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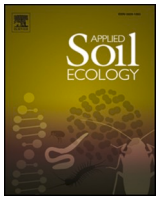

Long-term glyphosate application and its effects on soil total nitrogen and microbial composition two years after application stopped in biochar-amended soil☆

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ABSTRACT

Glyphosate is widely used in horticultural land management practices, but its environmental risks, especially in biochar-amended soil, remain poorly understood. This study aimed to evaluate the effects of repeated glyphosate applications on biochar-amended- soils, focusing on changes in soil nitrogen (N) cycling, soil microbial diversity, and community structure. The experiment was conducted in a macadamia orchard where wood-based biochar had been applied for five years prior to our study. Simultaneously, glyphosate (Roundup®) had been applied at the recommended label rate (4 L ha^{-1}) with active ingredient of glyphosate of 360 g L^{-1}, in a strip under tree canopy for 12 years, until its use was stopped two years prior to our sample collection. Thus, biochar and glyphosate were applied concurrently for three years before glyphosate use was stopped. Soil samples were collected from three areas, including: under the tree canopy with and without a history of glyphosate application; and areas outside the tree canopy with no history of glyphosate application. Changes in foliar and soil total carbon (TC), total nitrogen (TN) and soil N isotope composition ($\delta^{15}N$), and microbial community composition, were subsequently assessed. Our findings revealed that soil TN and $\delta^{15}N$ were significantly higher under tree canopy with a history of glyphosate application compared with areas without glyphosate application history likely due to the die back of weed because the other sections were mechanically managed. Glyphosate residues were also found under and outside tree canopy where no glyphosate was applied. Therefore, higher TN and $\delta^{15}N$ under tree canopy could not directly be attributed to glyphosate application. Overall, neither glyphosate nor biochar influenced the soil microbial diversity and community structure. This study suggested that glyphosate application and farm management practices may have long-term implications for soil N cycling, even after application has stopped.

1. Introduction

Intensive cropping systems rely on organic and inorganic amendments to increase soil nutrients for plant growth and suppress weeds (De Silva et al., 2022; Omidvar et al., 2021, 2023). However, cropping systems often overlap with riparian zones, increasing fertiliser and herbicide runoff into waterways (Bartley et al., 2012; Mottes et al., 2017). Alternative soil ameliorants are sought to maximize weed control whilst minimizing nutrient loss. One approach that has gained global momentum is application of biochar, a carbon (C) rich material

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produced from the pyrolysis of organic material under limited oxygen and high temperatures (Joseph et al., 2021; Xu et al., 2015). Biochar improves soil quality (Bai et al., 2015a; He et al., 2017; Hannet et al., 2021; Song et al., 2016) and soil physicochemical properties by changing the structure of soil, increasing soil water retention, and maximising soil nitrogen (N) retention by altering soil cation and anion exchange capacities, soil moisture and pH (Clough et al., 2013; Hannet et al., 2021; Nguyen et al., 2018; Omidvar et al., 2025; Slavich et al., 2013). Wood biochar immobilises and then slowly releases soil N for plant assimilation leading to long-term benefits for crop yields (Bai et al., 2022; Nguyen et al., 2017). Biochar may also change the biological properties of soil increasing soil microbial abundance (Joseph et al., 2017; Lehmann et al., 2006). This is associated with; (1) increased availability of labile organic matter on the biochar surface (Bruun et al., 2012), (2) the porosity structure of biochar (Lu et al., 2014), which provides refuge, food and optimal niche ecological conditions for microbial activity, and (3) increased water retention and pH alteration (Lu et al., 2014; Razzaghi et al., 2020). Simultaneously, microbial communities and structure are impacted by biochar and soil characteristics, pyrolysis temperature used, soil pH, soil organic carbon (SOC), and soil total N (TN) (Biederman and Harpole, 2013; Mitchell et al., 2015; Rutigliano et al., 2014; Tian et al., 2016). Generally, biochar aging in soil decreases glyphosate adsorption in short term studies and has the ability to remobilise chemicals into soil over time which might have adverse effects on non-targeted organisms (Hall et al., 2018; Zhelezova et al., 2017; Zaparoli et al., 2023). However, in many cases, biochar-soil interactions overtime may not promote herbicide desorption and sometimes may not have any effect on sorption and desorption (Gámiz et al., 2019; Junqueira et al., 2020; Sharma and Lai, 2019). Hence, the long-term interaction of biochar with other land management practices such as chemical weed control and the associated impacts on soil TN and soil microbial communities remain unclear.

Glyphosate (N-(phosphonomethyl) glycine) is a commonly applied herbicide (Allegrini et al., 2015; Duke and Powles, 2008; Tzanetou and Karasali, 2020). Commercially known as Roundup®, it is categorised as a broad-spectrum, post-emergence, non-selective herbicide that inhibits the enzyme 5-enolpyruvylshikimate3-phosphate (EPSP) synthase in the shikimate pathway (Fernández-Escalada et al., 2016), which may also affect soil microorganisms (Nguyen et al., 2016). Long-term and repeated applications of glyphosate may induce changes in soil microbial composition and structure and subsequently affect N availability. However, the results from different studies are contradictory (Bai and Ogbourne, 2016). Most studies have focused on single applications of glyphosate (Bottrill et al., 2020), whereas repeated application of glyphosate is common in land management practices (Allegrini et al., 2017). Glyphosate residues have commonly been detected in soil, plant and water samples after glyphosate application, on some occasions over acceptable thresholds (Bai and Ogbourne, 2016). Glyphosate is typically found at concentrations below 2 g mL^{-1} and at a neutral pH, about 7.2, however, the presence of glyphosate depends on different conditions such as weather and temperature (De Araujo et al., 2023; Villamar-Ayala et al., 2019). Glyphosate residues may persist in soil when soil is rich in organic C (Bai and Ogbourne, 2016). Glyphosate persistence in biochar-amended soils, may also be influenced by diffusion into nanopore structure of biochar, which serves as a key mechanism of retention (Herath et al., 2016; Jiang et al., 2018). The strong binding between glyphosate and biochar is another factor to affect the mineralisation of glyphosate and glyphosate residue mitigation including aminomethylphosphonic acid (AMPA), one of its major metabolite (Hagner et al., 2015; Jiang et al., 2018). However, some microbes use glyphosate as a source of C and N, facilitating the glyphosate biodegradation (Zhan et al., 2018). As a result, the potential interaction between glyphosate and biochar on the soil microbial communities and subsequent N transformation processes is currently unknown.

Bacteria and fungi have key roles in the decomposition of organic matter compounds and N cycling within the soil ecosystem (Gangloff et al., 2016; Nicolás et al., 2019). Species of both the bacterial and fungal kingdoms are highly responsive to land management practices due to the induced changes in soil pH, nutrient content, and water availability, and soil aeration (Xia et al., 2020). Consequently, soil microbial communities have evolved different strategies with respect to land management changes. Microbial communities related to nitrification, such as ammonia-oxidising bacteria (AOB) and archaea (AOA) which carry the amoA gene encoding for the ammonia monooxygenase (Pester et al., 2012), are involved in N availability for plant uptake and N loss pathways (He et al., 2018). Several studies have demonstrated an increase in the abundance of AOA and AOB and thus increased soil nitrification following biochar application (Teutscherova et al., 2017; Yao et al., 2022). Moreover, both AOB and AOA are sensitive to a wide spectrum of chemicals and hence are suitable indicators in the environmental risk assessment of pesticides (Hoshino et al., 2011; Karpouzas et al., 2016; Wessen and Hallin, 2011). However, the information on the effects of glyphosate on ammonia-oxidising microbes, and the results from available studies are too scarce to draw any conclusion (Sim et al., 2022; Zabaloy et al., 2016). To our knowledge, the interactions between biochar-amended soil and repeated application of glyphosate on soil N cycling and soil microbial communities are not well documented. Therefore, this study aims to investigate the impacts of long-term repeated application of glyphosate interacted with biochar on soil N cycling more specifically the abundance of nitrifying populations and the overall soil microbial diversity and community structure (both fungi and bacteria), two years after the herbicide application stopped.

2. Materials and methods

2.1. Site description and experimental design

The experimental site was conducted in a macadamia orchard at Beerwah (26 °50014.1600S 152 °56049.9600E) in Bluegum Creek approximately 88 km north of Brisbane Australia (Fig. S1). The region has a humid subtropical climate (Cfa; Köppen-Geiger climate classification) (Peel et al., 2007) with an average annual temperature of 20 °C, ranging from 14.3 °C to 30.4 °C, and an annual rainfall of 116.9 mm during the period of study in 2018. The orchard established in 2003, consists of macadamia (*Macadamia integrifolia* variety 741) trees planted at 4 m × 9 m spacing (Bai et al., 2015b).

The study used a randomized complete block design with six replicates across 24 trees. Wood biochar derived from pine feedstock via slow pyrolysis at 550 °C was surface applied at two different rates of 10 t ha^{-1} (B10) and 30 t ha^{-1} (B30) in 2012 (Bai et al., 2015b). The B10 and B30 plots received 16 kg and 48 kg dry weight biochar respectively (Bai et al., 2015b). Although biochar is typically incorporated into soil, it was not feasible in this orchard setting (e.g. macadamia) since disturbing the soil would significantly damage the established root system (Bai et al., 2015b). Before biochar application, it was mixed with top soil collected from same area in a ratio of 1:1.5 (w/w; based on dry weight) to minimise wind and rain erosion (Bai et al., 2015b; Asadyar et al., 2021). The control plots were established with the same amount of soil but without any biochar, namely 10 t ha^{-1} (C10) and 30 t ha^{-1} (C30). However, the possibility of prior glyphosate residue testing was not assessed before application. The biochar characteristics and available nutrients are presented in Table S1. Weeds were controlled mechanically outside tree canopy areas using a lawnmower from early establishment of the orchard onwards. Glyphosate was applied under the tree canopy at the label rate (4 L ha^{-1}, active ingredient of glyphosate of 360 g L^{-1}) and where mechanical weed control was not possible. This was applied in a 1 m wide strip approximately 0.5 m from the tree base between 2003 and 2015 (for 12 years). No glyphosate was applied two years before sample collection. The orchard was conventionally fertilised with 50 % urea, 25 % ammonium (NH_4^+-N), and 25 % nitrate (NO_3^--N) (with a total rate equivalent to 120 kg N ha^{-1} yr^{-1}) (Bai et al., 2015b).

2.2. Soil sample collection and chemical analysis

Soil samples were collected in February 2018, 64 months after biochar application. The soil was collected at a depth of 0–5 cm from three positions: (1) 0.5 m from the tree base and under the canopy where there was a history of glyphosate application; (2) 1 m perpendicular away from the tree base under canopy where there was no history of glyphosate application; and (3) 4.5 m perpendicular away from the tree base and outside of the canopy where there was no history of glyphosate application (Fig. 1). The soil samples were collected from both biochar-amended and control areas where no biochar treatment existed. Hence, the treatments were structured as follows. In the biochar-amended area (B10 and B30): (1) biochar + glyphosate (under tree canopy); (2) biochar + no glyphosate (under tree canopy); (3) no biochar + no glyphosate (outside tree canopy); and in the control area (C10 and C30): (4) no biochar + glyphosate (under tree canopy); (5) no biochar + no glyphosate (under tree canopy); and (6) no biochar + no glyphosate (outside tree canopy).

In total, 72 soil samples were collected. A sub-sample from each treatment and control plots was taken (using an auger with an internal diameter of 5 cm), air-dried, and sieved using a 2-mm sieve in the laboratory before chemical analysis. Another sub-sample of soil was immediately placed on the ice container and transported to the laboratory and stored at −20 °C before the molecular analysis. Air-dried and sieved soil samples were dry ground to a fine powder with a Rocklabs™ ring grinder. Approximately 20 mg of the homogenised powder of each sample was then transferred into 8 mm × 5 mm tin capsules for measuring total C (TC), total N (TN), and N isotope composition ($\delta^{15}N$) using an isotope ratio mass spectrometer (GV Isoprime, Manchester, UK). Soil pH was measured using deionised water with a 1:5 (soil: water ratio). A sub-subsample of soils collected from plots with no biochar was also analysed for glyphosate and aminomethylphosphonic acid (AMPA) residues using an Agilent 1290 uHPLC coupled with an Agilent 6470 QQQ MS system (Fig. S2).

2.3. Soil DNA extraction and real-time quantitative PCR (qPCR) of ammonia-oxidising microorganisms

Total genomic DNA was extracted from 0.25 g of each soil sample using a PowerSoil® Kit (QIAGEN, Germany) following the manufacturer's instructions. The quality and quantity of DNA was assessed via NanoDrop spectrophotometric analysis (NanoDrop™ 2000, ThermoFisher Scientific). Genomic DNA samples were stored at −20 °C before determining microbial properties through molecular analyses.

The abundance of functional genes involved in N cycling, such as AOA *amoA* and AOB *amoA* genes were quantified using optical 96 well plates on a BIO-RAD CFX96 real-time PCR detection system (Bio-Rad laboratories) and reactions were prepared according to the manufacturer's instructions. Each qPCR reaction was performed in triplicate and a Non-Template Control (NTC) was included with each combination of primers to identify any possible contamination from genomic DNA and/or primer dimer.

Each 20.0 μL reaction consisted of 10.0 μL of SYBR® Premix Ex Taq™ (TaKaRa Biotech, Dalian, China), 0.2 μL forward primer (10 μM), 0.2 μL reverse primer (10 μM), 2.0 μL of soil template DNA, and 7.6 μL of sterile DNase/RNase free water (Zhang et al., 2016). The specific forward and reverse primers utilised for determining the abundance of functional gene are listed in Table S2. Each primer set was validated using both melting curve analysis and gel electrophoresis to confirm specific amplification. The amplification process involved an initial denaturation step at 95 °C for 5 min; followed by 40 cycles of denaturation at 95 °C for 20 s, 55 °C for 30 s, 72 °C for 45 s (fluorescence reading), and then followed by a melt curve analysis at 65–95 °C every 0.5 °C for 10 s. Standard curves for the quantification of the target sequences in the qPCR amplification were generated using purified plasmids containing the respective genes, with a 10-fold serial dilution ranging from 10^1 to 10^8 times and a blank control containing sterile ddH_2O as the template was included in each reaction set (Zhang et al., 2016). The amplification efficiencies of target genes were 96.47–104.94 %, with R^2 values of 0.991–0.994.

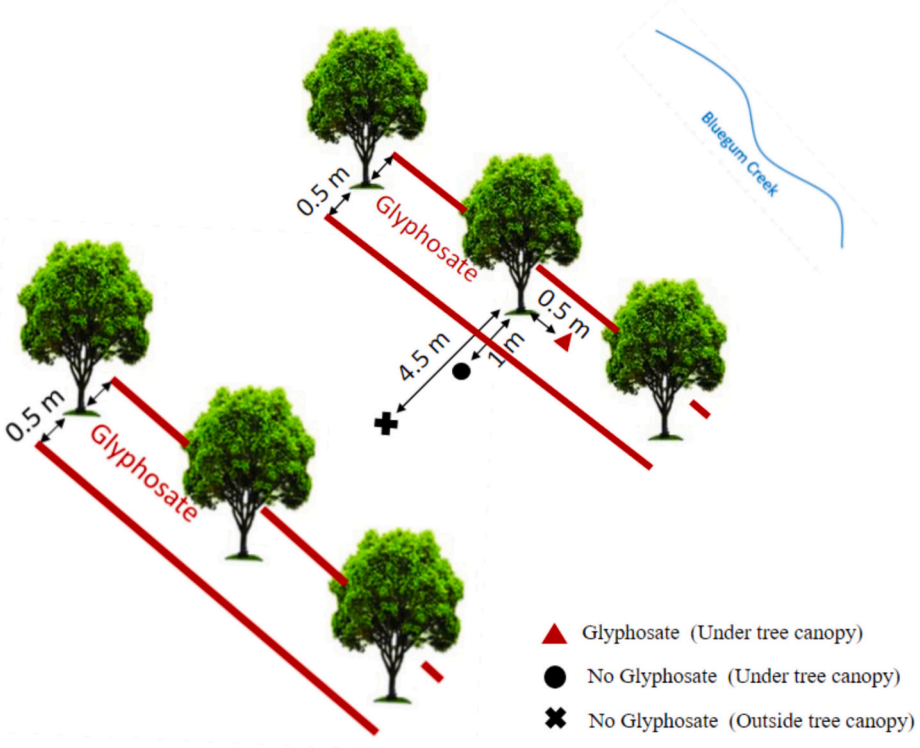

Fig. 1. A schematic to show the location of glyphosate application and soil sample collection points.

2.4. The Illumina MiSeq sequencing

Next-generation sequencing (NGS) library preparation and Illumina MiSeq sequencing were conducted by using GENEWIZ® kits (GENEWIZ, Biotechnology Co., Ltd., Suzhou, China) according to standard protocols described by (Cai et al., 2018; Han et al., 2018; Huang et al., 2019; Qiao et al., 2018). In brief, a total of 30–50 ng of each soil gDNA sample was used to generate amplicons using a MetaVx™ Library Preparation kit (GENEWIZ, Inc., South Plainfield, NJ, USA) according to standard protocols (Han et al., 2018).

2.5. Leaf sample collection and chemical analyses

Three fully expanded leaves from the outermost layer of the middle canopy (1/3 plant heigh) of each of the 24 macadamia trees were collected in February 2018. The collected leaves from each tree were pooled and dried in an oven at 50 °C until reaching a constant weight. Subsequently, they were finely ground into a powder using a Rocklabs™ ring grinder. Approximately 5 mg of the homogenised powder was then transferred into a tin capsule (8 mm × 5 mm) for the measurement of TC, TN, and $\delta^{15}N$ using an isotope ratio mass spectrometer (GV Isoprime, Manchester, UK).

2.6. Statistical analysis

Two-way analyses of variance (ANOVA) were conducted where biochar and glyphosate application history were assessed as the main effects on soil pH, moisture, TN, TC, and $\delta^{15}N$, AOA *amoA* and AOB *amoA*. One-way analyses of variance (ANOVA) were conducted where biochar and biochar application rate were assessed as the main effect on leaf TC, TN, and $\delta^{15}N$. Data were checked for normality and homoscedasticity, and variables were transformed when needed to meet model assumptions. The IBM SPSS Statistics (version 26) software was used for all of the statistical analyses.

The Quantitative Insights into Microbial Ecology (QIIME) package was used for 16S rRNA and ITS rRNA data analysis whereby low-quality reads and sequence length shorter than 200 bp were discarded (Han et al., 2018; Huang et al., 2019). The sequences were then aligned with the reference database (RDP Gold database) using the UCHIME algorithm to detect and remove chimeric sequences (Han et al., 2018). The effective remaining sequences were used in the final analysis.

The operational taxonomic units (OTUs) with 97 % similarity were grouped using the clustering program VSEARCH (v.1.9.6) by comparing against the SILVA119 and UNITE ITS database (https://unite.ut.ee/) (Han et al., 2018; Huang et al., 2019). The Ribosomal Database Program (RDP) classifier was applied to assign a taxonomic category to all OTUs at a confidence threshold of 0.8 (Han et al., 2018). The bacterial and fungal alpha diversity indices were calculated using QIIME. The Shannon index was employed for analysing diversity, while the ACE and Chao1 indices were used to assess richness (Huang et al., 2019). Non-metric multidimensional scaling (NMDS) was performed to visualize similarities among bacterial and fungal communities using the vegan package in R software (v.3.3.1) based on the beta diversity distance matrix. The heatmap analysis was performed using MeV (v.4.2) to visualize similarities within and between the bacterial and fungal species under different weed control methods. To visualize the bacterial and fungal communities across various treatments, a bipartite interaction network was constructed. This network was based on the relative proportions of fungi or bacteria within each treatment using the 'bipartite' package in R (Dormann et al., 2009). Linear Discriminant Analysis (LDA) effect Size (LefSe) method was used to characterise the bacterial and fungal community features.

3. Results

3.1. Soil chemical properties

Two-way ANOVA analyses showed that soil properties including soil moisture, TC, TN, and soil $\delta^{15}N$ were influenced by glyphosate application (Table 1). Under glyphosate application, soil TN, TC, and soil $\delta^{15}N$ were significantly higher compared with areas with no application of glyphosate (Table 1). On the other hand, glyphosate did not affect soil pH, which ranged between 6.1 and 6.8 (Tables 1 and 2). Biochar was not significantly associated with any of the soil chemistry changes assessed (Table 1). No significant interactions between biochar and glyphosate on soil TC and TN were detected.

3.2. Abundance of AOA and AOB

The abundance of the AOB genes varied from 1.16×10^5 to 1.06×10^6 and was generally one or two orders of magnitude higher than for the AOA genes (1.22×10^3 to 5.16×10^4) across all treatments (Table 3). No significant differences were observed in the abundance of AOA genes with the application of biochar and glyphosate (Table 4). Conversely, biochar and glyphosate application did significantly increase the abundance of AOB genes compared with plots without biochar and glyphosate applications (Table 4). No significant interactions were detected among application rates of biochar or glyphosate application on either AOA or AOB gene abundances (Table 4).

3.3. Soil bacterial and fungal community composition

A total of 1,850,432 16S DNA sequences reads were observed with 43,071–63,153 sequences derived per sample and with an average read length of 452 bp. Of these, 15,195 were bacterial OTUs with 97 % sequence similarity. The whole OTUs were derived from 38 individual bacterial phyla, 901 genes, and 937 species. The most dominant bacterial phyla were *Proteobacteria* (39.00 %), *Actinobacteria* (20.72 %), and *Acidobacteria* (16.94 %) (Fig. 2). These accounted for 76.66 % of the total number of bacterial species detected. No significant differences in bacterial diversities were observed within samples from different treatments, and the bacterial Shannon diversity indices ranged from 10.35 to 10.91 (Table 5). Bacterial richness, estimated by the Chao1 index, was not significantly different among the treatments (Table 5). The NMDS results showed that the bacterial communities of the treatments with no glyphosate and no biochar (outside of the tree canopy) were segregated from the other treatments under the tree canopy (Fig. 3a).

A total of 1,814,451 high-quality sequences were observed from the ITS region with 42,902–77,267 sequences detected per sample and an average read length of 350 bp. These presented 19 fungal phyla, 320 genera, and 518 species (Fig. 4). The most abundant fungal phyla represented were the *Mortierellomycota* (25.11 %), *Basidiomycota* (21.90 %), and *Mucoromycota* (20.68 %), which accounted for 67.69 % of the total number of fungal-associated sequences identified and *Mortierellomycota* was the most abundant phylum detected outside the tree canopy (Fig. 4). No significant differences in fungal diversity were observed between treatments and the fungal Shannon diversity index ranged from 4.54 to 5.89 across treatments (Table 5). Similarly, the NMDS results showed that the fungal communities of the treatments with no glyphosate and no biochar (outside of the tree canopy) were segregated from the other treatments under the tree canopy (Fig. 3b).

The most abundant bacterial species detected among the treatments were *Bacillus* and uncultured bacterium with *Acidobacter* the more abundant bacterial species in the areas treated with glyphosate, irrespective of biochar application (Fig. S3). Also, lower abundances of *Flavobacterium* were detected outside the tree canopy than under the canopy, irrespective of glyphosate or biochar application (Fig. S3). The 30 most abundant species across all treatments belonged to *Mortierella*,

Table 1
Two-way analysis of variation (ANOVA) on the impacts of biochar and glyphosate on soil chemical properties in a macadamia orchard.

	df	Soil moisture %		pH		TC (%)		TN (%)		δ^{15}N (‰)	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Biochar	1	0.10	ns	1.42	ns	0.31	ns	0.70	ns	0.00	**
Glyphosate	1	6.74	*	3.28	ns	30.12	**	36.29	**	28.96	**
Biochar × glyphosate	1	0.12	ns	0.51	ns	0.29	ns	0.47	ns	0.14	ns

ns = not significant ($P > 0.05$).
* $P < 0.05$.
** $P < 0.001$.

Table 2
Soil moisture, pH, total C (TC), total N (TN) and N isotope composition (δ^{15}N) in a macadamia orchard following application of biochar and glyphosate. Mean standard errors are presented in the brackets. Letters represent significant differences using Tukey's post hoc test following two-way ANOVA at $P < 0.05$.

Glyphosate application position	Biochar application	Soil moisture (%)		pH		TC (%)		TN (%)		δ^{15}N (‰)	
Glyphosate (under tree canopy)	B10	24.15	(4.60)a	6.45	(0.03)b	5.93	(0.98)a	0.40	(0.07)a	7.31	(0.51)a
	B30	27.02	(4.59)a	6.22	(0.30)b	5.58	(0.95)a	0.37	(0.07)a	6.94	(0.62)a
	C10	27.19	(4.55)a	6.31	(0.13)b	7.16	(0.98)a	0.50	(0.07)a	6.93	(0.53)a
	C30	21.63	(3.30)a	6.27	(0.09)b	5.21	(1.21)a	0.35	(0.08)a	7.11	(0.63)a
No glyphosate (under tree canopy)	B10	20.43	(2.55)ab	6.30	(0.02)b	4.42	(0.38)b	0.28	(0.03)b	6.16	(0.61)b
	B30	19.99	(1.93)ab	6.47	(0.11)b	4.05	(0.22)b	0.24	(0.01)b	6.19	(0.28)b
	C10	20.57	(1.89)ab	6.16	(0.12)b	4.12	(0.43)b	0.27	(0.03)b	6.13	(0.33)b
	C30	18.02	(1.23)ab	6.17	(0.16)b	3.68	(0.46)b	0.25	(0.04)b	5.98	(0.50)b
No glyphosate (outside tree canopy)	B10	21.80	(1.30)b	6.65	(0.11)a	3.26	(0.26)b	0.23	(0.02)b	3.52	(0.67)c
	B30	19.82	(1.37)b	6.81	(0.08)a	3.47	(0.21)b	0.24	(0.02)b	3.41	(0.42)c
	C10	20.74	(1.84)b	6.60	(0.06)a	3.99	(0.26)b	0.28	(0.02)b	4.11	(0.34)c
	C30	22.87	(1.56)b	6.62	(0.11)a	3.47	(0.24)b	0.25	(0.02)b	3.80	(0.49)c

Biochar applied at 10 t ha^{-1} (B10) and 30 t ha^{-1} (B30), control, received no biochar (C10 and C30).

Table 3
Abundances of AOA and AOB genes (number of gene copies g^{-1} soil) resulting from treatments of biochar application and glyphosate. Mean standard errors are presented in the brackets. No letter means no statistically significant difference.

Glyphosate application position	Biochar application	AOB *amoA*		AOA *amoA*	
Glyphosate (under tree canopy)	B10	6.28×10^5	(2.72×10^5)	3.27×10^4	(1.85×10^4)
	B30	1.06×10^6	(1.62×10^5)	1.44×10^4	(7.57×10^3)
	C10	3.60×10^5	(1.78×10^5)	7.91×10^4	(7.20×10^4)
	C30	2.58×10^5	(1.20×10^5)	4.58×10^3	(4.42×10^3)
No glyphosate (under tree canopy)	B10	4.67×10^5	(8.65×10^4)	2.31×10^3	(1.49×10^3)
	B30	5.78×10^5	(3.35×10^5)	5.16×10^4	(4.98×10^4)
	C10	4.00×10^5	(6.57×10^4)	5.54×10^3	(4.32×10^3)
	C30	1.16×10^5	(5.67×10^4)	1.22×10^3	(5.52×10^3)
No glyphosate (outside tree canopy)	B10	1.86×10^5	(9.45×10^4)	3.93×10^3	(2.12×10^3)
	B30	3.59×10^5	(2.11×10^5)	2.04×10^4	(2.02×10^4)
	C10	2.27×10^5	(8.64×10^4)	3.20×10^4	(2.99×10^4)
	C30	1.33×10^5	(9.07×10^4)	4.18×10^3	(1.90×10^4)

Biochar applied at 10 t ha^{-1} (B10) and 30 t ha^{-1} (B30), control, received no biochar (C10 and C30).

Table 4
Two-way analysis of variation (ANOVA) of biochar and glyphosate on abundance of AOA and AOB in a macadamia orchard.

	df	AOB *amoA*		AOA *amoA*	
		F	*P*	*F*	*P*
Biochar	1	9.26	**	1.77	ns
Glyphosate	2	4.28	*	0.64	ns
Biochar × glyphosate	2	1.73	ns	0.64	ns

ns = not significant ($P > 0.05$).
* $P < 0.05$.
** $P < 0.001$.

Mucor, and unclassified fungi (Fig. S4).

The bacterial co-occurrence patterns and network analysis at the phylum level showed that *Acidobacteria* and *Actinobacteria* were more abundant in the glyphosate treated areas, irrespective of biochar treatment (Fig. S5). However, *Proteobacteria* were most abundant in areas without the application of glyphosate (Fig. S5). The phylogenetic diversity of soil fungal communities indicated that *Ascomycota* was more abundant in the glyphosate treated areas with lower abundance in areas without glyphosate application (Fig. S6). Conversely, the abundances of *Mortierelomycota* and *Mucoromycota* were higher in areas without the application of glyphosate (Fig. S6). The LEfSe analysis, identified bacterial and fungal abundance differences across treatments and were depicted as colored dots, with each dot representing different taxonomic levels (Fig. S7).

3.4. Foliar chemical properties

No significant differences were observed in foliar TC and TN as well as foliar δ^{15}N among the treatments (Table 6). Foliar TC ranged between 48.10 % and 48.43 % with the highest TC detected in B30 treatments and the lowest observed in C30 treatments. Foliar TN varied between 1.30 and 1.40 among treatments (Table 6).

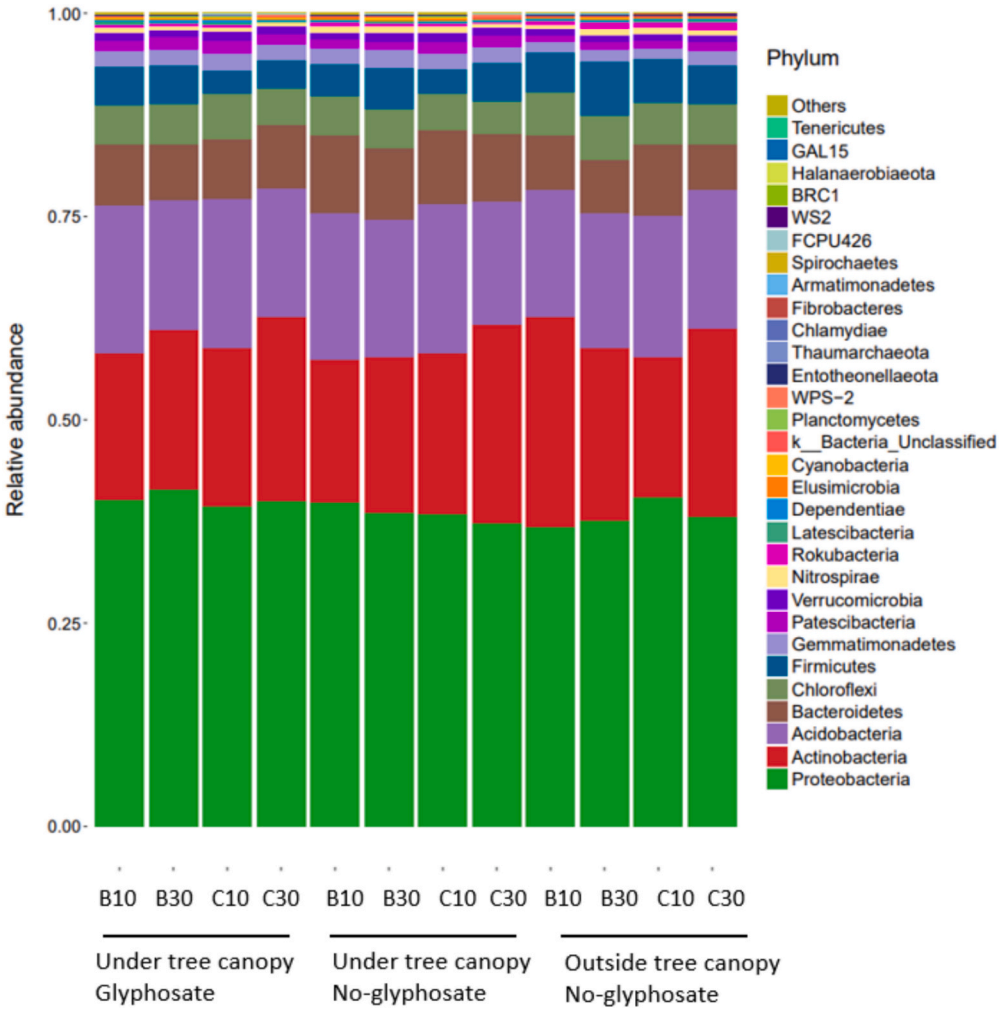

Fig. 2. Soil bacterial community structure at the phylum level at the Beerwah site. Relative abundance was based on the proportional frequencies of DNA sequences that could be classified. Bacteria phylum identified are shown as individual colors and listed in the legend.

Table 5
Alpha diversity statistics of soil bacteria and fungi in different treatments.

Glyphosate application position	Biochar application	Bacteria				Fungi			
		Chao		Shannon		Chao		Shannon	
Glyphosate (under tree canopy)	B10	7144.70	(128.96)	10.84	(0.01)	974.43	(39.57)	5.8827	(0.31)
	B30	6512.77	(388.96)	10.54	(0.23)	955.55	(71.96)	5.8003	(0.49)
	C10	6861.33	(260.73)	10.80	(0.07)	922.73	(66.09)	5.6283	(0.22)
	C30	6734.24	(226.06)	10.68	(0.03)	986.84	(52.69)	6.087	(0.21)
No glyphosate (under tree canopy)	B10	6891.68	(341.87)	10.83	(0.06)	853.69	(95.45)	4.5493	(0.99)
	B30	7319.59	(74.46)	10.91	(0.01)	794.16	(121.72)	4.772	(1.37)
	C10	6725.81	(331.73)	10.73	(0.12)	820.66	(40.65)	5.7737	(0.07)
	C30	6660.35	(307.80)	10.67	(0.05)	841.91	(51.79)	5.693	(0.31)
No glyphosate (outside tree canopy)	B10	6173.54	(213.88)	10.58	(0.11)	692.46	(41.15)	5.0367	(0.26)
	B30	6161.12	(68.63)	10.59	(0.04)	744.48	(5.24)	4.7953	(0.22)
	C10	6329.54	(87.83)	10.35	(0.29)	738.97	(21.28)	5.323	(0.18)
	C30	6267.91	(194.08)	10.55	(0.03)	755.17	(84.39)	5.0773	(0.05)

Biochar applied at 10 t ha^{-1} (B10) and 30 t ha^{-1} (B30), control, received no biochar (C10 and C30).

4. Discussion

4.1. Effects of glyphosate and biochar on soil TN

Soil TN was significantly higher in glyphosate areas compared to untreated areas. Glyphosate induces desiccation of roots and plant residues, which enhances microbial access to residual constituents and thus increases soil N mineralisation (Snapp and Borden, 2005). Glyphosate degradation is predominantly microbially mediated, providing C and N sources that support soil microbial growth and activity (Wang et al., 2021). It has been reported that glyphosate decomposition in soil is relatively fast (Padilla and Selim, 2020; Tang et al., 2019) with the half-life being within 0.8–151 days (Bai and Ogbourne, 2016). Heterotrophic soil microorganisms require C and N for their growth (Soong et al., 2020). Eventually, excess N of microbial demand is released in the soil as an inorganic form of N leading to increased soil N pools (Schleuss et al.,

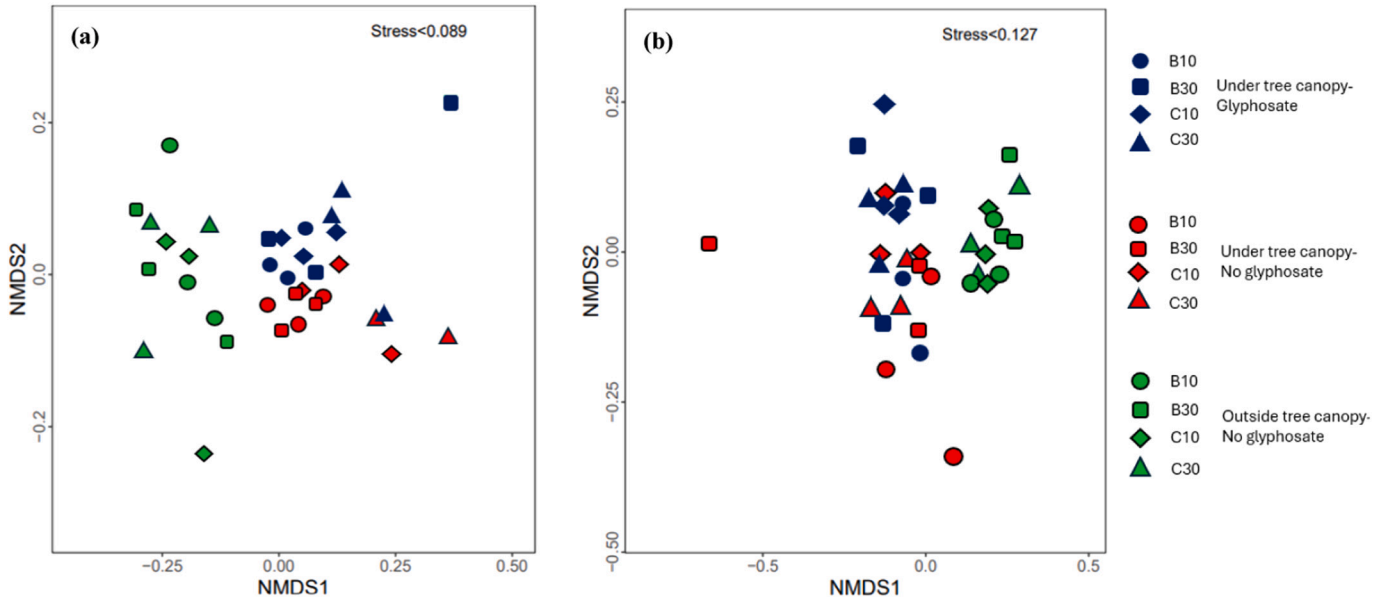

Fig. 3. NMDS ordinations derived from the Bray-Curtis dissimilarity matrices showing changes in the community structure of (a) soil bacteria and (b) soil fungi in different treatments including under tree canopy (glyphosate), under tree canopy (no glyphosate), and outside tree canopy (no glyphosate).

2021). Since glyphosate had not been applied for two years before this study, we would suggest that it is unlikely that the observed TN increase was directly related to ongoing glyphosate degradation. Additionally, in this study, areas with a history of glyphosate application had higher soil TC than untreated areas, which could be attributed to dieback of weeds and roots leading to increased availability of easily degradable organic matter (Imparato et al., 2016). The increased soil C could further provide a substrate to stimulate microbial activity leading to an increase in soil TN. In our study, the non-glyphosate areas were mowed mechanically, removing weed and organic matter residues. Additionally, orchard floor-sweeping during nut harvesting likely contributed to further organic matter removal. Therefore, lower TC in non-glyphosate areas (under and outside tree canopy) may be related to the mechanical removal of organic matter due to nut harvest or weed control. The combination of organic matter removal from non-glyphosate areas and degradation of weed residues within the glyphosate areas could partly explain higher TC in the glyphosate area than those with no history of glyphosate application.

Conversely, the application of biochar did not affect soil TN in this study. An increased soil TN has been reported after five years of poultry litter biochar application, most likely due to TN mineralisation of biochar over time (Bai et al., 2015a). Generally, biochar stimulates N transformation processes, primarily affecting inorganic N forms rather than TN through various mechanisms over time such as decreased N loss, increased soil nitrification or decreased leaching (Bai et al., 2015a; Nguyen et al., 2018; Asadyar et al., 2021). However, aged biochar is also able to retain soil N into organo-mineral complexes leading to increased soil N retention (Joseph et al., 2021). However, the wood-based biochar used in this study contained very low TN concentrations to be released into the soil as a result of mineralisation (Bai et al., 2015a; Nguyen et al., 2018; Asadyar et al., 2021). However, it was efficient to increase soil inorganic N concentrations (Asadyar et al., 2021). In the current study, we did not measure soil inorganic N and potential nitrification, the higher abundance of AOB in biochar-treated areas compared to non-biochar areas suggests that biochar stimulated N transformation processes.

4.2. Microbial changes after glyphosate and biochar application

In this study, glyphosate-treated areas showed a significantly higher abundance of AOB compared with non-glyphosate areas, while AOA abundance remained unaffected. As previously reported, AOB is more responsive to toxicants such as fungicides (mancozeb) (Feld et al., 2015) and microcystins (Corbel et al., 2015) than AOA. The high level of ribosomal content and cell activity in AOB (Hatzenpichler et al., 2008; Wagner et al., 1995; Carey et al., 2016) may make it more susceptible to external environmental factors (Zhang et al., 2014). With regards to glyphosate, there is a lack of information about the effect of glyphosate on *amoA* abundance and the existing results are contradictory. It is likely to find glyphosate and AMPA residues in soil after one or two years and it is likely to be moved by both wind and water (Bento et al., 2017; Silva et al., 2018). We detected both glyphosate and AMPA residues at this site even where it was not applied (Fig. S2). Therefore, the increased abundance of AOB observed in the glyphosate treated areas was not a direct effect of the glyphosate application. AOB is responsive to soil C (Sun et al., 2019). Potentially, increased TC concentrations under tree canopy favored AOB abundance.

Our study showed that biochar had a significantly higher abundance of AOB compared with areas with no biochar, however, no significant changes were observed in the abundance of AOA. An increase in the abundance of AOB after the application of biochar has previously been reported (Bi et al., 2017; Lin et al., 2017). The different result in this study may be influenced by soil pH, and differences in feedstock, and pyrolysis temperature in the biochar manufacture process. In particular, soil pH plays a key role in the structure and abundance of ammonia-oxidising microorganisms (Nicol et al., 2008) and biochar usually increases the abundance of AOB *amoA* gene copy in acidic soil (Xiao et al., 2019). The addition of biochar in our study did not significantly change soil pH and hence the increase in AOB was not driven primarily by pH changes. A recent meta-analysis has shown that wood-based biochar increases the abundance of AOB by 140.6 % (Xiao et al., 2019), likely because it increases soil porosity and aeration (Lei and Zhang, 2013). This then promotes the growth of nitrifying bacteria, and more specifically, AOB (Xiao et al., 2019). Additionally, high biochar pyrolysis temperature (i.e. >500 °C) has also been shown to increase the abundance of AOB according to a recent meta-analysis study (Xiao et al., 2019). We also used biochar produced at 550 °C. Therefore, the higher AOB in the biochar areas compared with the non-biochar areas in this study could be partly explained by biochar characteristics and soil physical changes that occurred after biochar application.

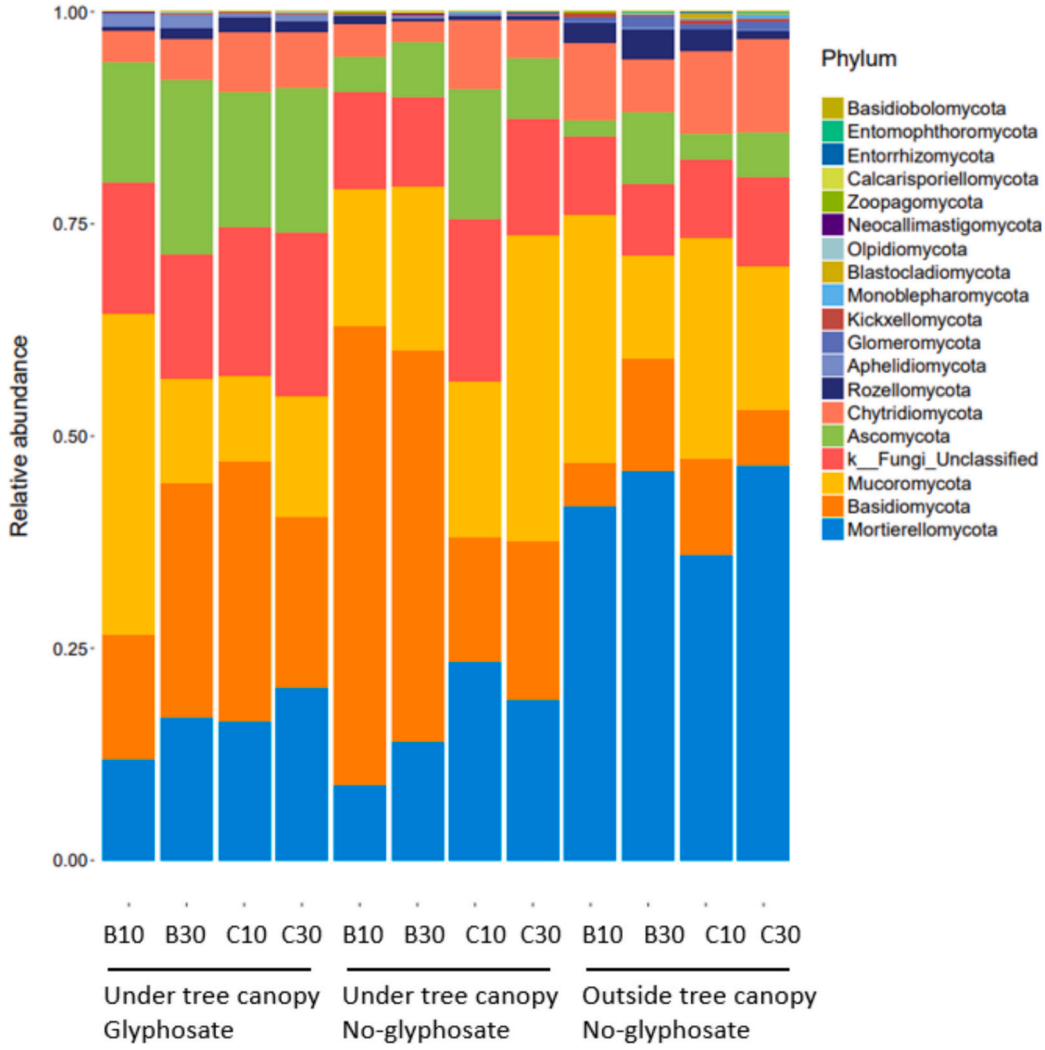

Fig. 4. Soil fungal community structure at the phylum level at the Beerwah site. Relative abundance was based on the proportional frequencies of DNA sequences that could be classified. Fungal phylum identified are shown as individual colors and listed in the legend.

Table 6
Total C (TC), total N (TN) and N isotope composition ($\delta^{15}N$) in macadamia leaf following 64 months of application of biochar. Mean standard errors are presented in the brackets. No letter means no statistically significant difference.

Treatments	TC (%)		TN (%)		$\delta^{15}N$ (‰)	
B10	48.08	(0.14)	1.37	(0.03)	3.19	(0.57)
B30	48.43	(0.18)	1.36	(0.05)	2.33	(0.61)
C10	47.20	(0.63)	1.40	(0.03)	3.10	(0.64)
C30	48.10	(0.38)	1.30	(0.05)	3.00	(0.75)

Biochar applied at 10 t ha^{-1} (B10) and 30 t ha^{-1} (B30), control, received no biochar (C10 and C30).

No significant impact of glyphosate was found on soil microbial diversity and community structure for either the bacterial or fungal phyla. This is in agreement with a previous study and proposed to be because some prokaryotic and fungal species are resilient and able to metabolize glyphosate, protecting other susceptible species within the same communities (Kepler et al., 2020). Meanwhile, higher application rates of glyphosate and immediate microbial community profiling have previously been shown to increase microbial resistance tolerating extreme changes (Kepler et al., 2020). In this study, we did not have soil samples taken before or during glyphosate application. Therefore, we could not distinguish whether the microbial population was initially high but had recovered during the period of two years since glyphosate application was stopped or whether glyphosate did not affect the microbial community in the first place. It is not uncommon to find glyphosate and AMPA residues in soil after one or two years and it is likely to be moved by both wind and water (Bento et al., 2017; Silva et al., 2018). In the current study, it was likely that extreme rainfall events had led to moving glyphosate and AMPA to non-glyphosate area. It should be also noted that a concentration of glyphosate or AMPA in soil is considered from low to medium when the concentrations vary between 0.05 and 0.5 mg kg^{-1} (Silva et al., 2018). In the current study, the concentrations of both glyphosate and AMPA residues were well below 0.05 mg kg^{-1} which might partly explain our observation where no effects of glyphosate application on soil microbes were found.

Previous studies have demonstrated no response in bacterial community structure following long-term biochar application (i.e. > 2 years) (Quilliam et al., 2012; Anderson et al., 2014). In the current study, biochar was applied 64 months (i.e. > 5 years) prior to sampling and also did not result in any changes to the bacterial community structure. This was contrary to what was expected as biochar aging is expected to alter physical and chemical properties including smaller surface areas, changes in functional surface molecules particularly carboxylic and hydroxyl groups and pH (Mia et al., 2017). Aged biochar may also decrease the available space for the growth of microorganisms due to the build-up of many organic and inorganic compounds on the biochar

surface (Jindo et al., 2012; Weng et al., 2017). Soil pH plays a critical role in the soil biochemical process and is one of the driving factors affecting soil microbial diversity and community structure (Fan et al., 2018; Rousk et al., 2010; Wang et al., 2021). This study showed that biochar did not change soil pH over the monitoring period, which might explain why we did not observe any changes in the soil microbial community associated with the biochar treatment.

It should be noted that macadamia farms commonly employ sweeping or blowing techniques every year to collect nuts from the orchard ground (Dalby et al., 2010). However, these practices may lead to soil degradation, depletion and root exposure in macadamia farms (Dalby et al., 2010). One of the reasons for applying glyphosate close to tree trucks was the inaccessibility of these areas to weed mowing. Moreover, the areas near the tree bases are less prone to lose top soil during harvesting. A combination of farm management practices, including increased organic matter accumulation due to the die back of weeds around the tree bases and improved soil moisture retention, could have contributed to enhanced soil N transformation in areas with a history of glyphosate application. Therefore, understanding the implications of glyphosate use on soil health and nutrient dynamics in tree crop orchards is essential for fostering sustainable farming practices and maintaining soil fertility. Long term observations coupled with a more detailed analysis of glyphosate fate could provide a more comprehensive understanding of the persistence and long-term effects of glyphosate residues in biochar-amended soils. This study focused on soil N cycling, microbial diversity, and community structure. Examination of functional genes or key microbial enzyme activities related to N cycling could provide a more understanding of the system.

5. Conclusion

This study examined the effects of repeated application of glyphosate and its interaction with biochar on soil N cycling and soil microbial communities two years after glyphosate was last applied. Overall, glyphosate areas had higher soil TN and TC than other areas which was likely related to die back of weeds. The glyphosate and biochar did not show any effects on soil microbial structure. Glyphosate residues were, however, detected in areas where it had not been applied, even after application has stopped which may have long-term implications for soil N cycling. Our study suggested that farm management practices may have long term implications on soil health.

CRediT authorship contribution statement

Negar Omidvar: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Zhihong Xu:** Writing – review & editing. **Steven M. Ogbourne:** Writing – review & editing. **Rebecca Ford:** Writing – review & editing. **Prabhakaran Thanjavur Sambasivam:** Writing – review & editing. **Trong D. Tran:** Writing – review & editing, Data curation. **Babak Salehin:** Writing – review & editing, Data curation. **Ruby N. Michael:** Writing – review & editing. **Fang Wang:** Writing – review & editing, Data curation. **Iman Tahmasbian:** Writing – review & editing, Data curation. **Rachele S. Wilson:** Writing – review & editing, Visualization. **Shahla Hosseini Bai:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2025.106266.

Data availability

Data will be made available on request.

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