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► **To cite this version:**

Yilu Xu, Balaji Seshadri, Nanthi Bolan, Binoy Sarkar, Yong Sik Ok, et al.. Microbial functional diversity and carbon use feedback in soils as affected by heavy metals. *Environment International*, 2019, 125, pp.478-488. 10.1016/j.envint.2019.01.071 . hal-02628778

HAL Id: hal-02628778

<https://hal.inrae.fr/hal-02628778v1>

Submitted on 26 May 2020

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Microbial functional diversity and carbon use feedback in soils as affected by heavy metals

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ARTICLE INFO

Keywords:

Heavy metals

Soil organic carbon

Microbial carbon decomposition

Microbial activity

Microbial community composition

PLFAs

ABSTRACT

Soil microorganisms are an important indicator of soil fertility and health. However, our state of knowledge about soil microbial activities, community compositions and carbon use patterns under metal contaminations is still poor. This study aimed to evaluate the influences of heavy metals (Cd and Pb) on soil microorganisms by investigating the microbial community composition and carbon use preferences. Metal pollution was approached both singly and jointly with low (25 and 2500 mg kg⁻¹) and high (50 and 5000 mg kg⁻¹) concentrations of Cd and Pb, respectively, in an artificially contaminated soil. In a laboratory incubation experiment, bio-available and potentially bio-available metal concentrations, selected soil properties (pH, electrical conductivity, total organic carbon and total nitrogen), and microbial parameters (microbial activity as basal respiration, microbial biomass carbon (MBC) and microbial functional groups) were determined at two sampling occasions (7 and 49 days). Metal contamination had no effect on the selected soil properties, while it significantly inhibited both microbial activity and MBC formation. Contaminated soils had higher microbial quotient (qCO_2), suggesting there was higher energy demand with less microbially immobilized carbon as MBC. Notably, the efficiency of microbial carbon use was repressed as the metal concentration increased, yet no difference was observed between metal types ($p > 0.05$). Based on the microbial phospholipid fatty acids (PLFA) analysis, total PLFAs decreased significantly under metal stress at the end of incubation. Heavy metals had a greater negative influence on the fungal population than bacteria with respective 5–35 and 8–32% fall in abundances. The contaminant-driven (metal concentrations and types) variation of soil PLFA biomarkers demonstrated that the heavy metals led to the alteration of soil microbial community compositions and their activities, which consequently had an adverse impact on soil microbial carbon immobilization.

1. Introduction

Soil is a heterogeneous mixture of physical, chemical and biological components that determine soil function and ecosystem services (Doran and Parkin, 1996). Soil can deliver nutrients, and provide habitats and support for living organisms (Lehmann and Kleber, 2015). It can also be

a large sink for organic and inorganic compounds, including heavy metals and metalloids (Seshadri et al., 2015). Both natural and anthropogenic processes can lead to the release of heavy metals into the ecosystem (Khan et al., 2010; Margesin et al., 2011). The biohazard nature of metals has captured community attention, leading to increasing public awareness and research regarding the metal toxicity

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<https://doi.org/10.1016/j.envint.2019.01.071>

Received 27 August 2018; Received in revised form 26 January 2019; Accepted 28 January 2019

Available online 13 February 2019

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and persistence in terrestrial ecosystems (Bolan et al., 2014).

Soil microorganisms are the essential driving force for many critical ecosystem processes (Carney and Matson, 2005; Yang et al., 2015), especially the active microorganisms which regulate carbon (C) and nutrient cycling in soils (Blagodatskaya and Kuzyakov, 2013; Creamer et al., 2015; Farrell et al., 2014). In order to adapt to the variation in environmental conditions, microorganisms may shift their energy management strategies by altering their C use preferences (Tripathy et al., 2014). Thus, soil microorganisms can be a promising indicator for monitoring the soil fertility and health conditions (Igalavithana et al., 2017a). Microbial metabolic quotient (qCO_2) is defined as the microbially respired CO_2 -C per unit of MBC, serving as an ecophysiological indicator for soil microbial activity status (Anderson and Domsch, 1993). Additionally, various microbial functional groups have important roles in dictating various soil biochemical reactions (Wertz et al., 2007).

The microbial population is highly sensitive to soil environmental changes and stresses, leading to microbial C use patterns variation related to the change in environmental parameters (Frey et al., 2001). While some heavy metals serve as micro-nutrients and are necessary for maintaining biological functions of microorganisms, excessive quantities of heavy metals lead to bio-toxicity, inhibit microbial activity and alter the community composition (Choppala et al., 2014; Khan et al., 2007). The interaction between soil microorganisms and heavy metals can also affect metal functional groups, leading to metal mobilization, dissolution, leaching and redox transformation, or immobilization through organic-metal binding and precipitation (Gadd, 2004). Due to metal stress, soil microorganisms alter energy management by diverting more resources into maintenance rather growth (Dilly, 2005; Tripathy et al., 2014).

Numerous earlier studies focussed on the physicochemical interactions between metals and soil components (e.g., organic matter, clay minerals, oxidic particles) (Adriano et al., 2004), but little attention was given to the specific and systemic elucidation of the impact of metals on soil microbiota, and most importantly, alteration of microbial C use patterns in soils. The identification of phospholipid fatty acids (PLFAs) has been introduced as a relatively effective way to reveal critical information on the negative effects of metal contamination because it can identify microbial community structure alteration due to metal toxicity, which helps to interpret the microbial C mediation strategy (Lenart and Wolny-Kołodka, 2013). An understanding of the relationship between heavy metals and microbial properties, including activity and community composition, will contribute to the interpretation of microbial C use patterns under metal contamination, which is scarcely reported in the literature. In addition, there have been studies on the effect of high concentration of heavy metals (2500 and 5000 mg Pb kg^{-1} soil) on phytoremediation performance of plants (Epelde et al., 2008), but the lack of soil microbial information leaves a huge knowledge gap about the underground soil bio-chemical reactions under such high level of metal contamination (Chenery et al., 2012; Wuana and Okieimen, 2011), which the current study aims to address.

As such, we included Cd and Pb (as comparatively mobile and less mobile metals) with different spiking concentrations, singly and together. Following ageing of the metal-spiked soils for 4 weeks, microbial parameters and the bio-availability of metals were investigated twice during a 49 day incubation period. Such a controlled situation experiment allowed us to investigate soil microbial C use strategy by reducing the impacts of soil types and pollutant interactions, which otherwise is a highly heterogeneous environment (Ahmad et al., 2016). We hypothesised that: (i) metal toxicity would vary depending on the metal types and contamination levels, (ii) microbial activity, MBC and qCO_2 would vary due to heavy metal stress, and (iii) microbial functional groups composition would show change under different metal contaminations.

2. Materials and methods

2.1. Soil preparation, spiking and analysis

A fresh soil sample was collected in July 2014. The sampling site was selected to be away from roots and vegetation influence located in the Barossa Valley region, South Australia (138°57'37"E, 34°27'48"S). The region is identified as Mediterranean climate with the major soil type as Sodosol, according to the Australian Soil Classification system (Isbell, 1996). Sodosols are identified as relatively highly sodic yet low acidity (pH > 5.5) soils. Those features indicate Sodosols are very vulnerable to erosion, especially with the absent of soil microorganisms under contamination (Mine, 2014). The highest annual average temperature is 22.3 °C while the lowest average is 12.2 °C, and the average annual rainfall is 437 mm (Xu et al., 2018). The soil was processed by removing all fine roots and debris, air drying, homogenising and passing through < 2 mm sieve. Soil pH and EC were determined in 1:5 (w/v) soil suspension in deionized water with a pH/conductivity meter (smartCHEM-LAB Laboratory Analyser, TPS Pty Ltd., Springwood, QLD, Australia). Soil texture was determined following the micro-pipette method (Miller and Miller, 1987). Soil cation exchange capacity (CEC) was determined by extracting the soil with NH_4^+ , followed by the determination of the concentration of NH_4^+ (Ross and Quirine, 1995) on a Continuous Flow Analyser (San⁺⁺, Skalar Analytical B.V., Breda, Netherlands). Soil total organic C (TOC) and Total nitrogen (TN) were measured by Leco C/N Analyser (Leco TruMac[®] CNS/NS Analyser, LECO Corporation, Osaka, Japan). In brief, 0.2 g soil sample was weighed and combusted at 1300 °C with an O_2 flow for 5 s. For calibration, a standard weight of Leco EDTA reference material (containing 95.7 g N kg^{-1} and 410 g C kg^{-1}) was added every 10 samples. The experimental soil was slightly acidic (pH = 5.51) with EC value of 27.53 mS cm^{-1} , and CEC value of 32.71 cmol (p^+) kg^{-1} . The initial TOC and TN contents of the studied soil were 2.29% and 0.14%, respectively (Xu et al., 2018). The soil was silty loam in texture, containing 32, 26 and 42% of clay, silt and sand, respectively. Soil water holding capacity (WHC, the water held between field capacity and permanent wilting point, i.e., available water in this research) was measured before air drying in order to adjust all the samples to 50% of the initial WHC. Then soils were incubated at 25 °C and 28% relative humidity for a week before conducting the microbiological analysis.

In this research, soil was spiked with low and high levels of Cd (NO_3)₂ and Pb(NO_3)₂. To demonstrate the relationship between metal types and their toxicities, the metals were added into soils both separately and jointly. Control soil portions were prepared with the absence of metal contamination, but similarly amended with KNO_3 to compensate the amount of nitrate added to the polluted soil (Leita et al., 1993). Briefly, both metal and KNO_3 solutions were sprinkled evenly on the soil spread on a polyethylene sheet. To achieve homogenisation, soils were stirred and mixed thoroughly on an end-over-end shaker (Lamb et al., 2016). Then soils were air-dried, aged for 4 weeks at room temperature, and passed through a 2-mm sieve. The final concentrations of metals in the spiked soils for each treatment are listed in Supplementary Material (SM. 1). All experiments were conducted in triplicate.

2.2. Potentially available and bio-available heavy metals

Bio-available and potentially available heavy metal concentrations were measured by extracting the amended and unamended soils with 0.01 M $CaCl_2$ and 0.05 M EDTA solutions, respectively, (1:10 w/v, with 60 min reaction time) (Sparks et al., 1996). The metal concentration in 0.01 M $CaCl_2$ extractions presents a distinguishing indicator for metal bio-availability in soils (Ok et al., 2011b). The bio-available and potentially available metals were measured on day 7 and again at the end of the incubation (day 49). The extracts were filtered through a 0.45 μ m nylon filter before analysis by inductively coupled plasma mass

spectrometry (ICP-MS, Agilent 7900, Agilent Technologies Ltd., Santa Clara, CA, USA). Uncontaminated soils (without Cd and Pb spiking) were set up as control treatments. The treatments and respective sample abbreviations are as follows: uncontaminated soils are referred as control, soils spiked with low and high Cd concentrations are referred as CL and CH, respectively, soils spiked with low and high Pb concentrations are referred as PL and PH, respectively, soils spiked with both Cd and Pb at low concentrations are referred as CPL, while soils spiked with both Cd and Pb at high concentrations are referred as CPH.

2.3. Microbial properties

The microbial activity of soils was monitored by measuring the basal respiration (CO_2 evolution) from the samples (El-Naggar et al., 2015). In brief, 10 g soil was incubated in Schott bottles at 25 °C and 28% relative humidity, in dark for a 49 days incubation period. 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. Ten milliliters of freshly prepared alkali was replaced every time. Sampling and titration were done on days 1, 3, 5, 7, 11, 15, 20, 25, 32, 39 and 49 of the soil incubation. For each set, three blank Schott bottles (without soil) with NaOH were incubated and titrated as described above as control. The amount of evolved CO_2 was thus measured, and the microbial respiration was calculated using Eq. 1:

$$MR = \{MW\text{CO}_2(V_b - V_s) \times M \times 1000\} / (DW \times T \times 2) \quad (1)$$

where, MR is the microbial respiration ($\text{mg CO}_2\text{-C kg}^{-1}$ soil h^{-1}), $MW\text{CO}_2$ is the molecular weight of CO_2 , V_b is the volume (mL) of HCl for the blank titration, V_s is the volume (mL) of HCl for the sample titration, M is the concentration of HCl (0.03 M), DW is the dry weight of the soil (kg), T is the time of incubation (h), and 2 is the factor that accounts for the fact that two OH^- are consumed by one CO_2 .

Measurement of MBC followed the method of Vance et al. (1987). In brief, 10 g dry weight equivalent soil was placed in 50 mL beakers with the presence of 50 mL ethanol-free chloroform in a vacuum desiccator. Simultaneously, a chloroform free set was prepared. To extract the C after incubation, 40 mL of 0.5 M K_2SO_4 was mixed with soils by an end-over-end shaker for 1 h. Samples were then centrifuged and filtered through Whatman #40 filter papers. C content in the filtrates was analysed by a Total Organic C (TOC) Analyser (TOC-LCSH, Shimadzu Corporation, Kyoto, Japan). MBC was calculated using Eq. 2:

$$MBC = E_c / K_c \quad (2)$$

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

Phospholipid fatty acids (PLFAs) were used as biomarkers for determining certain microbial functional groups abundances. PLFAs were extracted following the method described by Frostegård et al. (1993), and modified by Bossio et al. (1998). In brief, 8 g freeze dried soil was extracted with one phase extraction mixture (Bligh and Dyer, 1959), 1:2:0.8 of chloroform: methanol: citrate buffer solvent. After shaking (2 h) and centrifugation (4500 rpm), the upper solution was decanted into non-transparent vials, and vortexed before standing over night. The solution thus would change into two separated layers due to density differences. The left-over liquid was dried by purging with pure N_2 gas at 32 °C. The thin solid phase left in vials was re-dissolved in chloroform, and transferred with chloroform, followed by acetone and methanol into a separation phase extraction (SPE) column. After drying the leaching solution, 0.5 mL of 1:1 (v/v) of methanol:toluene and 0.5 mL of 0.2 M methanolic KOH were added in the glass tube. PLFAs were converted into fatty acid methyl esters (FAMES) with mild alkaline methanolysis at 37 °C for 30 min. After the samples cooled back to room temperature, 1 mL deionized water, 0.15 mL 1 M acetic acid and 1 mL

hexane were added. The mixture was vortexed and settled before the upper layer was transferred into GC vials by a pipette.

FAMES were analysed by gas chromatography combined with mass selective detector (GC-MS, Model 7890B/5977B, Agilent Technologies Ltd., USA; AxION iQT with Cold EI Source, Perkin Elmer, Waltham, MA, USA) with an RTX-5MS fused silica capillary column (60 m, 250 $\mu\text{m} \times 0.25 \mu\text{m}$ film thickness) (Supelco, Sigma-Aldrich, Castle Hill, NSW, Australia). Methylnonadecanoate (19:0) was added to each sample as an internal standard. A Supelco 37 standard mixture (Supelco, Bellefonte, Pa.) was used as standards to compare with retention times of each PLFA peak. The specific microbial group was described as nmol signature PLFA g^{-1} soil. The biomarkers for Gram-negative (G⁻) bacteria were C16:1 ω 7c, C16:1 ω 9c; biomarkers for Gram-positive (G⁺) bacteria were iC15:0, aC15:0, C15:0, iC16:0, C16:0, iC17:0, aC17:0, C17:0; biomarkers for actinobacteria were 10MeC16:0, 10MeC17:0, 10MeC18:0; and biomarkers for fungi were C18:2 ω 6c, C18:1 ω 9 (Frostegård et al., 1993; Zelles, 1999).

2.4. Statistical analysis

The Shapiro-Wilk test was used for normal distribution determination, and the Levene's homogeneity of variance test was also employed. One factor ANOVA was used to test the significant differences among uncontaminated and various metal contaminated samples. In order to test the differences between Cd and Pb treatments two-factor ANOVA was used to test the main factor between the two metal types. The spearman correlation coefficients were used among soil and microbial parameters. The least significant difference (LSD) test was used and the significant differences accepted at $p < 0.05$. Principal components analysis (PCA) was used for microbial PLFA data to elucidate the major variation and covariation both for individual PLFA and microbial functional groups using varimax rotation. All the statistical analyses were performed in IBM SPSS version 23.0 (SPSS Inc., Chicago, USA).

3. Results

3.1. Soil properties and metal analyses

3.1.1. Soil pH, EC, total nitrogen and organic carbon

We determined the soil physiochemical characteristics twice during the incubation period (7 and 49 days) in order to study the variations in soil chemical properties under metal contamination. Soil pH increased from an initial value 5.51 to final 6.02 in the uncontaminated soil (SM. 2a). Microbial decomposition of organic matter led to a soil pH increase at the initial incubation period. After 49 days of incubation, soil pH decreased due to metal amendments. The lowest pH values were found in CPH soil (pH = 5.09 and 4.94, respectively). From day 7 to day 49, soil pH slightly increased in samples spiked with Cd (CL and CH), while it decreased in samples in the presence of Pb (PL and PH), also decreased in CPL and CPH. The pH difference was by 0.04 units between CL and CH, whereas by 0.23, 0.21, 0.03 and 0.15 units in PL, PH, CPL and CPH samples, respectively. The changes of soil electrical conductivity (EC) are shown in SM. 2b.

Compared to control, after 7 days of incubation, the lowest EC value (10.16 dS m^{-1}) was in PL, followed by PH (11.32 mS cm^{-1}) and CPH (12.50 mS cm^{-1}). The lowest EC was still in PL (9.66 mS cm^{-1}) after 49 days of incubation, followed by CL (11.61 mS cm^{-1}) and CPH (13.12 mS cm^{-1}). There were no significant ($p > 0.05$) correlation between soil EC values and different metal concentrations. Higher metal concentration showed relatively higher soil EC compared to lower metal concentration. But with the inhibitory of microbial activity, such as nitrogen metabolism repress and disruption of metabolism due to metal toxicity, soil EC values are were not directly related to metal concentrations.

We measured TOC and TN contents after 49 days of incubation (Table 1). After 49 days of incubation, CL and PL had the lowest TOC

Table 1

Multiple comparisons of soil and microbial carbon contents under Cd, Pb and Cd + Pb contamination at different concentrations after 49 days of incubation. Means \pm SE ($n = 3$).

Heavy metal	Sample	Soil organic carbon (%)	Soil nitrogen (%)	C/N	C_{min}/C_{org}
Cd	Control	2.73 \pm 0.08 ^a	0.27 \pm 0.003 ^b	10.21 \pm 0.23 ^a	14.78 \pm 0.22 ^b
	CL	2.84 \pm 0.05 ^a	0.23 \pm 0.003 ^a	12.53 \pm 0.36 ^b	4.03 \pm 0.17 ^a
	CH	2.68 \pm 0.01 ^a	0.25 \pm 0.002 ^b	10.57 \pm 0.05 ^a	2.87 \pm 0.66 ^a
Pb	Control	2.73 \pm 0.08 ^a	0.27 \pm 0.003 ^b	10.21 \pm 0.23 ^a	14.78 \pm 0.22 ^b
	PL	2.64 \pm 0.00 ^a	0.22 \pm 0.002 ^a	11.83 \pm 0.14 ^a	3.50 \pm 0.30 ^a
	PH	2.59 \pm 0.04 ^a	0.23 \pm 0.005 ^a	11.34 \pm 0.27 ^a	2.89 \pm 0.15 ^a
Cd + Pb	Control	2.73 \pm 0.08 ^b	0.27 \pm 0.003 ^a	10.21 \pm 0.23 ^a	14.78 \pm 0.22 ^b
	CPL	2.46 \pm 0.02 ^a	0.25 \pm 0.005 ^a	9.77 \pm 0.45 ^a	2.95 \pm 0.36 ^a
	CPH	2.67 \pm 0.05 ^b	0.23 \pm 0.004 ^a	11.42 \pm 0.30 ^b	2.26 \pm 0.11 ^a

Note: Abbreviation in table as: CL and CH indicate the soils spiked with low or high Cd concentration, PL and PH indicate the soils spiked with low or high Pb concentration, CPL and CPH indicate the soils spiked with low or high Cd and Pb concentration. Different letters in one treatment indicates significance ($p < 0.05$) among metal concentrations.

content (2.46%), and the lowest C/N ratio (9.77), whereas PL had the smallest TN content (0.22%). The largest value of TN was in the uncontaminated soil (0.27%), and the ratio of MBC to TOC had the lowest value in CPH, while the greatest value was in the uncontaminated soil (14.78 in control).

3.1.2. Bio-available and potentially available metal concentrations

Except CL, the bio-available Cd and Pb decreased with the incubation period (Fig. 1). By the end of incubation (49 days), the bio-availability of Cd significantly decreased by 59% (CH), 58% (CPL) and 58% (CPH) compared to those after 7 days (Fig. 1a). While comparing Pb bio-availabilities at day 7 and day 49 of the incubation, it reduced by 100, 37, 23 and 30% in PL, PH, CPL and CPH, respectively (Fig. 1b). In spite of the same initial input rates, the Cd bio-availability was significantly higher in CPH (11.82 and 5.04 mg kg⁻¹ soil at day 7 and 49, respectively) than CH (3.83 and 1.57 mg kg⁻¹ soil at day 7 and 49, respectively). Also, Pb bio-availability was significantly higher in Cd–Pb co-contaminated samples (122.79 and 95.26 mg kg⁻¹ soil at day

7 and 49, respectively) than single Pb contamination (105.15 and 66.42 mg kg⁻¹ soil at day 7 and 49, respectively).

The potentially available (0.05 M EDTA extracted) Cd concentration slightly increased from 2.58 to 3.09 mg kg⁻¹ soil in CL, while decreased from 6.06 to 5.55 mg kg⁻¹ soil in CH (Fig. 1c). The reduction was even more noticeable in CPL (from 16.04 to 3.44 mg kg⁻¹ soil) and CPH (from 48.18 to 12.44 mg kg⁻¹ soil) (Fig. 1c). Compared to the concentration on day 7, the potentially bio-available Pb increased in all treatments by 22, 26, 66 and 45% in PL, PH, CPL and CPH, respectively, on day 49 (Fig. 1d). Compared to the single Cd polluted soils, potentially available Cd contents were much higher in the co-polluted soils on day 7 (2.58 and 16.04 mg kg⁻¹ soil in CL and CPL, respectively, and 6.05 and 40.15 mg kg⁻¹ soil in CH and CPH, respectively). However, such significant differences disappeared on day 49 ($p > 0.05$, Fig. 1).

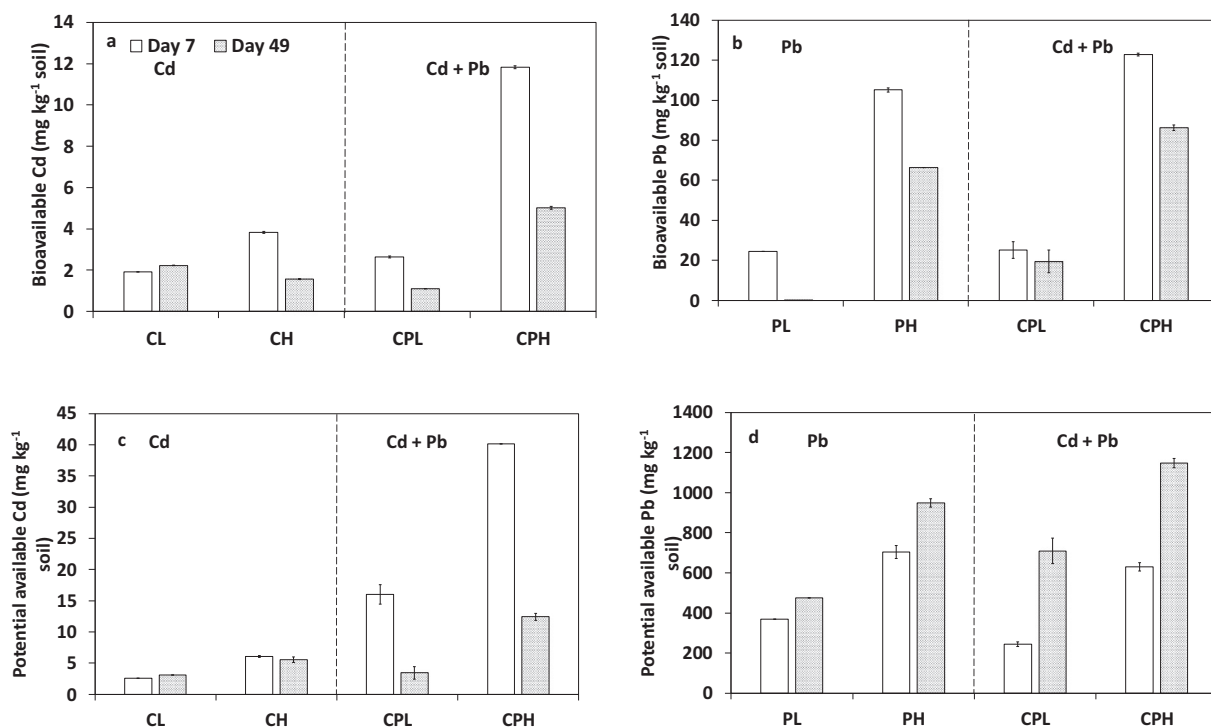


Fig. 1. Bio-available Cd (a) and Pb (b), potentially bio-available Cd (c) and Pb (d) concentrations under different treatments. Data are displayed as means, bars indicate SE ($n = 3$). CL and CH indicated the soils spiked with low or high Cd concentrations, PL and PH indicated the soils spiked with low or high Pb concentrations, CPL and CPH indicated the soils spiked with low or high Cd and Pb concentrations.

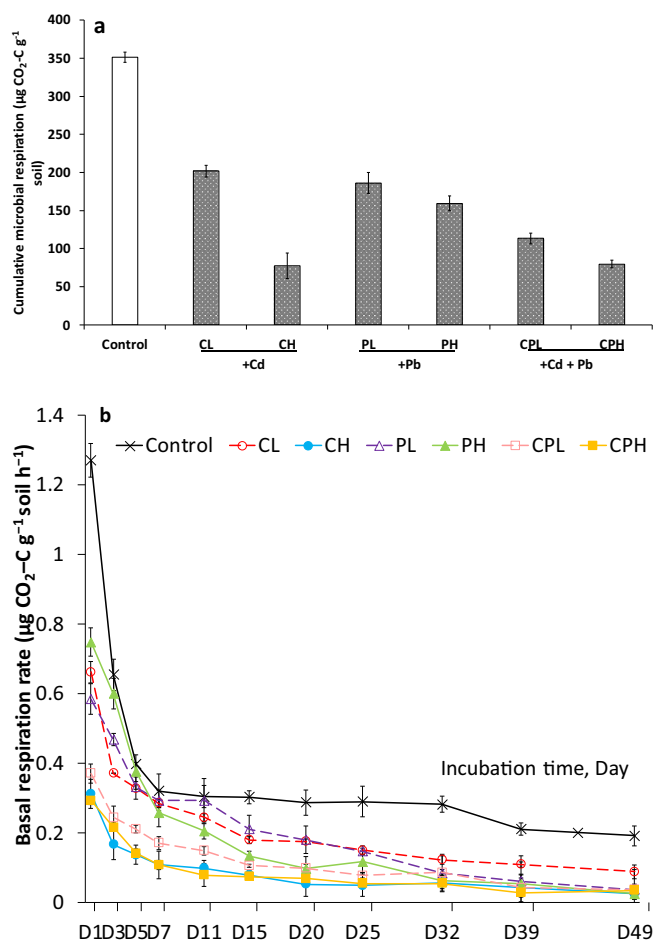


Fig. 2. Cumulative respired CO₂-C with different heavy metal contamination (a), and microbial respiration rate variation (b) during 49 days of incubation. Control indicated un-contaminated soils, CL and CH indicated the soils spiked with low or high Cd concentrations, PL and PH indicated the soils spiked with low or high Pb concentrations, CPL and CPH indicated the soils spiked with low or high Cd and Pb concentrations. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

3.2. Effect of heavy metals on microbial carbon use

3.2.1. Effect of heavy metal toxicity on microbial activity

Microbial basal respiration rate and total respired CO₂ were analysed to determine the soil microbial activity under metal stress (Fig. 2). By the end of incubation, total respired CO₂-C differed distinguishably among different metal treatments (Fig. 2a). Microbial respiration rates in all the treatments reached the highest peak on day 1 followed by a gradual decline over the incubation period (Fig. 2b). However, the respiration rate in the uncontaminated soil (black solid line in Fig. 2b) was higher than those in the contaminated soils (coloured lines) throughout the incubation period. The respiration rates in metal-spiked soils on day 1 followed the order: CPH < CH < CPL < PL < CL < PH (0.29, 0.31, 0.37, 0.58, 0.66 and 0.75 µg CO₂-C g⁻¹ soil h⁻¹) in the respective treatments. After 49 days of incubation, the largest cumulative CO₂-C production was in the control soil (351.41 µg CO₂-C g⁻¹ soil) (Fig. 2a). The cumulative CO₂-C productions were different between PL and PH, but there was significant difference between CL and CH. At low metal concentrations, the cumulative microbial respiration was decreased by 43, 47 and 68% in CL, PL and CPL treatments, respectively, as compared to the control. At relatively higher metal concentrations, such decreases were 78, 55 and 77% in CH, PH and CPH, respectively (compared to the control). The lowest cumulative CO₂-C value was in CH (77.52 µg CO₂-C g⁻¹ soil), followed by CPH (79.88 µg

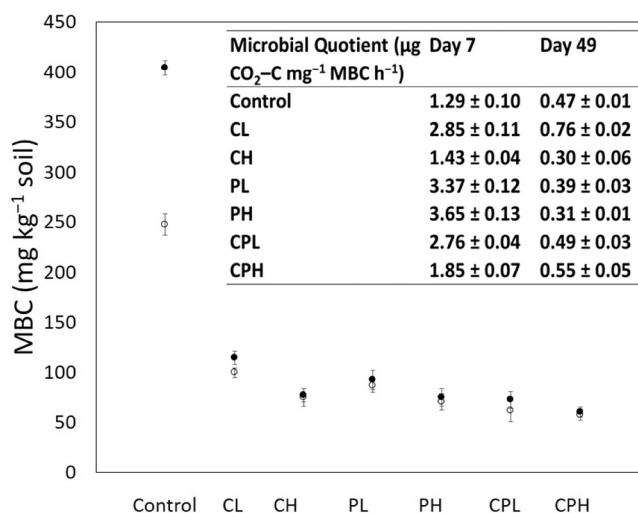


Fig. 3. Microbial biomass carbon and microbial quotient (inset table) in different treatment soils. Data are displayed as means, bars indicate SE (n = 3). Control indicated un-contaminated soils, CL and CH indicated the soils spiked with low or high Cd concentrations, PL and PH indicated the soils spiked with low or high Pb concentrations, CPL and CPH indicated the soils spiked with low or high Cd and Pb concentrations.

CO₂-C g⁻¹ soil) by the end of the incubation period.

3.2.2. Microbial biomass carbon and carbon use

Soil MBC was inhibited by the metal toxicity (Fig. 3). Values of MBC in contaminated soils were significantly lower than uncontaminated soil both on days 7 and 49. The MBC values in CL was 33% higher than CH, in PL was 23% higher than PH, and in CPL was 7% high than CPH after 7 days of incubation. The lowest MBC values were found in CPH (57.80 mg kg⁻¹ soil), followed by CPL (61.64 mg kg⁻¹ soil) and PH (70.48 mg kg⁻¹ soil). At the end of the incubation, compared to the uncontaminated soil, MBC values were 72, 81, 77, 81, 82 and 85% less in CL, CH, PL, PH, CPL and CPH, respectively. However, in general MBC values increased at the end of incubation compared to those on day 7. The largest increase was in the uncontaminated soil, from 247.40 to 403.86 mg kg⁻¹ soil. The differentiation of MBC is connected to microbial metabolism. Therefore, in order to understand microbial C process, we calculated the microbial qCO₂. In comparison to day 7, microbial qCO₂ values in all treatments decreased at the end of incubation (Fig. 3 inset). On day 7, the highest qCO₂ values were found in the PL and PH treatments, while the uncontaminated soil had the lowest qCO₂. Although the qCO₂ in PL and PH at day 7 were higher than the other treatments, the values dropped by the end of incubation. Despite the fact that Cd might exert a larger toxicity level due to its higher mobility (Neethu et al., 2015), microbial qCO₂ did not differ between single Cd and single Pb spiked soils in this study.

3.3. Microbial community composition

3.3.1. Total microbial PLFA

Microbial PLFAs were quantified to investigate specific microbial functional groups and investigate the shift of microbial community composition with heavy metal influences. After 7 days of incubation, total PLFA did but not differ from the uncontaminated soil (41.72 nmol g⁻¹ soil) than in the contaminated soils (37.97, 34.23, 35.63, 32.11, 30.69 and 27.84 nmol g⁻¹ soil in CL, CH, PL, PH, CPL and CPH, respectively) (SM. 3). By the end of incubation (49 days), the total PLFA in the uncontaminated soil was significantly larger than in the contaminated soils. Compared to the control at day 49, total PLFA decreased by 53, 48, 44, 42, 38 and 36% in CL, CH, PL, PH, CPL and CPH, respectively. However, total PLFA contents were not different among

Table 2
Spearman correlation coefficients ($n = 7$) among soil and microbial parameters as affected by heavy metals.

	G+/G-	B/F	Total PLFA	G+	G-	Fungi	MBC	TOC	TN
G+/G-	1	0.71	0.18	0.14	-0.75	-0.36	-0.18	0.50	-0.36
B/F		1	0.39	0.54	-0.39	-0.71	-0.39	-0.21	0.50
Total PLFA			1	0.93**	0.57	0.89**	0.99**	0.71	0.29
G+				1	0.54	0.89**	0.93**	0.68	0.04
G-					1	0.75	0.57	0.07	0.29
Fungi						1	0.89**	0.50	0.07
MBC							1	0.71	0.29
TOC								1	0.14
TN									1

Note: Abbreviation in table as: CL and CH indicate the soils spiked with low or high Cd concentration, PL and PH indicate the soils spiked with low or high Pb concentration, CPL and CPH indicate the soils spiked with low or high Cd and Pb concentration. G+ and G- as Gram positive and negative bacteria, respectively, B/F as ratio of bacterial and fungi, MBC as microbial biomass carbon, TOC as total organic carbon, TN as total nitrogen.

** Correlation is significant at 0.05 level.

the spiked soils. There was no significant ($p > 0.05$) correlation between specific microbial functional groups with soil organic C content based on Spearman correlation analysis (Table 2). However, G+ bacteria were significantly and positively correlated to both MBC and total PLFA.

3.3.2. Microbial community structure

Each specific microbial functional group abundance is presented in Table 3. After 49 days of incubation, the uncontaminated soil had the largest biomarker values (fungi: 5.10 nmol g^{-1} soil, actinomycetes: $104.92 \text{ nmol g}^{-1}$ soil, G+ bacteria: $89.44 \text{ nmol g}^{-1}$ soil, and G- bacteria: 1.65 nmol g^{-1} soil). On day 49, the smallest PLFA value was for fungi (1.42 nmol g^{-1}) in PH, while values of 31.60 and 0.54 nmol g^{-1} soil for G+ and G- bacteria, respectively, occurred for the CPH treatment. Cd–Pb co-contaminated soils had the lowest actinomycetes values too on day 49. The actinomycetes in uncontaminated soils over-numbered CPL and CPH by 65 and 62%, respectively. After 49 days, the microbial population increased, with the uncontaminated soils having the largest values overall either at the beginning or at the end of incubation. On day 7, microbial biomarker concentrations were 1.69 , 43.16 , 37.42 and 0.42 nmol g^{-1} soil for fungi, actinomycetes, G+ and G- bacteria, respectively. On day 7, the smallest fungi (1.10 nmol g^{-1} soil), G+ ($25.38 \text{ nmol g}^{-1}$ soil) and G- (0.25 nmol g^{-1} soil) populations were found in CPH. The ratio of G+/G- and bacteria/fungi (B/F) values both decreased at day 49 as compared to day 7 of the incubation.

Compared to uncontaminated soils, all microbial functional groups decreased in metal-polluted soils. However, the microbial populations

varied, which was shown by the ratio of G+/G- bacteria and B/F values. By the end of incubation (day 49), the largest G+/G- ratio was in PL (64.42), followed by PH and CPL, while the lowest ratio was in CH (36.87). In most cases, the B/F values were significantly increased under metal contamination as compared to the uncontaminated soil.

The principal component score plot based on PLFA biomarkers under metal stresses showed a distinguishable difference among treatments (Fig. 4). This indicated a shift in the microbial community structure that was strongly impacted by heavy metal treatments. In addition, certain biomarkers for bacteria and fungi were suppressed under metal-pollution (Fig. 4 and Table 3). On day 49, biomarkers for G+ and G- bacteria (aC17:0, iC17:0, aC15:0, iC16:0, C16:0, iC15:0, C15:0) and fungi (C18:1 ω 9) were generally positively positioned on PC1 and negatively on PC2 values, while actinomycetes biomarkers (10MeC16:0, 10MeC18:0 and 10MeC17:0) were positioned along the PC2 positive axis, especially 10MeC16:0 was remarkably positioned on the negative value on PC1 (Fig. 4a). This indicated that soil microbial populations were being driven more by G+ and G- negative bacteria than fungi and actinomycetes at the end of incubation. However, at the beginning of the incubation, microbial signature profiles showed significantly different patterns than on day 49 (Fig. 4b). Still, positive values of PC1 were generally assigned to bacteria. The most prominently difference was observed for G+ bacteria bio-signature iC17:0 and fungi bio-signature C18:1 ω 9. They were PC1 positive and PC2 negative on day 49, but PC1 negative and PC2 positive on day 7. This suggested the negative effect of heavy metals on microbial populations. The reduction of fungal and G+ bacterial PLFAs contributed to the

Table 3
Comparison of Gram-positive bacteria (G+ bacteria), Gram-negative bacteria (G- bacteria), fungi and actinomycetes as obtained through respective PLFA profiles (nmol g^{-1} soil). B/F is the ratio between bacterial and fungal PLFA values. Means \pm SE ($n = 3$).

Sample	G+ bacteria	G- bacteria	Fungi	Actinomycetes	G+/G- bacteria	B/F
Day 49						
Control	89.44 ± 5.51	1.65 ± 0.09	5.10 ± 0.25	104.92 ± 6.46	54.19 ± 3.40	17.87 ± 1.01
CL	43.15 ± 2.39	0.83 ± 0.06	2.15 ± 0.09	49.08 ± 2.18	51.81 ± 2.01	20.44 ± 1.21
CH	39.14 ± 3.69	1.06 ± 0.08	1.86 ± 0.24	45.55 ± 2.39	36.87 ± 2.39	21.60 ± 0.71
PL	37.73 ± 3.06	0.59 ± 0.04	1.87 ± 0.26	43.06 ± 3.85	64.42 ± 3.85	20.51 ± 0.54
PH	33.75 ± 2.39	0.56 ± 0.04	1.42 ± 0.17	38.74 ± 3.02	60.22 ± 3.02	24.14 ± 0.67
CPL	34.37 ± 2.36	0.57 ± 0.06	1.69 ± 0.16	39.38 ± 2.15	60.76 ± 2.15	20.64 ± 0.67
CPH	31.60 ± 2.74	0.54 ± 0.06	1.59 ± 0.16	36.70 ± 2.44	58.70 ± 2.44	20.27 ± 0.81
Day 7						
Control	37.42 ± 1.54	0.42 ± 0.02	1.69 ± 0.11	43.16 ± 2.37	89.97 ± 5.50	22.44 ± 2.12
CL	34.42 ± 1.52	0.34 ± 0.04	1.60 ± 0.15	38.96 ± 4.21	101.22 ± 3.35	21.77 ± 1.53
CH	30.97 ± 1.23	0.31 ± 0.02	1.19 ± 0.05	35.40 ± 1.34	100.65 ± 5.49	26.28 ± 1.69
PL	32.15 ± 1.20	0.32 ± 0.01	1.53 ± 0.05	36.64 ± 1.69	100.64 ± 7.02	21.23 ± 1.56
PH	29.13 ± 1.58	0.32 ± 0.04	1.18 ± 0.10	33.04 ± 1.77	90.77 ± 3.73	24.94 ± 1.40
CPL	27.65 ± 2.76	0.35 ± 0.03	1.23 ± 0.07	31.59 ± 2.26	79.03 ± 8.73	22.67 ± 1.90
CPH	25.38 ± 2.60	0.25 ± 0.03	1.10 ± 0.06	28.47 ± 1.61	103.18 ± 7.64	23.26 ± 0.51

Note: Abbreviation in table as: CL and CH indicate the soils spiked with low or high Cd concentration, PL and PH indicate the soils spiked with low or high Pb concentration, CPL and CPH indicate the soils spiked with low or high Cd and Pb concentrations.

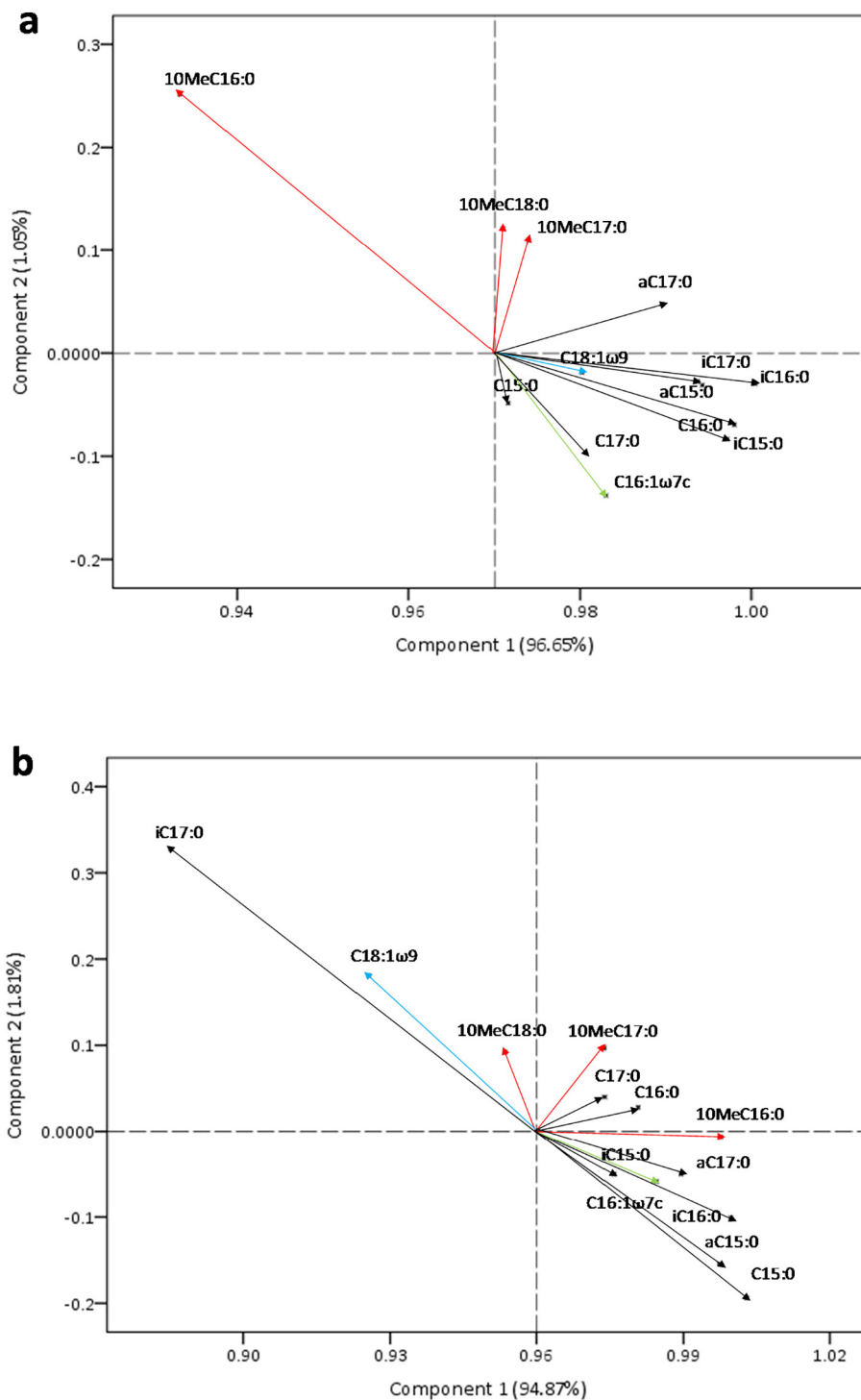


Fig. 4. Plot of principal component analysis (PCA) showing individual PLFA loading score variation based on phospholipid fatty acid (PLFA) patterns after 49 (a) and 7 days (b) of incubation. Arrows with different colours indicate biomarkers for Gram+ (black), Gram- (green), Fungi (blue) and Actinomycetes (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

overall microbial community shift in the metal contaminated soils. In addition, the biomarkers of G- bacteria and actinomycetes did not show a significant change.

4. Discussion

4.1. Soil conditions and metal bio-availability during incubation

Metals such as Cd and Pb are preferentially absorbed via passive

uptake as they are not essential elements to organisms. This type of metal bio-availability is more dependent on chemical (diffusion or mass flow) than physiological processes (Kim et al., 2015). There was no difference between Cd and Pb bio-availabilities at the end of incubation based on the two-factor ANOVA analysis. However, Cd²⁺ generally showed less potential availability in comparison with Pb²⁺ in the soil solution. Soil pH is a critical factor in determining metal speciation and mobility in soils (Bolan et al., 2014; Ok et al., 2011a). With increased soil pH, the heavy metal bio-availability was reduced (Houben et al.,

2013). However, Lenart and Wolny-Kołodka (2013) demonstrated that pH differentiation had limited effects on microbial abundance in heavy metal contaminated soils. While Chodak et al. (2013) argued that soil pH was the determining factor for soil bacterial community composition.

There were conflicting reports on soil pH changes with organic amendment addition/decomposition in soils. The plausible reasons for soil pH increase in the initial period of incubation in this study are: (i) proton consumption and adsorption onto exchange sites due to organic anion oxidation, (ii) production of NH_3 and basic cations due to organic matter decomposition, (iii) replacement of hydroxyl ions by organic anions on sesquioxide surfaces, and (iv) increased microbial biomass leading to the development of reducing conditions (Noble et al., 1996; Yan et al., 1996; Pocknee and Sumner, 1997). Towards the end of the incubation, Na^+ in the slightly acidic Sodosol were likely replaced by H^+ in the clay complexes creating an increase of exchangeable acidity (Noble and Randali, 1999).

Previous research demonstrated that soil EC affected metal bio-availability (Farrell et al., 2010; Salimi et al., 2012). Therefore, soil EC may alter both the bioremediation and phytoremediation in contaminated soil. Because the concentration of Ca, Mg and Na are the main factors that affects soil EC, the two metals in this research did not lead to significant differences in soil EC values. The differences between potentially available Cd and Pb contents in soils could be attributed to their relative mobility in soils. Generally, the relative mobility of Cd in soil solution is higher than Pb (Kim et al., 2015; Ok et al., 2011a, 2011b). In the current study, the potential availability of metals was significantly affected by the spiked metal concentrations, which consequently affected the soil microbial activities. Similar conclusions were drawn by Khan et al. (2010). The metal toxicity mechanisms mainly include protein denaturation, cell membrane disruption, inhibition of cell division, DNA transcription and enzyme activity (Gupta et al., 2016; Roane et al., 2015). Therefore, metal charges and/or valence might have a larger impact in terms of the ion replacing/binding potential on microbial cell membranes/biomolecules as compared to metal types (Roane et al., 2015). Bio-available metal concentrations were significantly higher in co-contaminated soils than single-metal contaminated soils. In spite of the possibility that there was relatively higher metal concentration in co-contaminated soils than single-metal contaminated soil, previous research proved that soil organic matter could serve as adsorbents to reduce metal bio-availability and mobility (Bolan et al., 2014). So the competition among metals for the adsorption sites could result in the greater metal bio-availability in co-polluted soils than a singly metal-contaminated soil.

In a moderately acidic soil as used in this study, metal hydroxides (M-OH^+) can be formed via covalent bonding. This indicated that the mobility of Cd and Pb based on metal charges and/or valence are associated with organic matter, including microbial biomass (Kim et al., 2015). Soil organic matter and/or soil clays could serve as negatively charged sorption sites for cations like Cd^{2+} and Pb^{2+} (Seshadri et al., 2017). The differential metal availability under sole or conjoint metal contaminations could be ascribed to the competition among metal ions for the immobilization sites (Ming et al., 2016; Seshadri et al., 2017). In addition, soil TOC is highly mediated by active microorganisms and their role as organic decomposer (Blagodatskaya and Kuzyakov, 2013). Chen et al. (2014) suggested that the metal bio-availability could potentially modulate soil biochemical processes, and the metal concentrations in 0.01 M CaCl_2 extraction solution are highly related to soil biochemical reactions (Ok et al., 2011b). Bio-available metals might influence microbial activity and C utilization patterns (Wang et al., 2007), and consequently affect the metal-organic complex formation and metal release in turn.

The bio-available metal concentration decreased at the end of the incubation compared to day 7 (Fig. 1). Lu et al. (2005) investigated the residence time effects on soil metal transformation and found that the bio-availability of metals decreased with residence time. The binding

between organic matter and metals may contribute to the bio-availability reduction. In the current study, there was significant negative impact of metal concentration on microbial abundance and MBC formation (Table 3). But, the reduced bio-availability might lead to less toxicity by the end of the incubation period. As a result, the microbial abundance increased on day 49. Therefore, the toxicity had negative influences on soil living microorganisms, and consequently affected the soil C dynamics.

4.2. Microbial carbon use under metal stress

Due to metal toxicity, it is not surprising to see suppressed microbial activity due to metal pollution. The suppression was not only in the form of total released $\text{CO}_2\text{-C}$, but also in the form of a reduced microbial respiration rate (Fig. 2). The inhibition of respiration could be ascribed to the metal negative influences on microbial enzymatic processes, cell functions and nuclei formation (Jiang et al., 2010; Tchounwou et al., 2012). As a result, soil microorganisms might show metabolic dysfunction or community alteration, resulting in lower microbial reproduction but relatively higher energy consumption (Gupta and Diwan, 2017). The microbial respiration was apparently suppressed in the current study, which suggested that metals were instantly inhibitory to microbial bio-processes. Therefore, metals imperil soil microbial population, leading to dysfunction and lower activity (Jiang et al., 2010).

Interestingly, some research found increased microbial activity under metal stress (Markowicz et al., 2016; Shi and Ma, 2017). This might be related to the energy re-location from MBC production to cell maintenance when microorganisms were facing stress (Killham, 1985), leading to an elevated energy demand of microbial functional groups in order to survive under undesirable living conditions (Lu et al., 2013; Zhang et al., 2010). In addition, some microbial functional groups might show tolerance to heavy metals (Fließbach et al., 1994; Giller et al., 2009).

Soil MBC is an essential part of soil organic C pool which simultaneously regulates soil nutrient transformation and C flow (Dai et al., 2004). As found in the current study, compared to the prompt response of microbial activity (respiration) to metals, changes of MBC might be slower (Knight et al., 1997). Similar effects can also be expected for microbial functional group based C use pattern changes.

In the comparison of soils without metal pollution, the low value of the ratio of MBC to soil organic C in CPH could be ascribed to the decrease of the formation of MBC. Khan et al. (2010) and Zhang et al. (2008) also noticed that MBC decreased significantly with increasing metal concentrations. This indicated that the MBC was repressed in metal polluted soils. In addition, there was no significant ($p > 0.05$) correlation between TOC or TN contents and MBC in this study (Table 2). The connection between soil organic C and MBC was most likely constrained by the toxic stress of heavy metal in the soil.

Heavy metal toxicity evolves due to displacement or substitution of essential elements by toxic metals either in the extracellular enzymes or in nuclear proteins of microorganisms, damaging cell membrane or DNA structure (Choppala et al., 2014; Tchounwou et al., 2012). The formation of MBC was suppressed due to the presence of excess metals in the contaminated soils, impeding the microbial population to multiply. However, this would not mean less organic C mineralization, rather, an increased $\text{CO}_2\text{-C}$ release per unit of MBC might be observed. The current research found higher cost of maintenance (higher $q\text{CO}_2$) in polluted soils, indicating an inhibitory influence of heavy metals on MBC efficiency while unitizing TOC (Tripathy et al., 2014). The increase in microbial $q\text{CO}_2$ indicated that soil microorganisms demanded increased energy for repair and maintenance under the metals stress (Fließbach et al., 1994).

On the other hand, microbial processes, including metal bio-leaching, biosorption and bio-precipitation, might also contribute to metal immobilization and bio-translocation (Gadd, 2004). Certain

microorganisms might develop adaptation and tolerance to metal toxicity by altering their C use preference in the long term (Khan et al., 2010; Zhang et al., 2010). In treatment CPL and CPH, the microbial qCO_2 values increased at day 49 compared to day 7, implying the trade-offs between metal impacts and microbial substrate uses.

4.3. Microbial community profiles

In this study, MBC was significantly correlated with microbial total PLFA (Table 2) because both of these parameters were a reflection of the total microbial abundance (Bailey et al., 2002; Frostegård et al., 2011; García-Orenes et al., 2013). Wang et al. (2013) reported that the G+/G- bacterial ratio was significantly positively correlated to the annual soil respiration rate, while the G+ bacteria were significantly negatively correlated to the same. Therefore, the G- bacterial population might contribute to a large proportion of soil microbial respiration compared to other microbial functional groups. In the current study, the correlation between TOC and specific microbial populations was insignificant (Table 2). However, Kallenbach et al. (2016) demonstrated a positive relation between C use efficiency and fungal population size. We also found the reduced fungi abundance in metal-polluted soil, which also had the highest microbial qCO_2 . Together with the common assumption of fungi have higher C use efficiency than bacteria, our results demonstrated by altering microbial functional groups, the metal pollution subsequently changes microbial community C use patterns. Therefore, the microbial communities ascribed to C dynamics were true to some extent.

Depending on metal types and concentration, metals could have inhibitory or even toxic effect on soil microbial populations (Mudhoo and Kumar, 2013). Microbial PLFA fingerprints provided the alteration of microbial biomarkers (Fig. 4) and the impact on microbial community (Fig. 5) due to metal stresses. The total microbial PLFA decreased in heavy metal contaminated soils, and the CPH had more negative influences compared to un-contaminated and singly polluted soils (Table 3). Some microbial functional groups might have higher metal tolerance, and consequently grow better than other functional groups, leading to microbial species replacement or diversity loss (Chodak et al., 2013; Tipayno et al., 2018). Bacteria and fungi comprise the majority of soil MBC and have important functional roles in soil organic C degradation (Rinnan and Bååth, 2009; Six et al., 2006). In the current

study, fungal population was significantly suppressed in metal-polluted soils (Table 3). These results confirmed that fungi had more sensibility under metal stress than bacteria (Liu et al., 2012). On the other hand, bacterial population showed higher tolerance to metals than fungi.

The shifts in microbial PLFA compositions might regulate the C use patterns (Liu et al., 2012; Schimel et al., 2007). Compared to bacteria, fungi have a lower C/N component and C turnover rate (Six et al., 2006). Hui et al. (2012) found no effect of Pb on bacterial richness and diversity in a boreal forest soil. They suggested that bacteria showed a higher capability for avoiding Pb toxicity than fungi. A shift of fungal and bacteria abundances could lead to C use differentiation in the perspective of whole microbial community (Liu et al., 2012). In the current research, fungal population and fungal PLFAs showed a greater decrease under metal contaminated soils than bacteria. Consequently, the microbial C use patterns and microbial C storage performances in soils were also shifted.

The variation of microbial PLFA profiles based on PCA indicated the metal influence on certain biomarkers. Microbial PLFAs spread with the two principal components in Fig. 4, which demonstrated that 10MeC16:0 showed significantly different response compared to other PLFAs. Earlier studies demonstrated that the common biomarkers negatively correlated with metals were: 16:1 ω 5c, 17:1 ω 8c, 18:1 ω 9c, 18:1 ω 6c, i14:0, i18:0, 18:0 3OH, 16:0 10Me, 18:0 10Me, a15:1, i16:1, and 18Me18:1 ω 7c (Hinojosa et al., 2004; Igalavithana et al., 2017b). In the current research, the most affected PLFA biomarkers were: C18:1 ω 9, iC17:0, C17:0, aC17:0, C16:0 and 10MeC16:0. However, even with the PCA analysis, it was not conclusive whether one single environmental factor was responsible for the effect because soil physico-chemical properties are highly inter-related (Fernández-Calviño et al., 2010). For instance, Chodak et al. (2013) suggested that soil pH was the dominant influence on microbial community composition, while the toxic influence of metal was relatively low.

The loading plot demonstrated the effect of metal pollution on microbial community structure (Fig. 4). The un-contaminated soils were discrete from those of contaminated soils. A higher metal concentration had more suppressive influence on microbial PLFAs, leading to a smaller microbial population (Khan et al., 2010). The co-pollution of Cd and Pb could have a greater negative influence on microbial enzymatic activities (Khan et al., 2010). Caliz et al. (2012) suggested that toxicity of Cd to soil microbiota was slightly higher than Pb. The current study also showed that the combination of Cd and Pb contaminated soil had more influence on microbial profiles, which might be due to the fact that co-contaminated soils had a larger metal concentration than the singly metal polluted soils.

5. Conclusions

The determination of microbial community compositions by assaying biomarkers, such as microbial PLFAs, was able to address the different responses of individual functional groups targeting certain metal stresses. The current study demonstrated that not only microbial population varied due to metal contamination, microbial C use were suppressed in soils because of increased energy requirement under metal stresses. Microbial functional groups also varied with different metal concentrations and types. Noteworthy, heavy metal toxicity had a greater negative influence on fungal population than other microbial functional groups, leading to a reduced F/B ratio. Our results indicate that the soil microbial communities had adapted to the heavy metals in the soils, as evidenced by the shifting of functional groups. Metal toxicity to microbial C use preferences and microbiota abundances were more affected by metal concentrations than metal type, while microbial PLFA biomarkers and community populations were influenced both by the metal concentration and metal type. Results of this study have implication in the assessment of phyto- or microbial remediation performance or microbial C use alteration potential at a metal contaminated site, and thus to improve the quality of human health by

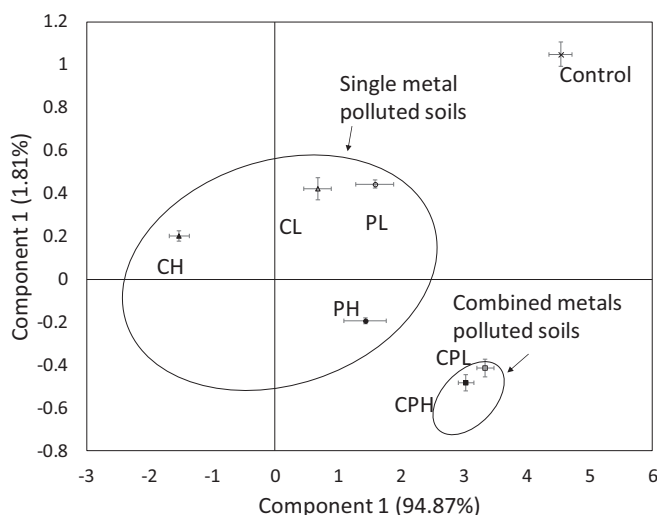


Fig. 5. Principal component (PC) score plot based on PLFA biomarkers profiling soil microbial community composition under metal stress at the end of incubation. Control indicated un-contaminated soils, CL and CH indicated the soils spiked with low or high Cd concentrations, PL and PH indicated the soils spiked with low or high Pb concentrations, CPL and CPH indicated the soils spiked with low or high Cd and Pb concentrations.

reducing the chances of heavy metal's entry into the food chain. However, the soil sample used in this research was homogenized before conducting the chemically and microbiology measurements. It should be noted this might lead to loss of ecologically information and the interpretation of in situ remediation. Also, future research should investigate the different microbial responses due to incubation length, especially include the possibility of microbial adaptation due to community shifts among different functional groups.

Acknowledgements

Yilu Xu is thankful to the University of Newcastle and Department of Education and Training, Government of Australia, for awarding her PhD Scholarship. This research was partly supported by an Australian Research Council Discovery–Project (DP140100323).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2019.01.071>.

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