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Seasonal variation is a bigger driver of soil faunal and microbial community composition than exposure to the neonicotinoid acetamiprid within *Brassica napus* production systems

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ABSTRACT

Neonicotinoid pesticides are widely used within agroecosystems. Due to their systemic nature and high solubility, neonicotinoids are frequently recorded in soil, water, untreated plant matter and non-target organisms. Studies have demonstrated their capacity to induce invertebrate mortality, however, very little research has been conducted beyond pollinator exposure, particularly under field conditions. Typically, many neonicotinoids are applied via seed-dressings, reducing their direct contact with pollinators, but offering an unintended soil-exposure pathway. Soil biology underpins many vital functions, from regulating water and gas flow, to maintaining physical soil structure. In this study we investigated the effect of a commercial neonicotinoid pesticide (Insyst®) on the abundance, richness, and composition of both the mesofaunal and microbial communities and associated metabolome during oilseed rape (*Brassica napus* L.) production. Our results showed that over a single growing season, foliar application of Insyst® (250 g ha^{-1}, 50 g ha^{-1} of the active ingredient, acetamiprid) had no significant effect ($P > 0.05$) on the measured soil biological indexes. Seasonal variation was a significantly greater driver in regulating biological communities within the soil than Insyst® application. In addition, we showed that the active ingredient (acetamiprid) was rapidly degraded by the soil microbial community (theoretical half-life = 119 days) during the summer cropping season. These results help highlight the need for realistic field studies, as agricultural pesticides are never pure, often containing surfactants, adjuvants, or emulsifiers which alter their behaviour and ecotoxicity. Understanding the biological interactions of vital soil fauna with necessary pesticide usage will enable proper risk alleviation measures to maintain soil biological and ecological health.

1. Introduction

Soil biology underpins many essential soil functions and processes, from maintaining organic matter stocks and cycling to improving soil structure, and regulating air and water flow through the soil profile (Behan-Pelletier, 1999; Bottinelli et al., 2010; Lin et al., 2019; Wu et al., 2021). The quality and health of soil is often described by its functional capacity, and ability to provide vital ecosystem services (Karlen et al., 1997; Hou et al., 2020). Sustainable soil health is at the cornerstone of maintaining global food production (Comerford et al., 2013; Kopittke et al., 2019), however, achieving this remains a major challenge. The continued drive to increase crop production to support a growing global population has led to a reliance on crop protection agents. Recent studies have shown that increased agrochemical usage can significantly affect soil-dwelling communities, from altering earthworm survival and longevity (Cang et al., 2017; Fang et al., 2018), to influencing keystone microbial taxa (Edlinger et al., 2022; Yu et al., 2020; Wu et al., 2021). Changes in these populations within the soil have been shown to alter various biologically driven processes and ecosystem services within the soil; for example, reductions in earthworm populations have been linked to reductions in litter decomposition in agricultural settings, which can subsequently lead to the immobilisation of nutrients, reduction in crop emergence and potential increases in the prevalence of crop pests (Basley and Goulson, 2017; Pearsons and Tooker, 2021).

Whilst there has been increasing understanding of the impacts of farm management strategies (e.g., tillage regime (Haddaway et al.,

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2017), plastic usage (de Souza Machado et al., 2019), organic amendment (Luo et al., 2018)) and their possible impacts on soil function, our understanding of the effect of agrochemicals on the biological community and associated biochemical interactions in soil communities remains relatively poor (Pisa et al., 2021). Changes to the relative abundances and composition of organisms in soil after exposure to chemicals can be difficult to quantify due to a wide range of external and confounding factors, including but not limited to – soil type, climate, chemical used (including surfactants, additives, adjuvants, emulsifiers, and active ingredients), as well as historic land-use and underlying geogenic properties (Horswell et al., 2014; George et al., 2017, 2019; Uwizeyimana et al., 2017).

Recent years have seen an upsurge in the use of highly selective and systemic insecticides, such as neonicotinoids, to control pests. These types of pesticides accounted for approximately 33% of the global insecticide market in 2015 (Simon-Delso et al., 2015) and have been at the forefront of pest management practices in recent years, with over half of soybean seeds and almost all maize seeds being treated with neonicotinoids in the United States of America (Douglas and Tooker, 2015). Their highly selective neurotoxic mechanism, targeting the acetylcholine receptors, initially made neonicotinoids a staple insecticide, as, due to their lower mammalian toxicity levels, they were deemed environmentally safe and low risk for human contact (Tomizawa et al., 2007; Kimura-Kuroda et al., 2016; Casida, 2018). However, since their release in the early 1990's neonicotinoids have been continually linked to declines in pollinator insects, as well as songbird mortalities and ground water contamination (Whitehorn et al., 2012; Gilburn et al., 2015; Lopez-Antia et al., 2015; Schaafsma et al., 2015; Mogren and Lundgren, 2016).

Often incorporated in soil through seed dressings, soil drenches, irrigation or secondary ploughing of treated crop stubble (Jones et al., 2014; Bonmatin et al., 2015; Zaller et al., 2016), neonicotinoids are highly water soluble and have been shown to persist in soil for over 2.5 years (Baskaran et al., 1999; Sarkar et al., 2001; Rexrode et al., 2003; European Commission, 2004; Gupta et al., 2008; Fernández-Bayo et al., 2009; DeCant and Barrett, 2010; European Chemicals Agency, 2015). This prolonged persistence provides the perfect conditions for soil-borne neonicotinoids to interact with and influence soil biology, on both a macro, meso and micro scale. To date, most neonicotinoid research has focussed on above-ground pollinator impacts and the use of pure active ingredients, where the negative effects of neonicotinoid exposure have been well documented (Jin et al., 2015; Williams et al., 2015; Sánchez-Bayo et al., 2017; Tavares et al., 2017). However, the consequences of neonicotinoid application below-ground remain largely undocumented.

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

To date, there have been very few studies focussing on the impact of acetamiprid, a neonicotinoid active ingredient of several insecticides, on soil biology, particularly using commercial formulations, under field conditions. Here, we used a field-based study to assess the impacts of a single application of the high mobility (Potts et al., 2022) neonicotinoid acetamiprid-based foliar spray (Insyst®) on soil physicochemistry and key soil biological groups, across a range of trophic levels, in a typical oilseed rape (canola) cropping system. We were particularly interested in the influence of neonicotinoid pesticides on the abundance and composition of mesofauna groups such as Collembola and Acari. These two groups of soil-dwelling mesofauna play important roles in maintaining soil functions such as their involvement in litter decomposition and supporting soil microstructures, and their abundance and diversity have been well documented to be impacted by various human activities, making them useful indicator species (Rusek, 1998; Pearsons and Tooker, 2021).

The aims of this present study were to; i) quantify the degradation rate of a field relevant level of Insyst® under field conditions, ii) assess the production of any significant metabolites as a result of their degradation or changes in soil biochemical pathways, and iii) monitor any changes in the abundance and community composition of soil mesofauna and microbial communities. We hypothesised that the acetamiprid pesticide treatment will i) have a negative impact on mesofauna abundance and community composition, and ii) significantly change the soil microbial community composition and associated metabolite profile, as a proxy for biological functioning.

2. Materials and methods

2.1. Experimental setup

2.1.1. Site

The experimental field site was located at the Henfaes Agricultural Research Station, Bangor University, Abergwyngregyn, North Wales, UK (53°14′N, 4°01′W). The trial was undertaken during summer 2019 (May–September; full sampling timetable in Table 1). The field site has a temperate oceanic climate with a mean average temperature of 10 °C and an average annual rainfall of 1060 mm. The soil is classified as a sandy clay loam textured Eutric Cambisol, developed on a mixed glacial till parent material. The site has no previous record of neonicotinoid use.

2.1.2. Field design

In March 2019, a split-plot design was established creating four replicated split-plots, each half was randomly assigned either treatment or control ($n = 4$). Each combined plot (3 × 3 m) was contained by plastic boards sunk 20 cm into the ground in order to prevent lateral water flow and movement of chemicals and soil fauna between plots. All plots were subsequently hand-sown with spring oilseed rape (*Brassica napus* L.) at a rate of 150 seeds m^{-2}, the plots were later thinned to average 80 plants m^{-2} (Roques and Berry, 2016). Fertiliser was applied in accordance with national guidelines (RB209; AHDB, 2019), with 50 kg N ha^{-1} (NH_4NO_3) applied.

2.1.3. Acetamiprid treatment

One commercially available neonicotinoid product containing acetamiprid (N-[(6-chloropyridin-3-yl)methyl]-N′-cyano-N-methylethanimidamide) was tested. Insyst® (Certis UK Crop Protection, Great Abington, UK) is a specialist agricultural formulation sold as a dissolvable powder with application rates of 200–250 g ha^{-1} (liquid dose 200–600 l ha^{-1}). Insyst® is a formulation of 20% w/w acetamiprid in combination with benzenesulfonic acid, mono-C10-13-alkyl derivatives, and sodium salts. The Insyst® insecticide treatment was mixed to a final concentration by dissolving it in ultrapure water (resistivity = 18.2 MΩ-cm; total organic carbon <5 μg l^{-1}). The density of foliar soil cover, while not directly quantified here, was representative of a typical oilseed rape crop (~80 plants m^{-2}). The treatment was applied using a knapsack hand-held sprayer at a rate of 250 g ha^{-1} (equivalent to maximum application rates), protecting the control plots with plastic sheeting to avoid direct spray-drift.

2.2. Mesofauna extraction and identification

Soil mesofauna were extracted from soil cores using the Tullgren funnel methodology (Rusek, 1998; Behan-Pelletier, 1999). Soil cores (ø = 10 cm, depth = 10 cm) were left on the funnel array for seven days and extracted samples were collected in tubes containing 70% industrial methylated spirit (IMS). This process was repeated three times throughout the duration of the experiment (Table 1). Invertebrate

Table 1
Summary of the timeline associated with the oilseed rape field cropping season and associated sampling regime.

	May		Jun				Jul				Aug				Sept				Oct
	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1
Crop establishment	■																		
Insyst® application									■										
Tulgren funnel		■							■			■							
16S rRNA metabarcoding								■	■	■		■				■			■
Soil metabolomics								■	■	■		■				■			■
^{14}C degradation									■	■	■	■	■	■	■	■		■	■
Soil physicochemistry								■	■	■		■				■			

samples were refrigerated until visual taxonomic binning. Upon identification, individuals were separated into Collembola (springtails), Acari (mites), Coleoptera (beetles), Diptera (flies), Nematoda (roundworms), and "Other" (which included unidentifiable larvae and one-off individuals). Although, we note that Tullgren funnels are not the most appropriate technique for extracting nematoda, so their numbers may be underestimated. Further consideration was given to the mesofauna samples of Acari and Collembola, sub-dividing further into orders and families, allowing for further examination due to their importance and proportional dominance within the soil communities.

2.3. 16S rRNA metagenomic sequencing analyses

Soil samples were collected periodically (see Table 1) using an auger (0–10 cm) and immediately stored at −80 °C, to quench metabolic activity and prevent microbial community change. The samples were then lyophilised, ground using a stainless-steel ball mill (MM200, Retsch GmbH, Haan, Germany) and shipped on dry ice (−78.5 °C) to Microbiome Insights (Vancouver, British Columbia, Canada) to conduct the 16S rRNA metagenome extraction and analysis.

Soil was extracted using a MoBio PowerMag Soil DNA Isolation Bead Plate (QIAGEN, Hilden, Germany). DNA was extracted following MoBio's instructions using a KingFisher flex robot (Thermo Fisher Scientific Corp, Waltham, MA). Bacterial 16S rRNA genes were PCR-amplified with dual-barcoded primers targeting the V4 region (515F 5′-GTGCCAGCMGCCGCGGTAA-3′, and 806R 5′-GGACTACHVGGGT WTCTAAT-3′), as per the protocol of Kozich et al. (2013). Amplicons were sequenced with an Illumina MiSeq using the 300-bp paired-end kit (v.3). Sequences were denoised, taxonomically classified using Silva (v. 138) as the reference database, and clustered into 97%-similarity operational taxonomic units (OTUs) with the mothur software package (v. 1.44.1) (Schloss et al., 2009).

The potential for contamination was addressed by co-sequencing DNA amplified from specimens and from template-free controls (negative control) and extraction kit reagents processed the same way as the specimens. A positive control consisting of cloned SUP05 DNA, was also included. Operational taxonomic unit were considered putative contaminants (and were removed) if their mean abundance in controls reached or exceeded 25% of their mean abundance in specimens. Sequencing read files analysed in this study can be accessed from the National Center for Biotechnology Information (project PRJNA931246).

2.4. Metabolomic analysis

Additional soil samples for metabolomic analyses were gathered at the same time as those used for the 16S rRNA analysis and treated identically, methods followed those described in Brown et al. (2021). Samples were taken at random from across each plot (n = 10) and homogenised to obtain a representative sample from each plot. Samples were subsequently stored in sterile clip-top glass jars at −80 °C. The samples were then prepared in the same manner as those for 16S rRNA, through lyophilising followed by ball milling. Samples were then shipped on dry ice (−78.5 °C) to the West Coast Metabolomics Center (UC David Genome Center, Davis. California, USA) for untargeted primary metabolic analysis using automated liner exchange cold injection system gas chromatography time of flight mass spectrometry (ALEX-CIS GCTOF MS).

The extraction of the untargeted primary metabolites involved vortexing a 1:0.025 (w/v) soil-to-3:3:2 (v/v/v) MeCN/IPA/H_2O solution, followed by shaking for 5 min at 4 °C. The sample solutions were then centrifuged, and an aliquot of the supernatant removed for analysis. Metabolomic analysis was achieved using a 689- GC (Agilent Technologies) coupled to a Pegasus IV TOF MS (Leco Corporation, St. Joseph, MI, USA), injected via a Gerstel CIS4 with dual MPS Injector (Gerstel, Muehlheim, Germany), following the parameters laid out by (Fiehn et al., 2008). Data pre-processing was conducted without smoothing, using; 3 s peak width, baseline subtraction just above the noise level, and automatic mass spectral deconvolution and peak detection at signal/-noise levels of 5:1 throughout the chromatogram using ChromaTOF vs.2.32 (Leco Corporation, St. Joseph, MI, USA). The data set was then validated, aligned and filtered using the BinBase algorithm as described in Fiehn et al. (2008) and Fiehn (2016). The final compiled results were reported as peak heights and internal standards were added to the extracts for quality control and peak correction and normalisation purposes. As is common practice for untargeted metabolomics, the data presented in this study are therefore are semi-quantitative and the compounds are only tentatively identified (Gertsman and Barshop, 2018).

2.5. ^{14}C-labelled acetamiprid mineralisation

To determine the acetamiprid degradation rate in the soil, small areas of each treatment plot were encased within sterile 50 ml plastic tubes with open bottoms, inserted 2 cm into the soil, these were excluded from Insyst® treatment. ^{14}C-acetamiprid [pyridyl-2,6–^{14}C; 1850 MBq mmol^{-1}] was purchased from the Institute of Isotopes Co. Ltd., Hungary. An Insyst® solution (250 g ha^{-1}) was spiked with ^{14}C-labelled acetamiprid (3.5 kBq sample^{-1}) and pipetted evenly to the surface of the soil within the tube. A 4 M NaOH trap (1 ml) was placed within each of the tubes and sealed, allowing for the respired $^{14}CO_2$ to be captured and used to calculate total ^{14}C-labelled acetamiprid mineralisation in soil under field conditions. The traps were sampled and replaced periodically over nine weeks (sampling regime in Table 1).

The amount of ^{14}C in the NaOH traps was determined by mixing 0.25 ml from each trap with Optiphase HiSafe 3 liquid scintillation cocktail (PerkinElmer Inc., Waltham, MA, USA) and placing it on a Wallac 1404 scintillation counter (Wallac EG&G, Milton Keynes, UK) with automated photon quench correction.

2.6. Soil physicochemical analysis

Available soil ammonium (NH_4^+) and nitrate (NO_3^-) were extracted from the sample using a 1:5 (w/v) soil-to-0.5 M K_2SO_4 extract (200 rev min^{-1}, 1 h). The concentrations were determined colorimetrically using the salicylate-based procedure of Mulvaney (1996) and vanadate-based procedure of Miranda et al. (2001), respectively. Available soil phosphate was measured after extraction with 1:5 (w/v) soil-to-0.5 M acetic acid suspension (200 rev min^{-1}, 1 h) and quantified colorimetrically using the molybdate blue method of Murphy and Riley (1962). Soil water content was determined by oven drying the soils at 105 °C for 24 h pH and EC (electrical conductivity) were determined in a 1:5 (w/v) soil: distilled H_2O suspension after shaking (200 rev min^{-1}, 10 min) and using standard electrodes (Hanna Instruments Ltd, Bedfordshire, UK).

2.7. Data analysis

The 16S rRNA and metabolomic analyses were conducted in the R environment (v 4.1.1; R core team, 2022), and the '*ggplot 2*' package was used for graphical data visualisation (Wickham, 2016). Mesofauna data was analysed using ANOVA (repeated measures and one-way as appropriate) and post-hoc packages in JASP (v. 0.14.1; JASP Team, 2020), unless stated otherwise. OTUs defined at 97% sequence similarity are loosely estimated as a species. OTUs were considered putative contaminants (and were removed) if their mean abundance in controls reached or exceeded 25% of their mean abundance in specimens. OTUs were also filtered if they had fewer than 3 counts and occurred in fewer than 10% of the samples. Alpha diversity was estimated with the Shannon index on raw OTU abundance tables after filtering out contaminants. Differences in taxonomic diversity were tested, using ANOVA. Bray-Curtis indices were used to calculate beta (β) diversity across samples, using the '*vegan*' package (Oksanen et al., 2020). For all analyses the significance threshold was set at $P \leq 0.05$. We visualized β diversity, using non-metric multidimensional scaling (NMDS) ordination of the OTU community composition. Variation in community structure was partitioned by permutational multivariate analyses of variance (PERMANOVA; Anderson et al., 2013) with insecticidal treatment as a fixed factor, using 999 permutations for significance testing. Heatmap analysis of metabolomic data was performed on log_{10} transformed and pareto-scaled data in '*metaboanalyst 4.0*' (Chong and Xia, 2018; Chong et al., 2018; Pang et al., 2020). Pareto transformed and log scaled data was used for NMDS analysis and PERMANOVA, as described above.

3. Results

3.1. Mesofauna Tullgren funnel extracts

A total of 4250 invertebrate individuals were counted and identified throughout the study period. There was a significant increase ($F_{(2,18)} = 21.598$, $P < 0.001$) in total invertebrate abundance across the sampling season with 382, 1412, and 2456 individuals extracted in May, July, and August respectively.

3.1.1. Mesofauna abundance

Across all sampled invertebrate groups acetamiprid exposure was found to have no significant effect on the number of any measured invertebrate groups (Collembola (springtails), Acari (mites), Coleoptera (beetles), Diptera (flies), Nematoda (roundworms), and "Other" (which included unidentifiable larvae and individuals only identified once) (Table 2). There was no significant difference in total invertebrate counts between the two treatment scenarios ($F_{(1,18)} = 0.193$, $P = 0.67$).

Collembola were generally found to be the most common mesofauna group, on average accounting for between 34.5 and 50.3% of the mesofauna individuals across the sampling season. Coleoptera were the least represented group in both treatment scenarios across the sampling season, accounting for between 0.7 and 1.3% of recorded individuals (Fig. 1A). Across the sampling season there was an increase in the number of Collembola extracted from the soil samples (Fig. 2A).

Table 2
Repeated measures ANOVA results on the influence of season and Insyst® application (50 g ha^{-1} active ingredient) on the abundance of five major mesofauna groups. Statistical analysis refers to absolute number of extracted individuals.

	Time of sampling		Treatment	
Species	$F_{(2,10)}$	P	$F_{(1,5)}$	P
Collembola	1.51	0.267	0.239	0.646
Nematoda	4.07	0.051	0.461	0.527
Diptera	1.59	0.263	0.061	0.815
Coleoptera	21.70	0.004	0.191	0.680
Acari	0.15	0.864	1.628	0.258
Other	3.71	0.062	0.017	0.900

Entomobryoidea dominated the Collembola (Fig. 1B). Mesostigmata were the most common Acari, accounting for an average of 65 ± 4.7% of all individuals, across both treatments (Fig. 1C). Astigmata mites were continuously the least recorded Acari, accounting for on average 14 ± 4.0% 'of all individuals. While the number of Astigmata was comparable between the neonicotinoid treatment and the control plots, the number of Mesostigmata increased significantly across the season (Count- $F_{(2,12)} = 8.414$, $P = 0.005$; Proportional abundance- $F_{(2,12)} = 12.570$, $P = 0.001$; Fig. 1C). Many of the results do, however, show an increase in absolute abundance across the study period (Fig. 2).

3.1.2. Mesofauna community composition and structural diversity

Shannon diversity changed across the sampling season ($F_{(2,18)} = 7.624$, $P = 0.004$) reaching its maximum in July (Tukey post-hoc multiple comparison analysis- July:August, $P = 0.013$; July:May, $P = 0.007$). The neonicotinoid acetamiprid at the concentration of 50 g ha^{-1} was not found to have a significant effect on mesofaunal diversity ($F_{(1,18)} = 0.895$, $P = 0.43$; Fig. 1D). The Shannon diversity values of the recorded taxa were 1.28 ± 0.07, 1.56 ± 0.03, and 1.31 ± 0.05 for May, July, and August respectively (Mean ± SEM; $n = 4$).

The NMDS ordination only revealed seasonal structuring, but none, due to application of the Insyst® insecticide (Fig. 3). The mesofaunal communities were more similar in July and August than in May. PERMANOVA analysis showed whilst acetamiprid treatment did not explain the observed variance in mesofauna β-diversity (PERMANOVA: $F_{(1,23)} = 0.56$, $P = 0.75$); however, season did ($F_{(2,23)} = 6.6809$, $P < 0.001$).

3.2. Bacterial community composition and structure

In total, 13594 OTUs at 97% sequence identity were identified from the 16S rRNA reads. Twenty distinct phyla were identified, with seven distinct phyla (Firmicutes, Verrucomicrobiota, Proteobacteria, Actinobacteriota, Acidobacteriota, Chloroflexi, and Planctomycetota) accounting for 75.8 ± 0.3% of these counts. We therefore categorised the OTUs into eight distinct groups, seven of which were the aforementioned phyla, and "Other", which consisted of unclassified bacteria and phyla with <2.5% average abundance.

Acetamiprid exposure was found to have no significant impact on any of the distinct microbial phyla, although it did have a significant effect on the "other" category ($F_{(1,36)} = 4.654$, $P = 0.038$; Table 3) with the proportion of OTUs classified as "other" increasing significantly across the first three sampling points (Fig. 4A). However, since 72.5 ± 1.0% of the "other" category is dominated by unclassified bacteria, we are unable to specify the exact impacts of the acetamiprid exposure on this group. Of the major identified phyla, the most abundant identified phylum across the season was Proteobacteria, accounting for 22.3 ± 0.3% of OTUs. Chloroflexi was constantly the least identified of the major microbial phyla across this study, accounting for an average of 4.1

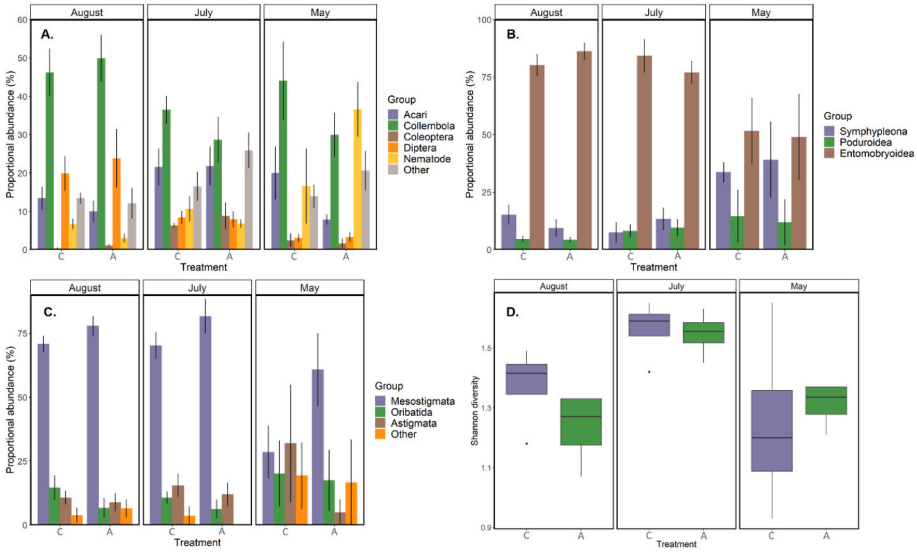

Fig. 1. Proportional abundance of the mesofauna collected from Tullgren funnel extractions in response to the addition of the neonicotinoid pesticide Insyst® to oilseed rape over a field season. Neonicotinoid pesticide (50 g ha^{-1} active ingredient, acetamiprid) was applied on the 14th July. Values are averaged across the replicated field plots ($n = 4$). A) Major mesofauna groups. B) Major Collembola families. C) Major Acari orders. D) Shannon diversity averaged across replicated plots ($n = 4$).

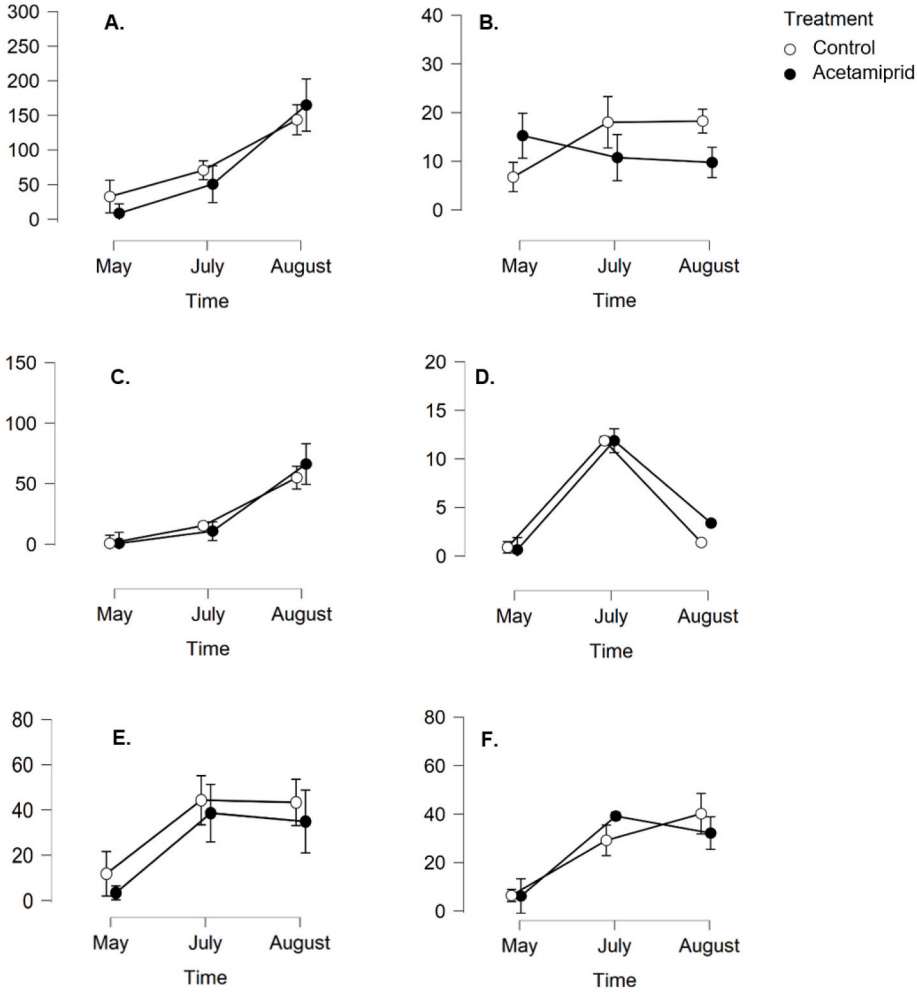

Fig. 2. Changes in total number of mesofauna in response to the addition of neonicotinoid pesticide Insyst® to oilseed rape over a field season. Neonicotinoid pesticide (50 g ha^{-1} active ingredient, acetimiprid) was applied on the 14th July, therefore May sampling represents pre-neonicotinoid application and July and August represent post-neonicotinoid application. A: Collembola, B: Nematoda, C: Diptera, D: Coleoptera, E: Acari, F: Other. Mean ± SEM ($n = 4$). N.B. different scales on the y-axis, units represent individuals per Tullgren funnel (volume = 0.015 m^3). May sampling represented pre-pesticide application, July sampling represented pre-pesticide application, post-seeding and August sampling represent post-pesticide application.

± 0.15% of OTU counts across the season (Fig. 4A).

Shannon diversity increased across the sampling season ($F_{(5,36)} = 32.681$, $P < 0.001$), with diversity in May being significantly lower than the rest of the growing season (Tukey post-hoc analysis- Baseline values: Rest of season $P < 0.001$). Acetamiprid treatment was not found to have any significant influence on the diversity values ($F_{(1,36)} = 0.021$, $P = 0.884$; Fig. 4B). Average Shannon diversity values were 5.62 ± 0.03, 5.88 ± 0.01, 5.93 ± 0.01, 5.93 ± 0.02, 5.85 ± 0.1, and 5.88 ± 0.02 at the different analysed time points, respectively (Mean ± SEM; $n = 4$).

NMDS analysis was used to show the clustering of soil-borne

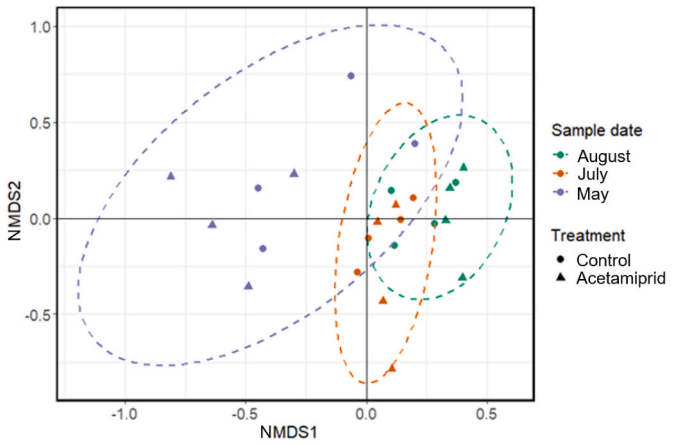

Fig. 3. Non-metric multidimensional scaling (NMDS) ordination plot of major mesofauna group composition in response to the addition of neonicotinoid pesticide Insyst® to oilseed rape over a cropping season. Neonicotinoid pesticide (50 g ha^{-1} active ingredient, acetamiprid) was applied on the 14th July, therefore May sampling represented pre-pesticide application, July sampling represented pre-pesticide application, post-seeding and August sampling represent post-pesticide application.

Table 3
ANOVA results across the abundance of seven major microbial phyla determined by 16S rRNA metabarcoding, assessed as influenced by time (since application of the neonicotinoid pesticide Insyst®) and treatment (neonicotinoid pesticide application).

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

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

3.3. Soil metabolomics

We identified 87 distinct metabolites, including a selection of amino acids, fatty acids, and saccharides. Using heatmap analysis, there are no obvious effects of neonicotinoid application on the soil metabolome (Fig. 6). There is, however, an obvious shift in metabolite concentrations between mid and end of July (Fig. 6).

3.4. Soil analysis

3.4.1. In-situ mineralisation of ^{14}C-labelled acetamiprid

The level of mineralisation of ^{14}C-labelled acetamiprid across all plots increased significantly towards autumn ($F_{(9,27)} = 178.74$, $P < 0.001$; Fig. 7), despite great variation among the replicate plots ($F_{(3,8)} = 6.022$, $P = 0.019$) (Fig. 7). An average of 33.1 ± 3.4% of the ^{14}C-labelled acetamiprid was mineralised across the study period of approximately 2.5 months. Assuming a linear rate of mineralisation, with no external confounding factors, this equates to an average mineralisation rate of 0.42% day^{-1}, resulting a theoretical half-life of 119 days during the warmer half of the vegetation period.

3.4.2. Changes in mineral nutrient levels

Neonicotinoid application was found to have no significant effect on the level of any of the analysed nutrients (ammonium; $F_{(1,6)} = 3.903$, $P = 0.096$, nitrate; $F_{(1,6)} = 1.924$, $P = 0.215$, phosphate; $F_{(1,6)} = 0.103$, $P = 0.759$). All nutrients were found to change significantly with the growing season (ammonium; $F_{(4,24)} = 4.556$, $P = 0.007$, nitrate; $F_{(4,24)} = 5.808$, $P = 0.002$, phosphate; $F_{(4,24)} = 50.069$, $P < 0.001$; Fig. S2).

3.4.3. pH and electrical conductivity

Soil pH did not vary significantly over time or across treatment scenarios (Treatment: $F_{(1,30)} = 0.113$, $P = 0.739$; Time: $F_{(4,30)} = 1.869$, $P = 0.142$). Average soil pH was found to be 7.07 ± 0.06. Electrical conductivity was found to significantly change throughout the study period ($F_{(1,30)} = 5.151$, $P = 0.003$), dropping significantly between 30th July and 12th August (Tukey post-hoc $P = 0.023$; Table S2), however, there was no difference between treatments.

4. Discussion

4.1. Mesofaunal communities

Total mesofaunal abundance and diversity were both lower in the second half of the growing season. Whilst total abundance values rose across the season, and were highest in August; the diversity was significantly higher in July, the middle of the growing season, beyond which diversity dropped to values similar to the initial diversity. Total abundance was primarily affected by the abundant Collembola. Collembola numbers continued to increase throughout the sampling season, with the August count being significantly higher than any of the previous sampling points. These results appear to counter the accepted collembola population growth, as Collembola are often noted as reaching their lowest numbers during the driest part of the summer (Rusek, 1998). This change in numbers may be explained by dense vegetation cover of the oilseed rape crop, with the Collembola utilising areas of refuge provided by this cover. Collembola are regarded as highly specialised feeders, with mouth parts and related prey/food sources varying across and amongst species (Rusek, 1998; Marie Kristiansen et al., 2021). Significant shifts in Collembola family composition, could therefore be as a result of resources changing across the season, with increases in Entomobryoidea numbers and decreases in Symphypleona counts responding in kind.

Acari peaked in July, afterwards dropping slightly towards August. Across the sampling season the structure of the Acari community remained relatively similar, with Mesostigmata always accounting for the majority of the individuals. Neonicotinoid application affected neither the total count of Acari, nor their proportional abundance. Though not statistically significant, the proportion of Oribatid mites decreased substantially towards autumn in soil samples of neonicotinoid-treated plots. Oribatid mites are generally sensitive to agricultural practices and disturbances, primarily due to their low fecundity and relatively long generational times (Behan-Pelletier, 1999; George et al., 2017). Despite these decreases, research by de Lima e Silva et al. (2017) found that when exposed to neonicotinoids imidacloprid and thiacloprid a species of *Oppia nitens*, an Oribatid mite was not affected by their exposure. *Oppia nitens* was shown to be essentially tolerant to levels of neonicotinoids exceeding 1000 mg kg^{-1} in soil over 35 days (de Lima e Silva et al., 2017). It was also found that in this case thiacloprid is more toxic for *Oppia nitens* than imidacloprid. These results support our findings of no change in the total abundance of Acari as a result of neonicotinoid application, suggesting that drops in Oribatida

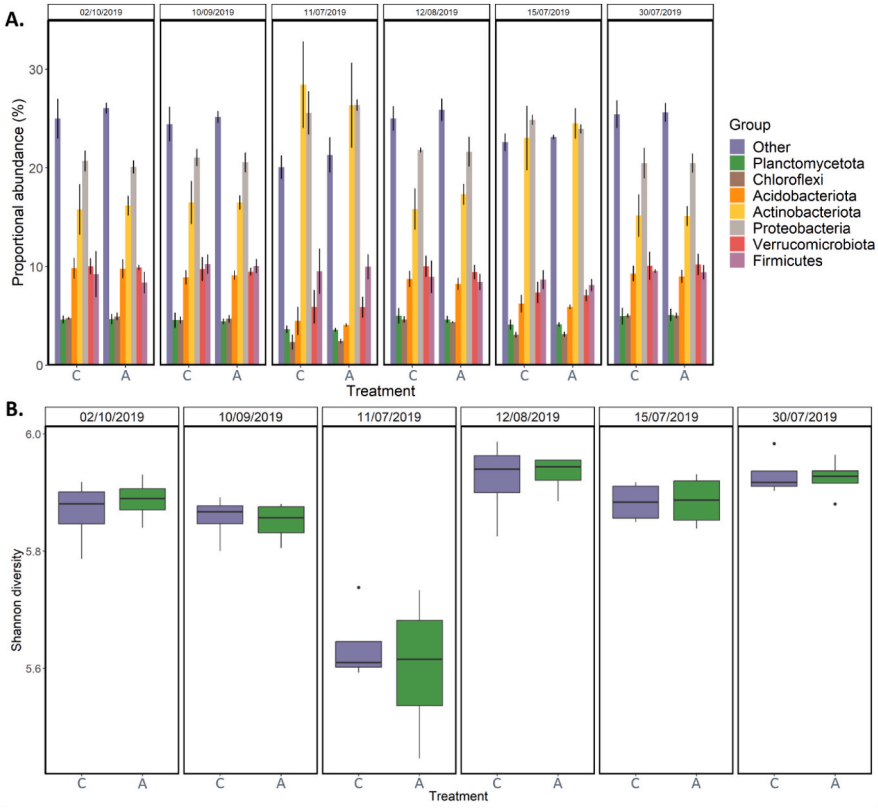

Fig. 4. Response of 16S rRNA metabarcoding microbial community OTUs to neonicotinoid pesticide addition to oilseed rape over a cropping season. A) Proportional abundances of bacterial phylum-level operational taxonomic units (OTU) after application of the neonicotinoid insecticide Insyst® (active ingredient, acetamiprid) as assessed by weekly to monthly sampling from mid-July until the beginning of October. B) Shannon diversity of bacterial phyla in soil after the application of the neonicotinoid. Neonicotinoid pesticide (50 g ha^{-1} active ingredient) was applied on the 14th July.

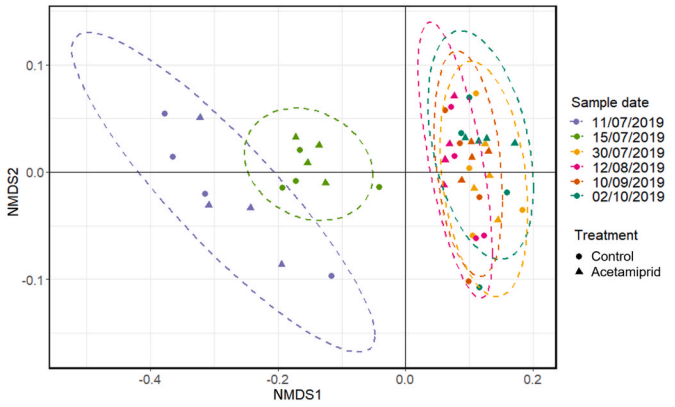

Fig. 5. Non-metric multidimensional scaling (NMDS) ordination of the soil community composition of bacterial OTUs based on 16S rRNA sequences in response to neonicotinoid pesticide (Insyst®) addition to oilseed rape over a cropping season. Neonicotinoid pesticide (50 g ha^{-1} active ingredient, acetamiprid) was applied on the 14th July. Overlapping ellipses indicate no significant differences in community composition and structure.

numbers may be due to reasons other than direct lethal toxicity. The only faunal group that showed significant interaction between time and acetamiprid were nematoda, however as mentioned above, the extraction techniques used was not the most effective extraction and quantification method for nematoda which may have introduced bias. Further research is required to confirm the effect of acetamiprid on nematoda.

Therefore, whilst the acetamiprid treatment may have no apparent direct impact on the survival and mortality of mesofauna, it could be that additional ingredients of insecticide mixtures may cause changes in the abundance of sensitive taxa. Most additional ingredients of insecticides can be categorised as either additives, adjuvants, emulsifiers, or surfactants, often assisting in the mode of action, ease of application or even improving the aesthetics or smell of the commercial formulation (Peña et al., 2011; Pescatore et al., 2020). The addition of these ingredients can sometimes be linked to changes in the microflora of the soil (Pescatore et al., 2020). Oribatid mites are fungivores and it is therefore suggested that the decrease in their presence could be as a result of a decrease in food resources as a result of detrimental effects of additional ingredients besides the insecticides.

Despite significant changes in mesofauna, abundances and community structure over the growing season, there is no evidence in this study of any significant changes to soil mesofauna as a result of exposure to the neonicotinoid Insyst® applied by spraying. These results are in opposition to those of Penn and Dale (2017), who found that imidacloprid-coated seeds, impaired the locomotion of ants and caused their death, demonstrating the importance of assessing the sublethal impacts of pesticide exposure as well as quantifying the lethal effects. There is much research to suggest that acetamiprid is "less toxic" than its forebear neonicotinoids, namely imidacloprid, clothianidin and thiamethoxam (Grimm et al., 2012; Amirzade et al., 2014; Pang et al., 2020). Differences in chemistry and thus mode of action could explain the apparent lack of non-target effects on the soil mesofauna. However, the study of Penn and Dale (2017) used neonicotinoid-coated seeds when assessing the impacts on soil mesofauna and biological activity. When applying neonicotinoids as seed dressings it is likely that no more than 2% of the applied pesticides is systemically absorbed by the seedling, with the remaining <98% of the chemical is often leached into the surrounding soil (Tapparo et al., 2012). In these cases, areas of high concentration, localised exposure can be expected, with population crashes and changes in faunal activity mirroring the insecticidal effect. However, due to changes in EU regulations regarding neonicotinoid use, only certain neonicotinoids, including acetamiprid and thiacloprid, are still registered for outdoor use (European Commission, 2004, 2018a, 2018b). Both acetamiprid and thiacloprid are seldom used for seed coating and are instead often applied through a foliar spray, as used in this study. The use of a foliar spray therefore decreases the amount and

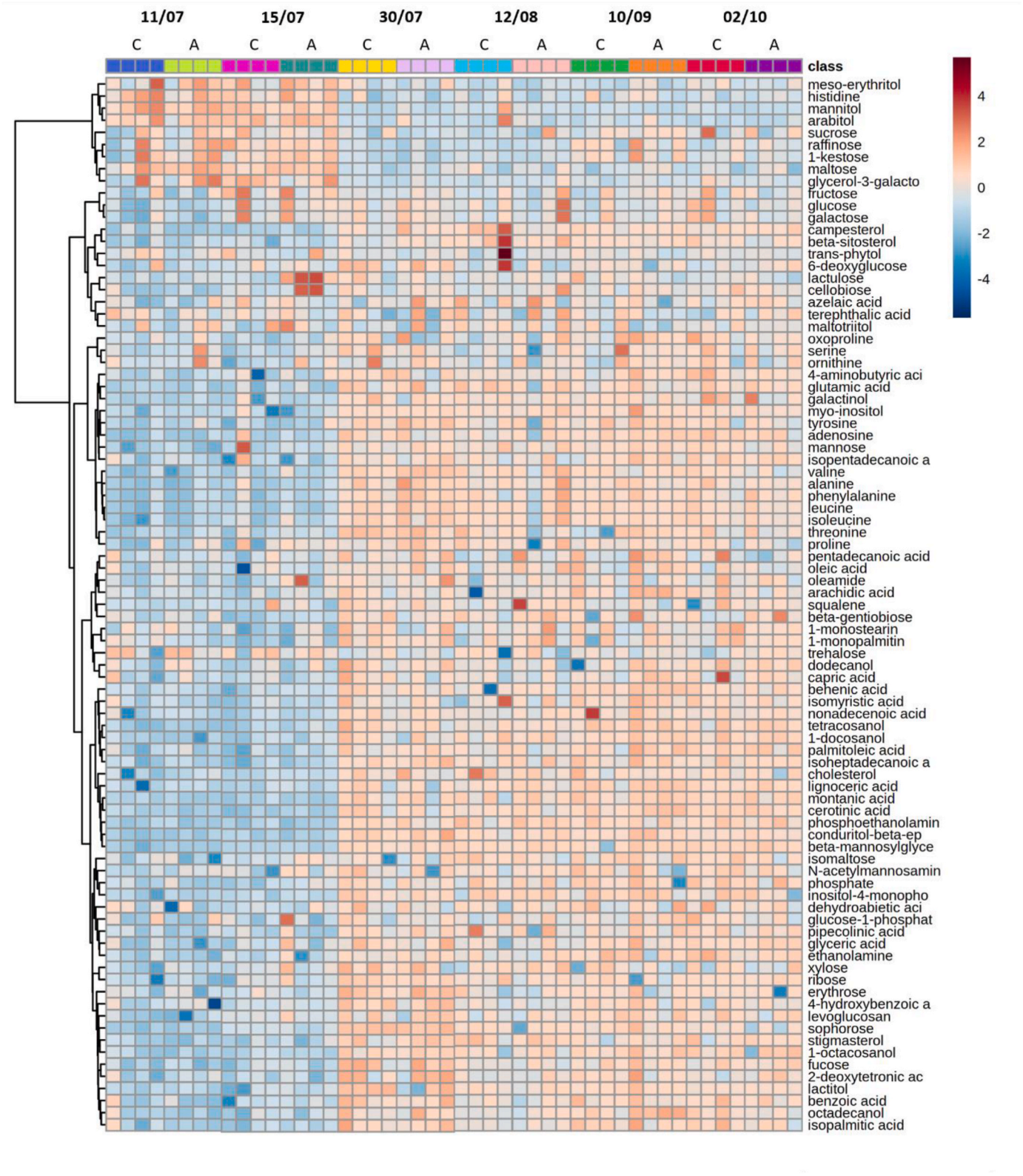

Fig. 6. Heat map of changes in relative soil metabolite concentrations in response to neonicotinoid pesticide (Insyst®) treated oilseed rape plots, clustered using Euclidean distance and Ward linkage. Neonicotinoid pesticide (50 g ha^{-1} active ingredient, acetamiprid) was applied on the 14th July. C- control, P- pesticide. Data were normalised using a log transformation and Pareto scaling. The colouring of the z-scores denotes the deviation of the individual metabolites from the mean across all samples in standard deviations.

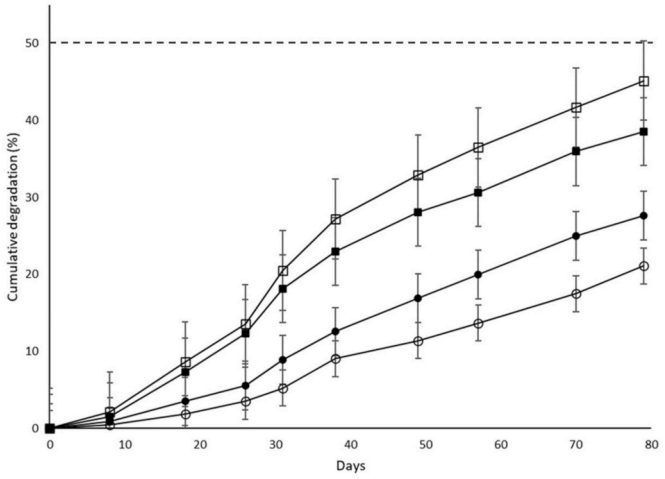

Fig. 7. Cumulative degradation of the ^{14}C-labelled acetamiprid pesticide (50 g ha^{-1} active ingredient within the neonicotinoid pesticide Insyst®) when applied to soil in an oilseed rape field over a 79-day period. From this the half-life was approximated to be 119.3 days. Lines represent the replicated plots, mean ± SEM, $n = 3$.

incidence of chemical contact and incorporation into the soil. Therefore, whilst the field application levels were representative of maximum rates, the amount that eventually came into contact with the soil and soil fauna may have been negligible.

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

4.2. Microbial community structure

Soil microorganisms are often considered to be the most sensitive bioindicators to changes in soil quality (Lau and Lennon, 2012; Pescatore et al., 2020; George et al., 2021). Their ability to rapidly respond to changes in their environment can often result in substantial changes in ecosystem function and services (Lehman et al., 2015; Bünemann et al., 2018). The results from this study demonstrate that changes in microbial community structure and therefore the function of soils are likely more affected by seasonal changes than by exposure to neonicotinoid insecticides.

Previous studies in this area have often yielded conflicting data. The findings of Wu et al. (2021) showed that the direct incorporation of thiamethoxam into soil significantly altered the bacterial abundance, diversity and community structure over a period of 60 days under controlled indoor conditions. They demonstrated substantial decreases in the growth promoting rhizosphere bacteria, actinobacteria, implying possible future challenges in sustaining soil fertility (Wu et al., 2021). Whereas, a study conducted by Li et al. (2018), performed under realistic field conditions, found that imidacloprid and clothianidin treated seeds did not negatively impact the richness or diversity of the rhizosphere bacterial communities. They did, however, find that species richness across both the bacterial and fungal communities were suppressed during the seedling stage due to neonicotinoid treatment. Despite the recovery of the community later in the growing season an early shift in community structure could alter plant development (Li et al., 2018). Therefore, studies using agriculturally relevant formulations applied at realistic rates under realistic field conditions seem necessary for risk assessments.

4.3. Metabolomic analysis

The metabolomics data agrees well with the rest of the data, showing a strong seasonal change, but no discernible effect of the neonicotinoid acetamiprid. The seasonal shift in primary metabolites may well reflect changes in plant growth and development of oilseed rape (Tarpley et al., 2005; Blancaflor et al., 2014), potentially due to variation in root exudates with the oilseed rape's ontogenetic stage (Canarini et al., 2019; Mavrodi et al., 2021). There was also a substantial increase in a range of amino acids (e.g., serine, ornithine, valine) towards autumn. These compounds are often used as proxies for increases in bacterial growth (Bastviken and Tranvik, 2001; Sasse et al., 2018; Zampieri et al., 2019; Braissant et al., 2020). Additionally, typical stress related compounds (namely, proline and trehalose), did not increase after acetamiprid application, suggesting there was little stress induced by acetamiprid application. We, therefore think that the change in metabolites reflects changes in microbial community structure changes, as detected by 16S rRNA sequencing; theorising that these changes in chemical pathways and detected compounds are strongly influenced by and correlate with the changes in microbial abundance and community composition.

4.4. ^{14}C-labelled acetamiprid mineralisation

Our results suggest that the active ingredient of Insyst®, acetamiprid was rapidly degraded in the soil during the summer cropping season of an oilseed rape crop, resulting in a theoretical half-life of 119 days (Fig. 7). Neonicotinoids may be applied to the field in a variety of ways, with many causing direct (seed coatings) or indirect (foliar spray) exposure to the soil (Jones et al., 2014; Bonmatin et al., 2015; Zaller et al., 2016). Temperature and exposure to ultraviolet (UV) light are two major factors governing neonicotinoid breakdown (with warmer temperatures (increasing microbial metabolic rates) and higher UV levels increasing surface mineralisation rates) (Acero et al., 2019; Pang et al., 2020). This assay took place under the plant canopy within field during the summer (i.e., representative conditions), and while temperatures may have been conducive to biotic breakdown, abiotic (UV) breakdown may have been limited. Long term persistence of acetamiprid may therefore likely, particularly when conditions are not conducive to biotic or abiotic breakdown (Bonmatin et al., 2015). While no effect on soil biology was shown here over one season, in a field that had not previously had a history of neonicotinoid use, future work should focus on the potential effect of neonicotinoid (and their associated breakdown products) persistence on soil biology and function particularly in a long-term field setting.

5. Conclusion

This study showed that a single spray application of the acetamiprid-containing insecticide, Insyst®, at the maximum legal rate in the EU to a field with no history of previous neonicotinoid use, had no significant direct effect on meso- and micro-fauna. Instead, seasonal variation was much greater in the soil faunal communities. It may be that relatively rapid degradation of acetamiprid limits its toxicological non-target effect in soil. This can be ascribed to the relatively rapid degradation of the acetamiprid which limits its toxicological potential, from one application. Soil metabolomics data support this notion with no changes in compound abundance as a result of exposure to acetamiprid, but instead large seasonal variation in the soil fauna. Our results demonstrate the need to make assessments at realistic dose rates of relevant insecticide formulations under true agricultural conditions. However, future follow-up work in this subject area should test the multi-season and multi-application effect to verify that there is also no long-term detrimental effect through the accumulation of pesticides and degradation products.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2023.109088.

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