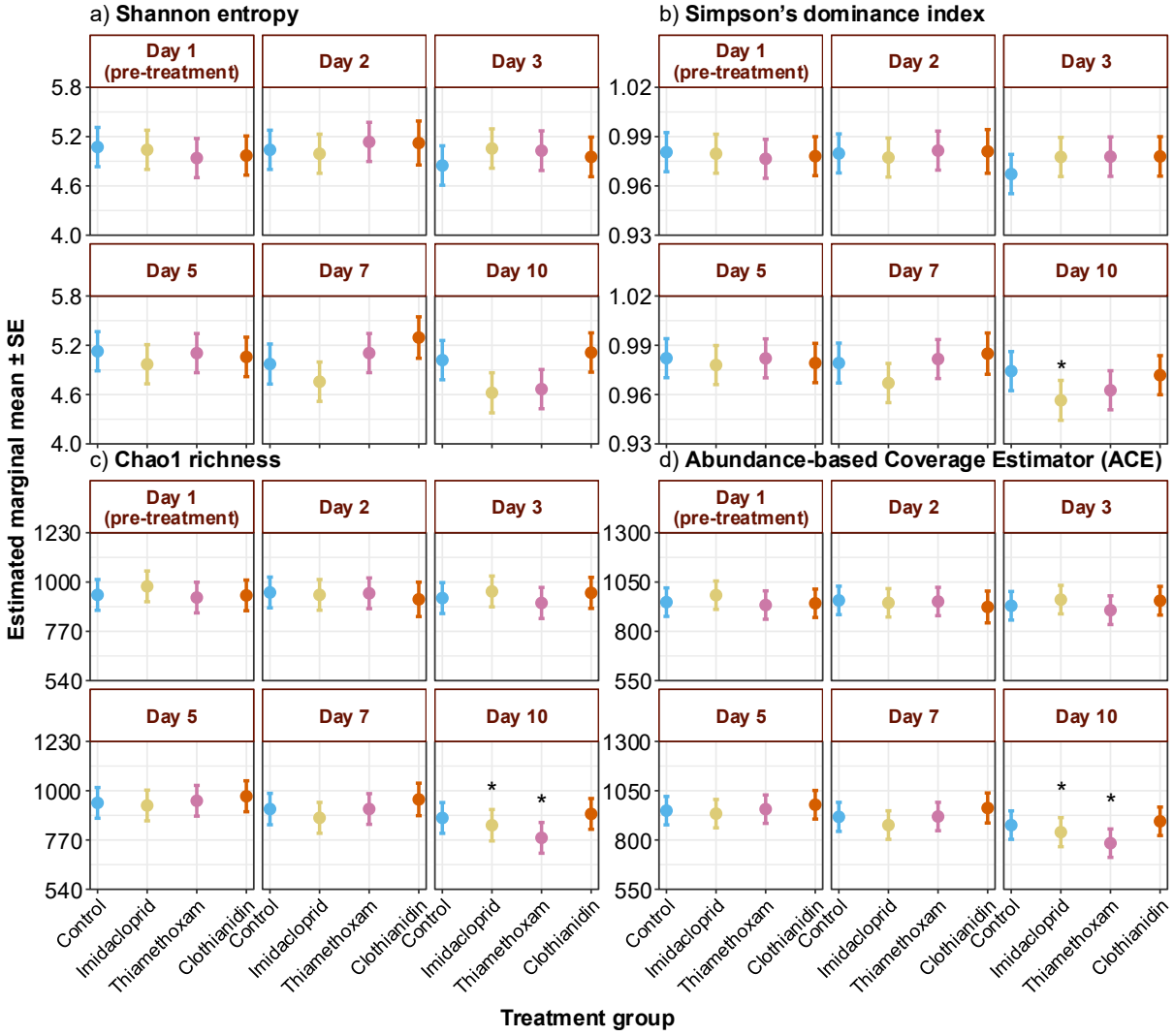

Figure D. 2: Estimated marginal means (± SE) of fungal alpha diversity metrics across neonicotinoid treatments and sampling days (n = 5). Panels show (a) Shannon entropy, (b) Simpson's dominance index, (c) Chao1 richness, and (d) Abundance-based Coverage Estimator (ACE) richness. Points represent model-estimated marginal means and error bars indicate standard errors. Significant contrasts between Day 1 (pre-treatment) and subsequent sampling days within each treatment, identified using post hoc estimated marginal means comparisons, are indicated by asterisks ('*' $P < 0.05$).

Appendix E: Supplementary materials for Chapter 7

Table E. 1: Summary statistics of sequencing depths across all samples.

Statistic	Value
Min.	1249
1st Qu.	29342
Median	42132.5
Mean	40463.2
3rd Qu.	51732.7
Max.	78220

Table E. 2: Results of Type II Wald chi-square tests for linear mixed-effects models assessing the effects of exposure group, soil texture, time and their interaction on fungal taxonomic diversity (Chao1 richness estimate and Pielou's evenness) and phylogenetic diversity (Faith's PD and MPD). Replicate was included as a random effect in each model. Significant effects are indicated as follows: '*' $P \leq 0.05$, '' $P \leq 0.01$, and '***' $P \leq 0.001$**

Factor	Response variable	χ^2	Df	*P* value
Chao1 richness estimate	Exposure group	2.78	1	0.09
	Soil texture	352.09	2	$< 2.2 \times 10^{-16}$***
	Time (days)	72.05	4	8.4×10^{-15}***
	Exposure group × Soil texture	0.50	2	0.78
	Exposure group × Time (days)	10.63	4	0.03*
	Soil texture × Time (days)	21.13	8	0.01**
	Exposure group × Soil texture × Time (days)	14.40	8	0.07
Pielou's evenness	Exposure group	0.23	1	0.63
	Soil texture	43.80	2	3.1×10^{-10}***
	Time (days)	145.54	4	$< 2.2 \times 10^{-16}$***
	Exposure group × Soil texture	3.09	2	0.21
	Exposure group × Time (days)	2.039	4	0.73
	Soil texture × Time (days)	18.53	8	0.02*
	Exposure group × Soil texture × Time (days)	7.51	8	0.48
Faith's PD	Exposure group	1.71	1	0.07
	Soil texture	294.80	2	$< 2.2 \times 10^{-16}$***
	Time (days)	56.18	4	1.3×10^{-13}***

Factor	Response variable	χ^2	Df	*P* value
Faith's PD	Exposure group × Soil texture	0.25	2	0.87
	Exposure group × Time (days)	16.91	4	0.03*
	Soil texture × Time (days)	17.19	8	0.06
	Exposure group × Soil texture × Time (days)	14.73	8	0.14
MPD	Exposure group	6.57	1	0.59
	Soil texture	404.04	2	$< 2.2 \times 10^{-16}$***
	Time (days)	7.57	4	$< 2.2 \times 10^{-16}$***
	Exposure group × Soil texture	3.41	2	0.074
	Exposure group × Time (days)	1.58	4	0.921
	Soil texture × Time (days)	16.50	8	1.1×10^{-06}***
	Exposure group × Soil texture × Time (days)	3.86	8	0.33

Table E. 3: Results of partial Mantel tests assessing correlations between fungal community structure (Bray–Curtis distances) and individual soil physicochemical properties, controlling for exposure group and time.

Property	Mantel statistics	*P* value	R^2	FDR *P*
pH	0.48	0.001	0.23	0.001
EC	0.34	0.001	0.11	0.001
OM	0.42	0.001	0.17	0.001
C	0.42	0.001	0.18	0.001
N	0.24	0.001	0.06	0.001
C:N	0.33	0.001	0.11	0.001
P	0.17	0.001	0.03	0.001
Ca	0.49	0.001	0.24	0.001
Mg	0.47	0.001	0.22	0.001
K	0.32	0.001	0.10	0.001
ECEC	0.53	0.001	0.29	0.001
ESP	0.45	0.001	0.20	0.001
Ca:Mg	0.41	0.001	0.17	0.001

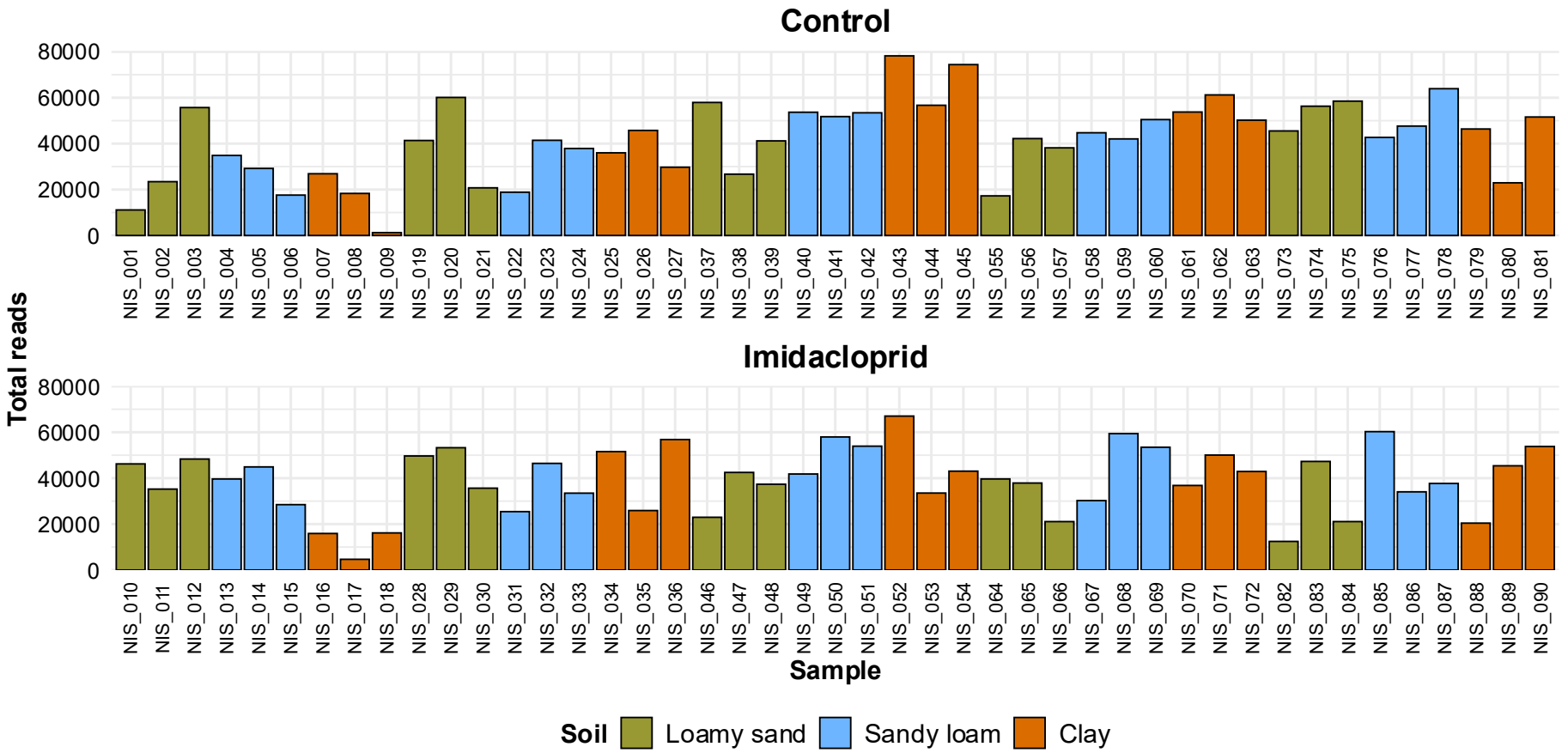

Figure E. 1: Barplot showing sequencing depth for each sample. Samples were not rarefied.

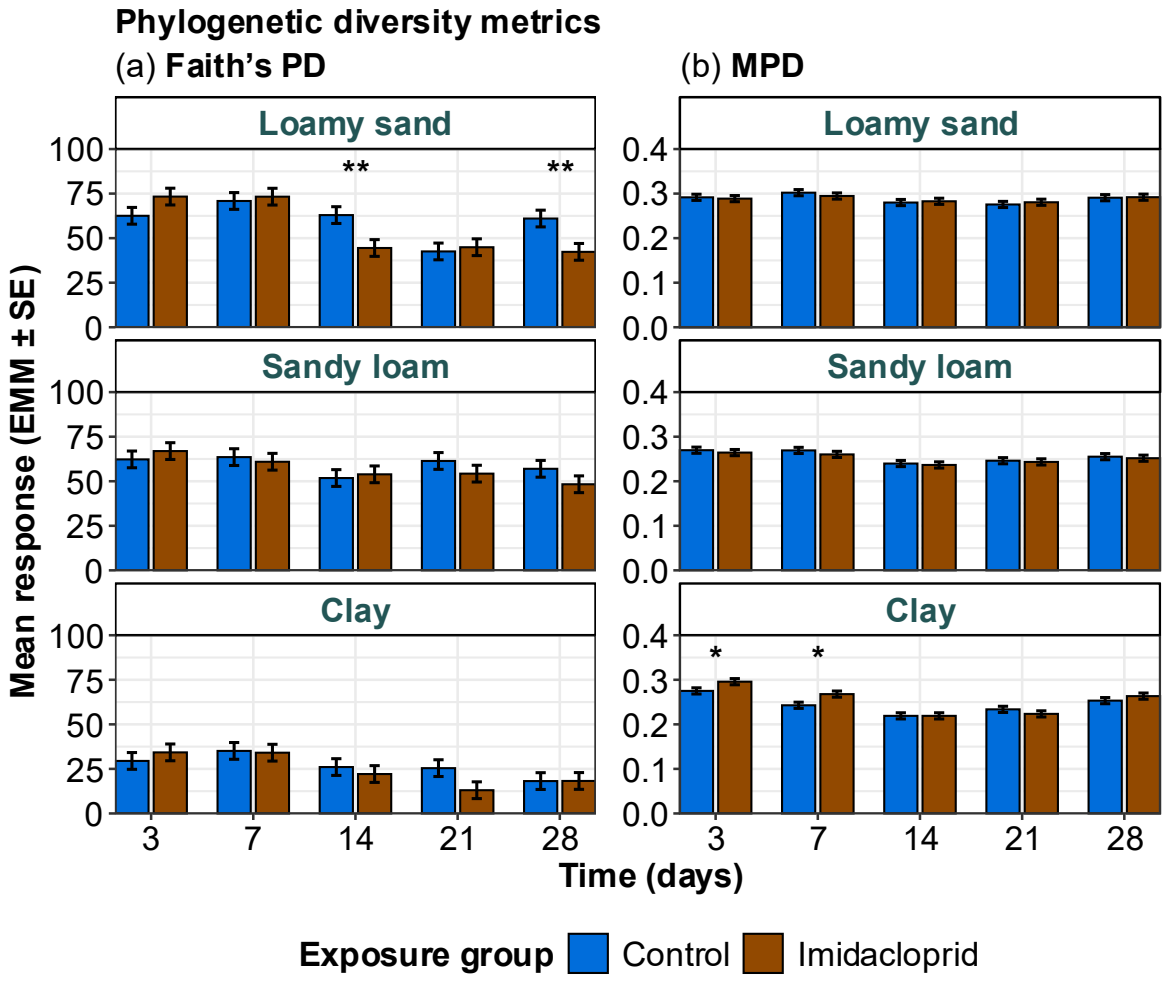

Figure E. 2: Responses of fungal phylogenetic diversity metrics across three soil textures under imidacloprid exposure. (a) Faith's phylogenetic diversity, and (b) Mean Pairwise Distance (MPD) are presented as estimated marginal means (EMM ± SE; n = 3).

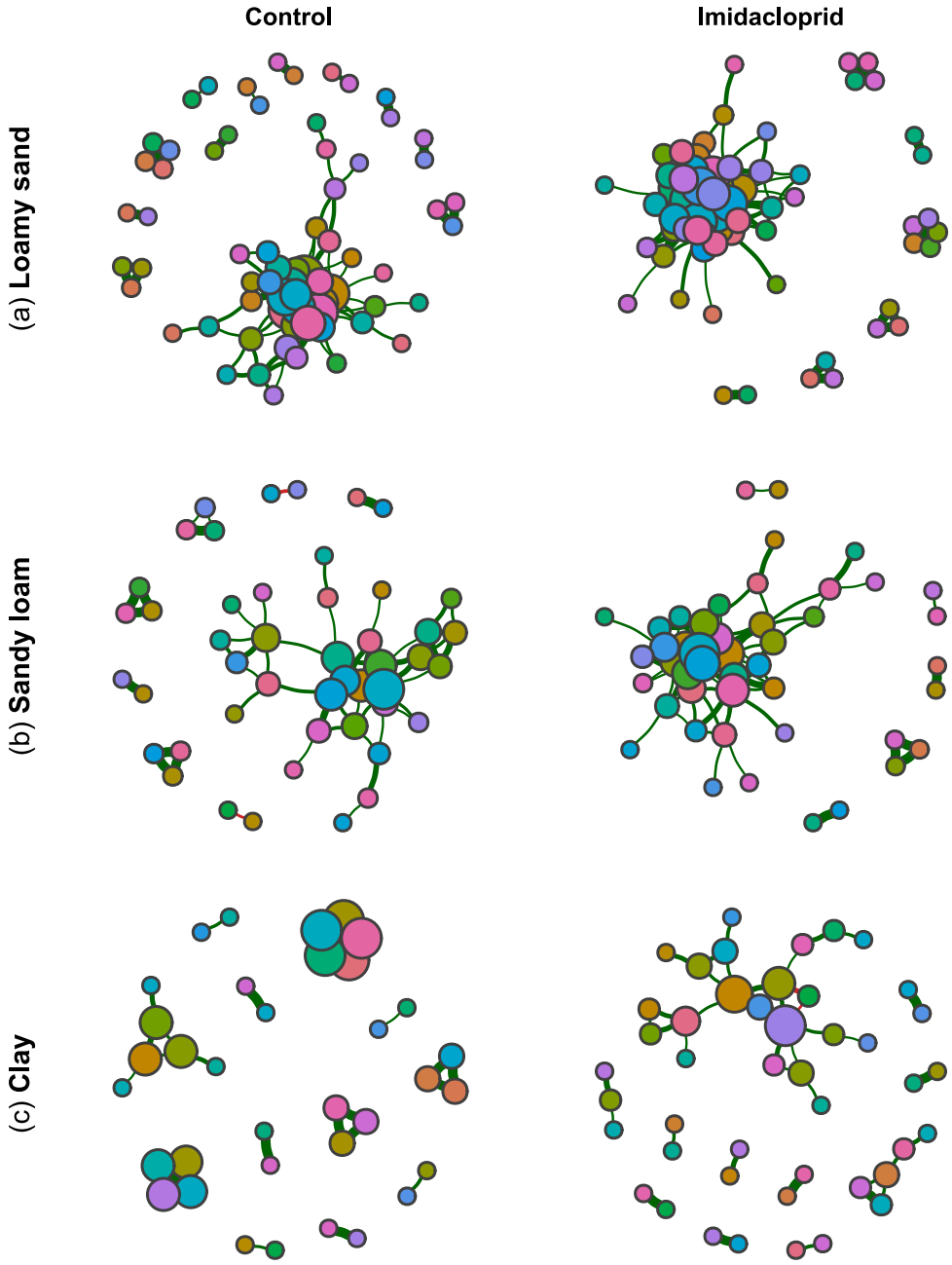

Figure E. 3: Exposure-specific co-occurrence networks of fungal taxa in three different soil textures. Nodes are taxa, edges denote significant pairwise associations (Spearman's ρ).