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Impact of twenty pesticides on soil carbon microbial functions and community composition

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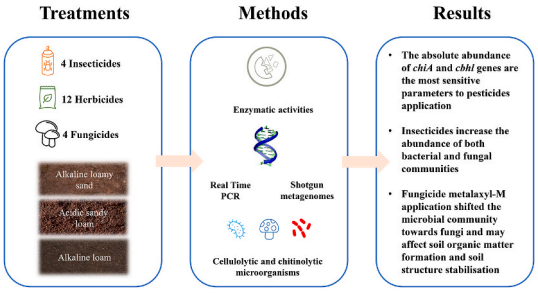
HIGHLIGHTS

- Effects of 20 pesticides on carbon cycle related soil microbiota are investigated.
- Pesticide application stimulates cellulolytic and chitinolytic activities.
- Pesticide effects are influenced by both the pesticide class and soil properties.
- Insecticides significantly increase the abundance of microbial communities.
- Metalaxyl-M soil application shifts the microbial community towards fungi.

GRAPHICAL ABSTRACT



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ABSTRACT

Pesticides are known to affect non-targeted soil microorganisms. Still, studies comparing the effect of multiple pesticides on a wide range of microbial endpoints associated with carbon cycling are scarce. Here, we employed fluorescence enzymatic assay and real-time PCR to evaluate the effect of 20 commercial pesticides, applied at their recommended dose and five times their recommended dose, on soil carbon cycling related enzymatic activities (α-1,4-glucosidase, β-1,4-glucosidase, β-D-cellobiohydrolase and β-xylosidase), and on the absolute abundance of functional genes (*cbhl* and *chiA*), in three different South Australian agricultural soils. The effects on cellulolytic and chitinolytic microorganisms, and the total microbial community composition were determined using shotgun metagenomic sequencing in selected pesticide-treated and untreated samples. The application of insecticides significantly increased the *cbhl* and *chiA* genes absolute abundance in the acidic soil. At the community level, insecticide fipronil had the greatest stimulating effect on cellulolytic and chitinolytic microorganisms, followed by fungicide metalaxyl-M and insecticide imidacloprid. A shift towards a fungal dominated microbial community was observed in metalaxyl-M treated soil. Overall, our results suggest that the application of pesticides might affect the soil carbon cycle and may disrupt the formation of soil organic matter and structure stabilisation.

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1. Introduction

Carbon is the building block of life, and the cycling of this element has profound implications on soil fertility and climate. Half of the photosynthetic carbon products are sequestrated into biomass and soil organic matter (SOM), while the remaining is released back into the atmosphere as carbon dioxide through plant or microbial respiration and SOM decomposition (Cheng and Gershenson, 2007). Soil organic matter, which consists of plant, animal, and microbial-derived residues in various stages of decomposition, holds the largest organic carbon pool in the terrestrial biosphere (Gougoulias et al., 2014). Perturbations in the SOM stabilisation processes may, therefore, be expected to have serious consequences to terrestrial ecosystems and global organic carbon stocks.

With the extensive application of pesticides in modern agriculture, their potential impacts on soil carbon cycling have been explored (Maddela and Venkateswarlu, 2018; Niemi et al., 2009; Prashar et al., 2016). When pesticides are applied to soil, the abundance and diversity of different macro- and micro- organisms might be positively (stimulation) or negatively (inhibition) affected, and microbial enzymatic activities in soil may also be altered. Pesticides could have beneficial effects on the soil microbiota. For instance, the most commonly used herbicide in Australia, glyphosate, has been reported to increase the soil microbial biomass carbon (SMBC; used to describe the total mass of living microorganisms in soil (Brookes, 1995)) in nine different soils (Arora and Sahni, 2016). The insecticide cypermethrin has also been found to increase the soil bacterial population and enhance cellulase activities in different studies (Prashar et al., 2016; Gundi et al., 2007). However, pesticides have also been reported to inhibit soil microbial activities. For example, application of the insecticide imidacloprid decreased the total bacterial population in a loamy sand soil (Cycoń et al., 2013), while the fungicides metalaxyl and mefenoxam inhibited the enzymatic activities of β-glucosidase (the enzyme responsible for the conversion of cellobiose to glucose which is the final step of cellulose hydrolysis (Monkiedje et al., 2002)). Either way, these pesticide effects disturb the soil microbiome and may impair soil carbon cycling.

Unlike the nitrogen cycle, where some functions are regulated by specialist organisms, the carbon cycle is mostly driven by a wide range of non-specialist microorganisms (Domeignoz-Horta et al., 2021). Therefore, it is important to assess not only the effect of pesticides on carbon cycle related functional genes, but also the potential effects of pesticides on wider microbial enzymatic activities, indirectly associated or affected by C cycling, and the whole microbial community composition. To date, we lack studies looking the specific effects of multiple pesticides on carbon cycle related microbial functions and relevant microbial communities. Most of the studies performed have been incremental, providing information on a limited number of functional microbial endpoints in one or two soils without trying to link functional endpoints to diversity endpoints. Furthermore, most studies have only compared the effect of one or a few pesticides (Bishnu et al., 2012; Wang et al., 2015). For example, Gundi et al. investigated the effect of three insecticides on carbon cycle associated microbial functions in two agricultural soils but their study was limited to measurements of cellulase enzymatic activities and culturable cellulolytic microorganisms (Gundi et al., 2007).

There are several factors that influence the potential effects of pesticides on soil microbial communities and their related functions. Such factors include the chemical structure, concentration and toxicity of the applied pesticide and the soil properties. The latter seem to affect both pesticide persistence but also the composition and function of the soil microbiome (Chowdhury et al., 2008). Different pesticides will cause different effects in different soil types and studies focusing on a single endpoint may oversimplify the effect of a pesticide effects on the soil ecosystem. Hence, the aim of this study was to use high-throughput methods to investigate the comparative effect of 20 commercial agricultural pesticides on a range of carbon cycle related soil microbial functions in three different South Australian soil types. The 20 pesticides selected represent different pesticide categories and modes of actions and were applied at their recommended field dose (RD) and five times the recommended field dose (5RD). Here, we measured several different microbial endpoints covering a range of microbial processes in carbon cycling including (*i*) the activity of four soil microbial exoenzymes associated with carbon decomposition (α-1,4-glucosidase (AG), β-1, 4-glucosidase (BG), β-D-cellobiohydrolase (CB) and β-xylosidase (XYL), (*ii*) the absolute abundance of carbon cycle related functional genes (*cbhI* (gene encoding fungal (GH7 family) cellobiohydrolase) and *chiA* (gene encoding group A bacterial chitinase)), and (*iii*) the absolute abundance of total bacteria (16S rRNA) and fungi (nuclear Internal Transcribed Spacer (ITS) region). Finally, based on the measurements of the above endpoints, we applied shotgun metagenomics to selected samples to determine the effects of pesticides on the total soil microbial community and microbial groups known to be associated with carbon cycling. We tested the hypotheses that the effect of pesticides on soil carbon cycling (*i*) varies across soil types and pesticide category (*ii*) is dose dependent and (*iii*) induces shifts in microbial community composition.

2. Materials and methods

The experimental details for the set-up of the pesticide-soil incubation study have been described previously (Sim et al., 2022). Briefly, 20 pesticides that are commonly used in Australian broadacre cropping were applied individually at their RD and 5RD to three South Australian agricultural soils. The 20 pesticides were (i) insecticides: chlorpyrifos, fipronil, alpha-cypermethrin and imidacloprid; (ii) herbicides: chlorsulfuron, imazamox, atrazine, trifluralin, propyzamide, prosulfocarb, metolachlor, pyroxasulfone, isoxaflutole, clopyralid, paraquat and glyphosate, and (iii) fungicides: flutriafol, metalaxyl-M, penflufen and azoxystrobin. The three South Australian soils used were an alkaline loam, an alkaline loamy sand and an acidic sandy loam, collected from the surface layer (0–10 cm depth) of the agricultural fields at Hart Field Site in the Mid-North region (Lat: 33°45′39.2"S, Long: 138°24′50.7"E), near Minnipa on the Eyre Peninsula (Lat: 32°50′54.3"S, Long: 134°30′13.2"E) and from Wolseley in the South East region of SA (Lat: 36°18′28"S, Long: 140°55′48"E), respectively. Each of the 20 pesticides was applied individually to 300 g of soil in a glass jar (in triplicate), with one soil sample remaining untreated as the control, homogenised and incubated in the dark for 28 days at 25 °C while maintaining the maximum water holding capacity (WHC) at 60% with regular additions of deionised water (Test, 2000). The concentrations of pesticides used, soils physico-chemical properties, and the experimental design of the soil microcosm can be found in Supplementary Tables S1 and S2 and Supplementary Figure S1. Additional details regarding the pesticide's chemical properties can be found in our previous study (Sim et al., 2022).

2.1. Enzymatic activities

Extracellular activities of AG, BG, CB and XYL of the soil samples were determined straight after sampling using substrates fluorescently labelled with 4-methylumbelliferone (MUB) as described by Bell et al. (2013). In brief, 2.75 g of soil (dry weight) were mixed with 91 mL of pH-adjusted buffer solution to match the soil pH. The blended soil slurries were amended with 0.2 mM fluorescently labelled substrate and incubated for 3 h at 25 °C in the dark. A range of MUB solutions with concentrations from 0 to 100 μM were prepared in each sample plate to establish standard curves and act as quality control. Post incubation, the plates were centrifuged, and the supernatant was analyzed for fluorescence intensity using a FLUOstar Optima (BMG Labtech, Cary, NC) microplate absorbance/fluorescence reader at 355 nm excitation and 460 nm emission. The enzymatic activities were then calculated according to the standard curve fluorescent data.

2.2. DNA extraction and real-time PCR

DNA was extracted directly from the frozen soil samples using the DNeasy PowerSoil™ Pro DNA kit (Qiagen, Hilden, Germany) and was quantified using a DS-11 Series Spectrophotometer/Fluorometer (DeNovix, Wilmington, Delaware, United States). The DNA concentration was ranged from 10 to 100 ng μL^{-1}, while the DNA quality was all within 1.8–2.2 according to the ratios of A_{260}/A_{280} and A_{260}/A_{230}. All real-time qPCR analyses were carried out on a LightCycler® 480 Instrument II (Roche Life Science) with appropriate standard curves and primer sets as shown in Table 1.

The qPCR amplification conditions were as follows: 95 °C for 10 min, 40 cycles at 95 °C for 10 s and annealing temperature as per Table 1 for 60 s. An extension step at 72 °C for 20 s was applied for all SYBR Green I assays. Additional details regarding the qPCR conditions can be found in our previous study (Sim et al., 2022).

2.3. Shotgun metagenomic sequencing and data processing

Shotgun metagenomic sequencing was performed on six pesticide-treated soil samples and the corresponding untreated soil sample (all from the acidic Wolseley soil). Whole metagenome shotgun libraries were constructed based on the metagenomic DNA preparations extracted from each soil samples. Analysis of pesticide treated soil metagenomes was performed through the metagenomics RAST server using default parameters (Meyer et al., 2008). Shotgun metagenomics produced 20–80 million raw sequence reads per sample.

For the annotation of functional subsystem, the read counts of cellulase and chitinase associated activities were derived from SEED subsystem level 3 (cellulosome) and 4 (chitinase) (Overbeek et al., 2005; Luo et al., 2014), while the read counts were derived using RefSeq generated taxonomic annotations for literature selected microorganisms involved in cellulolytic and chitinolytic activities and high abundant microbial communities at 1% cut-off level (genus level) (Paczian et al., 2019; Randle-Boggis et al., 2016). These read counts were first divided by the ITS read counts derived from shotgun metagenomics sequencing and were then multiplied by the respective qPCR ITS abundance for normalization to produce absolute abundances and be comparable between the functional attributes and microorganisms. ITS counts normalization was performed considering that fungi are known primary degraders and major contributors of several carbon cycle steps (Kohler et al., 2015). Parallel BLAT (Wang and Kong, 2019) was used for identifying ITS sequence reads throughout the dataset while testing the reads against Silva v138 or UNITE v8.3 database (Yilmaz et al., 2014).

2.4. Statistical analysis

Multivariate statistical analyses were performed using R version 4.1.3 (Team, 2021). The influence of soil type (Hart, Minnipa and Wolseley), pesticide category (insecticides, herbicides and fungicides), dose (RD and 5RD) and the interactions between these factors on the microbial enzymatic activities, and the absolute abundance of functional genes and total microbial communities involved in carbon cycle were evaluated with Permutational analysis of variance (PERMANOVA), using the adonis2 function in the vegan package (Anderson et al., 2017). To identify statistically significant effects between untreated sample with each pesticide category and dose, Bonferroni-corrected p values was calculated using the pairwise.adonis function in the pairwiseAdonis package (pairwiseAdonis: Pairwise multilevel comparison using, 2020). Two-way analysis of variance (ANOVA) and Dunnett's post hoc test were then performed using GraphPad Prism 8.2.0 to identify the effect of each individual pesticide on each microbial functional marker in a specific soil using a significance level of $P < 0.05$. To visualize the effect of selected pesticides on SEED subsystems (i.e. functionally related microbial roles) and taxon abundances, heatmap analysis was performed using the R package of pheatmap (Team, 2021). The association analysis between SEED subsystem and RefSeq generated taxonomic annotations was performed using Pearson correlations.

3. Results

3.1. The effect of soil type, pesticide category and pesticide dose on carbon cycle related microbial endpoints

The combination of soil type (Hart, Minnipa, and Wolseley), pesticide category (insecticides, herbicides, and fungicides), and dose (RD and 5RD) explained 54.8% of the variation ($P = 0.001$) in carbon cycle related microbial endpoints (enzymatic activities and absolute functional gene abundance). Soil type explained most of the variation (variance = 53.3%; $P = 0.001$), while pesticide category (variance = 1.12%; $P < 0.01$) and dose (variance = 0.40%; $P < 0.05$) showed a much lower but still significant contribution (Table 2).

The interaction between pesticide category and dose in Wolseley soil explained the greatest degree of variation (18.2%), followed by Hart and Minnipa soils (5.56% and 2.46% variance, respectively) (Table 2), meaning that Wolseley was more affected by the application of pesticides compared to the Hart and Minnipa soils ($P = 0.001$). When considering the individual effect of pesticide category and dose for Wolseley soil, both pesticide category (variance = 11.6%; $P = 0.001$) and dose (variance = 6.58%; $P = 0.001$) had a significant effect on all carbon cycle related microbial endpoints (Table 2).

Given the significant effects we found in Wolseley soil following PERMANOVA, we performed further statistical analyses using pairwise-PERMANOVA to better understand which were the main drivers of these effects. Insecticides significantly affected carbon cycle related microbial endpoints ($P < 0.05$) (Supplementary Table S3) and this effect was driven by changes in the abundance of *cbhl* and *chiA* genes ($P < 0.05$) (Supplementary Table S4). When considering the effect of dose in the Wolseley soil, pesticide applications at 5RD showed a greater effect on overall carbon cycle related microbial endpoints ($P < 0.05$) compared to the RD and the untreated soil sample, with significant effects shown in the abundance of the *chiA* gene (Supplementary Table S3 & S5).

Table 1
Primers, annealing temperature, and reference strains used for the preparation of calibration curves in our q-PCR measurements.

Target genes (expected fragment size)	Primer	Primer sequence	Annealing temperature (°C)	Reference strains	Reference
cbhl (166–173 bp)	cbhIF cbhIR	ACCAACTGCTACACIGGCAA GCCTTCCCAIATGTCCATC	60	Cloned from soil	(Edwards et al., 2008; Butterly et al., 2016; Phillips et al., 2015)
chiA (450bp)	GA1F GA1R	CGTCGACATCGACTGGGARTDBCC ACGCCGGTCCAGCCNCKNCCRTA	63	*Escherichia coli K-12*	(Williamson et al., 2000; Yergeau et al., 2007)
16S rRNA (187 bp)	*331-F* *518-R*	TCCTACGGGAGGCAGCAGT ATTACCGCGGCTGCTGG	55	*Escherichia coli* ATCC 25922	(Valero et al., 2016; Lueders et al., 2004; Denman and McSweeney, 2006)
ITS (300-500bp)	*ITS1-F* *ITS2Deg2-R*	TCCGTAGGTGAACCTGCGG GCTRCGTTCTTCATCGATRC		*Candida albicans* ATCC CRM-10231	

bp: base pairs.

Table 2
Effect of soil type, pesticide category and pesticide dose on soil carbon cycle related microbial functional endpoints in Hart, Minnipa, and Wolseley soils.

Parameter	Soil	Variable	R-squared (variance explained)	F-value	P-value	Significance
All carbon cycle related microbial functional markers	All soils	Soil + Category + Dose	0.55 (54.8%)	73.42	0.001	***
		Soil	0.53 (53.3%)	213.82	0.001	***
		Category	0.01 (1.12%)	4.50	0.007	**
		Dose	0.004 (0.40%)	3.22	0.049	*
	Hart	Category + Dose	0.06 (5.56%)	1.75	0.109	
	Minnipa	Category + Dose	0.02 (2.46%)	0.75	0.611	
	Wolseley	Category + Dose	0.18 (18.2%)	6.58	0.001	***
	Wolseley	Category	0.12(11.6%)	5.60	0.001	***
	Wolseley	Dose	0.07 (6.58%)	9.50	0.001	***

Pesticide effects on all tested carbon cycle related microbial functional markers are analyzed based on soil types (Hart, Minnipa, and Soil), pesticide category (Fungicides, Herbicides, and Insecticides) and pesticide dose (recommended dose and five times the recommended dose) using PERMANOVA with associated p-values. *Indicates significant effect contributed by tested variable at $P < 0.05$; ** indicates significant effect contributed by tested variable at $P \leq 0.01$; *** indicates significant effect contributed by tested variable at $P \leq 0.001$.

3.2. Pesticide-specific effects on the absolute abundance of cbhl and chiA genes in Wolseley soil

Since the absolute abundance of *cbhl* and *chiA* genes in Wolseley soil was significantly affected by pesticides application, we performed further statistical analyses to better understand the effect of individual pesticide on these two genes (Fig. 1). The good efficiencies of the conducted qPCR assay are supported by the similar peaks for all tested samples shown in the melting curves for both genes (Supplementary Figure S2). Our ANOVA and Dunnett's post-hoc test supported the pairwise-PERMANOVA test results where pesticides significantly increased *cbhl* and *chiA* gene absolute abundances. We observed no significant decrease in the absolute abundance of the target genes in the samples treated with the 20 pesticides at the two dose rates ($P > 0.05$) (Supplementary Figure S3). Instead, three of the four insecticides (alpha-cypermethrin, chlorpyrifos and imidacloprid) stimulated the abundance of both *chiA* and *cbhl* genes (Fig. 1) when applied at 5RD. From the fungicides tested, flutriafol and azoxystrobin significantly increased the abundance of the *cbhl* gene when applied at 5RD, while the latter also showed significant stimulation at RD. Finally, from the herbicides tested chlorsulfuron stimulated the abundance of *chiA* at 5RD (Fig. 1).

3.3. Effect of selected pesticides on cellulase and chitinase associated activities and microorganisms in Wolseley soil

Based on the results of functional microbial endpoints, we selected samples which had been treated with herbicide (metolachlor), fungicides (metalaxyl-M and azoxystrobin) and insecticides (imidacloprid, fipronil and alpha-cypermethrin), at 5RD and an untreated soil sample and we analyzed their microbial functional metabolic potential with shotgun metagenomic sequencing. The three insecticides and the fungicide azoxystrobin were selected based on their significant stimulating effects on the abundance of *cbhl* and *chiA* genes, while the herbicide metolachlor and the fungicide metalaxyl-M showing negligible effects were chosen to compare with the former selected pesticides and to represent all three pesticide categories (insecticide, herbicide, and fungicide) in our assessment.

In all pesticide treated samples, we noted a greater absolute abundance of chitinase and cellulase associated activities compared to the untreated control sample. Fipronil-treated samples showed the highest abundance of chitinase and cellulase associated activities, followed by the samples treated with the insecticide imidacloprid and the fungicide metalaxyl-M. The samples treated with the insecticide alpha-cypermethrin, the herbicide metolachlor and the fungicide azoxystrobin had the lowest abundance of cellulase and chitinase associated activities compared to the other pesticides-treated samples (Fig. 2).

We then determined the effect of the six pesticides on selected cellulolytic and chitinolytic microorganisms. These microorganisms were selected based on their known cellulolytic and chitinolytic activities from previous literature and our results showing a strong correlation of these microorganisms (at genus level) with chitinase and cellulase associated activities (Supplementary Figure S4-S5). Pesticide effects on these selected cellulolytic and chitinolytic microorganisms was similar with the effects of pesticides on chitinase and cellulase associated activities, with all pesticide-treated samples showing a greater absolute abundance of selected cellulolytic and chitinolytic microorganisms compared to the untreated sample (Fig. 3). The greatest abundance of both cellulolytic and chitinolytic microorganisms was shown in fipronil-treated sample, followed by samples treated with metalaxyl-M and imidacloprid, while the lowest abundance of cellulolytic and chitinolytic microorganisms were shown in samples treated with insecticide alpha-cypermethrin, herbicide metolachlor and fungicide azoxystrobin. In the metalaxyl-M treated sample, a greater abundance of cellulolytic and chitinolytic fungal communities, compared to the bacterial communities, was observed. (Fig. 3).

3.4. Effect of selected pesticides on bacterial and eukaryotic microorganisms

Based on the metagenomic dataset, we determined the composition of the bacterial and eukaryotic microbial communities in the Wolseley soil. Bacteria dominated the soil metagenome, constituting 98% of the whole microbial community, while eukaryotes contributed 1%. *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, *Proteobacteria* and *Verrucomicrobia* were the dominant bacterial phyla in all samples analyzed, with *Actinobacteria* and *Proteobacteria* constituting more than 35% of the total bacterial abundance (Supplementary Fig. S6). For eukaryotes, *Apicomplexa*, *Arthropoda*, *Ascomycota*, *Bacillariophyta*, *Basidiomycota*, *Chlorophyta*, *Chordata*, *Cnidaria*, *Nematoda* and *Streptophyta* were the dominant phyla, with *Ascomycota* constituted almost half of the soil eukaryotic microbial community (ie. half of the normalized eukaryotic reads) (Supplementary Fig. S7). We, then determined the effect of pesticides on the dominant microorganisms at 1% cut-off level (genus level). In general, the absolute abundance of the dominant microorganisms (at genus level) in the soils treated with selected pesticides was greater than the untreated soil sample (Fig. 4).

The fipronil-treated samples again, showed the greatest absolute abundance of dominant bacteria and eukaryotes, while samples treated with the insecticide alpha-cypermethrin, the herbicide metolachlor and the fungicide azoxystrobin demonstrated the lowest absolute abundance of dominant bacteria and eukaryotes. Soil samples treated with imidacloprid and metalaxyl-M showed greater absolute abundance of most highly abundant microorganisms compared to alpha-cypermethrin, metolachlor and azoxystrobin, and this was more prominent for some eukaryotic microorganisms, especially *Pyrenophora, Phaeosphaeria,*

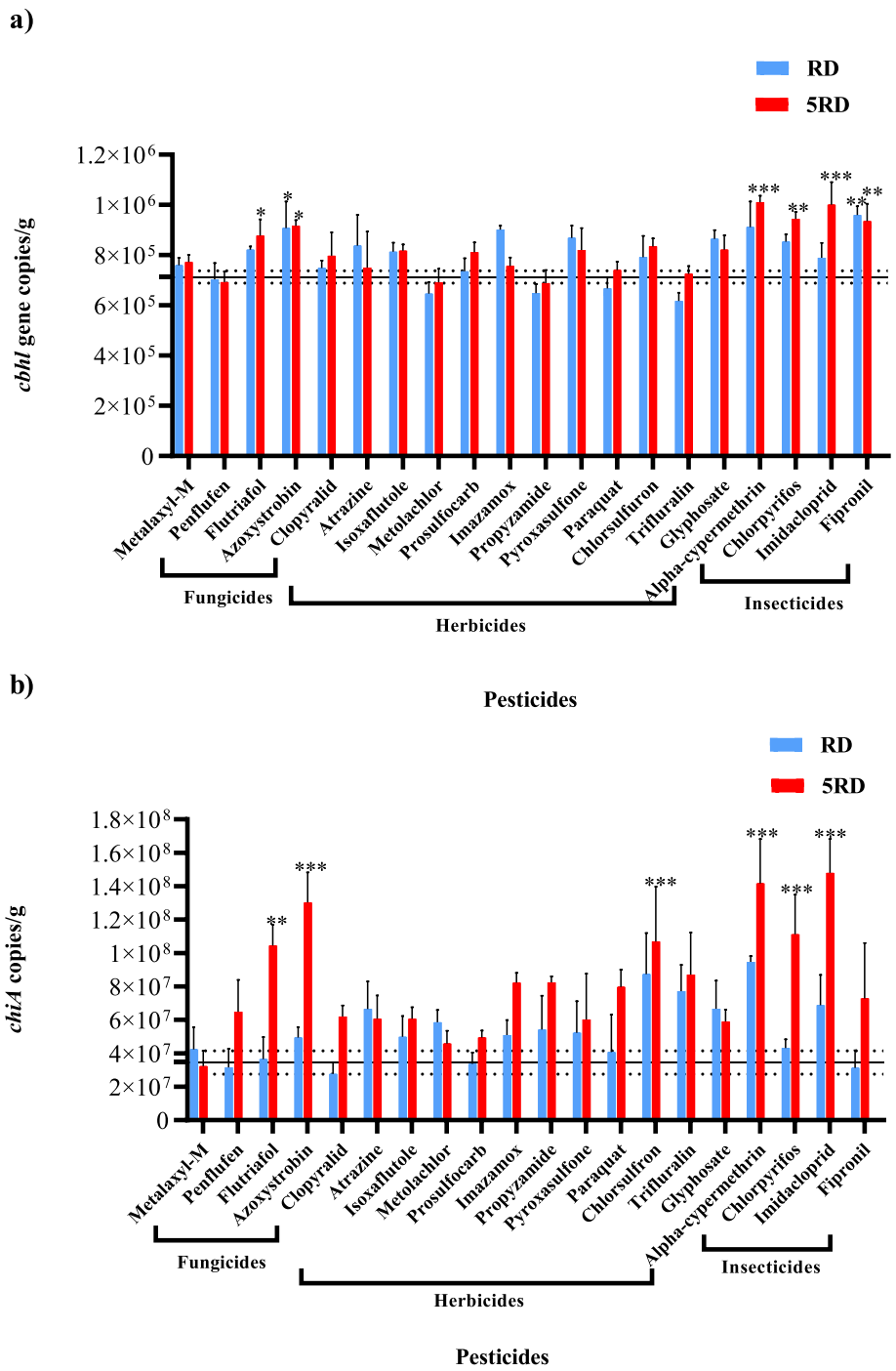

Fig. 1. The effect of 20 pesticides on the abundance of (a) cbhl and (b) chiA genes in Wolseley soil. Straight line represents the mean of controls (untreated soil samples) while the dotted lines represent the standard error of the mean. * Indicates gene abundance is significantly different to the control at $P < 0.05$; ** at $P < 0.01$; *** at $P < 0.001$. RD: recommended dose; 5RD: five times the recommended dose. cbhl – cellulase; chiA – chitinase.

Neurospora and *Xenopus* in imidacloprid-treated samples and *Neurospora*, *Chaetomium*, *Podospora*, and *Penicillium* in metalaxyl-*M*-treated samples. (Fig. 4).

4. Discussion

To the best of our knowledge, this is the first study giving insights into the effect of 20 pesticides, with different mode of actions, on multiple carbon cycling related microbial endpoints in three contrasting soil types. Herein, we provide a comprehensive understanding of the impact of pesticides/active ingredients on soil ecosystems at both functional and taxonomical levels, allowing more accurate prediction of the potential effects of pesticides on soil carbon cycling.

The effect of pesticides on soil carbon cycling is influenced by numerous environmental and abiotic soil factors (*e.g.* clay content) as well as pesticide properties (Reeve et al., 2010; Gan and Wickings, 2017). In the present study, we investigated the effect of soil type, pesticide category and pesticide application rate on carbon cycling related microbial endpoints and found soil type to be the major determinant of pesticide effects. The main difference between the three soils was their pH, with Hart (pH 8.4) and Minnipa (pH 8.8) soils being alkaline and Wolseley (pH 5.4) being acidic. Past studies have suggested

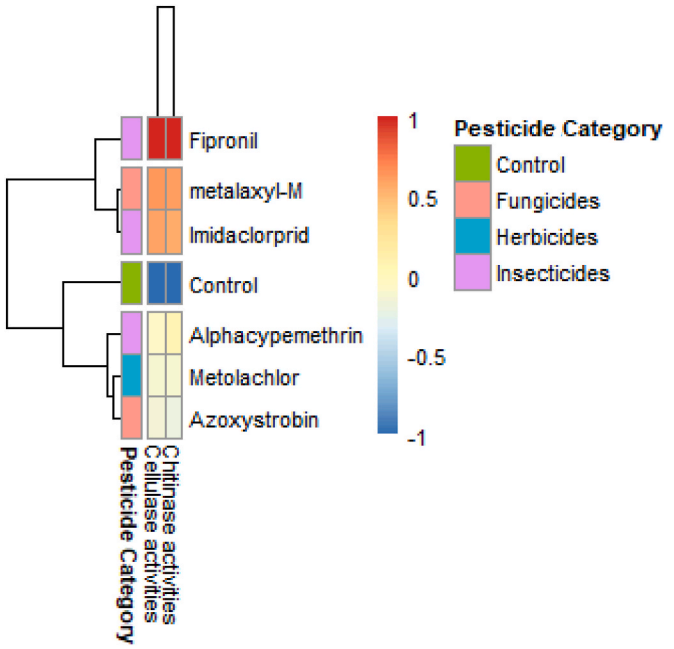

Fig. 2. Heatmap of the absolute abundances (relative abundances expressed as relative to the ITS read counts derived from shotgun metagenomics sequencing were multiplied by the respective qPCR ITS abundance) scaled from −1 to 1 (values presented here comprise the z-scores (number of standard deviations from the mean)) for microbial associated chitinase and cellulase activities (see blue to red key) throughout six selected pesticide treated soil samples plus untreated soil sample (control). The pesticide category of each sample is indicated by the green-pink-blue-purple column at the left according to the secondary key. Hierarchical clustering with the average linkage algorithm was performed for depicting microbial associated activities groupings. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

soil pH is one of the factors strongly affecting pesticide soil adsorption (Kodešová et al., 2011; Ping et al., 2010). The pesticides that showed stimulating effects on the absolute abundance of *cbhI* and *chiA* genes in Wolseley soil were mostly non-ionizable, such as insecticides chlorpyrifos, fipronil, imidacloprid and alpha-cypermethrin and the fungicide azoxystrobin, while the herbicide chlorsulfuron and the fungicide flutriafol are weak acids with dissociation constant (pKa) of 3.4 and 2.3, respectively (PPDB. Pesticide Properties Database, 2007). Previous studies have reported that the non-ionizable chlorpyrifos possesses greater adsorption affinity in acidic soils, which was attributed to its temporary polarization in soils with low pH (Rasool et al., 2022; Muhamad et al., 2004). A positive correlation has also been demonstrated between acidic pesticides and pesticide adsorption in soils with low pH (Muhamad et al., 2004). This may be attributed to the tendency of acidic pesticides to become protonated as pH decreases, hence reducing the repulsive forces between soil particles and dissociated pesticides (Palma et al., 2015; Stougaard et al., 1990). In our study, the greater adsorption ability of these pesticides may have decreased their adverse impacts, but the stimulatory effects on the abundance of *cbhI* and *chiA* genes warrants further analysis.

In addition to soil physicochemical characteristics, pesticide effects also varied between different pesticide categories, with insecticides application having a consistent and significant positive effect on *cbhI* and *chiA* gene abundances ($P < 0.05$). Notably, the widespread use of pesticides has caused soil microorganisms to develop pesticide degrading capacities (Ortiz-Hernández et al., 2013). Various bacterial and fungal strains such as *Acinetobacter*, *Bacillus*, *Flavobacterium*, *Pseudomonas*, *Streptomyces*, *Aspergillus*, and *Trichoderma* were reported to utilize pesticides, especially insecticides, as carbon sources (Singh et al., 2003; Zhao et al., 2014; Akbar and Sultan, 2016; Huang et al., 2018). However, the microbial ability to assimilate pesticides as energy sources is not limited to insecticides. The stimulatory effect of the insecticides on cellulase activity might be associated with their mode of actions which require further investigations. The effect of pesticides on soil carbon microbial functions was also dose-dependent ($P < 0.01$) with the higher the application dose, the greater the observed stimulatory effect on *cbhI* and *chiA* gene abundances. Our results agree with previous findings which also demonstrated significant stimulatory effects on microbial growth and enzymatic activities when insecticides and fungicides were applied to soil (Maddela and Venkateswarlu, 2018; Srinivasulu, 2015; Xie et al., 2009). For example, fungicides tridemorph and captan were found to stimulate invertase and cellulase enzymatic activities when applied at 2.5 and 5 kg ha^{-1}, a dose comparable to the one used in our study (5RD was 4 kg ha^{-1}), while the authors observed a significant inhibition at a higher dose of 7.5 kg ha^{-1} (Srinivasulu, 2015). Gundi et al. (2005) also showed that insecticide monocrotophos, quinalphos and cypermethrin applied at dose rates up to 25 μg g^{-1} significantly increased the abundance of total microbial population and cellulase enzymatic activities (Gundi et al., 2005). Hence, the effect of pesticide dose on soil microbial function might be dependent on the activity and the specificity of the pesticide/active ingredient used. Moreover, we suggest that pesticide effects on soil microbial functions are closely associated with soil type, pesticide category and pesticide dose. Our previous work studied the effects of the same 20 pesticides on the nitrogen cycle when applied to the same three soils (Sim et al., 2022). Overall, pesticides caused significantly more inhibition of N cycle endpoints compared to those for the C cycle observed in the current study where stimulatory affects were predominant. For example, the insecticide fipronil inhibited β-1,4-N-acetylglucosaminidase activity and potential nitrification in the alkaline loam Hart soil yet caused stimulation of cellulolytic and chitinolytic microorganisms in the acidic sandy loam Wolseley soil (Sim et al., 2022). This highlights the importance of incorporating multiple testing parameters in each study.

Microbial degradation of carbon is carried out by an extremely diverse set of microorganisms, and it involves a wide range of enzymes which on their own are characterized by high genotypic diversity (Hamid et al., 2013; Wilson DBJCoim, 2011). While most pesticides had minimal effects on soil carbon microbial functions, we identified *cbhI* and *chiA* absolute gene abundance as the most responsive markers to pesticide application. Six pesticides showed significant stimulation of both *chiA* and *cbhI* absolute gene abundance in the Wolseley soil while pesticide effects on AG, BG, CB and XYL enzymatic activities were negligible. The apparent discrepancy between enzymatic activity and gene abundance is mostly due to the different attributes being measured in each microbial endpoint; AG, BG, CB and XYL activities estimate the microbial functions in carbon degradation while *cbhI* and *chiA* genes absolute abundance quantify fungal CBH and group A chitinases (Williamson et al., 2000; Baldrian and Valášková, 2008). The higher sensitivity of *cbhI* and *chiA* genes to pesticide exposure may be due to the higher potential of the fungal community and *Streptomyces* for polysaccharide degradation and the faster response to cope with environmental changes, especially the fluctuation of carbon sources from pesticide applications, than enzymatic activities (Berlemont et al., 2015; Breitkreuz et al., 2021). Despite the often reported negative pesticide effects on soil enzymatic activities, Riah-Anglet et al. (2018) suggested that AG, BG, CB and XYL are not highly sensitive to fungicidal treatment (Riah-Anglet et al., 2018), which supports our findings. Hence, it is important to include both enzymatic activities and gene abundance as microbial parameters for a more thorough understanding of pesticide effects on the microbiological activities in soil carbon cycling.

Since there are opposing studies regarding the role of bacteria and fungi in labile and recalcitrant carbon decomposition, linking taxonomy to microbial function is important to assess how microbial communities involved in carbon cycling would respond to pesticide applications. According to the literature, the bacterial genera *Bacillus*, *Paenibacillus*,

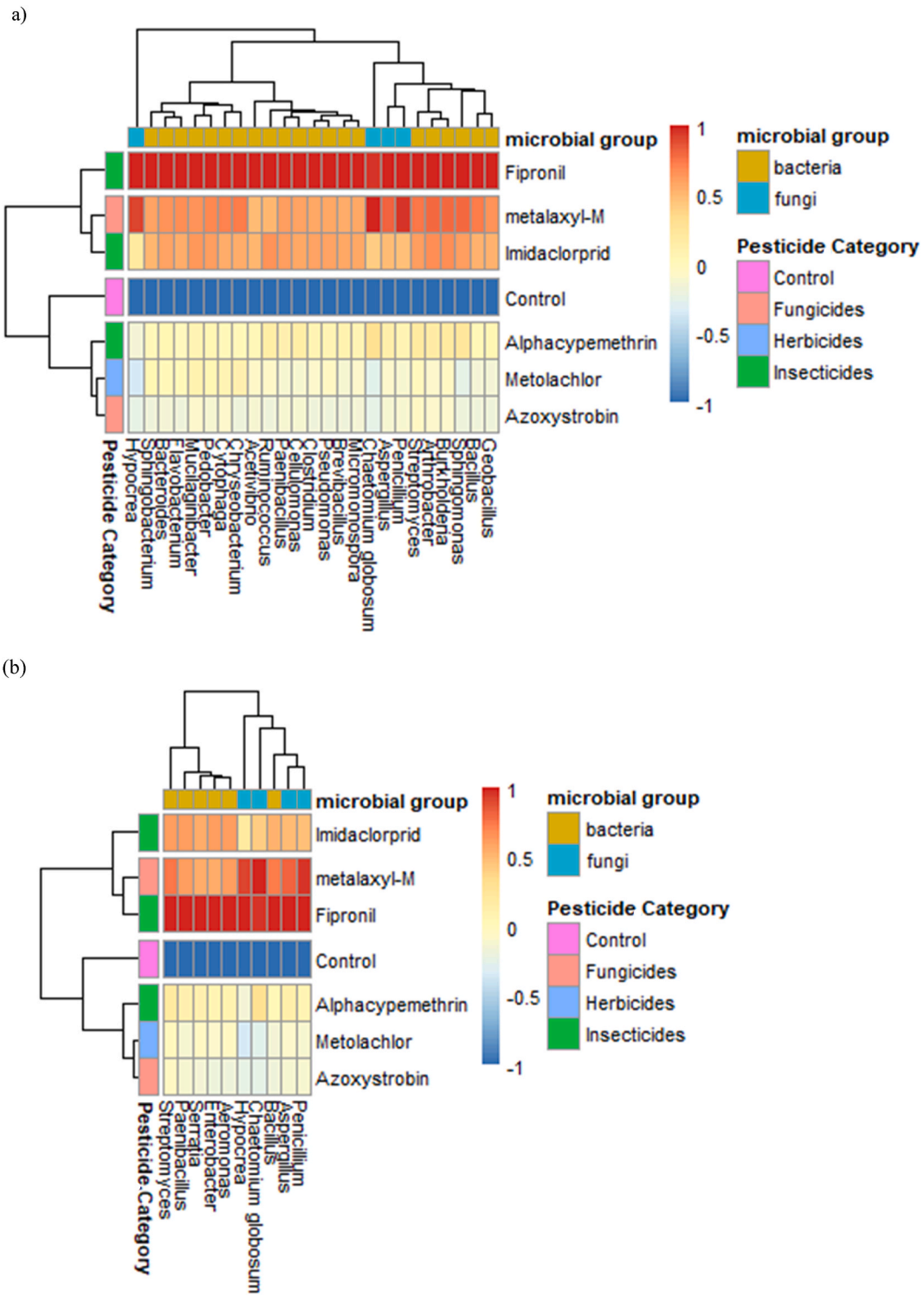

Fig. 3. Heatmap of the absolute abundances (relative abundances expressed as relative to the ITS read counts derived from shotgun metagenomics sequencing were multiplied by the respective qPCR ITS abundance) scaled from −1 to 1 (values presented here comprise the z-scores (number of standard deviations from the mean)) for each microorganism (see blue to red key) throughout six selected pesticide treated samples plus untreated sample (control) of literature known (a) cellulolytic and (b) chitinolytic microorganisms at genus level. The pesticide category of each sample is indicated by the green-pink-blue-purple column at the left according to the secondary key. Hierarchical clustering with the average linkage algorithm was performed for depicting microorganism groupings. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Streptomyces and the fungal genera *Aspergillus*, *Penicillium*, *Hypocrea* and *Chaetomium globosum* harboured potential in both cellulose and chitin catalytic activities (el Zahar Haichar et al., 2007; Coronado-Ruiz et al., 2018; Liang et al., 2014; Bhattacharya et al., 2007; Hoster et al., 2005). Chitin and cellulose are reported as structural homologues and in fact, most carbon sources display high intragenomic redundancy (Beier and Bertilsson, 2013). Therefore, each microbial strain may possess traits that can degrade cellulose, chitin, and other carbon sources. All these microorganisms, together with other cellulolytic and chitinolytic microorganisms reported previously were shown to correlate positively

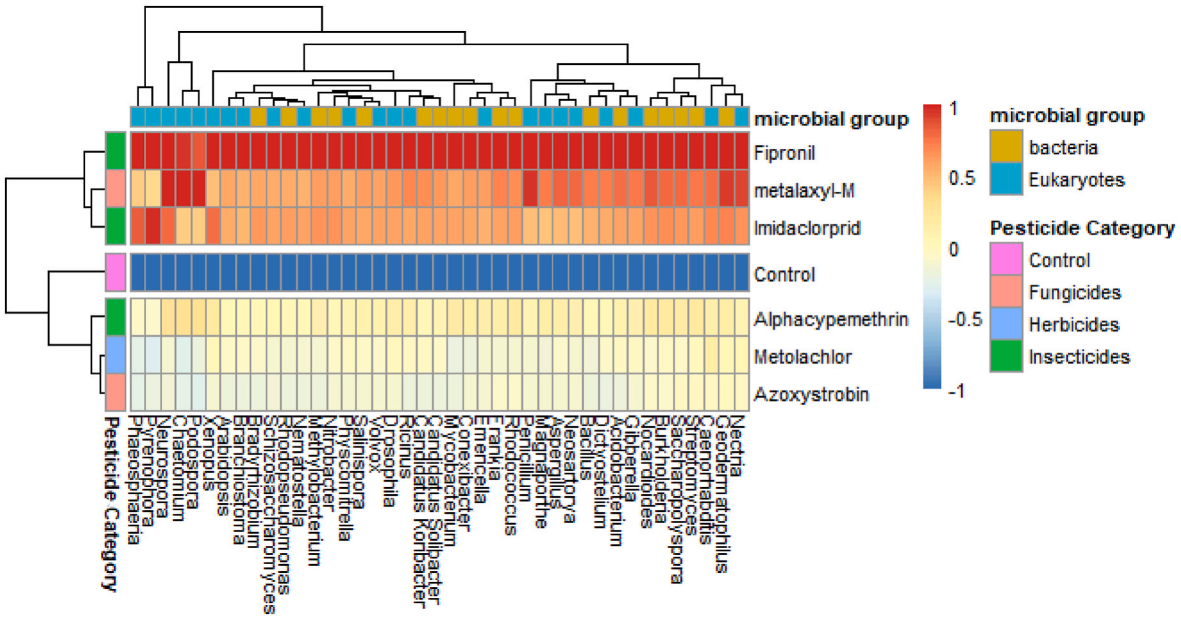

Fig. 4. Heatmap of the absolute abundances (relative abundances expressed as relative to the ITS read counts derived from shotgun sequencing were multiplied by the respective qPCR ITS abundance) scaled from −1 to 1 (values presented here comprise the z-scores (number of standard deviations from the mean)) for each microorganism (see blue to red key) throughout six selected pesticide treated samples plus untreated sample (control) of bacteria and eukaryotes at 1% cut-off level (genus level). The pesticide category of each sample is indicated by the green-pink-blue-purple column at the left according to the secondary key. Hierarchical clustering with the average linkage algorithm was performed for depicting sample and microorganism groupings. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with microbial associated cellulase and chitinase activities, respectively in the acidic soil sample treated with the selected pesticides in our study. Apart from these functional specific taxonomic groups, we further investigated the effect of selected pesticides on high abundant bacteria and eukaryotes genera to prevent the probability that the observed pesticide's effect on microbial communities may be due to functional redundancy. Our results demonstrated an overall increase on the abundance of microorganisms in all six-pesticide treated acidic soils compared to the control soil. Remarkably, the insecticides fipronil, alpha-cypermethrin and imidacloprid have been well established as carbon and energy source for many soil microorganisms and several studies have reported an increase in total microbial communities in soil treated with these insecticides (Zhou et al., 2021; Lupwayi et al., 2009; Tejada et al., 2015; Wang et al., 2008; Zhang et al., 2009). The increase in total microbial communities further proves that carbon cycle is driven by various non-specialist microorganisms, unlike nitrogen cycle which is performed by functional microbial guilds (Sim et al., 2022).

A microbial community shift towards fungi in response to the fungicide metalaxyl-M was observed in the present study with a greater abundance of fungi compared to bacteria, especially in cellulolytic and chitinolytic fungi such as *Hypocrea*, *Penicillium*, *Aspergillus* and *Chaetomium* and the highly abundant eukaryotes *Neurospora* and *Podospora*. *Neurospora* and *Podospora*, although not known for either cellulolytic or chitinolytic capabilities, are reported to be essential in pectin and lignin assimilation (Bourdais et al., 2012; Wu et al., 2020). The greater increase in fungal community is in line with a previous study conducted by Wakelin et al. (2008) who reported a greater adaptability of fungi (*Fusarium)* towards metalaxyl-M with a significant increase in its relative abundance in metalaxyl-M treated soil. This may be due to the suppressing effect of metalaxyl-M on the pathogenic fungi or the development of metalaxyl-M resistance in fungal community due to its widespread use for the control of *Phytophthora* and other oomycetes (Wang et al., 2020; Pérez et al., 2009).

The observed shift in the overall community from bacteria to fungi in metalaxyl-M treated soil may affect soil carbon cycling and the stability of soil structure. Bacteria and fungi are known to work interdependently on organic matter formation with fungi being the major force by forming macroaggregates which are recalcitrant, while bacteria stabilize microaggregates which are more labile (Rashid et al., 2016; Malik et al., 2016). It is thus, important to maintain the balance of soil bacterial and fungal communities for stable soil organic matter and soil structure formation.

Although this study tested a wide range of pesticides in three contrasting soil types, there were still some limitations. There was an imbalance in the number of each category of pesticide tested. More herbicides were tested in the present study than insecticides or fungicides as herbicides are the largest group of pesticide products, with more than 50% of agricultural pesticides annual sales in Australia (APVMA, 2020). However, the aim of the study was to determine if there were significant differences between pesticides with different modes of action, which was achieved with the current experimental design. Although the three South Australian agricultural soils used in this study had contrasting soil textures, nutrient content and soil pH, the conclusions are not necessarily applicable to all soil types. Therefore, it would be beneficial to perform these experiments across an even wider range of soil types, as soil properties strongly influence the potential negative effects of pesticides on soil microbial functions.

5. Conclusion

This study investigated the effects of twenty commercial pesticides on soil carbon cycling parameters in three contrasting agricultural soils. *Cbhl* and *chiA* gene abundances were the most sensitive microbial endpoints to pesticide application. No negative effects on functional microbial endpoints associated with carbon cycling by any of the pesticides tested were observed. Instead, we noted pesticide stimulatory effects which were associated with soil type, specifically soil pH, pesticide group and the pesticide dose. Shotgun metagenomic analysis of selected pesticide-treated and untreated samples of the acidic soil showed an overall stimulation of the microbial community upon application of the insecticides fipronil, imidacloprid, alpha-cypermethrin, the fungicides metalaxyl-M, azoxystrobin, and the herbicide metolachlor. However,

the stimulation imposed by the application of metalaxyl-M favoured a shift of the microbial community towards fungi, with potential unwanted effects on soil organic matter formation. Altogether our results support all three hypotheses that the effect of pesticides on soil carbon cycling (i) varies across soil types and pesticide category (ii) is dose dependent and (iii) induces shifts in microbial community composition. Our results also suggest that the application of pesticides might affect the soil carbon cycle and may disrupt the formation of soil organic matter and structure stabilisation. Furthermore, our findings provide novel insights into the assessment of pesticide effects on SOM degradation and formation as well as carbon cycling.

Credit author statement

Jowenna X. F. Sim: Conceptualization, Investigation, Formal analysis, Data Curation, Writing – Original Draft Barbara Drigo: Conceptualization, Validation, Data Curation, Supervision, Writing – Review & Editing Casey L. Doolette: Conceptualization, Validation, Supervision, Writing – Review & Editing Sotirios Vasileiadis: Methodology, Formal analysis, Data Curation, Writing – Review & Editing Dimitrios G. Karpouzas: Writing – Review & Editing Enzo Lombi: Conceptualization, Validation, Supervision, Writing – Review & Editing.

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

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.135820.

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