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

3

4 Yilu Xu¹, Balaji Seshadri¹, Binoy Sarkar^{2,3}, Hailong Wang⁴, Cornelia Rumpel⁵, Donald
5 Sparks⁶, Mark Farrell⁷, Tony Hall⁸, Xiaodong Yang^{1,9,10}, and Nanthi Bolan^{1,11,12*}

6

7 ¹Global Center for Environmental Remediation, University of Newcastle, Callaghan, NSW
8 2308, Australia

9 ²Department of Animal and Plant Sciences, The University of Sheffield, Sheffield, S10 2TN,
10 UK

11 ³Future Industries Institute, University of South Australia, Mawson Lakes, SA 5095,
12 Australia

13 ⁴School of Environment and Chemical Engineering, Foshan University, Foshan, Guangdong
14 528000, China

15 ⁵CNRS, Institute of Ecology and Environment Paris, IEES, CNRS-INRA-UPMC-UPEC-
16 IRD, Thiverval-Grignon 78850, France

17 ⁶Department of Plant and Soil Sciences, Delaware Environmental Institute, University of
18 Delaware, Newark 19711, USA

19 ⁷CSIRO Agriculture & Food, Locked Bag 2, Glen Osmond, SA 5064, Australia

20 ⁸Sprigg Geobiology Centre & Department of Earth Sciences, University of Adelaide,
21 Adelaide, SA 5005, Australia

22 ⁹Key Laboratory of Oasis Ecology, Urumqi 830046, China

23 ¹⁰Institute of Resources and Environment Science, Xinjiang University, Urumqi 830046,
24 China

25 ¹¹Cooperative Research Centre for Contamination Assessment and Remediation for the
26 Environment, University of Newcastle, Callahan, NSW 2308, Australia

27 ¹²International Centre for Balanced Land Use, University of Newcastle NSW 2308, Australia

28

29

30 *Corresponding author:

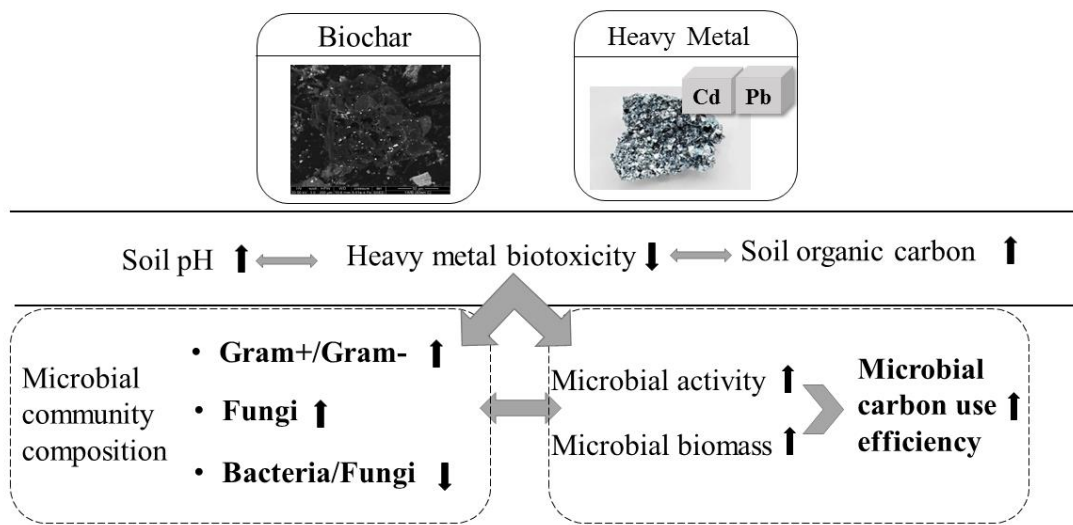
31 Prof Nanthi Bolan

32 University of Newcastle

33 Email: Nanthi.Bolan@newcastle.edu.au

34 Tel: +61 2 49138750

35 **Graphical abstract**



36

37

38 **Highlights**

39

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

45 **Abstract:**

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

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

66

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

69

70 **1. Introduction**

71 Biochar has been acknowledged as an effective material to sequester terrestrial carbon, while
72 at the same time improving microbial habitat in soil (Lehmann et al., 2011; Quilliam et al.,
73 2013). Biochar can play an important role in the biogeochemical cycling of carbon and other
74 elements in soils (Kuzyakov et al., 2009; Bolan et al., 2012). In addition to improving soil
75 fertility and water holding capacity (Paetsch et al., 2017), applications of biochar have
76 attracted a rising attention due to the possibility of the remediation of heavy metal
77 contaminated soils (Rees et al., 2014). Dominance of oxygen-containing functional groups in
78 the highly porous structure of biochar makes the material suitable for the adsorption of a
79 range of contaminants including heavy metals (Bolan et al., 2014; Mandal et al., 2017).
80 Heavy metal(loid)s are among the most toxic and widespread contaminants in our
81 environment because of their persistent nature and high bioaccumulation potential. Some
82 metal elements (e.g., Fe, Zn, Cu, Mn) are involved in many biochemical reactions, but metals
83 like Cd, Pb and Ag have no biological role. They are rather potentially toxic to micro- and
84 macroorganisms (Bruins et al., 2000). The key mechanism of metal toxicity to
85 microorganisms evolves due to the displacement or substitution of essential elements by toxic
86 elements either in the extracellular enzymes or even in nuclear proteins, which consequently
87 may lead to enzyme synthesis inhibition and metabolic process dysfunction (Tchounwou et
88 al., 2012; Baumann et al., 2013). Additionally, when present at a high concentration, even the
89 essential metal elements may lead to adverse consequences (e.g., damage to cell membranes
90 and DNA structure and oxidative stress) (Kachur et al., 1998; Tchounwou et al., 2012).
91 Therefore, toxic levels of heavy metal(loid)s may give rise to the deterioration of soil
92 microbial populations and their metabolic activities through denaturing the protein structure
93 and impairing cell membrane functions (Jiang et al., 2010).

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

104 Biochar is reported to recover microbial activities in metal contaminated soils (Yang et al.,
105 2016). Such improvement in microbial activities could be attributed to: (i) improvement of
106 soil physiochemical properties (increase of soil aeration, moisture content and pH), (ii)
107 immediate supplement of soil carbon pools, especially the recalcitrant pool, (iii) supply of
108 nutrients, and (iv) modification of microbial habitat and ecological niche (Jones et al., 2011).

109 Soil microbiota and their carbon utilisation preferences could be significantly altered by
110 biochar amendments (Farrell et al., 2013; 2015). However, due to the complexity of soil and
111 ecosystem diversity, there is a lack of understanding about biochar modulated microbial
112 responses in metal polluted environments (Pan et al., 2016). Microbial carbon use efficiency
113 (CUE) is defined as the conversion of the organic carbon assimilated into the microbial
114 biomass in the net carbon sequestration process (Rousk and Bååth, 2011). Different
115 approaches of microbial CUE measurement and interpretation of results may involve some
116 discrepant assumptions (Frey et al., 2001), but it can be used as a reference for the microbial
117 carbon utility preference in soils (Sinsabaugh et al., 2013; Blagodatskaya et al., 2014). Some
118 microbial species, especially fungi often positively respond to biochar addition (Warnock et

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

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

137

138 **2. Materials and methods**

139 **2.1. Soil sampling and preparation**

140 A natural surface soil (0-10 cm) sample was collected from the Barossa Valley region, South
141 Australia (138°57'37''E, 34°27'48''S). The region is characterised by Mediterranean
142 climate, with an average summer temperature range of 26-29°C (daytime) and 12-14°C (night
143 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 O₂-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 NH₄⁺, then extracting and analysing the exchanged NH₄⁺ 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 (HNO₃: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

169 (Leco TruMac[®] CNS/NS Analyser, LECO Corporation). Soil samples (0.2 g) were weighed
170 and combusted at 1300°C with an O₂ flow for 5 sec. The instrument was calibrated at every
171 10 samples by analysing standard weights of Leco EDTA reference material (containing 95.7
172 g N kg⁻¹ and 410 g C kg⁻¹). Soil and biochar physico-chemical characteristics are presented
173 in **Table 1**.

174 In addition, the specific surface area and pore size of the biochar sample were measured by
175 conducting N₂ adsorption-desorption experiments by BET method on a NOVA 1000e
176 Analyser (Quantachrome Instruments, USA). Functional groups in the biochar sample were
177 studied through Fourier Transform Infrared (FTIR) spectroscopy on a Cary 660 FTIR
178 Analyser (Agilent Technologies Ltd., USA). Morphological features and pore structures of
179 the biochar sample were examined by a Quanta 450 FEG environmental scanning electron
180 microscope (SEM) (FEI Company, USA). The elemental composition of the biochar was
181 determined by an energy dispersive X-ray (EDX) spectrometer attached with the SEM
182 equipment.

183

184 **2.3. Soil spiking, biochar amendment and incubation experiment**

185 The experiment approach, including experiment design and quantification of microbial
186 response were detailed in **SI. 1**. In the present study, the experimental soil was spiked with 50
187 and 5000 mg kg⁻¹ of Cd²⁺ and Pb²⁺, respectively. These concentrations were chosen to reflect
188 a contamination level above the sensitivity threshold of the respective metals in order to
189 detect responses of microbial carbon use patterns under metal stresses in the soil (Sobolev
190 and Begonia 2008; Smolders et al., 2009). Two concentrations of Cd(NO₃) and Pb(NO₃) were
191 mixed with the soil separately, and also in combination (Table 1). Briefly, metal solutions
192 were sprinkled evenly on the soil spread on a polyethylene sheet. To achieve homogenisation,
193 soils were then stirred and mixed thoroughly on an end-over-end shaker. Soils were then air-

194 dried, and passed through a 2 mm-sieve again. The final concentrations of metals in the
195 spiked soils and abbreviations for each treatment are listed in **Table 2**.
196 Biochar was added at 5% *w/w* into 200 g soils. The 5% addition is equivalent to 12.75 tons
197 ha⁻¹ biochar addition in the field (based on 2.5 cm depth incorporation with a bulk density of
198 around 1020 kg m⁻³). The reduction of available metal concentration due to biochar addition
199 (dilution effect) ranged from 1-10% (Houben et al., 2013). Glucose (100 g L⁻¹ in H₂O) was
200 applied to a separate set of samples to achieve the same total carbon content (3.71%) as of the
201 added biochar. A separate treatment without any amendment was prepared as control. Metal-
202 spiked and biochar/glucose treated soils were transferred into plastic containers, and
203 incubated at 25°C and 28% room humidity for 49 days. The moisture content of the soil was
204 maintained at 60% of the WHC throughout the incubation experiment. All experiments were
205 conducted in triplicate.

206

207 **2.4. Bioavailability of heavy metals**

208 Bioavailable heavy metal concentrations were measured by extracting the biochar/glucose-
209 amended and unamended soils with 0.01 M CaCl₂ solution (1:10 *w/v*) for 60 min reaction
210 time (Sparks et al., 1996). The extracts were filtered through 0.45 µm syringe filter before
211 analysing the metal elements on an ICP-MS instrument (ICP-MS, Agilent 7900, Agilent
212 Technologies Ltd., USA).

213

214 **2.5. Microbial properties**

215 2.5.1. Microbial activity

216 The microbial activity of soils was monitored by measuring the rate of CO₂ evolution from
217 the samples. Sealed soil microcosms (10 g) in Schott bottles having different treatments as
218 stated above were incubated for 49 days in dark at 25°C and 60% WHC. Three blank Schott

219 bottles without any soil were set as controls. A 20 mL open-top vial containing 10 mL of 0.05
220 M NaOH solution was used to trap the evolved CO₂ within the sealed Schott bottles.
221 Periodically, the alkali was decanted into an Erlenmeyer flask and rinsed with deionised
222 water three times. The small vial was replaced with 10 mL of fresh alkali every time. The
223 collected alkaline aliquot was then titrated against 0.03 M HCl in the presence of
224 phenolphthalein indicator following the addition of 5 mL of 0.5 M BaCl₂. The amount of
225 evolved CO₂ was thus measured, and the microbial respiration was calculated using (Eq. 1):

$$226 \quad MR = \{MWCO_2(V_b - V_s) \times M \times 1000\} / (DW \times T \times 2) \quad \text{Eq. 1}$$

227 where, *MR* is the microbial respiration (mg CO₂-C kg⁻¹ soil h⁻¹), *MWCO₂* is the molecular
228 weight of CO₂, *V_b* is the volume of HCl for the blank titration, *V_s* is the volume of HCl for the
229 sample titration, *M* is the concentration of HCl, *DW* is the dry weight of the soil, *T* is the time
230 of incubation, and 2 is the factor that accounts for the fact that two OH⁻ are consumed by one
231 CO₂.

232

233 2.5.2. Microbial biomass carbon

234 Microbial biomass carbon (MBC) was determined using the fumigation-extraction method
235 (Vance et al., 1987). Soils (10 g, dry weight basis) were placed in 50 mL beakers within a
236 vacuum desiccator containing 50 mL of ethanol free chloroform. The desiccator was tightly
237 sealed and pumped until chloroform was vaporised. Soils were thus incubated in chloroform
238 vapour for 48 h within the desiccator. Another set of un-fumigated soils were maintained
239 simultaneously. After 48 h, both the fumigated and un-fumigated soils were mixed with 40
240 mL of 0.5 M K₂SO₄, and shaken on an end-over-end shaker for 1 h. Samples were then
241 centrifuged and filtered through Whatman #40 filter papers. Carbon content in the filtrates
242 was analysed by a Total Organic Carbon (TOC) Analyser (TOC-LCSH, Shimadzu
243 Corporation, Japan). MBC was calculated using Eq. 2:

244 $MBC = E_c/K_c$ Eq. 2

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

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

250 $M_C = \Delta MBC / (\Delta MBC + \Sigma CO_2-C)$ Eq. 3

251 where, *M_C* is microbial CUE measured as microbial biomass variation, Δ MBC is the change
252 of microbial biomass carbon, Σ CO₂-C is cumulative CO₂-C as microbial respiration.

253

254 2.5.3. Microbial community composition

255 Phospholipid fatty acid (PLFA) patterns were used to estimate the relative abundance of
256 bacteria, fungi and actinomycetes in the biochar/glucose-amended and unamended soils.

257 Microbial PLFAs were extracted by standard methods (Frostegård et al., 1993; Bossio et al.,
258 1998).

259 In brief, soil samples were first freeze dried at –45°C and at less than 1 millibar (0.8 mbar)
260 pressure. Then, freeze dried soils (5 g) were extracted with one-phase extraction solvent. The
261 one-phase solvent was a mixture of chloroform, methanol and citrate buffer (1:2:0.8, v/v/v),
262 while the citrate buffer was made of citric acid and sodium citrate (3:1, v/v) with pH adjusted
263 at 3.6 (Bligh and Dyer, 1959). After shaking on an end-over-end shaker, the mixture was
264 centrifuged twice at 4500 rpm for 30 min. The supernatant was decanted into a non-
265 transparent vial, and vortexed before standing overnight. The upper layer of the standing
266 liquid was removed, and the remaining bottom portion was dried under N₂ flow at 32°C.
267 Following drying, the thin solid phase left at the bottom of the vial was re-dissolved in
268 chloroform (1 mL), and transferred into a solid phase extraction (SPE) column. To set up the

269 column, 0.5 g of silica was packed, followed by conditioning with chloroform thrice (1+1+1
270 mL). Then, the sample transfer in the SPE column included three steps: chloroform (2+1+1+1
271 mL), acetone (2+1+1+1 mL) and methanol (2+1+1+1 mL). The final leaching solution was
272 dried with continuous N₂ flow at 32°C. To the pellet obtained, 0.5 mL of 1:1 (v/v) of
273 methanol:toluene and 0.5 ml of 0.2 M methanolic KOH (by dissolving 0.28 g KOH in 25 mL
274 of methanol) were added. The mixture was incubated at 37°C for 30 min, and then cooled to
275 room temperature. The PLFAs were thus converted into fatty acid methyl esters (FAMES)
276 with mild alkaline methanolysis. Following incubation, 1 mL of deionised water, 0.15 mL of
277 1 M acetic acid, and 1 mL of hexane were added to the mixture, vortexed for 30 sec, and
278 centrifuged at 4500 rpm for 30 min to separate the solution into two layers. The upper layer
279 was carefully transferred into a Gas Chromatography (GC) vial with a pipette. This
280 separation procedure was repeated twice with the addition of fresh extractants. Finally, the
281 extract was concentrated by continuous N₂ flow, and stored at -20°C in total darkness before
282 further analysis.

283 An internal standard (methyl nonadecanoate, C19:0) (10 ng) was added to all samples as a
284 quality control measure. The FAMES were analysed by gas chromatography-mass
285 spectrometry (GC-MS) (Model 7890B/5977B, Agilent Technologies Ltd., USA; AxION iQT
286 with Cold EI Source, Perkin Elmer, USA). A RTX-5MS fused silica capillary column (60 m,
287 250 µm × 0.25 µm film thickness) (Supelco, Sigma-Aldrich, Australia) was used. Sample (1
288 µL) was injected in splitless mode with an injector temperature of 250°C, and helium carrier
289 gas at a constant flow rate of 1.4 mL min⁻¹. The temperature program was set as follows:
290 column temperature initially at 60°C for 1 min, then increased to 180°C at a rate of 12°C
291 min⁻¹, then increased to 300°C at a rate of 4°C min⁻¹ and kept at 300°C for 4 min. Electron
292 energy in the detector was set 70 eV. Data was acquired in scan mode from 50 to 400Da at 3
293 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

319 addition (**Fig. 1**). For example, the pH increased by 0.3 and 0.1 units after 7 days of
320 incubation in soils spiked with Cd and Pb, respectively, while it increased by 0.3 units in soils
321 spiked with both metals. The soil pH did not show any significant drop at the end of 49 days
322 of experimental period. Acidic soils lead to a higher metal biotoxicity risk and subsequent
323 carbon depletion than alkaline soils (Bolan et al., 2014; Sheng et al., 2016; Dai et al., 2017).
324 Soil pH is also critical in determining the various forms of Cd and their toxicities to certain
325 microorganisms (Bolan et al., 2014). The naturally-released Pb in mining deposits are less
326 mobile, but they may become more soluble and mobile if soils are moderately acidic (John
327 and Leventhal, 1995). The increase in pH values in this study, although small but potentially
328 significant, was brought about by biochar addition, and we speculate this may reduce the
329 mobility and availability of metals to soil microorganisms (Rees et al., 2014). Liang et al.
330 (2014) also noticed a rise in soil pH value due to biochar addition, and they suggested that the
331 pH variation could cause a shift in the soil microbial population, such as bacteria and fungi.
332 The alkaline feature of formed metal oxides, hydroxides and carbonates admixed with the
333 biochar during the pyrolysis process might have increased the soil pH (Novak et al., 2009).

334

335 3.1.2. Heavy metal immobilisation by biochar

336 Bioavailability is critical in the determination of accessibility and toxicity of metals to soil
337 microorganisms (Wang et al., 2007). In this study, both Cd and Pb bioavailabilities were
338 significantly ($p < 0.01$) reduced due to the biochar amendment, but not in the glucose-amended
339 soils (**Fig. 2a and b**). Metal remediation ability of biochar based on the elevated soil pH
340 theory may be envisaged through the following two mechanisms: (i) elevated pH may
341 contribute to metal (co-)precipitation with carbonates, and (ii) it may increase the net
342 negative charges that favour the formation of metal-organic complexes. In the current study,
343 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 ($-\text{OH}$, $-\text{COOH}$ or $\text{C}-\text{OH}$) (**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 $\text{C}=\text{O}$ and aromatic
354 $\text{C}=\text{C}$ 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

369 respective biochar-amended soils (PB and CPB). The bioavailable metal concentrations were
370 decreased remarkably, indicating that biochar application reduced the metal mobility.

371 Investigations have shown that the specific morphology and chemical features may support
372 the metal sorption potential of biochar (Igalavithana et al., 2017). In addition, a discrepancy
373 was observed in Cd and Pb bioavailability decline patterns due to biochar application.

374 Bioavailability of Cd was decreased by 49% and 59% in CB and CPB, respectively (**Fig. 2a**),
375 while that of Pb was decreased by 23% and 13% in PB and CPB, respectively (**Fig. 2b**). The
376 decrease of Cd bioavailability was larger than Pb, which interestingly was consistent with the
377 slightly greater pH rise in Cd-spiked soil than the Pb-spiked soil. The rapid adsorption of
378 metals to biochar functional groups during the incubation period might have attributed to
379 their decreased mobilities in biochar-amended soils (Houben et al., 2013). The incubation
380 duration (ageing) was essential for the formation of effective adsorption bonds between
381 biochar surfaces and metal ions. In addition, soil type and clay content could often play an
382 important role in metal immobilisation by biochar. For example, Shen et al. (2016) suggested
383 that the biochar-amended clayey soils was not satisfactory for adsorption of Pb. Therefore,
384 the effect might become more prominent in a light-textured soil as used in the present study.

385 The specific surface area of the biochar sample was $202.49 \text{ m}^2 \text{ g}^{-1}$ (**Table 1**). This feature of
386 biochar along with its highly porous structure (**SI. 3a and b**) supported the existence of large
387 quantity of organic functional groups on the surface (**SI. 3c and SI. 4**), and consequently
388 their electrostatic as well as specific interactions with metal cations (**SI. 4**). The metal
389 adsorption ability can vary depending upon the properties of biochar as affected by the
390 pyrolysis conditions and feedstock sources (Park et al., 2011; Uchimiya et al., 2011). Results
391 of the current study also showed that the bioavailability of metals were slightly higher
392 ($p > 0.05$) when Cd and Pb coexisted in the system than the single metal-spiked soil. The
393 bioavailability of Cd was 1.40 mg kg^{-1} dry soil in CB against 2.08 mg kg^{-1} dry soil in CPB

394 (0.68 mg kg⁻¹ difference), while the bioavailability of Pb was 32.46 mg kg⁻¹ dry soil in PB
395 and 37.78 mg kg⁻¹ dry soil in CPB (5.32 mg kg⁻¹ difference). This might be due to the
396 competition among metal cations for the adsorption sites on biochar surfaces. Moreover, Rees
397 et al. (2014) demonstrated that the metal adsorption to organic materials may be partially
398 irreversible with multiple and element-dependent mechanisms, which could imply that
399 biochar might play a more prominent role in a long-term soil remediation approach.

400

401 **3.2. Influence of biochar on soil microbiota under metal stress**

402 3.2.1. Microbial activity

403 In all cases, the microbial activity was gradually decreased after the peak value on day 1 (**Fig.**
404 **3a**). Despite the patterns of respiration were similar irrespective of the treatments, the
405 cumulative respiration dropped significantly ($p < 0.01$) in metal spiked soils in comparison to
406 un-spiked soils (**Fig. 3b**). This demonstrated that the metal toxicity caused a reduction of the
407 soil microbial activity. Compared to the control soil, microbial respiration rate was
408 significantly ($p < 0.01$) stimulated immediately after biochar addition (**Fig. 3a**). The
409 respiration rates in uncontaminated soils with biochar amendment were 1.78 $\mu\text{g CO}_2\text{-C g}^{-1}$
410 dry soil h⁻¹ on day 1, and 1.11 $\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil h⁻¹ on day 3. These values were greater
411 than the control soil (uncontaminated and without biochar) on the respective days (1.27 μg
412 $\text{CO}_2\text{-C g}^{-1}$ dry soil h⁻¹ on day 1, and 0.65 $\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil h⁻¹ on day 3). Afterwards,
413 the respiration rate decreased likely because of the depletion of readily available organic
414 carbon supply. The respiration rate in the control soil was slightly higher than biochar-
415 amended soils on day 25 ($p > 0.05$), and this trend continued until the end of incubation. The
416 cumulative microbially respired CO₂-C values at the end of incubation were 351.41 and
417 379.56 $\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil in the control and biochar-amended soils, respectively (**Fig.**
418 **3b**). The difference in cumulative CO₂-C release between them (28.15 $\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil)

419 is consistent with most of the previous reports that biochar addition could increase the
420 microbial activity and CO₂-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 $\mu\text{g CO}_2\text{-C g}^{-1}$ dry soil) due to
431 biochar application compared to un-spiked control soil. The CO₂-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 $\mu\text{g CO}_2\text{-C g}^{-1}$ 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 CO₂-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 CO₂-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

493 amendment. Certain Gram-negative bacterial species show resilience to metal stresses, and
494 even exhibit capability of metal bioremediation in contaminated sites (Kang et al., 2016).
495 Sheng et al. (2016) suggested that the ratio of Gram-positive and Gram-negative bacteria
496 (G+/G-) is a promising indicator for predicting carbon sequestration in soils. A higher G+/G-
497 ratio may lead to a positive soil carbon depletion (Sheng et al., 2016). In the current study,
498 Gram-positive bacteria showed a higher tolerance to metal pollution than Gram-negative
499 bacteria and fungi. An increased Gram-positive bacteria population with a decreased fungi
500 population was also earlier reported with increasing metal concentrations (Aoyama and
501 Tanaka, 2013). In addition, the G+/G- ratio was increased by biochar addition, meaning that
502 biochar would favour the Gram-positive bacteria more than the Gram-negative bacteria to
503 grow in a heavy metal contaminated soil. The fungal abundance was increased significantly
504 ($p < 0.01$) because of biochar addition, while it was negatively affected by metal toxicity. In
505 spite of the fact that an elevated soil pH should support the bacterial population more than
506 fungi, the carbon and nutrients supplied by biochar might favour fungi to grow better than
507 bacteria (Liang et al., 2014). Biochar was reported to alter soil microbial community
508 composition, and a fungi dominated soil might lead to a higher resistance and resilience when
509 facing environmental stresses (Paz-Ferreiro et al., 2015). In this study, however, compared to
510 the bacterial groups, metal toxicity induced much severe inhibition of the fungal populations.
511 Such difference in microbial species was in consistence with earlier reports (Hinojosa et al.,
512 2005; Deng et al., 2015). Biochar in the present study had more prominent positive effect on
513 fungi than bacteria. Chen et al. (2013) also noticed a microbial community composition shift
514 after biochar addition to soils, and fungi communities were benefited more than bacteria.
515 There was no consistent pattern of metal types that influenced the soil microbiota, but a
516 slightly lower population of G+, G- bacteria, fungi and actinomycetes was noticed in Cd-
517 spiked soils than Pb-spiked soils. There were controversial reports on metal toxicity

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

521 A change in the microbial PLFA pattern with metal toxicity and biochar-mediated
522 remediation indicated a shift in the microbial community structure. This was shown by the
523 PLFA patterns from different treatments through principal component analysis (PCA, **Fig. 6**).

524 The first axis, which accounted for 80% of the variation in the PLFA data, separated different
525 treatments. In a reasonable agreement with the hypothesis that biochar addition would
526 modulate the soil microbial community under heavy metal stress, the community composition
527 after biochar-mediated remediation showed a certain distance from those without biochar
528 amendment, and the individual components remained close to each other. In addition,
529 uncontaminated soils with biochar addition were separated to the far on the right in the PC
530 analysis, and thus had a different PLFA pattern than the metal-spiked soils. The glucose- and
531 biochar-amended uncontaminated soils were grouped together, indicating that they had
532 similar PLFA patterns. It has been shown recently that the effect of biochar on the soil
533 microbiome is modulated by time and site (Thies et al., 2015). Therefore, further
534 investigations on biochar parameters and monitoring the duration effect are necessary for
535 interpreting the microbial population variation.

536

537 **3.3. Influence of biochar on soil and microbial carbon**

538 3.3.1. Soil organic carbon and nutrient pool

539 Soil TOC was measured at the end of 49 days incubation. Results showed that TOC value
540 decreased in the control soil as the microbial mineralisation increased (**Table 4**). With the
541 addition of biochar, soil organic carbon amount increased, contributing to heavy metal
542 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 CO₂ (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 CO₂-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 CO₂ 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 CO₂ 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 CO₂, 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. Biototoxicity 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.

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

619

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624

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837 **List of figures**

838

839 **Fig. 1.** Soil pH responses to biochar and glucose amendments in different types of metal
840 contaminated soils; (a) soil with Cd, (b) soil with Pb, (c) soil with combined Cd and Pb, (d)
841 soil without any heavy metal. Closed symbols indicate treatments with biochar application,
842 open symbols indicate treatments without biochar. D1, D7 and D49 indicate data on day 1, day
843 7 and day 49 of the incubation, respectively. Values show means \pm SE. * indicates significant
844 difference between glucose and biochar amendments ($p < 0.01$).

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

849 **Fig. 3.** Microbial respiration rate (a) and cumulative CO₂-C respired (b) in different treatment
850 soils. Data are displayed as means, bars indicate SE (n=3), * indicates significant difference
851 between glucose and biochar amendments ($p < 0.01$). S: control soil without any amendment; B:
852 soil applied with biochar; CB: soil applied with Cd + biochar; CG: soil applied with Cd +
853 glucose; PB: soil applied with Pb + biochar; PG: soil applied with Pb + glucose; CPB: soil
854 applied with Cd + Pb + biochar; CPG: soil applied with Cd + Pb + glucose.

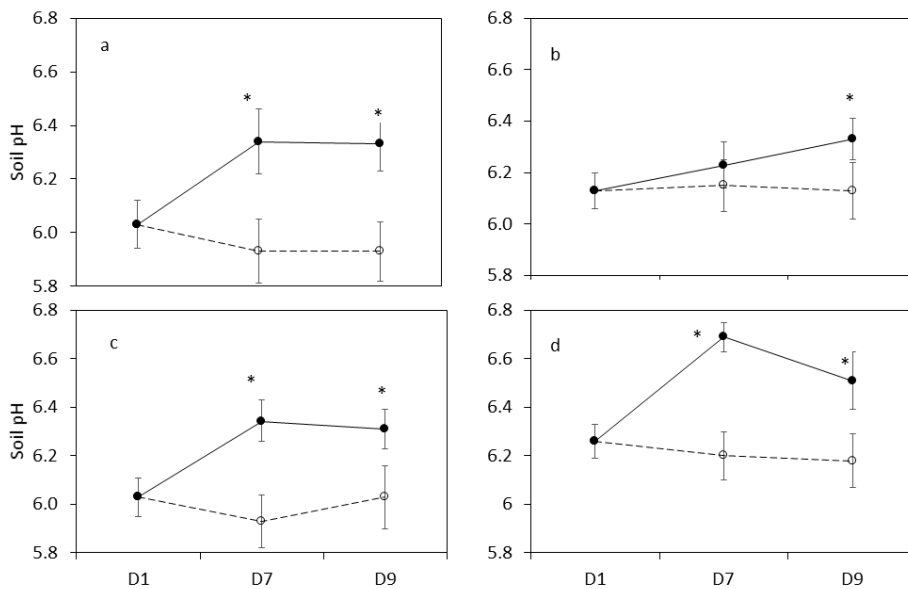
855 **Fig. 4.** Microbial biomass carbon in different treatment soils. Data are displayed as means, bars
856 indicate SE (n=3). S: control soil without any amendment; B: soil applied with biochar; CB:
857 soil applied with Cd + biochar; CG: soil applied with Cd + glucose; PB: soil applied with Pb +
858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

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

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

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

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872 **Fig. 1.**

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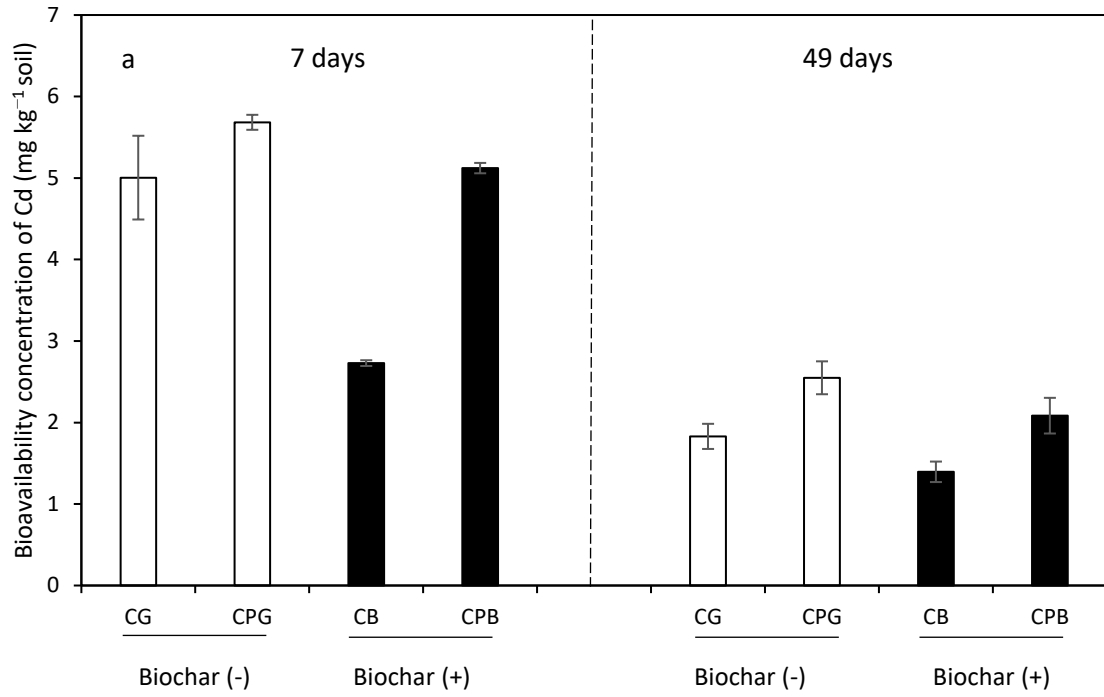
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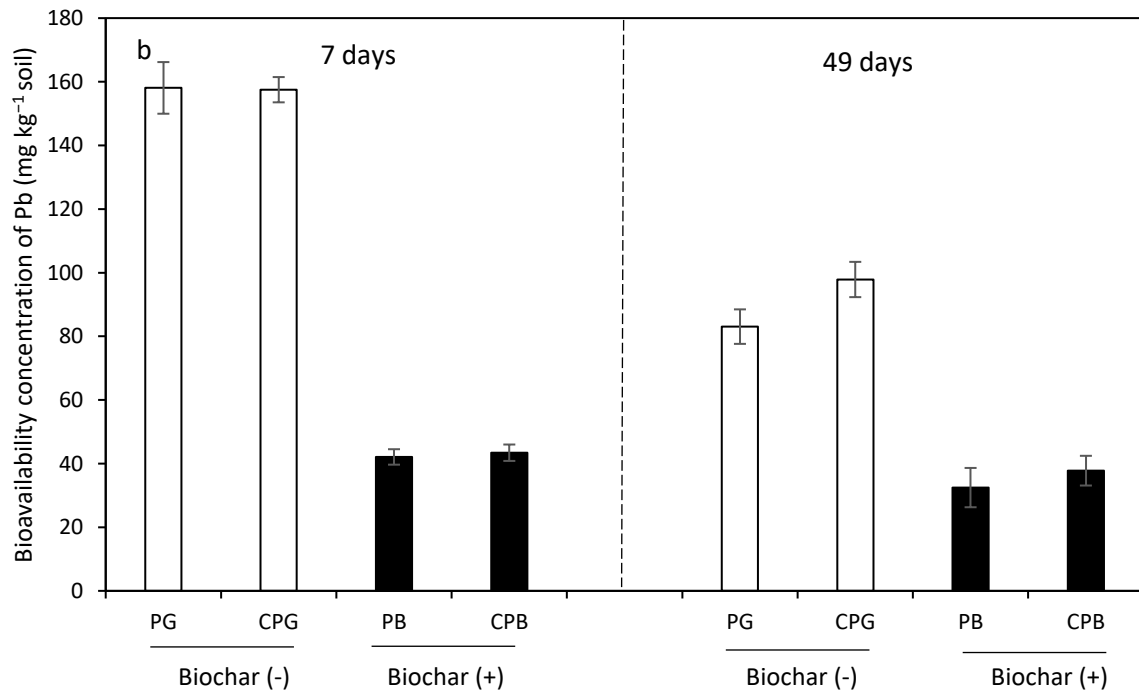
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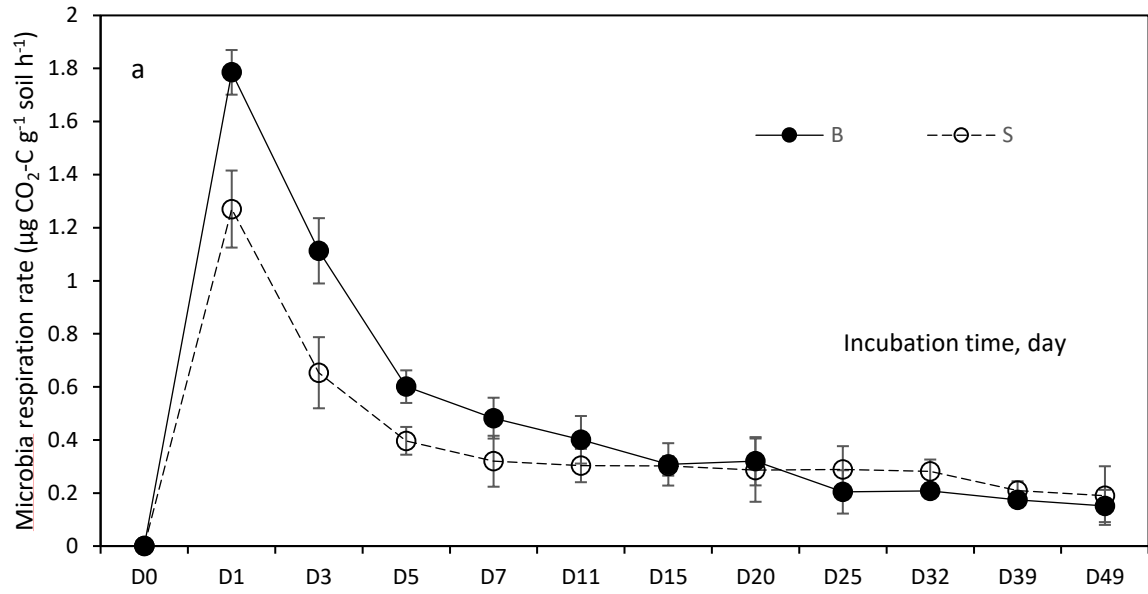
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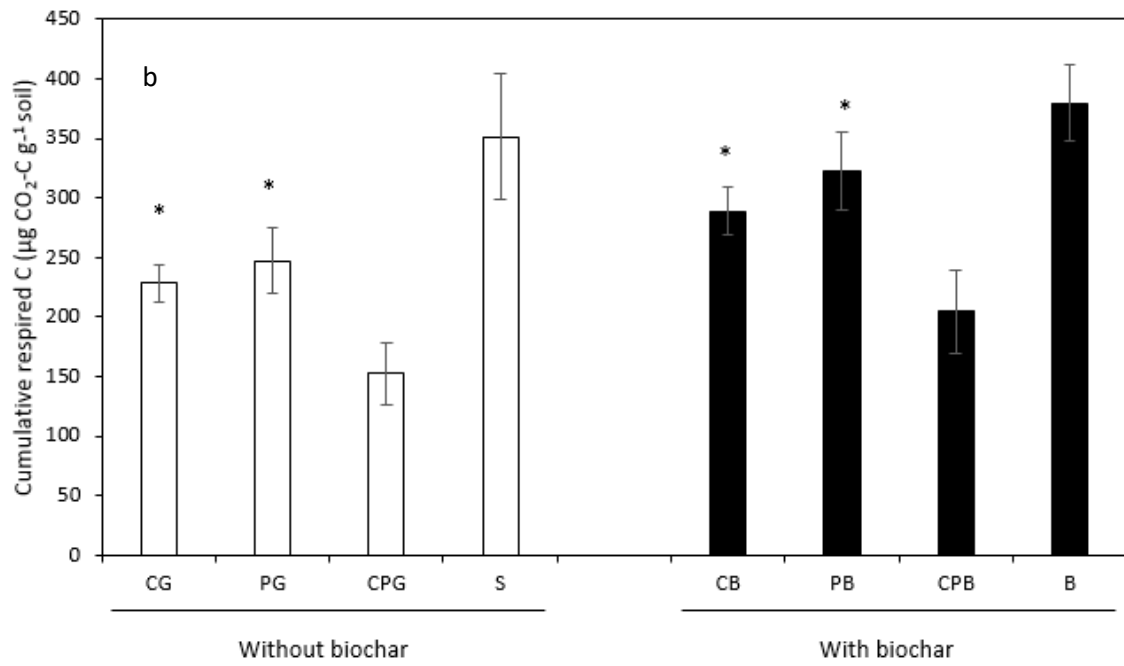
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882 **Fig. 2.**

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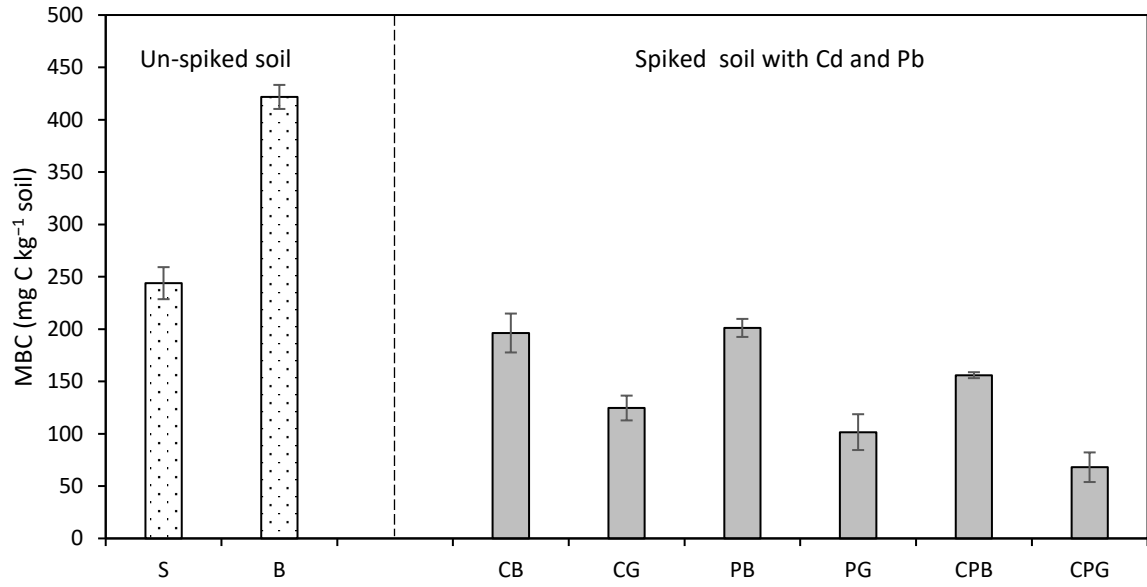
887 **Fig. 3.**

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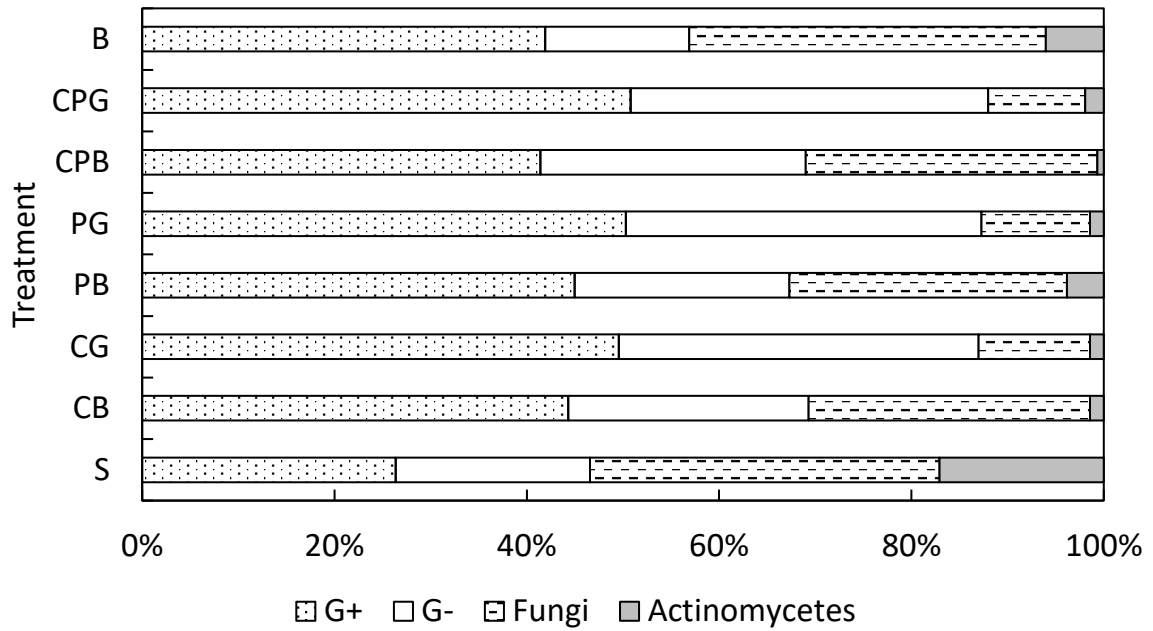
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893 **Fig. 4.**

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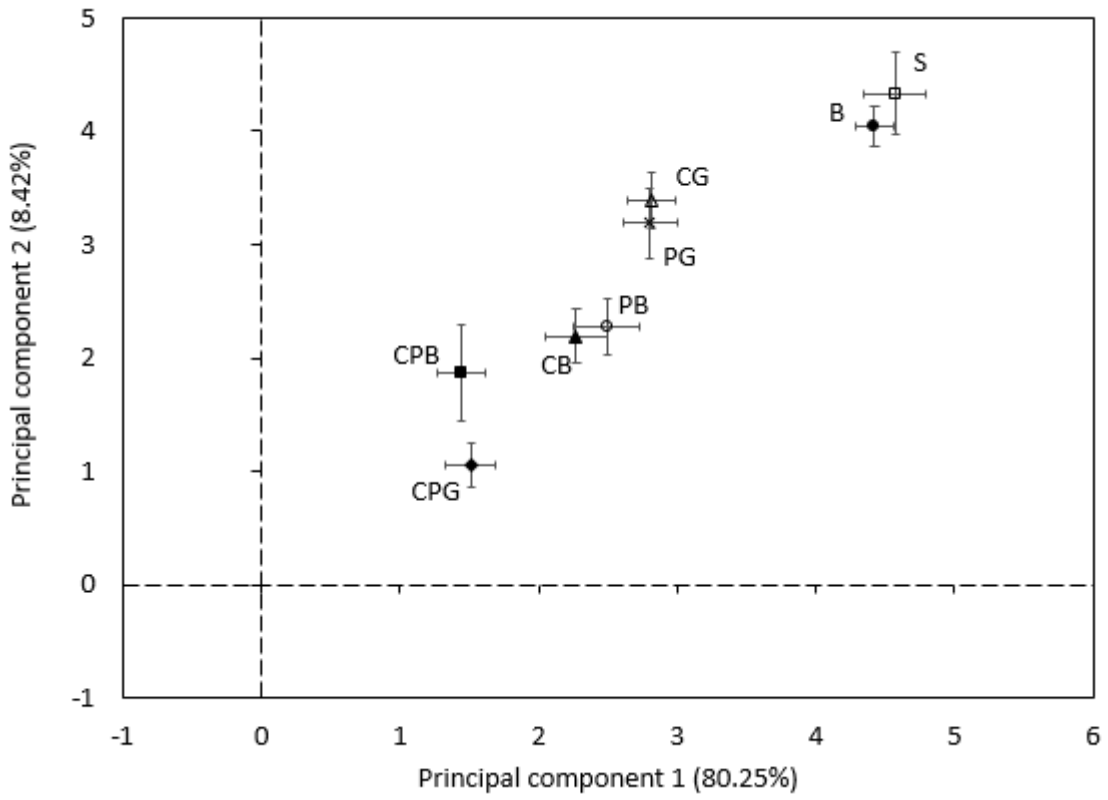


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896 **Fig. 5.**

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901 **Fig. 6.**

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903 **List of tables**

904 **Table 1.** Selected properties of the soil and macadamia nutshell biochar samples

905 **Table 2.** Soil spiking rate and final metal concentrations. Mean \pm SE, n=3

906 **Table 3.** Comparison of Gram-positive bacteria (G+ bacteria), Gram negative bacteria (G-
907 bacteria), fungi and actinomycetes as obtained through respective PLFA profiles (nmol g⁻¹ dry
908 soil). Mean values followed by different letters indicated significant difference (p<0.05) among
909 treatments

910 **Table 4.** Comparison of total organic carbon (TOC), total nitrogen (TN), and ratio of C:N in
911 soils after 49 days incubation. Means \pm SE (n=3)

912 **Table 5.** Effect of heavy metal toxicity on microbial carbon use efficiency. Means \pm SE (n=3)
913 of total PLFA, PLFA diversity, ratio of Gram-positive and Gram-negative bacteria, ratio of
914 bacteria and fungi. Mean values followed by the same letter are not significant among
915 treatments according to ANOVA ($p>0.05$)

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924 **Table 1**

925 Selected properties of the soil and macadamia nutshell biochar samples

<i>Soil property</i>	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

<i>Biochar</i>	pH	EC (mS cm ⁻¹)	Pyrolysis temperature (°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

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931 **Table 2**

932 Soil spiking rate and final metal concentrations. Mean \pm SE, n=3

Sample	Cd (mg kg ⁻¹ soil)	Pb (mg kg ⁻¹ soil)	Biochar (%)	Glucose (%)	Cd concentration (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% \pm 7.5%	-
Cd + Glucose	50	-	-	16	41.65	-	83.3% \pm 7.5%	-
Pb + Biochar	-	5000	5	-	-	4605	-	92.1% \pm 1.8%
Pb + Glucose	-	5000	-	16	-	4605	-	92.1% \pm 1.8%
Cd + Pb + Biochar	50	5000	5	-	43.85	4687.5	87.7% \pm 4.6%	93.75% \pm 2.5%
Cd + Pb +Glucose	50	5000	-	16	43.85	4687.5	87.7% \pm 4.6%	93.75% \pm 2.5%
Soil + Biochar	-	-	5	-	-	-	-	-

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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 applied*			Biochar applied			
	glucose/biochar							
	Uncontaminated soil	Soil + Cd	Soil + Pb	Soil + Cd + Pb	Uncontaminate d 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
<i>Microbial</i>								
<i>species feature</i>								
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

945 **Table 4**

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

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

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

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955 **Table 5**

956 Effect of heavy metal toxicity on microbial carbon use efficiency. Means \pm SE (n=3) of total PLFA, PLFA diversity, ratio of Gram-positive and
 957 Gram-negative bacteria, ratio of bacteria and fungi. Mean values followed by the same letter are not significant among treatments according to
 958 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	
<i>Microbial CUE</i>	0.41 \pm 0.02b	0.35 \pm 0.04b	0.29 \pm 0.02a	0.31 \pm 0.03a	0.53 \pm 0.01d	0.40 \pm 0.01b	0.38 \pm 0.02b	0.43 \pm 0.01c	
	Biochar applied: Glucose applied				Heavy metal: Biochar				
	CB:CG	PB:PG	CPB:CPG	CB:B	PB:B	CPB:B			
<i>CUE ratio</i>	1.15 \pm 0.06	1.32 \pm 0.05	1.40 \pm 0.01	0.77 \pm 0.12	0.73 \pm 0.10	0.82 \pm 0.04			

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961 Supplementary information for:
962 **Biochar modulates heavy metal toxicity and improves microbial carbon use efficiency in**
963 **soil**

964

965 Yilu Xu¹, Balaji Seshadri¹, Binoy Sarkar^{2,3}, Hailong Wang⁴, Cornelia Rumpel⁵, Donald
966 Sparks⁶, Mark Farrell⁷, Tony Hall⁸, Xiaodong Yang^{1,9,10}, and Nanthi Bolan^{1,11,12*}

967

968 ¹Global Center for Environmental Remediation, University of Newcastle, Callaghan, NSW
969 2308, Australia

970 ²Department of Animal and Plant Sciences, The University of Sheffield, Sheffield, S10 2TN,
971 UK

972 ³Future Industries Institute, University of South Australia, Mawson Lakes, SA 5095,
973 Australia

974 ⁴School of Environment and Chemical Engineering, Foshan University, Foshan, Guangdong
975 528000, China

976 ⁵CNRS, Institute of Ecology and Environment Paris, IEES, CNRS-INRA-UPMC-UPEC-
977 IRD, Thiverval-Grignon 78850, France

978 ⁶Department of Plant and Soil Sciences, Delaware Environmental Institute, University of
979 Delaware, Newark 19711, USA

980 ⁷CSIRO Agriculture & Food, Locked Bag 2, Glen Osmond, SA 5064, Australia

981 ⁸Sprigg Geobiology Centre & Department of Earth Sciences, University of Adelaide,
982 Adelaide, SA 5005, Australia

983 ⁹Key Laboratory of Oasis Ecology, Urumqi 830046, China

984 ¹⁰Institute of Resources and Environment Science, Xinjiang University, Urumqi 830046,
985 China

986 ¹¹Cooperative Research Centre for Contamination Assessment and Remediation for the
987 Environment, University of Newcastle, Callahan, NSW 2308, Australia

988 ¹²International Centre for Balanced Land Use, University of Newcastle NSW 2308, Australia

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991 *Corresponding author:

992 Prof Nanthi Bolan

993 University of Newcastle

994 Email: Nanthi.Bolan@newcastle.edu.au

995 Tel: +61 2 49138750

996 **List of supplementary information**

997

998 **SI. 1.** Schematic diagram showing the experiment approach

999

1000 **SI. 2.** Phospholipid fatty acid (PLFA) biomarkers used to characterise microbial communities
1001 in the experimental soils (Frostegård et al., 1993)

1002

1003 **SI. 3.** Morphological and surface chemical characteristics of macadamia nutshell biochar,
1004 including scanning electron micrographs (SEM) (a, b) and energy dispersive spectrum (c), and
1005 presenting the elements composition information of tested biochar area in (b).

1006

1007 **SI. 4.** Fourier transformed infrared (FTIR) spectrum showed the functional group of the
1008 macadamia nutshell biochar used in this research.

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1010 **SI. 5.** Detected microbial PLFA data under different treatments after 49 days of incubation
1011 (nmol g⁻¹ soil)

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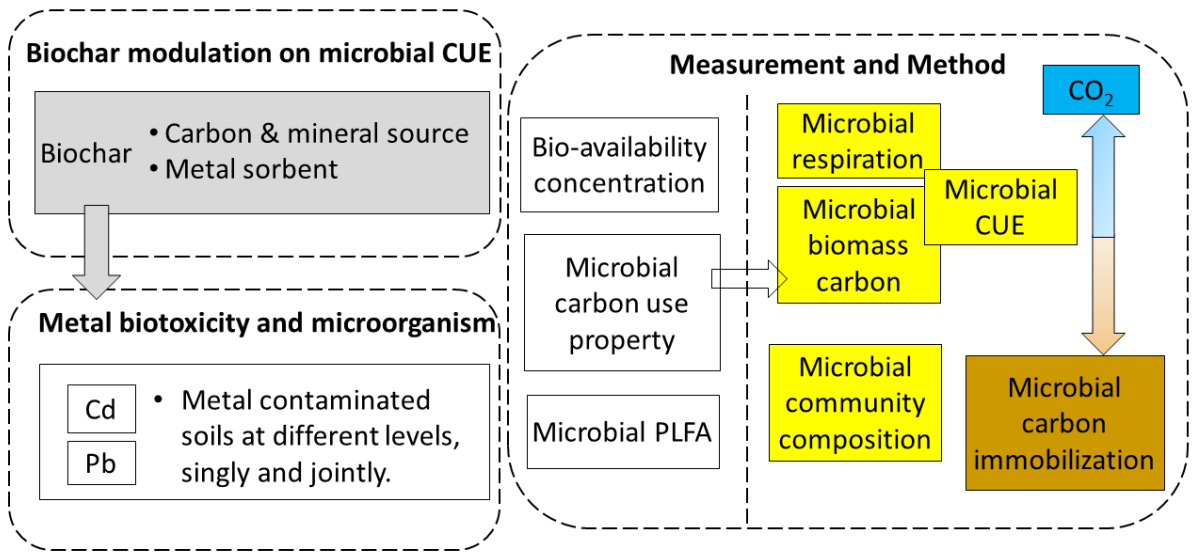
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1021 **SI. 1.** Schematic diagram showing the experiment approach

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1037 **SI. 2.**

1038 Phospholipid fatty acid (PLFA) biomarkers used to characterise microbial communities in the
1039 experimental soils

Microbial group	Biomarker PLFAs
Gram-negative bacteria	C16:1 ω 7c
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	C18:2 ω 6c, C18:1 ω 9c

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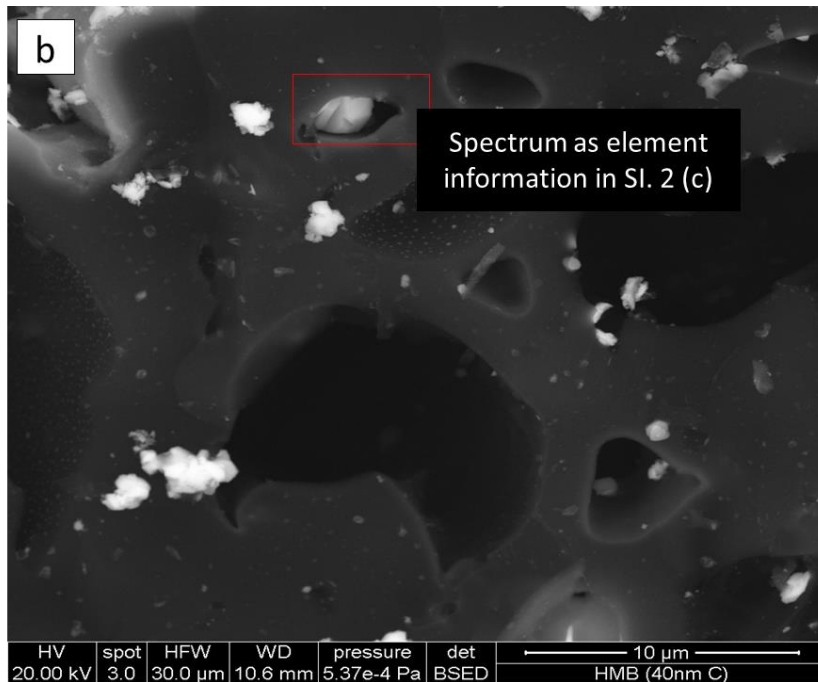
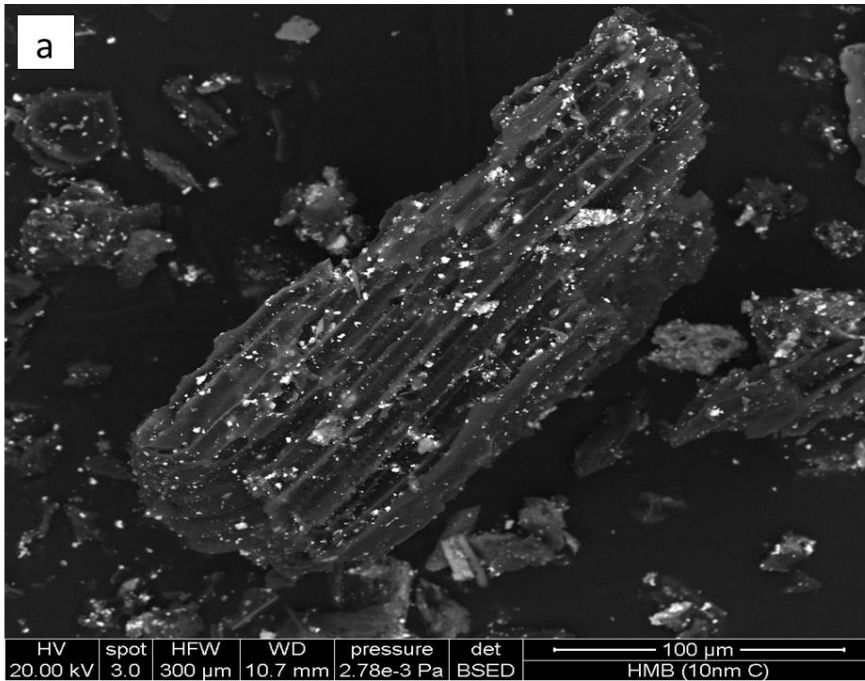
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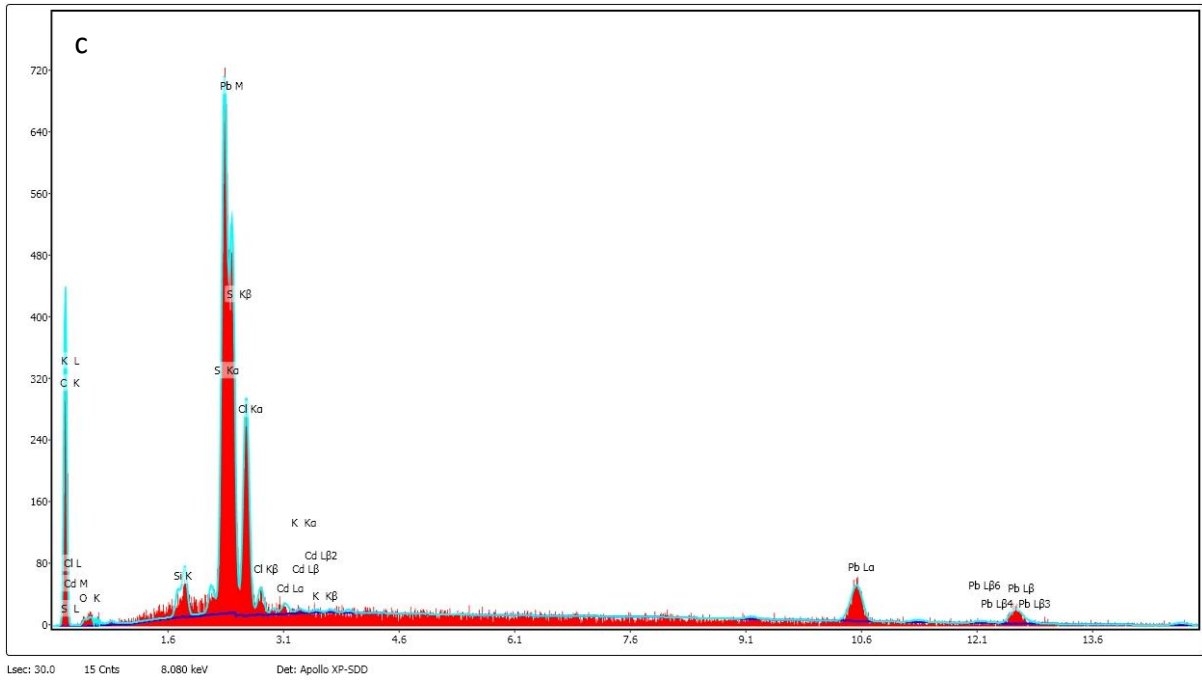
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1054 **SI. 3.** Morphological and surface chemical characteristics of macadamia nutshell biochar,
 1055 including scanning electron micrographs (SEM) (a, b) and energy dispersive spectrum (c), and
 1056 presenting the elements composition information of tested biochar area of SI. 2. (b).

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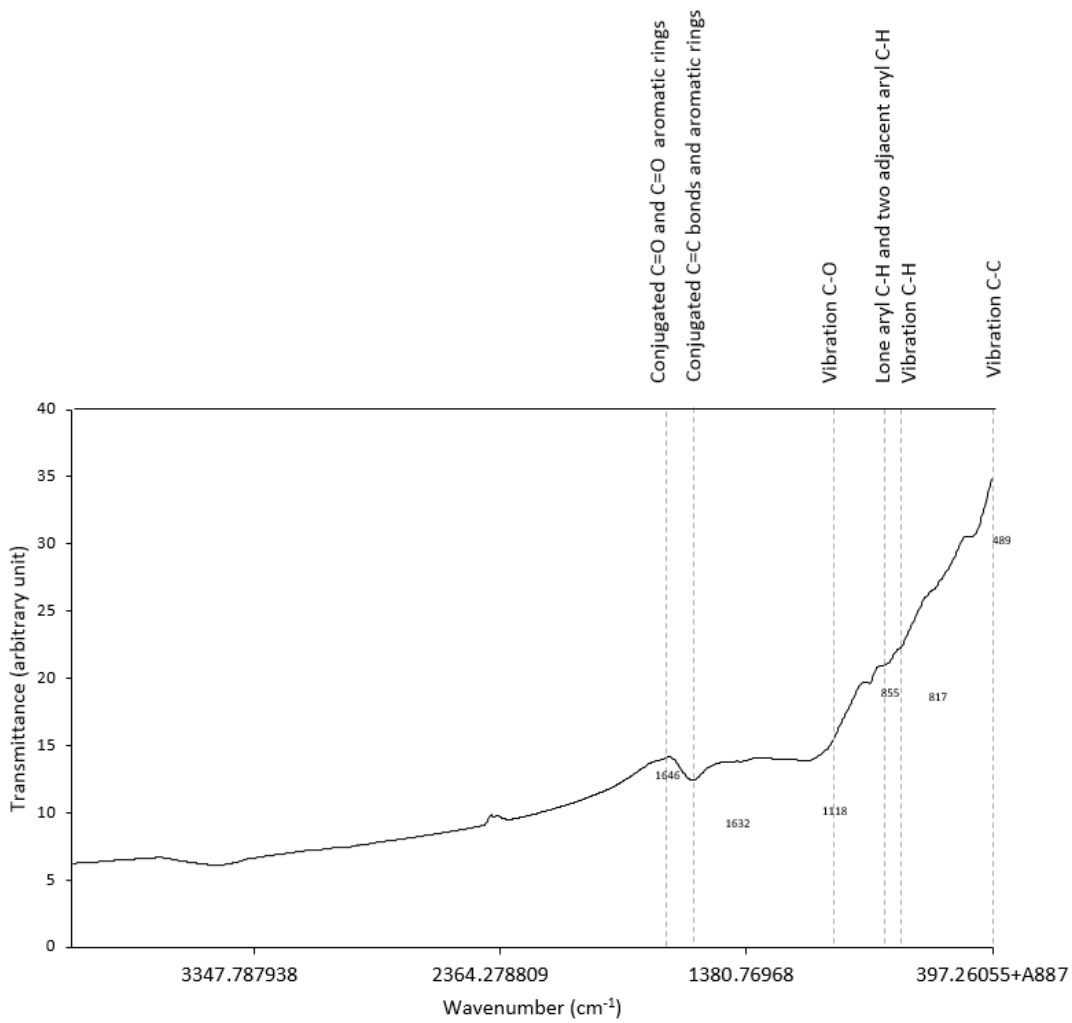
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1069 **SI. 4.** Fourier transformed infrared (FTIR) spectrum showed the functional group of the
 1070 macadamia nutshell biochar used in this research.

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1073 **SI. 5.**

1074 Detected microbial PLFA data under different treatments after 49 days of incubation (nmol g⁻¹
1075 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
C16:1 ω 7c	20.56	22.41	26.44	21.52	27.73	22.15	23.77	18.33
C18:2 ω 6c	7.59	2.16	1.88	5.28	3.45	3.06	4.06	8.57
C18:1 ω 9c	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

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