Biochar modulates heavy metal toxicity and improves microbial carbon use efficiency in soil

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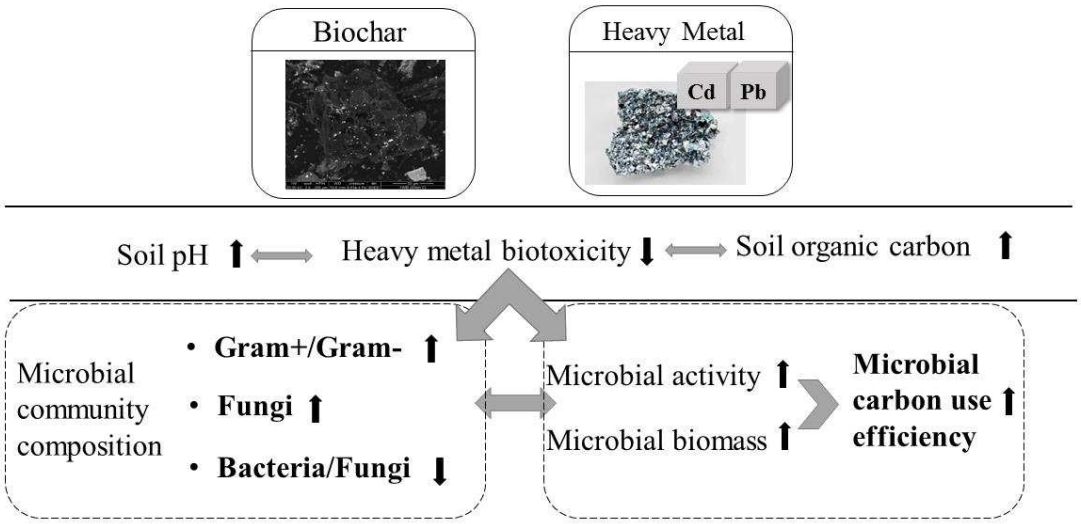
35 **Graphical abstract**



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Highlights

- Biochar addition reduced metal toxicity to microorganisms in contaminated soils
- It improved microbial activity, biomass, and microbial carbon use efficiency Biochar shifted soil microbial community as evidenced by PLFA biomarkers
- Overall, biochar enhanced microbial ability in immobilization soil carbon under contaminated soils

Abstract:

Soil organic carbon is essential to improve soil fertility and ecosystem functioning. Soil microorganisms contribute significantly to the carbon transformation and immobilisation processes. However, microorganisms are sensitive to environmental stresses such as heavy metals. Applying amendments, such as biochar, to contaminated soils can alleviate the metal toxicity and add carbon inputs. In this study, Cd and Pb spiked soils treated with macadamia nutshell biochar (5% w/w) were monitored during a 49 days incubation period. Microbial phospholipid fatty acids (PLFAs) were extracted and analysed as biomarkers in order to identify the microbial community composition. Soil properties, metal bioavailability, microbial respiration, and microbial biomass carbon were measured after the incubation period. Microbial carbon use efficiency (CUE) was calculated from the ratio of carbon incorporated into microbial biomass to the carbon mineralised.

Total PLFA concentration decreased to a greater extent in metal contaminated soils than uncontaminated soils. Microbial CUE also decreased due to metal toxicity. However, biochar addition alleviated the metal toxicity, and increased total PLFA concentration. Both microbial respiration and biomass carbon increased due to biochar application, and CUE was significantly ($p<0.01$) higher in biochar treated soils than untreated soils. Heavy metals reduced the microbial carbon sequestration in contaminated soils by negatively influencing the CUE. The improvement of CUE through biochar addition in the contaminated soils could be attributed to the decrease in metal bioavailability, thereby mitigating the biotoxicity to soil microorganisms.

Keywords: Biochar; Heavy metal toxicity; Microbial carbon use efficiency; PLFA; Soil carbon sequestration

1. Introduction

Biochar has been acknowledged as an effective material to sequester terrestrial carbon, while at the same time improving microbial habitat in soil (Lehmann et al., 2011; Quilliam et al., 2013). Biochar can play an important role in the biogeochemical cycling of carbon and other elements in soils (Kuzyakov et al., 2009; Bolan et al., 2012). In addition to improving soil fertility and water holding capacity (Paetsch et al., 2017), applications of biochar have attracted a rising attention due to the possibility of the remediation of heavy metal contaminated soils (Rees et al., 2014). Dominance of oxygen-containing functional groups in the highly porous structure of biochar makes the material suitable for the adsorption of a range of contaminants including heavy metals (Bolan et al., 2014; Mandal et al., 2017). Heavy metal(loid)s are among the most toxic and widespread contaminants in our environment because of their persistent nature and high bioaccumulation potential. Some metal elements (e.g., Fe, Zn, Cu, Mn) are involved in many biochemical reactions, but metals like Cd, Pb and Ag have no biological role. They are rather potentially toxic to micro- and macroorganisms (Bruins et al., 2000). The key mechanism of metal toxicity to microorganisms evolves due to the displacement or substitution of essential elements by toxic elements either in the extracellular enzymes or even in nuclear proteins, which consequently may lead to enzyme synthesis inhibition and metabolic process dysfunction (Tchounwou et al., 2012; Baumann et al., 2013). Additionally, when present at a high concentration, even the essential metal elements may lead to adverse consequences (e.g., damage to cell membranes and DNA structure and oxidative stress) (Kachur et al., 1998; Tchounwou et al., 2012). Therefore, toxic levels of heavy metal(loid)s may give rise to the deterioration of soil microbial populations and their metabolic activities through denaturing the protein structure and impairing cell membrane functions (Jiang et al., 2010).

Soil microorganisms have important roles in developing soil structure, maintaining its stability, and also in carbon and other nutrient cycling processes (Lehmann et al., 2011). A high microbial community diversity is critical to maintain various soil functions. Microorganisms are also a central part of the soil contaminant remediation strategies through the global biogeochemical cycling of different elements. However, variations in soil environments (e.g., pH, redox potential, toxic elements, etc.) may affect the microbial populations and their activities, and thus may alter the state of soil remediation and/or carbon sequestration (Pan et al., 2016). The sensitive responses of microorganisms to soil environmental changes may serve as the indicators of any restoration progress in a contaminated site and its risk assessment.

Biochar is reported to recover microbial activities in metal contaminated soils (Yang et al., 2016). Such improvement in microbial activities could be attributed to: (i) improvement of soil physiochemical properties (increase of soil aeration, moisture content and pH), (ii) immediate supplement of soil carbon pools, especially the recalcitrant pool, (iii) supply of nutrients, and (iv) modification of microbial habitat and ecological niche (Jones et al., 2011). Soil microbiota and their carbon utilisation preferences could be significantly altered by biochar amendments (Farrell et al., 2013; 2015). However, due to the complexity of soil and ecosystem diversity, there is a lack of understanding about biochar modulated microbial responses in metal polluted environments (Pan et al., 2016). Microbial carbon use efficiency (CUE) is defined as the conversion of the organic carbon assimilated into the microbial biomass in the net carbon sequestration process (Rousk and Bååth, 2011). Different approaches of microbial CUE measurement and interpretation of results may involve some discrepant assumptions (Frey et al., 2001), but it can be used as a reference for the microbial carbon utility preference in soils (Sinsabaugh et al., 2013; Blagodatskaya et al., 2014). Some microbial species, especially fungi often positively respond to biochar addition (Warnock et

al., 2007; O'Neill et al., 2009). However, metabolic features of the assimilated carbon in fungi and bacteria are different, which can potentially distinguish between the preferences of soil organic carbon decomposition patterns, and also the specific functional roles of respective microorganisms. The phospholipid fatty acid (PLFA) profiles of microorganisms can be a useful chemotaxonomic biomarker to interpret the microbial community composition and carbon utilisation differences in response to biochar addition to soils under metal stress (Birk et al., 2009).

To our knowledge, limited information is available in the literature on how soil microbial parameters, especially microbial population react to metal pollution in the presence of biochar (Ahmad et al., 2016). The current study not only quantified microbial carbon use patterns (as measured by the percentage of microbial biomass formation over substrate carbon uptake), but also coupled those information with microbial community compositions. We hypothesised that the microbial properties and CUE will be benefited by the metal remediation ability of biochar in contaminated soils. The objective of this research is to assess the magnitude to which biochar could modulate the soil microbiota underpinning terrestrial carbon sequestration under metal stress conditions. By using microbial community abundance and composition approaches, this study will help to better understand the mechanisms of carbon sequestration through biochar addition in metal contaminated soils.

2. Materials and methods

2.1. Soil sampling and preparation

A natural surface soil (0-10 cm) sample was collected from the Barossa Valley region, South Australia (138°57'37''E, 34°27'48''S). The region is characterised by Mediterranean climate, with an average summer temperature range of 26-29°C (daytime) and 12-14°C(night time), and winter range of 12-16°C (daytime) and 3-6°C (night time). The Barossa region

144 receives an average annual rainfall of 437 mm and the soil pattern is extremely variable with
145 the chief soils are Sodosol (Australia soil taxonomy). The soil was classified as silty loam
146 (USDA textural classification).
147 After sampling, the soil was homogenised and sieved (<2 mm). Fine roots and other plant
148 debris were carefully removed during the processing steps. Prior to the experiment, the soil
149 moisture content was adjusted to 50% (weight basis) of the water holding capacity (WHC),
150 and pre-incubated at 25°C, 28% relative humidity for 7 days in order to recover the microbial
151 activity. The biochar sample used in this study was prepared from macadamia nutshell by
152 pyrolysing the feedstock slowly at 465°C under O_2-limited environment, as described by
153 Khan et al. (2014).
154

155 **2.2. Soil and biochar characterisation**

156 Soil and biochar pH values in 1:5 (w/v) suspensions in deionised water following
157 equilibration on an end-over-end shaker for 2 h were determined by a pH/conductivity meter
158 (smartCHEM-LAB Laboratory Analyser, VWR International Pty Ltd., Australia). Soil texture
159 was determined by the micro-pipette method (Miller and Miller, 1987). The cation exchange
160 capacity (CEC) of the soil was determined by first saturating the exchange sites (positive
161 charges) with NH_4^+, then extracting and analysing the exchanged NH_4^+ on a Continuous Flow
162 Analyser (San ++, Skalar Analytical B.V., The Netherlands). For the total elemental analysis,
163 soil and biochar samples were mixed with 5 mL of aqua-regia (HNO_3:HCl at 1:3 v/v), and
164 digested in a micro-wave digestion oven (MARSXpress, CEM Corporation, USA). The
165 digested samples were decanted and filtered before analysing elements on an Inductively
166 Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Agilent 7900, Agilent
167 Technologies Ltd., USA). Total nitrogen (TN) and total organic carbon (TOC) in soil and
168 biochar samples were determined by dry combustion technique using a Leco C/N Analyser

(Leco TruMac® CNS/NS Analyser, LECO Corporation). Soil samples (0.2 g) were weighed and combusted at 1300°C with an O_2 flow for 5 sec. The instrument was calibrated at every 10 samples by analysing standard weights of Leco EDTA reference material (containing 95.7 g N kg^{-1} and 410 g C kg^{-1}). Soil and biochar physico-chemical characteristics are presented in **Table 1**.

In addition, the specific surface area and pore size of the biochar sample were measured by conducting N_2 adsorption-desorption experiments by BET method on a NOVA 1000e Analyser (Quantachrome Instruments, USA). Functional groups in the biochar sample were studied through Fourier Transform Infrared (FTIR) spectroscopy on a Cary 660 FTIR Analyser (Agilent Technologies Ltd., USA). Morphological features and pore structures of the biochar sample were examined by a Quanta 450 FEG environmental scanning electron microscope (SEM) (FEI Company, USA). The elemental composition of the biochar was determined by an energy dispersive X-ray (EDX) spectrometer attached with the SEM equipment.

2.3. Soil spiking, biochar amendment and incubation experiment

The experiment approach, including experiment design and quantification of microbial response were detailed in **SI. 1**. In the present study, the experimental soil was spiked with 50 and 5000 mg kg^{-1} of Cd^{2+} and Pb^{2+}, respectively. These concentrations were chosen to reflect a contamination level above the sensitivity threshold of the respective metals in order to detect responses of microbial carbon use patterns under metal stresses in the soil (Sobolev and Begonia 2008; Smolders et al., 2009).Two concentrations of $Cd(NO_3)$ and $Pb(NO_3)$ were mixed with the soil separately, and also in combination (Table 1). Briefly, metal solutions were sprinkled evenly on the soil spread on a polyethylene sheet. To achieve homogenisation, soils were then stirred and mixed thoroughly on an end-over-end shaker. Soils were then air-

dried, and passed through a 2 mm-sieve again. The final concentrations of metals in the spiked soils and abbreviations for each treatment are listed in **Table 2**.

Biochar was added at 5% w/w into 200 g soils. The 5% addition is equivalent to 12.75 tons ha^{-1} biochar addition in the field (based on 2.5 cm depth incorporation with a bulk density of around 1020 kg m^{-3}). The reduction of available metal concentration due to biochar addition (dilution effect) ranged from 1-10% (Houben et al., 2013). Glucose (100 g L^{-1} in H_2O) was applied to a separate set of samples to achieve the same total carbon content (3.71%) as of the added biochar. A separate treatment without any amendment was prepared as control. Metal-spiked and biochar/glucose treated soils were transferred into plastic containers, and incubated at 25°C and 28% room humidity for 49 days. The moisture content of the soil was maintained at 60% of the WHC throughout the incubation experiment. All experiments were conducted in triplicate.

2.4. Bioavailability of heavy metals

Bioavailable heavy metal concentrations were measured by extracting the biochar/glucose-amended and unamended soils with 0. 01 M $CaCl_2$ solution (1:10 w/v) for 60 min reaction time (Sparks et al., 1996). The extracts were filtered through 0.45 µm syringe filter before analysing the metal elements on an ICP-MS instrument (ICP-MS, Agilent 7900, Agilent Technologies Ltd., USA).

2.5. Microbial properties

2.5.1. Microbial activity

The microbial activity of soils was monitored by measuring the rate of CO_2 evolution from the samples. Sealed soil microcosms (10 g) in Schott bottles having different treatments as stated above were incubated for 49 days in dark at 25°C and 60% WHC. Three blank Schott

bottles without any soil were set as controls. A 20 mL open-top vial containing 10 mL of 0.05 M NaOH solution was used to trap the evolved CO_2 within the sealed Schott bottles. Periodically, the alkali was decanted into an Erlenmeyer flask and rinsed with deionised water three times. The small vial was replaced with 10 mL of fresh alkali every time. The collected alkaline aliquot was then titrated against 0.03 M HCl in the presence of phenolphthalein indicator following the addition of 5 mL of 0.5 M $BaCl_2$. The amount of evolved CO_2 was thus measured, and the microbial respiration was calculated using (Eq. 1):

$$MR = \{MWCO_2(V_b - V_s) \times M \times 1000\}/(DW \times T \times 2) \quad \text{Eq. 1}$$

where, MR is the microbial respiration (mg CO_2-C kg^{-1} soil h^{-1}), $MWCO_2$ is the molecular weight of CO_2, V_b is the volume of HCl for the blank titration, V_s is the volume of HCl for the sample titration, M is the concentration of HCl, DW is the dry weight of the soil, T is the time of incubation, and 2 is the factor that accounts for the fact that two OH^- are consumed by one CO_2.

2.5.2. Microbial biomass carbon

Microbial biomass carbon (MBC) was determined using the fumigation-extraction method (Vance et al., 1987). Soils (10 g, dry weight basis) were placed in 50 mL beakers within a vacuum desiccator containing 50 mL of ethanol free chloroform. The desiccator was tightly sealed and pumped until chloroform was vaporised. Soils were thus incubated in chloroform vapour for 48 h within the desiccator. Another set of un-fumigated soils were maintained simultaneously. After 48 h, both the fumigated and un-fumigated soils were mixed with 40 mL of 0.5 M K_2SO_4, and shaken on an end-over-end shaker for 1 h. Samples were then centrifuged and filtered through Whatman #40 filter papers. Carbon content in the filtrates was analysed by a Total Organic Carbon (TOC) Analyser (TOC-LCSH, Shimadzu Corporation, Japan). MBC was calculated using Eq. 2:

$$MBC = E_c/K_c \quad \text{Eq. 2}$$

where, MBC stands for microbial biomass carbon (MBC, mg C kg^{-1} soil), E_c stands for the value = (carbon extracted from fumigated soils – carbon extracted from non-fumigated soils), and K_c stands for the conversion factor (0.45) from chloroform flush carbon values into MBC (Anderson and Domsch, 1989).

The microbial carbon use efficiency (CUE) was estimated following Eq. 3.

$$M_C = \Delta MBC/(\Delta MBC + \Sigma CO_2\text{-C}) \quad \text{Eq. 3}$$

where, M_C is microbial CUE measured as microbial biomass variation, ΔMBC is the change of microbial biomass carbon, ΣCO_2-C is cumulative CO_2-C as microbial respiration.

2.5.3. Microbial community composition

Phospholipid fatty acid (PLFA) patterns were used to estimate the relative abundance of bacteria, fungi and actinomycetes in the biochar/glucose-amended and unamended soils. Microbial PLFAs were extracted by standard methods (Frostegård et al., 1993; Bossio et al., 1998).

In brief, soil samples were first freeze dried at −45°C and at less than 1 millibar (0.8 mbar) pressure. Then, freeze dried soils (5 g) were extracted with one-phase extraction solvent. The one-phase solvent was a mixture of chloroform, methanol and citrate buffer (1:2:0.8, v/v/v), while the citrate buffer was made of citric acid and sodium citrate (3:1, v/v) with pH adjusted at 3.6 (Bligh and Dyer, 1959). After shaking on an end-over-end shaker, the mixture was centrifuged twice at 4500 rpm for 30 min. The supernatant was decanted into a non-transparent vial, and vortexed before standing overnight. The upper layer of the standing liquid was removed, and the remaining bottom portion was dried under N_2 flow at 32°C. Following drying, the thin solid phase left at the bottom of the vial was re-dissolved in chloroform (1 mL), and transferred into a solid phase extraction (SPE) column. To set up the

column, 0.5 g of silica was packed, followed by conditioning with chloroform thrice (1+1+1 mL). Then, the sample transfer in the SPE column included three steps: chloroform (2+1+1+1 mL), acetone (2+1+1+1 mL) and methanol (2+1+1+1 mL). The final leaching solution was dried with continuous N_2 flow at 32°C. To the pellet obtained, 0.5 mL of 1:1 (v/v) of methanol:toluene and 0.5 ml of 0.2 M methanolic KOH (by dissolving 0.28 g KOH in 25 mL of methanol) were added. The mixture was incubated at 37°C for 30 min, and then cooled to room temperature. The PLFAs were thus converted into fatty acid methyl esters (FAMEs) with mild alkaline methanolysis. Following incubation, 1 mL of deionised water, 0.15 mL of 1 M acetic acid, and 1 mL of hexane were added to the mixture, vortexed for 30 sec, and centrifuged at 4500 rpm for 30 min to separate the solution into two layers. The upper layer was carefully transferred into a Gas Chromatography (GC) vial with a pipette. This separation procedure was repeated twice with the addition of fresh extractants. Finally, the extract was concentrated by continuous N_2 flow, and stored at −20°C in total darkness before further analysis.

An internal standard (methyl nonadecanoate, C19:0) (10 ng) was added to all samples as a quality control measure. The FAMEs were analysed by gas chromatography-mass spectrometry (GC-MS) (Model 7890B/5977B, Agilent Technologies Ltd., USA; AxION iQT with Cold EI Source, Perkin Elmer, USA). A RTX-5MS fused silica capillary column (60 m, 250 µm × 0.25 µm film thickness) (Supelco, Sigma-Aldrich, Australia) was used. Sample (1 µL) was injected in splitless mode with an injector temperature of 250°C, and helium carrier gas at a constant flow rate of 1.4 mL min^{-1}. The temperature program was set as follows: column temperature initially at 60°C for 1 min, then increased to 180°C at a rate of 12°C min^{-1}, then increased to 300°C at a rate of 4°C min^{-1} and kept at 300°C for 4 min. Electron energy in the detector was set 70 eV. Data was acquired in scan mode from 50 to 400Da at 3 scans per second. Quantification was conducted against a Supelco 37 standard mixture

294 (Supelco, Bellefonte, PA), and the C19:0 internal standard with a 6 point linearity curve
295 analysed in triplicate ($r^2 \geq 0.98$ for each component). Each PLFA peak was identified by
296 comparing the respective retention time and by their mass spectra. The isomers not included
297 in the standard mix were quantified against the relative response factor for C16:0, and were
298 individually identified by their mass spectra from a Cold EI TOF scanning analysis
299 conducted on a Perkin Elmer AxION iQT instrument. The specific microbial species were
300 identified by the signature PLFAs listed in **SI. 2**.
301

302 **2.6. Statistical analysis**

303 Significant differences among treatments were tested using one factor ANOVA followed by
304 the post-hoc least significant difference (LSD) test. Duncan's multiple range test was used to
305 compare the means of the treatments. Variability in the data was expressed as the standard
306 deviation, and a $p<0.05$ was considered to be statistically significant. Microbial PLFA data
307 were analysed with principal component analysis (PCA) to elucidate the major variation and
308 covariation both for individual PLFA and microbial species using varimax rotation. All
309 statistical analyses were performed using SPSS version 23.0 software packages (SPSS Inc.,
310 Chicago, USA) with significant differences as stated in specific cases.
311

312 **3. Results and discussion**

313 **3.1. Influence of biochar on heavy metal availability**

314 3.1.1. Influence of biochar-induced pH increase on heavy metal availability

315 The soil used in this study was slightly acidic in reaction (pH = 6.26). The pH of the
316 macadamia nutshell biochar was 10.29 (**Table 1**). The pH of the biochar-amended and
317 unamended soils was analysed 7 and 49 days after incubation. The pH value was found to be
318 increased significantly ($p<0.01$) throughout the incubation period as a result of biochar

addition (**Fig. 1**). For example, the pH increased by 0.3 and 0.1 units after 7 days of incubation in soils spiked with Cd and Pb, respectively, while it increased by 0.3 units in soils spiked with both metals. The soil pH did not show any significant drop at the end of 49 days of experimental period. Acidic soils lead to a higher metal biotoxicity risk and subsequent carbon depletion than alkaline soils (Bolan et al., 2014; Sheng et al., 2016; Dai et al., 2017). Soil pH is also critical in determining the various forms of Cd and their toxicities to certain microorganisms (Bolan et al., 2014). The naturally-released Pb in mining deposits are less mobile, but they may become more soluble and mobile if soils are moderately acidic (John and Leventhal, 1995). The increase in pH values in this study, although small but potentially significant, was brought about by biochar addition, and we speculate this may reduce the mobility and availability of metals to soil microorganisms (Rees et al., 2014). Liang et al. (2014) also noticed a rise in soil pH value due to biochar addition, and they suggested that the pH variation could cause a shift in the soil microbial population, such as bacteria and fungi. The alkaline feature of formed metal oxides, hydroxides and carbonates admixed with the biochar during the pyrolysis process might have increased the soil pH (Novak et al., 2009).

3.1.2. Heavy metal immobilisation by biochar

Bioavailability is critical in the determination of accessibility and toxicity of metals to soil microorganisms (Wang et al., 2007). In this study, both Cd and Pb bioavailabilities were significantly ($p<0.01$) reduced due to the biochar amendment, but not in the glucose-amended soils (**Fig. 2a and b)**. Metal remediation ability of biochar based on the elevated soil pH theory may be envisaged through the following two mechanisms: (i) elevated pH may contribute to metal (co-)precipitation with carbonates, and (ii) it may increase the net negative charges that favour the formation of metal-organic complexes. In the current study, the elevation of soil pH due to biochar addition was up to 0.3 units, which might have

344 imparted only a small effect on metal immobilisation. The SEM images and elemental
345 analysis clearly showed that heavy metal ions clustered or spread on the surface and pores of
346 the biochar (**SI. 3a, b and c**). Additionally, a highly porous structure of the biochar sample
347 was observed as a result of the pyrolysis process. The porous structure of biochar could
348 reduce the metal mobility and bioavailability (Puga et al., 2015). However, Han et al. (2013)
349 pointed out that the metal adsorption was not solely ascribed to biochar pore structure. The
350 adsorption of metal ions by biochar through its surface hydroxyl, carboxyl, and phenolic
351 functional groups (−OH, −COOH or C−OH) (**SI. 4**) might have imparted a more prominent
352 effect. The FTIR spectra show a variety of oxygen-containing functional groups which were
353 negatively charged. Strong bands at 1400 cm^{-1} and 875 cm^{-1} presented C=O and aromatic
354 C=C groups, respectively (Wang and Griffiths, 1985; Abdel-Fattah et al., 2015). Although
355 the functional groups composition could be affected by the parent feedstock and pyrolysis
356 temperature during biochar production (Hossain et al., 2011), the spectral features correlated
357 well with the elemental analysis of the material (**SI. 3c**), showing a relatively high carbon
358 content originating from the organic parent material (macadamia nutshell) (Chia et al., 2012).
359 The aging effect of metal immobilisation was also observed in the present study. In
360 comparison to the initial 7 days of incubation, both bioavailable Cd and Pb concentrations
361 were decreased at the end of incubation (49 days) (**Fig. 2a and b**). At that stage,
362 bioavailabilities of Cd were 1.83 and 2.55 mg kg^{-1} dry soil in glucose-amended Cd-spiked
363 soil (CG) and glucose-amended Cd-Pb-spiked soil (CPG), respectively, while these values
364 were significantly ($p<0.01$) lower in respective biochar-amended soils (1.40 and 2.08 mg kg^{-1}
365 dry soil in biochar-amended Cd-spiked soil (CB) and biochar-amended Cd-Pb-spiked soil
366 (CPB), respectively). The bioavailable concentrations of Pb were 83.05 and 97.86 mg kg^{-1}
367 dry soil in glucose-amended Pb-spiked (PG) and glucose-amended Cd-Pb-spiked soil (CPG),
368 respectively, while these values were reduced to 32.46 and 37.78 mg kg^{-1} dry soil in the

respective biochar-amended soils (PB and CPB). The bioavailable metal concentrations were decreased remarkably, indicating that biochar application reduced the metal mobility. Investigations have shown that the specific morphology and chemical features may support the metal sorption potential of biochar (Igalavithana et al., 2017). In addition, a discrepancy was observed in Cd and Pb bioavailability decline patterns due to biochar application. Bioavailability of Cd was decreased by 49% and 59% in CB and CPB, respectively (**Fig. 2a**), while that of Pb was decreased by 23% and 13% in PB and CPB, respectively (**Fig. 2b**). The decrease of Cd bioavailability was larger than Pb, which interestingly was consistent with the slightly greater pH rise in Cd-spiked soil than the Pb-spiked soil. The rapid adsorption of metals to biochar functional groups during the incubation period might have attributed to their decreased mobilities in biochar-amended soils (Houben et al., 2013). The incubation duration (ageing) was essential for the formation of effective adsorption bonds between biochar surfaces and metal ions. In addition, soil type and clay content could often play an important role in metal immobilisation by biochar. For example, Shen et al. (2016) suggested that the biochar-amended clayey soils was not satisfactory for adsorption of Pb. Therefore, the effect might become more prominent in a light-textured soil as used in the present study. The specific surface area of the biochar sample was 202.49 m^2 g^{-1} (**Table 1**). This feature of biochar along with its highly porous structure (**SI. 3a and b**) supported the existence of large quantity of organic functional groups on the surface (**SI. 3c and SI. 4**), and consequently their electrostatic as well as specific interactions with metal cations (**SI. 4**). The metal adsorption ability can vary depending upon the properties of biochar as affected by the pyrolysis conditions and feedstock sources (Park et al., 2011; Uchimiya et al., 2011). Results of the current study also showed that the bioavailability of metals were slightly higher ($p>0.05$) when Cd and Pb coexisted in the system than the single metal-spiked soil. The bioavailability of Cd was 1.40 mg kg^{-1} dry soil in CB against 2.08 mg kg^{-1} dry soil in CPB

(0.68 mg kg^{-1} difference), while the bioavailability of Pb was 32.46 mg kg^{-1} dry soil in PB and 37.78 mg kg^{-1} dry soil in CPB (5.32 mg kg^{-1} difference). This might be due to the competition among metal cations for the adsorption sites on biochar surfaces. Moreover, Rees et al. (2014) demonstrated that the metal adsorption to organic materials may be partially irreversible with multiple and element-dependent mechanisms, which could imply that biochar might play a more prominent role in a long-term soil remediation approach.

3.2. Influence of biochar on soil microbiota under metal stress

3.2.1. Microbial activity

In all cases, the microbial activity was gradually decreased after the peak value on day 1 (**Fig. 3a**). Despite the patterns of respiration were similar irrespective of the treatments, the cumulative respiration dropped significantly ($p<0.01$) in metal spiked soils in comparison to un-spiked soils (**Fig. 3b**). This demonstrated that the metal toxicity caused a reduction of the soil microbial activity. Compared to the control soil, microbial respiration rate was significantly ($p<0.01$) stimulated immediately after biochar addition (**Fig. 3a**). The respiration rates in uncontaminated soils with biochar amendment were 1.78 µg CO_2-C g^{-1} dry soil h^{-1} on day 1, and 1.11 µg CO_2-C g^{-1} dry soil h^{-1} on day 3. These values were greater than the control soil (uncontaminated and without biochar) on the respective days (1.27 µg CO_2-C g^{-1} dry soil h^{-1} on day 1, and 0.65 µg CO_2-C g^{-1} dry soil h^{-1} on day 3). Afterwards, the respiration rate decreased likely because of the depletion of readily available organic carbon supply. The respiration rate in the control soil was slightly higher than biochar-amended soils on day 25 ($p>0.05$), and this trend continued until the end of incubation. The cumulative microbially respired CO_2-C values at the end of incubation were 351.41 and 379.56 µg CO_2-C g^{-1} dry soil in the control and biochar-amended soils, respectively (**Fig. 3b**). The difference in cumulative CO_2-C release between them (28.15 µg CO_2-C g^{-1} dry soil)

419 is consistent with most of the previous reports that biochar addition could increase the
420 microbial activity and CO_2-C liberation from soils (Jones et al., 2011). The stimulation of soil
421 microbial activity resulted from biochar in uncontaminated soils could be attributed to the
422 higher organic carbon content and supplement of base nutrient elements (primarily Ca, Mg, K
423 and Na) (Novak et al., 2009; Houben et al., 2013).
424 The least microbial cumulative respiration was observed in Cd and Pb co-contaminated soils
425 (**Fig. 3b**). Compared to either Cd or Pb spiked soils, the cumulative microbial respiration was
426 reduced significantly ($p<0.01$) in the co-contaminated soils, but the effect did not differ
427 significantly ($p>0.05$) between Cd and Pb. Nwuche and Ugoji (2008) also noticed that the
428 combination of Zn and Cu amplified the negative influence on soil microbial activity. As
429 expected, biochar addition was found beneficial to improve the microbial activity. Soil
430 respiration was increased by 26% (from 152.21 to 204.58 µg CO_2-C g^{-1} dry soil) due to
431 biochar application compared to un-spiked control soil. The CO_2-C amount due to biochar
432 addition increased by 21% and 23% in Cd and Pb spiked soils, respectively (18.66 and 33.65
433 µg CO_2-C g^{-1} dry soil with glucose and biochar amendment, respectively). There were
434 similar patterns of microbial activities in the Cd and Pb singly spiked soils, meaning the
435 different metal types did not significantly affect microbial respiration rate in this study. The
436 respiration values were slightly higher in Pb-spiked soils than Cd-spiked soils, but not
437 significantly ($p>0.05$). Some previous reports, however, indicated that the level of biotoxicity
438 of Cd was larger than that of Pb to soil microorganisms at an equal molar concentration
439 because Cd was more bioaccessible than Pb owing to dissimilar solubilities of the respective
440 metal salts (Neethu et al., 2015). Microbially respired CO_2-C values in the contaminated soils
441 were increased by 8 and 10% in glucose and biochar treatments, respectively. It was
442 noteworthy that the biochar-amended contaminated soils respired a higher amount of CO_2-C
443 than the glucose-amended contaminated soils despite the fact that both the treatment groups

444 received an equal amount of carbon at the beginning of the experiment and carbon in glucose
445 was more easily mineralisable than that in biochar. This again confirmed that biochar
446 imparted a metal remediation effect on microorganisms in the contaminated soils. This study
447 thus demonstrated that the improvement of microbial activity was not only due to the organic
448 carbon supplied by biochar, but also due to its metal remediation ability.
449
450 3.2.2. Microbial biomass carbon
451 Compared to the control sample, MBC values were significantly ($p<0.01$) increased in
452 biochar-amended uncontaminated soils (**Fig. 4**). The values were 243.86 and 421.77 mg C
453 kg^{-1} dry soil in the control and biochar-amended soils, respectively, indicating a 42%
454 increase. The MBC values in the metal-spiked soils were significantly ($p<0.01$) lower than
455 the control soil due to the possible metal toxicity. A reduced MBC value due to heavy metal
456 toxicity of soils was also observed in numerous previous studies (Abaye et al., 2005; Li et al.,
457 2008).
458 The MBC values were 124.60, 101.55 and 68.02 mg C kg^{-1} dry soil in glucose amended Cd,
459 Pb and Cd + Pb spiked soils, respectively. These values in biochar-amended contaminated
460 soils were significantly ($p<0.01$) increased (37, 50 and 56% in Cd, Pb and Cd + Pb spiked
461 soils), demonstrating that the metal toxicity inhibited the microbial respiration as well as
462 MBC formation. However, the MBC value did not show any significant ($p>0.05$) difference
463 between the metal types. Nwuche and Ugoji (2008) noticed that the combination of Cu and
464 Zn pollution had a lower MBC content than the individual metal. However, the current study
465 did not indicate any significant difference in MBC due to the metal types, or in single or
466 metal co-contaminated situations.
467
468 3.2.3. Microbial community composition

469 Total microbial PLFA was decreased due to metal biotoxicity, while it was increased with
470 biochar application (**Table 3**). The toxicity of heavy metals had significant ($p<0.01$) negative
471 influence on the PLFA abundance. Total PLFA contents were decreased by 27%, 21% and
472 34% in unamended Cd, Pb and Cd + Pb spiked soils, respectively. Similar results were
473 reported earlier (Oliveira and Pampulha, 2006). Total PLFAs increased from 101.75 nmol g^{-1}
474 dry soil in the control soil to 122.22 nmol g^{-1} dry soil with uncontaminated biochar-amended
475 soil. The increased PLFA concentration due to biochar amendments could be attributed to the
476 increased carbon and nutrient availabilities as well as the alleviation of metal toxicity. The
477 highly porous structure of biochar could also provide a congenial habitat niche for soil
478 microorganisms (Quilliam et al. 2013; Dai et al., 2017).
479 The microbial community composition varied among the treatments (**Fig. 5**). A detailed
480 microbial marker concentration presented as individual PLFA data was presented in **SI. 5**.
481 The bacterial:fungal (B/F) ratio in uncontaminated soil was 1.28 without biochar addition,
482 and 1.53 with biochar addition (**Table 3**). Compared to the uncontaminated soil amended
483 with biochar, bacterial:fungal ratio was significantly higher ($p<0.01$) in metal-contaminated
484 soils, indicating that heavy metal toxicity could result in the variation of different microbial
485 species. Generally, fungi are more sensitive to environmental stress than bacteria (Lu et al.,
486 2015). A more distinct drop of fungi abundance compared to bacteria was also observed in
487 the current study. The uncontaminated soil applied with biochar had the largest fungi (45.33
488 nmol g^{-1} dry soil) and Gram-positive bacteria (51.21 nmol g^{-1} dry soil) abundances.
489 Therefore, fungal species were favoured by organic amendments such as biochar. The high
490 carbon and nutrient contents of biochar could support the fungal species that usually have a
491 relatively lower C:N ratio in their cell composition. Surprisingly, the largest Gram-negative
492 bacterial abundance (27.73 nmol g^{-1} dry soil) was shown in Pb-spiked soil without biochar

amendment. Certain Gram-negative bacterial species show resilience to metal stresses, and even exhibit capability of metal bioremediation in contaminated sites (Kang et al., 2016). Sheng et al. (2016) suggested that the ratio of Gram-positive and Gram-negative bacteria (G+/G-) is a promising indicator for predicting carbon sequestration in soils. A higher G+/G- ratio may lead to a positive soil carbon depletion (Sheng et al., 2016). In the current study, Gram-positive bacteria showed a higher tolerance to metal pollution than Gram-negative bacteria and fungi. An increased Gram-positive bacteria population with a decreased fungi population was also earlier reported with increasing metal concentrations (Aoyama and Tanaka, 2013). In addition, the G+/G- ratio was increased by biochar addition, meaning that biochar would favour the Gram-positive bacteria more than the Gram-negative bacteria to grow in a heavy metal contaminated soil. The fungal abundance was increased significantly ($p<0.01$) because of biochar addition, while it was negatively affected by metal toxicity. In spite of the fact that an elevated soil pH should support the bacterial population more than fungi, the carbon and nutrients supplied by biochar might favour fungi to grow better than bacteria (Liang et al., 2014). Biochar was reported to alter soil microbial community composition, and a fungi dominated soil might lead to a higher resistance and resilience when facing environmental stresses (Paz-Ferreiro et al., 2015). In this study, however, compared to the bacterial groups, metal toxicity induced much severe inhibition of the fungal populations. Such difference in microbial species was in consistence with earlier reports (Hinojosa et al., 2005; Deng et al., 2015). Biochar in the present study had more prominent positive effect on fungi than bacteria. Chen et al. (2013) also noticed a microbial community composition shift after biochar addition to soils, and fungi communities were benefited more than bacteria. There was no consistent pattern of metal types that influenced the soil microbiota, but a slightly lower population of G+, G- bacteria, fungi and actinomycetes was noticed in Cd-spiked soils than Pb-spiked soils. There were controversial reports on metal toxicity

variations when the metals were present singly or in combination. The competition for adsorption sites by metal cations could modify the respective metal bioavailability to soil microorganisms (Bur et al., 2012).

A change in the microbial PLFA pattern with metal toxicity and biochar-mediated remediation indicated a shift in the microbial community structure. This was shown by the PLFA patterns from different treatments through principal component analysis (PCA, **Fig. 6**). The first axis, which accounted for 80% of the variation in the PLFA data, separated different treatments. In a reasonable agreement with the hypothesis that biochar addition would modulate the soil microbial community under heavy metal stress, the community composition after biochar-mediated remediation showed a certain distance from those without biochar amendment, and the individual components remained close to each other. In addition, uncontaminated soils with biochar addition were separated to the far on the right in the PC analysis, and thus had a different PLFA pattern than the metal-spiked soils. The glucose- and biochar-amended uncontaminated soils were grouped together, indicating that they had similar PLFA patterns. It has been shown recently that the effect of biochar on the soil microbiome is modulated by time and site (Thies et al., 2015). Therefore, further investigations on biochar parameters and monitoring the duration effect are necessary for interpreting the microbial population variation.

3.3. Influence of biochar on soil and microbial carbon

3.3.1. Soil organic carbon and nutrient pool

Soil TOC was measured at the end of 49 days incubation. Results showed that TOC value decreased in the control soil as the microbial mineralisation increased (**Table 4**). With the addition of biochar, soil organic carbon amount increased, contributing to heavy metal immobilisation. The formation of metal-organic complexes on biochar surfaces could

543 contribute to the increased metal retention in biochar-amended soils (Bolan et al., 2014).
544 Biochar addition significantly ($p<0.05$) increased the soil carbon stock by 7% compared to
545 unamended control soil. However, SOC content was also high in glucose-amended soils even
546 under metal contamination. Because of the metal induced inhibition of microbial activity,
547 TOC content was slightly higher in the contaminated soils than uncontaminated soils.
548 Although exactly similar quantity of carbon was added to soils in the form of glucose and
549 biochar, SOC patterns in those soils after incubation were largely varied. With glucose
550 addition, TOC was increased by 6, 6 and 14% in CG, PG and CPG, while with biochar
551 addition it was increased by 17, 15 and 21% in CB, PB and CPB. This could be attributed to
552 the dominance of microbially resistant OC in biochar as well as the metal remediation
553 capability of the material following its application to soils.
554 Total nitrogen (TN) was the lowest in singly metal-spiked biochar amended soils, indicating
555 an acceleration of native N depletion. New organic carbon addition might have caused soil
556 microbial populations to deplete the native N, which is also known as the 'mining theory'
557 (Tian et al., 2016). As a consequence, the C:N ratio was significantly ($p<0.05$) higher in
558 singly metal-spiked biochar amended soils than unamended soils (**Table 4**). There was also a
559 discrepancy in C:N ratios among glucose and biochar treatments, ranging from 25.8 to 29.1
560 in glucose treatments, while 30.67 to 31.03 in biochar treatments.
561
562 3.3.2. Microbial carbon use efficiency in soil
563 Microbial CUE represents the ratio of carbon assimilated in microbial biomass over uptake,
564 which is an indicator of net carbon sequestration by soil microorganisms. In this study, both
565 microbial respiration and biomass carbon were significantly ($p<0.01$) reduced in heavy metal
566 contaminated soils. The microbial CUE was also reduced in a similar manner (**Table 5**).
567 Microbial CUE values in metal contaminated soils were 0.35, 0.29 and 0.31 in Cd, Pb and Cd

568 + Pb spiked soils, respectively, while it was 0.41 in uncontaminated soils. The inhibition of
569 microbial activity and proliferation due to metal biotoxicity was reported in many studies
570 (Liao et al., 2005; Sobolev and Begonia, 2008). Biochar addition however was able to
571 increase both microbial respiration and biomass carbon in soils even under heavy metal
572 stress. Due to biochar application, microbial CUE was increased by 0.05, 0.09 and 0.12 units
573 in Cd, Pb and Cd + Pb spiked soils, respectively. This indicated that a higher portion of
574 assimilated carbon was incorporated into the microorganisms rather than it was released as
575 CO_2 (Lehmann et al., 2011; Chen et al., 2017).
576 In spite of the same carbon amount added to soil with biochar and glucose, CUE ratios of
577 biochar:glucose in Cd, Pb and Cd-Pb-spiked soils were all larger than 1 (1.15, 1.32 and 1.40,
578 respectively) (**Table 5**). Compared to labile carbon source, such as glucose, a higher carbon
579 sequestration by microbiota was noticed in biochar-amended soils. Unlike biochar, glucose
580 induced a larger microbial respiration, but smaller carbon sequestration. The CUE ratios in
581 biochar-amended contaminated and uncontaminated soils were all less than 1. It indicated
582 that more CO_2-C was released in the metal contaminated soils than the healthy soils by
583 producing a similar amount of biomasses. The metal toxicity led to less carbon use efficiency
584 by microorganisms, and consequently less carbon sequestration ability in polluted soils.
585 Microbial CUE needs to take microbial community composition into account because the
586 differentiation of microbial species may contribute to MBC or CO_2 release, and also may
587 slow down the population turnover rates of fungi (Six et al., 2006). The alteration of
588 microbial community structure could modify the carbon dynamics, and consequently might
589 lead to either depletion or sequestration of terrestrial carbon (Malcolm et al., 2009; Compant
590 et al., 2010). In this study, the heavy metal toxicity had a more negative effect on fungi than
591 bacteria, and bacteria tended to release more CO_2 to form the same amount of biomass. Due
592 to biochar application, the abundance of fungal species was increased by 2, 60, 62 and 67%

593 in uncontaminated, Cd-spiked, Pb-spiked and Cd-Pb-spiked soils, respectively (**Fig. 5**). The
594 assimilated carbon was likely incorporated into microbes and their secondary metabolites
595 instead of being released as CO_2, and consequently contributing to increased CUE.
596 Modulation with biochar thus reduced the metal biotoxicity and altered the microbial
597 community composition, and consequently improved the microbial CUE. The microbial
598 community shift might have occurred as the results of biochar modulation (Cross and Sohi,
599 2011).
600

601 **4. Conclusions**

602 The present study demonstrated that biochar contributed to soil pH increase, metal
603 bioavailability reduction, and consequently heavy metal immobilisation. The SEM images,
604 EDX elemental analysis and IR spectra suggested binding of metals by biochar and thereby
605 potentially reducing their mobility in soils. However, there is a need to examine the long term
606 stability of metal immobilisation in soils through biochar application and the underlying
607 chemical interactions. This study also provided evidence that biochar improved the microbial
608 CUE by modulating heavy metal stresses in contaminated soils. Biochar application increased
609 the microbial activity, microbial biomass, and benefitted certain microbial populations, such
610 as Gram-positive bacteria and fungi, which were otherwise repressed under heavy metal
611 stresses. Microbial community populations were also shifted in response to metal stresses and
612 biochar modulation. Biotoxicity from heavy metals affected the soil carbon metabolism by
613 inhibiting the microbial activity. Biochar amendment increased both microbial respiration
614 and biomass, but most importantly it imparted positive influences on microbial CUE, thereby
615 improving microbial carbon assimilation rate. However, the biochar-modulated carbon
616 sequestration in metal contaminated soils might lead to a native N mining phenomenon.

Future research is needed to investigate the long-term shift of microbial populations under similar scenarios by monitoring the microorganisms' carbon source preferences.

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List of figures

Fig. 1. Soil pH responses to biochar and glucose amendments in different types of metal contaminated soils; (a) soil with Cd, (b) soil with Pb, (c) soil with combined Cd and Pb, (d) soil without any heavy metal. Closed symbols indicate treatments with biochar application, open symbols indicate treatments without biochar. D1, D7 and D49 indicate data on day 1, day 7 and day 49 of the incubation, respectively. Values show means ± SE. * indicates significant difference between glucose and biochar amendments ($p<0.01$).

Fig. 2. Bioavailable Cd (a) and Pb (b) concentrations in different treatments. Data are displayed as means, bars indicate SE (n=3). CB: soil applied with Cd + biochar; CG: soil applied with Cd + glucose; PB: soil applied with Pb + biochar; PG: soil applied with Pb + glucose; CPB: soil applied with Cd + Pb + biochar; CPG: soil applied with Cd + Pb + glucose.

Fig. 3. Microbial respiration rate (a) and cumulative CO_2-C respired (b) in different treatment soils. Data are displayed as means, bars indicate SE (n=3), * indicates significant difference between glucose and biochar amendments ($p<0.01$). S: control soil without any amendment; B: soil applied with biochar; CB: soil applied with Cd + biochar; CG: soil applied with Cd + glucose; PB: soil applied with Pb + biochar; PG: soil applied with Pb + glucose; CPB: soil applied with Cd + Pb + biochar; CPG: soil applied with Cd + Pb + glucose.

Fig. 4. Microbial biomass carbon in different treatment soils. Data are displayed as means, bars indicate SE (n=3). S: control soil without any amendment; B: soil applied with biochar; CB: soil applied with Cd + biochar; CG: soil applied with Cd + glucose; PB: soil applied with Pb + biochar; PG: soil applied with Pb + glucose; CPB: soil applied with Cd + Pb + biochar; CPG: soil applied with Cd + Pb + glucose.

Fig. 5. Proportion of fatty acids representing five microbial species (%). G+: Gram-positive bacteria; G-: Gram-negative bacteria; F: fungi; A: actinomycetes; S: control soil without any

amendment; B: soil applied with biochar; CB: soil applied with Cd + biochar; CG: soil applied with Cd + glucose; PB: soil applied with Pb + biochar; PG: soil applied with Pb + glucose; CPB: soil applied with Cd + Pb + biochar; CPG: soil applied with Cd + Pb + glucose.

Fig. 6. Score plot of principal component analysis (PCA) showing treatment variation based on phospholipid fatty acid (PLFA) patterns. S: control soil without any amendment; B: soil applied with biochar; CB: soil applied with Cd + biochar; CG: soil applied with Cd + glucose; PB: soil applied with Pb + biochar; PG: soil applied with Pb + glucose; CPB: soil applied with Cd + Pb + biochar; CPG: soil applied with Cd + Pb + glucose.

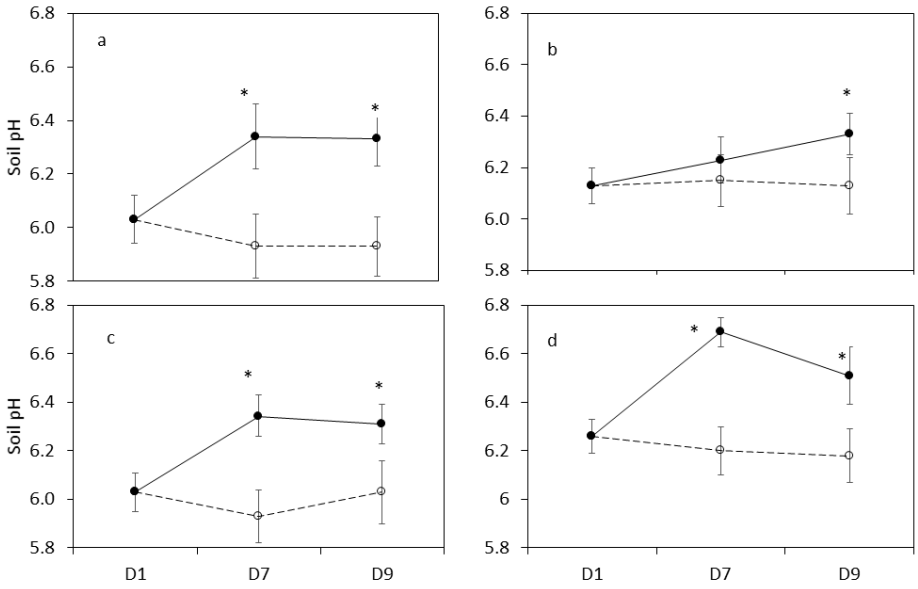

Fig. 1.

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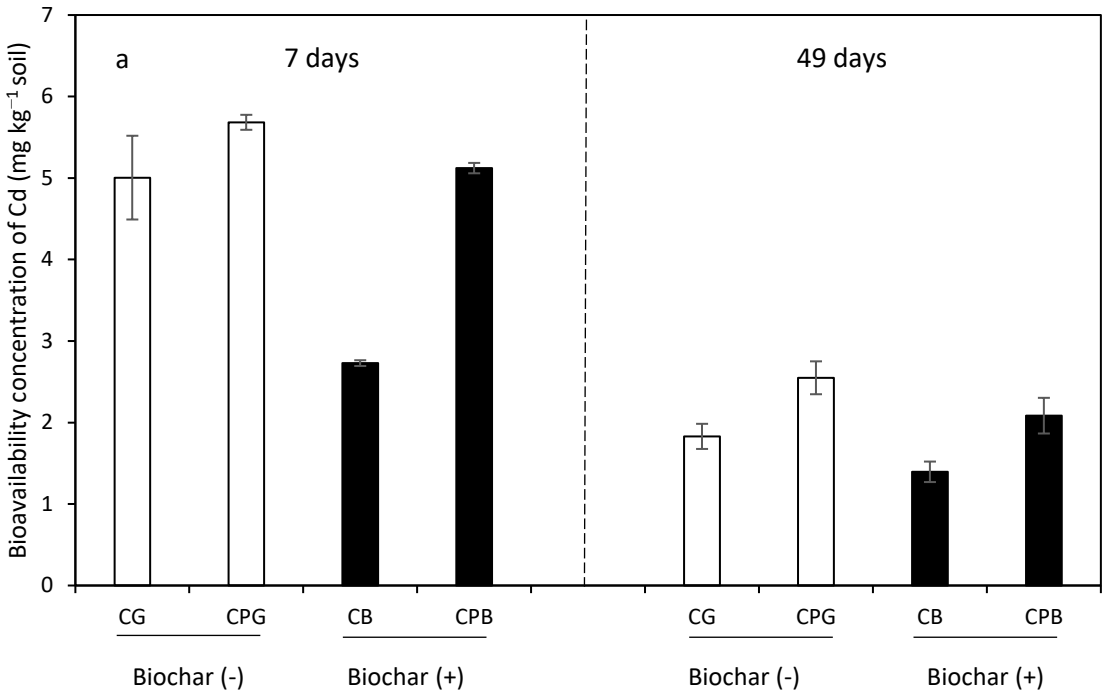

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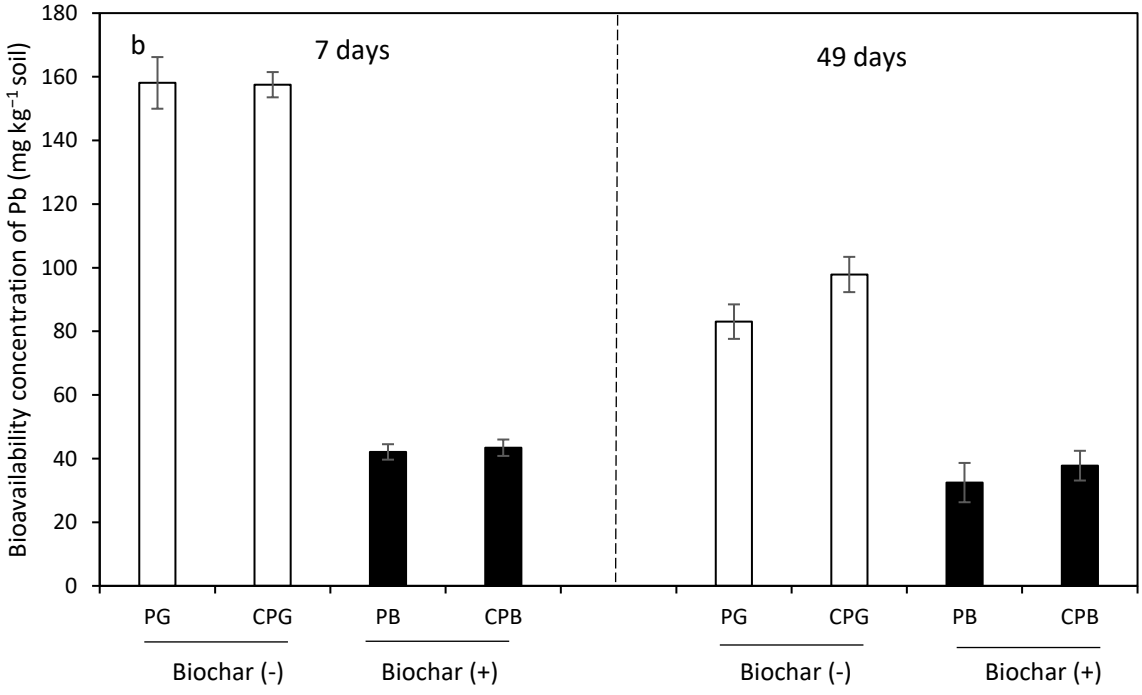

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882 **Fig. 2.**

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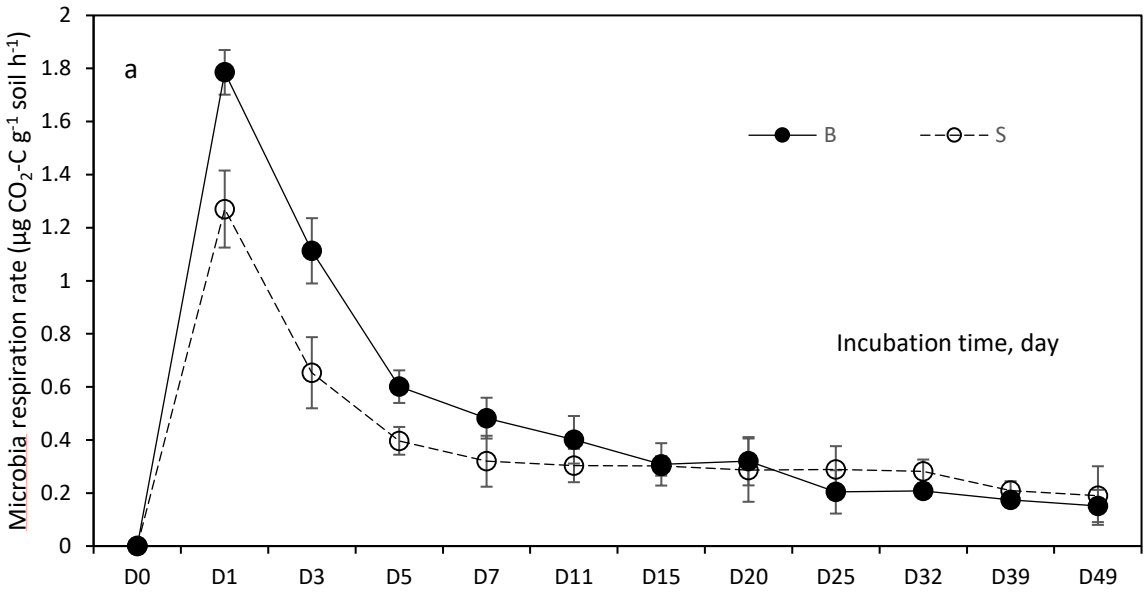

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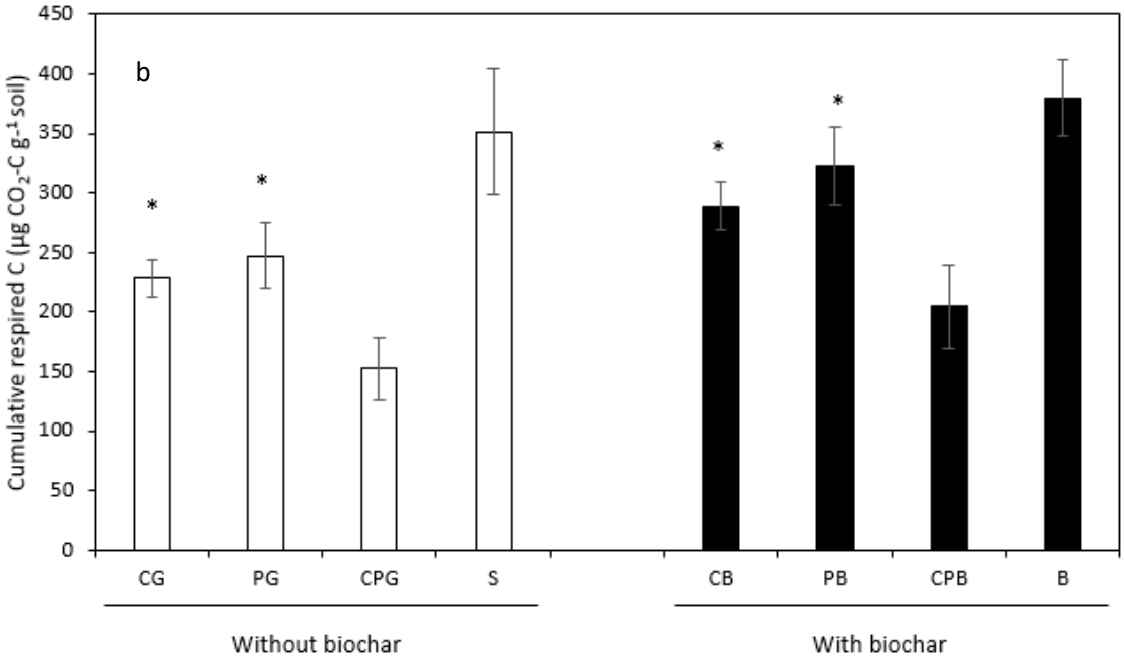

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887 **Fig. 3.**

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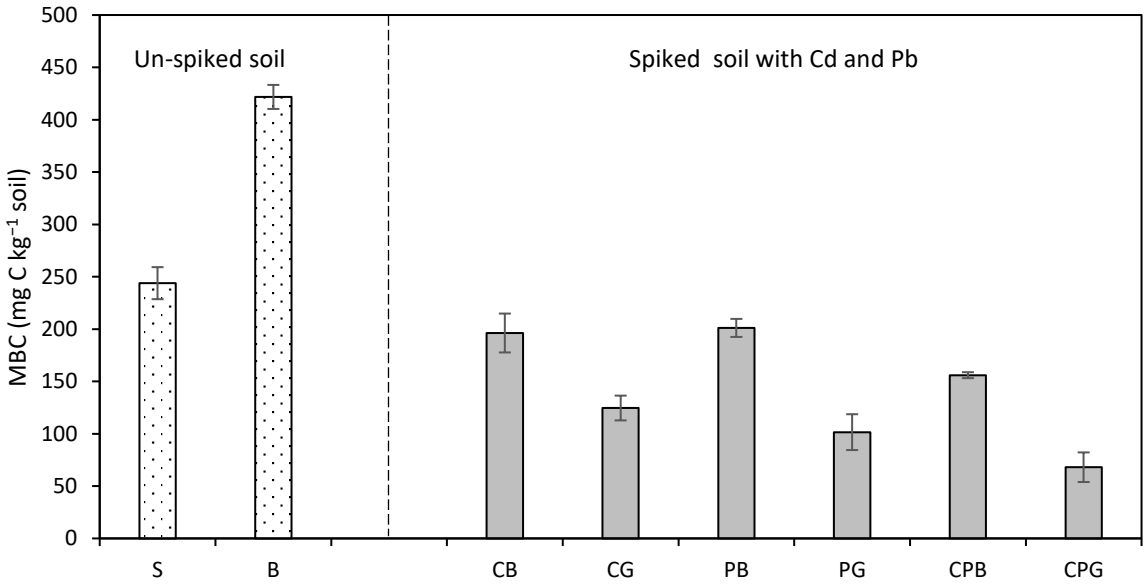
Un-spiked soil
Spiked soil with Cd and Pb
MBC (mg C kg^{-1} soil)
500
450
400
350
300
250
200
150
100
50
0
S
B
CB
CG
PB
PG
CPB
CPG

Fig. 4.

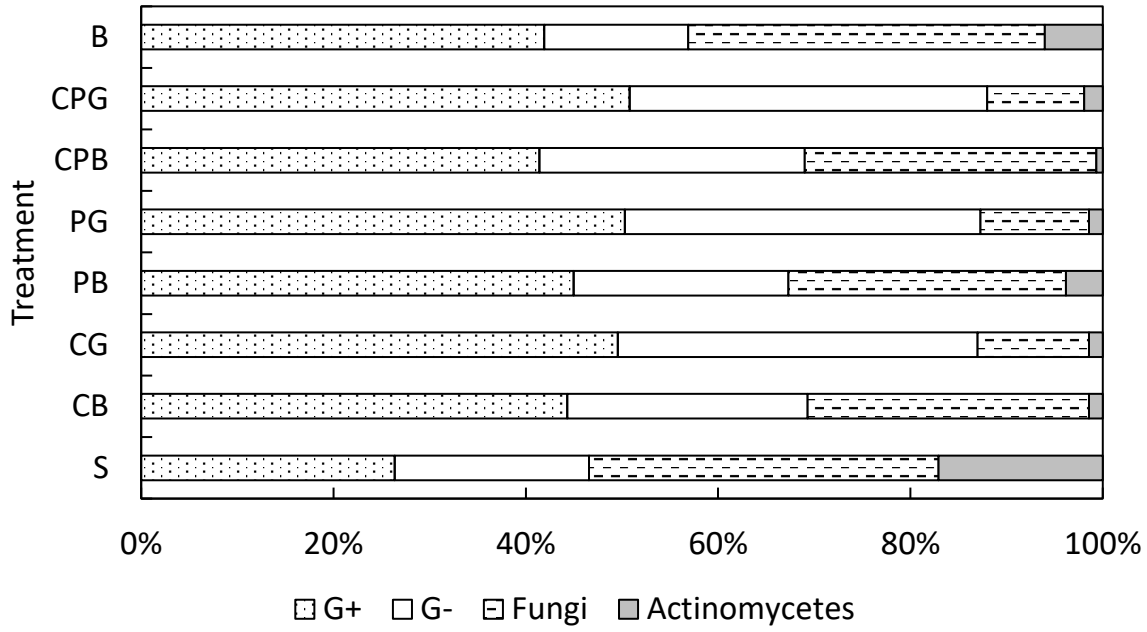
Treatment
B
CPG
CPB
PG
PB
CG
CB
S
0%
20%
40%
60%
80%
100%
G+
G-
Fungi
Actinomycetes

Fig. 5.

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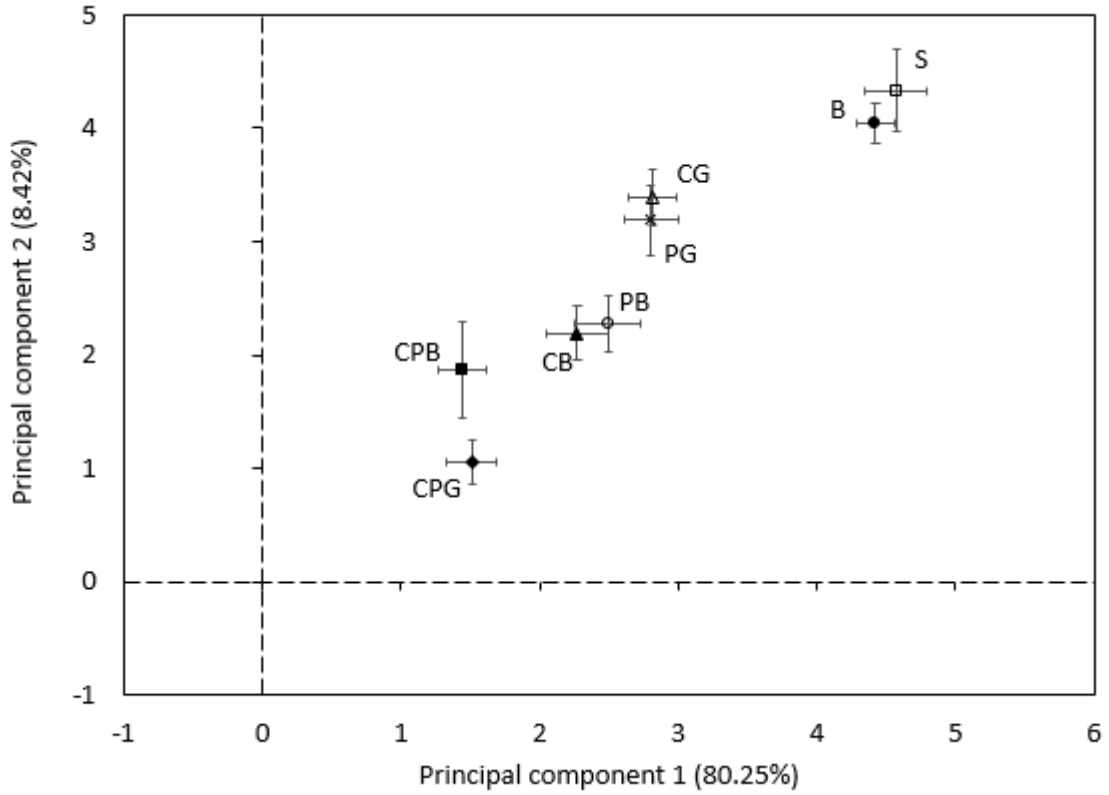

900

901 **Fig. 6.**

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List of tables

Table 1

Selected properties of the soil and macadamia nutshell biochar samples

Soil property	pH	EC(mS cm^{-1})	CEC (cmol (+) kg^{-1})	C (%)	N (%)	C:N	Clay (Wt. %)	Silt (Wt. %)	Sand (Wt. %)	
	6.26	27.53	32.71	2.29	0.14	16.36	25.73	41.56	20.17	
	Ca (g kg^{-1})	Cu (mg kg^{-1})	Fe (g kg^{-1})	K (g kg^{-1})	Mg (g kg^{-1})	Mn (mg kg^{-1})	P (g kg^{-1})	Zn (mg kg^{-1})	Na (g kg^{-1})	S (g kg^{-1})
	1.39	8.90	1.42	0.59	0.46	28.50	0.13	8.19	0.23	0.37

Biochar	pH	EC (mS cm^{-1})	Pyrolysis temperature (^{0}C)	C (%)	N (%)	P (%)	K (%)	S (%)	C:N	DOC (g Kg^{-1})	Specific surface area (m^2 g^{-1})	Pore volume (ml g^{-1})
	10.29	0.17	465	74.72	0.66	0.09	1.02	0.05	113.21	0.55	202.49	0.0085

Table 2

Soil spiking rate and final metal concentrations. Mean ± SE, n=3

Sample	Cd (mg kg^{-1} soil)	Pb (mg kg^{-1} soil)	Biochar (%)	Glucose (%)	Cd concertation (mg kg^{-1} soil)	Pb concentration (mg kg^{-1} soil)	Cd recovery rate (%)	Pb recovery rate (%)
Control soil	-	-	-	-	-	-	-	-
Cd + Biochar	50	-	5	-	41.65	-	83.3%±7.5%	-
Cd + Glucose	50	-	-	16	41.65	-	83.3%±7.5%	-
Pb + Biochar	-	5000	5	-	-	4605	-	92.1%±1.8%
Pb + Glucose	-	5000	-	16	-	4605	-	92.1%±1.8%
Cd + Pb + Biochar	50	5000	5	-	43.85	4687.5	87.7%±4.6%	93.75%±2.5%
Cd + Pb +Glucose	50	5000	-	16	43.85	4687.5	87.7%±4.6%	93.75%±2.5%
Soil + Biochar	-	-	5	-	-	-	-	-

940 **Table 3**

941 Comparison of Gram-positive bacteria (G+ bacteria), Gram negative bacteria (G- bacteria), fungi and actinomycetes as obtained through respective

942 PLFA profile (nmol g^{-1} dry soil). Means ± SE (n=3) of total PLFA, PLFA diversity, ratio of Gram-positive and Gram-negative bacteria, ratio of

943 bacteria and fungi. Mean values followed by different letters indicated significant difference ($p<0.05$) among treatments

	Without glucose/biochar	**Glucose applied***			**Biochar applied**			
	Uncontaminated soil	Soil + Cd	Soil + Pb	Soil + Cd + Pb	Uncontaminated soil	Soil + Cd	Soil + Pb	Soil + Cd + Pb
G+ bacteria	26.84±3.68a	35.05±2.21b	37.72±3.19c	32.49±2.26ab	51.21±1.79d	39.72±2.04c	43.28±1.73c	33.24±2.20b
G- bacteria	20.56±1.90ab	26.44±2.09b	27.73±2.09b	23.77±2.10b	18.33±2.06a	22.41±2.25ab	21.52±2.08ab	22.15±2.28ab
Fungi	36.97±1.03c	8.20±1.07a	8.48±0.90a	6.45±1.10a	45.33±0.97d	26.23±1.00b	27.78±1.06b	24.34±0.98a
Actinomycetes	17.38±0.67d	1.01±0.22a	1.07±0.48a	1.24±0.78a	7.35±0.29c	1.29±0.33a	3.69±0.31b	0.54±0.33a
Microbial species feature								
G+/G- ratio	1.31±0.19a	1.33±0.11a	1.43±0.15a	1.37±0.11a	2.79±0.09d	1.77±0.09b	2.01±0.08c	1.50±0.10a
B/F ratio	1.28±0.54a	7.50±0.40b	7.95±0.59bc	8.72±0.40c	1.53±0.40a	2.37±0.47a	2.33±0.36a	2.28±0.48a
Total PLFA (nmol g^{-1} dry soil)	101.75±17.44bc	70.70±11.00ab	77.01±10.96ab	63.95±10.10a	122.22±17.46c	89.64±13.75a	96.28±11.47b	80.27±12.81ab

944

Table 4

Comparison of total organic carbon (TOC), total nitrogen (TN), and ratio of C:N in soils after 49 days incubation. Means ± SE (n=3)

	Without glucose/biochar	**Glucose applied***			**Biochar applied**			
	Uncontaminated soil	Soil + Cd	Soil + Pb	Soil + Cd + Pb	Uncontaminated soil	Soil + Cd	Soil + Pb	Soil + Cd + Pb
TOC (g kg^{-1} soil)	27.68±0.68	29.42±0.98	29.36±1.24	32.12±1.88	33.22±0.92	33.51±0.91	32.71±1.04	34.96±1.12
TN (g kg^{-1} soil)	1.18±0.05	1.14±0.08	1.01±0.04	1.15±0.08	0.91±0.03	1.08±0.06	1.01±0.02	1.14±0.10
C:N	23.53±1.23	25.81±1.75	29.07±1.41	27.93±0.71	36.51±1.39	31.03±1.38	32.39±1.58	30.67±1.24

*Glucose applied at the same carbon loading rate as biochar.

Table 5

Effect of heavy metal toxicity on microbial carbon use efficiency. Means ± SE (n=3) of total PLFA, PLFA diversity, ratio of Gram-positive and Gram-negative bacteria, ratio of bacteria and fungi. Mean values followed by the same letter are not significant among treatments according to ANOVA ($p>0.05$)

	Without glucose/biochar	**Glucose applied***			**Biochar applied**			
	Uncontaminated soil	Soil + Cd	Soil + Pb	Soil + Cd + Pb	Uncontaminated soil	Soil + Cd	Soil + Pb	Soil + Cd + Pb
Microbial CUE	0.41±0.02b	0.35±0.04b	0.29±0.02a	0.31±0.03a	0.53±0.01d	0.40±0.01b	0.38±0.02b	0.43±0.01c
	Biochar applied: Glucose applied				Heavy metal: Biochar			
	CB:CG	PB:PG	CPB:CPG		CB:B	PB:B	CPB:B	
CUE ratio	1.15±0.06	1.32±0.05	1.40±0.01		0.77±0.12	0.73±0.10	0.82±0.04	

Supplementary information for:

Biochar modulates heavy metal toxicity and improves microbial carbon use efficiency in soil

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List of supplementary information

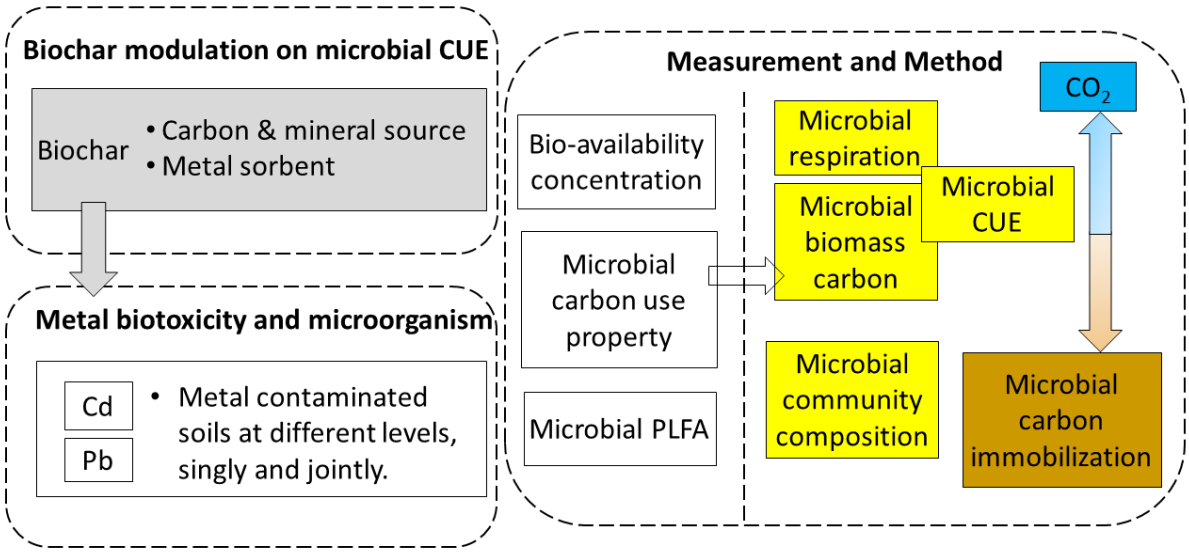
SI. 1. Schematic diagram showing the experiment approach

SI. 2. Phospholipid fatty acid (PLFA) biomarkers used to characterise microbial communities in the experimental soils (Frostegård et al., 1993)

SI. 3. Morphological and surface chemical characteristics of macadamia nutshell biochar, including scanning electron micrographs (SEM) (a, b) and energy dispersive spectrum (c), and presenting the elements composition information of tested biochar area in (b).

SI. 4. Fourier transformed infrared (FTIR) spectrum showed the functional group of the macadamia nutshell biochar used in this research.

SI. 5. Detected microbial PLFA data under different treatments after 49 days of incubation (nmol g^{-1} soil)



SI. 1. Schematic diagram showing the experiment approach

SI. 2.

Phospholipid fatty acid (PLFA) biomarkers used to characterise microbial communities in the experimental soils

Microbial group	Biomarker PLFAs
Gram-negative bacteria	C16:1ω7c
Gram-positive bacteria	i-C15:0, a-C15:0, C15:0, i-C16:0, iC-17:0, aC-17:0, C:170
Actinobacteria	10MeC16:0, 10MeC17:0, 10MeC18:0
Fungi	C18:2ω6c, C18:1ω9c

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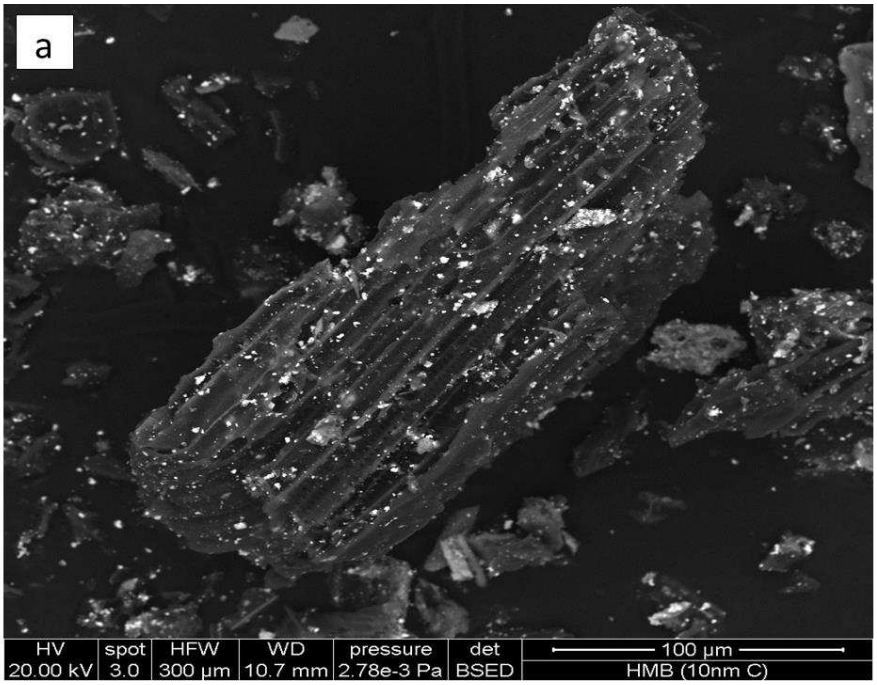
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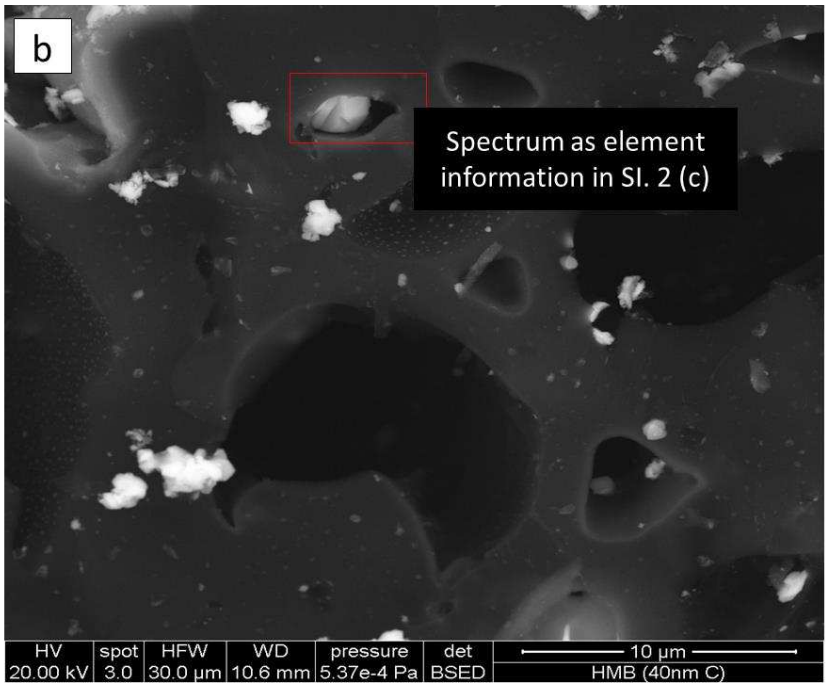
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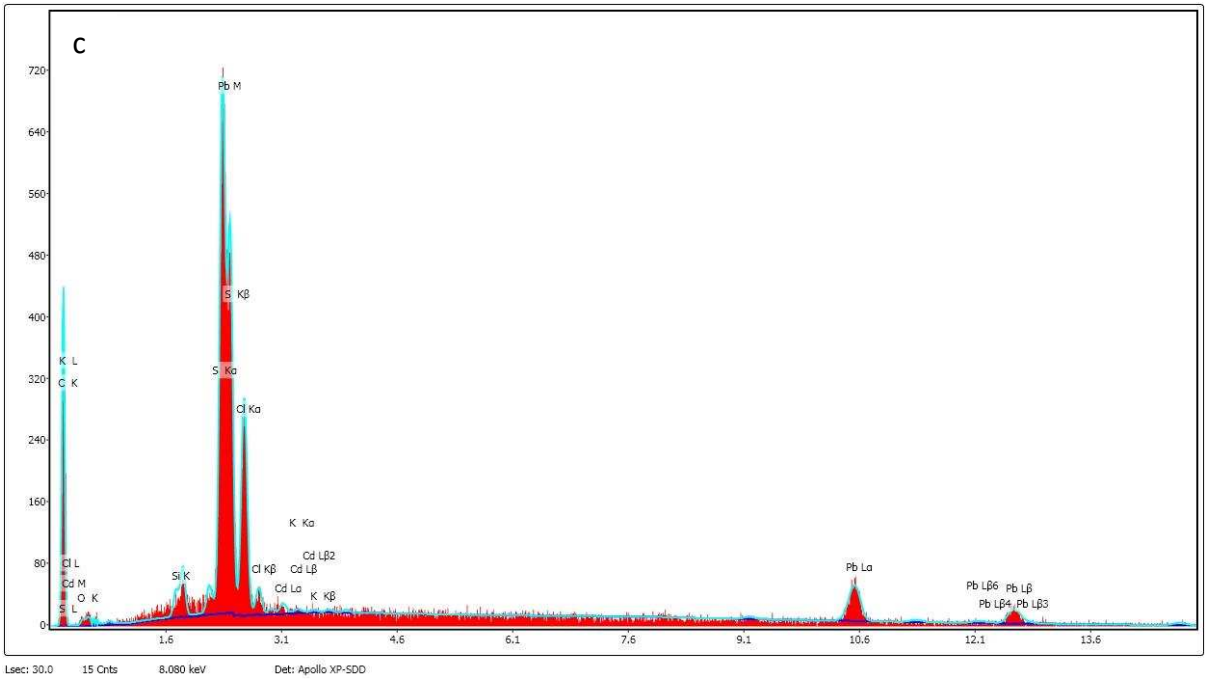

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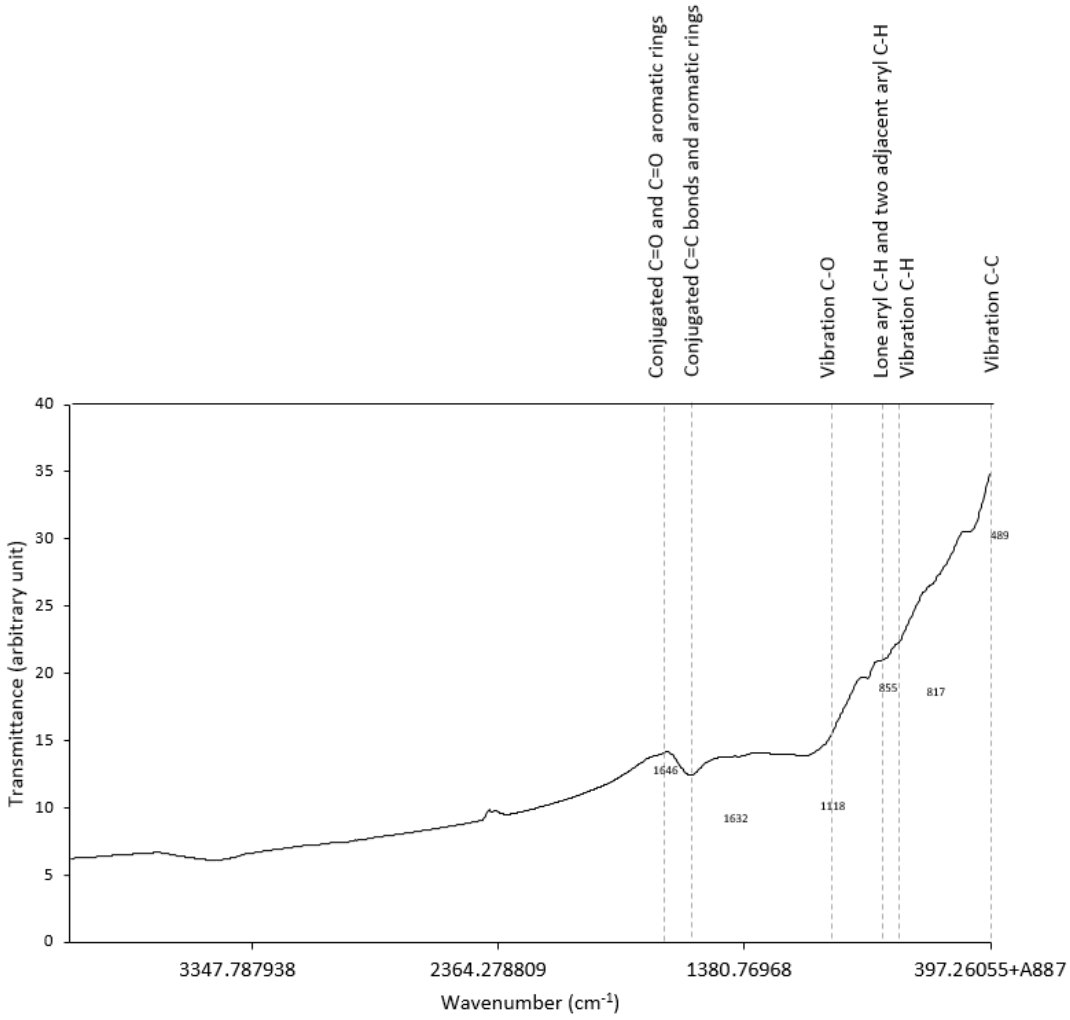

SI. 4. Fourier transformed infrared (FTIR) spectrum showed the functional group of the macadamia nutshell biochar used in this research.

SI. 5.

Detected microbial PLFA data under different treatments after 49 days of incubation (nmol g^{-1} soil)

PLFA	Control	CB	CG	PB	PG	CPB	CPG	B
iC15:0	2.07	3.54	1.07	2.81	2.22	1.95	1.83	4.57
aC15:0	0.93	1.59	0.50	1.81	0.95	1.25	0.83	1.79
C15:0	0.17	0.32	0.25	0.34	0.26	0.26	0.25	0.40
iC16:0	7.42	10.41	3.10	11.06	6.99	9.06	5.15	12.48
iC17:0	12.87	19.02	28.72	22.69	24.17	16.73	22.17	25.18
aC17:0	1.74	2.76	0.77	3.26	1.93	2.81	1.36	3.44
C17:0	0.17	0.28	0.28	0.30	0.28	0.28	0.26	0.39
C16:1ω7c	20.56	22.41	26.44	21.52	27.73	22.15	23.77	18.33
C18:2ω6c	7.59	2.16	1.88	5.28	3.45	3.06	4.06	8.57
C18:1ω9c	29.38	24.07	6.32	22.49	5.03	21.28	2.40	36.76
10MeC16:0	6.02	0.51	0.46	1.44	0.44	0.21	0.56	2.81
10MeC17:0	3.68	0.23	0.21	0.74	0.23	0.11	0.24	1.51
10MeC18:0	7.69	0.54	0.34	1.51	0.40	0.22	0.44	3.04
Total	101.75	89.65	70.70	96.27	75.00	80.27	63.95	122.22