A Study on Microbial Carbon Use Efficiency in Soil

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Xu, Y., Bolan, N.S., Seshadri, B., Farrell, M. 2016. Microbial carbon use efficiency (CUE) in heavy metal contaminated soil. Best Practice Ecological Rehabilitation of Mined Lands 2016 Conference. (**Best student poster winner**)

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ABSTRACT

Soil organic carbon (SOC) plays a critical role in soil health and also in maintaining its ecological service. The stabilization of SOC involves physical, chemical, and biological processes in soil. Soil microorganisms serve as a carbon (C) biological sink as well as biochemical agents in C transformation in soil. The plant litter inputs and root exudates provide microorganisms with both labile and recalcitrant C sources. The C availability and soil habitat environment alter microbiota, consequently impacting the organic C decomposition processes in soil. Anthropogenic disturbances such as organic amendments, contaminants, tillage and grazing practices impact soil 'biophysicochemical' properties. The addition of organic C sources such as manure composts and biochar can lead to processes such as priming effect and microbial population shifts. In metal contaminated soils, organic-metal bonding can be beneficial to the immobilization of heavy metals, thereby reducing their bioavailability and biotoxicity. Microorganisms also develop strategies for the purpose to adapt to soil environment stress conditions. These stress tolerance processes include alteration of microbial community composition, and the redistribution of energy between catabolism (respired CO_2) and anabolism (biomass C).

Although a number of studies have examined soil C biogeochemical dynamics, very few comprehensive studies have been reported on the role of soil microorganisms in relation to the mobilization and immobilization ((im)mobilization) processes of organic C dynamics. In this research, soil microbial function and community composition in relation to C dynamics as affected by environmental factors were investigated. The definition of 'microbial carbon use efficiency' (CUE) was introduced for the purpose of assessing the fraction of microbially decomposed organic C that is subsequently assimilated into microbial biomass. The specific objectives of this research include: (i) to determine microbial CUE involving different approaches in relation with various sources of C and nitrogen (N) inputs; (ii) to investigate the influence of land use practices on soil microbial functions in relation to CUE; (iii) to evaluate metal stress on microbial function in relation to CUE; and (iv) to examine the influence of biochar on metal toxicity in relation to microbial CUE.

The first experiment was aimed to compare four approaches to measure microbial CUE using isotopic labelled glucose as an organic C source. The first approach (C_s) for microbial CUE measurement was based on monitoring C depletion, while the second (C_m) and third (C_p) approaches were based on detecting of microbial biomass accumulation, the forth approach (C_r) was aimed at calculating the ratio of the increased microbial biomass to the decreased C content. The microbial CUE values varied amongst the four approaches, and the C_m values

were generally higher than other measurements. Because the main aims of the subsequent experiments were to understand the microbial mediation of soil C and the accumulation of C in microbial community, the microbial CUE measurement based on the accumulation of microbial biomass C (C_m) was used in the remaining chapters. In the first experiment, the ^{13}C labelled glucose was evenly applied to soils to trace the C flow as measured by the release of CO_2, C incorporation into microbial biomass, and C remaining as undecomposed C input. Microbial phospholipid fatty acids (PLFAs) were extracted and analysed as biomarkers in order to identify the microbial community composition. Results revealed that organic amendment coupled with mineral N [$(NH_4)_2SO_4$] stimulated both microbial activity and biomass, leading to a positive priming effect (PE). However, as different C:N ratios were introduced in this experiment, the PE intensity stimulated by different exogenous C and mineral N sources showed variation amongst C sources, similar to microbial CUE values as determined by above approaches. The labile C source (glucose) with low N contributed to relatively higher microbial PE. Microbial community varied with C input sources, the readily available C source (glucose) favoured bacteria community growth over fungi, while fungi population increased with mineral N application. In conclusion, microbial CUE measurements are related to the methods and parameters used, and the C use preference and community composition are highly dependent on the exogenous C and mineral sources.

Based on the microbial CUE measurement results of the first experiment, the second experiment used soils from three land use systems: cropping, pasture and natural forest soil. Three types of organic amendments were introduced: glucose as a labile C source, and wheat straw and macadamia nutshell biochar as a relatively recalcitrant C material. Microbial biomass C, and basal and substrate-induced respiration were measured to determine microbial CUE. Microbial community composition was determined based on the measurement of PLFAs. Land use history generally affects soil physiochemical and microbial properties. The natural forest soil had the highest organic C content while having relatively low soil nutrient contents. Because of constant disturbance and management, cropping soil had relatively lower values in microbial activity and biomass. Although there were no significant differences of microbial CUE values in soils from different land systems, the organic amendments lead to distinct microbial CUE values. Therefore, the exogenous C source applied to cropping land during cultivation played a more important role in terms of microbial C use preference. Glucose input significantly ($p > 0.05$) increased microbial respiration with less biomass formation, thereby resulting in a decrease in microbial CUE, while wheat straw and biochar inputs increased microbial CUE compared to glucose. However, microbial community composition differed among land use systems. Fungi was dominant in natural forest soil while bacteria population was larger in cropping and pasture soils. The type of organic amendment inputs

also altered microbial community composition. The addition of an easily degradable C source such as glucose stimulated a growth in Gram-positive bacteria, while biochar input favoured fungi population growth.

The biotoxicity of heavy metal(loid)s was evaluated by monitoring microbial CUE and community composition in soil samples spiked with Cd(II) and Pb(II), both individually and in combination. The bioavailable metal concentrations, soil properties, and microbial parameters including microbial respiration, biomass and microbial PLFAs were determined at two sampling periods during the 49 days incubation experiments. Microbial CUE was determined as the ratio of accumulated biomass to decomposed C amount. Metal contamination had no significant effect on ($p > 0.05$) on soil properties such as pH and EC, while significantly ($p < 0.05$) inhibiting microbial activity and biomass formation. Notably, the microbial CUE decreased due to metal contamination, and the higher heavy metal concentration lead to lower microbial CUE values. Both total PLFAs and PLFA diversity decreased under metal stress. The microbial community composition and PLFA patterns also differed among treatments. Heavy metal pollution had greater negative influences on fungi population compared to bacteria. This might result in a vulnerable soil ecosystem with less resilience ability.

Based on the third experiment, biochar was introduced as an effective method for the remediation of metal contaminated soils. In this fourth experiment, Cd and Pb spiked soils treated with macadamia nutshell biochar (5% *w/w*) were monitored during a 49 days incubation period. Soil properties, metal bioavailability, microbial respiration, and microbial biomass C were measured after the incubation period. Microbial CUE was calculated from the ratio of C incorporated into microbial biomass to the C mineralised. Microbial community composition was determined by measuring microbial PLFAs. Results showed that total PLFA concentration decreased to a greater extent in metal contaminated soils than uncontaminated soils. Microbial CUE also decreased due to metal toxicity. However, biochar addition alleviated the metal toxicity, and increased total PLFA concentration. Both microbial respiration and biomass C increased due to biochar application, and CUE was significantly ($p < 0.01$) higher in biochar treated soils than untreated soils. Heavy metals reduced the microbial C sequestration in contaminated soils by negatively influencing the CUE. The improvement of CUE through biochar addition in the contaminated soils could be attributed to the decrease in metal bioavailability, thereby mitigating the biotoxicity to soil microorganisms.

In conclusion, microbial properties are essential indicators in the determination of soil health. The microbial CUE values vary depending on the measurement adopted. As such, there is a need for a comprehensive conceptual understanding and unified method of determination of microbial CUE. For the purpose of this research, the microbial CUE measured based on the accumulation of microbial biomass was more appropriate to examine microbial function in

terms of microbial C utilization. Land use histories, organic amendments and environmental factor all alter the direction and dimension of microbial CUE, as well altering the microbial community composition. Especially certain microbial species such as bacteria and fungi could reveal soil functional status because of the difference in C use and allocation preference among these communities. Biochar could be beneficial to microbiota under metal stress, not only because of its high C content, but also because of its remediation ability as metal sorbents.

DECLARATION

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision.

The thesis contains published scholarly work of which I am a co-author. As the author of this Elsevier article, I retain the right to include it in a thesis or dissertation, provided it is not published commercially. The article in Science of the Total Environment Journal is the original source.

The thesis contains no other material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

Yilu Xu

Signed___ ____ Date___03/03/2018_____

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Chapter 1

INTRODUCTION

1.1 Biogeochemical carbon dynamics and soil as carbon reservoir

In terms of global scale, Carbon (C) cycle refers to biogeochemical processes of C transformation between gas phase (carbon dioxide, CO_2) and solid phase (organic or inorganic C) in several reservoirs (e.g., atmosphere, pedosphere, geosphere and biosphere reservoirs). The increase of greenhouse gas (GHG) emission, including CO_2 and CH_4, expedites the speed of global warming. The global average surface temperature has increased by 0.6 °C over the 20th century (Change, 2001). If a reservoir attributes to C sequestration, that is to say the atmosphere CO_2 is captured and stored with a relatively long mean residue time (MRT), and the amount of C input is larger than C release. Up to 1 m depth, global soil organic carbon (SOC) pool is estimated to be 1,550 Gt with the total C pool including inorganic and dissolved C at 2,300 Gt (Lal, 2004). As soil organic matter consists of *ca*. 50% organic matter (Toor and Shober, 2015), increasing SOC storage supports plants and soil fauna as an important nutrient source. Additionally, SOC also helps in maintaining ecosystem services, such as improving water hold capacity, enhancing soil resilience, mitigating climate change (Lal, 2014; Paterson et al., 2009) and preventing soil from erosion (Hartemink et al., 2014; Lal, 2014; Larsen et al., 2014).

There are three major sources for C inputs into soil system: (i) above-ground plant litter and its leachates (i.e., dissolved organic C washed by infiltrating rainfall), (ii) below-ground root exudates (collectively known as rhizodeposition), and (iii) dead roots (Gougoulias et al., 2014). The fate of C after entering terrestrial ecosystem can be totally different, it can be transferred into organo-mineral complexes and humic substances or incorporated into microbial biomass (Sulman et al., 2014). In a broad scale perspective, plant photosynthesis and soil respiration (root autotrophic respiration and heterotrophic microbial respiration) are the two major parts regulating terrestrial C balance (Foley et al., 2003). Ultimately, enhancing soil C pool stems from either increasing organic C inputs with a reduction in the breakdown of soil organic matter (Luo et al., 2003) or maintaining a longer MRT (Cheng et al., 2007; Jastrow et al., 2007). Actually, SOC is a continuum of C flow among a series of discrete C pools ranging from labile

to recalcitrant C pool. Although C turnover rate depends on several soil physico-chemical factors, such as organic material characterizations and soil mineral adsorption, the driving force is soil microbiota (Kuzyakov et al., 2003).

1.2 Soil carbon reservoir mediated by microorganisms

Terrestrial C content is determined by soil physical (i.e., aggregation, illuviation and erosion), chemical (i.e., flocculation, organo-mineral complexation and nutrient depletion) and biological processes (i.e., humification, biological assimilation and respiration) (Lal, 2004). However, these processes among different C forms are difficult to be distinguished or separated very clearly. A promising way to understand the mechanism of soil C dynamic is to evaluate soil microbiota, because it is well acknowledged that microorganisms are the key driving force in determining C biogeochemical dynamics (Hartemink et al., 2014; Xu et al., 2017a). To a certain extent, soil microorganisms are one of the major immobilizers of SOC by incorporating C into microbial biomass carbon (MBC) (O'Donnell, et al., 2001), while also contributing to a large part of soil respiration. Yet, there are many unsolved questions about soil microorganisms as major driving force of regulating C content. Globally, about 75 Pg C per year are released into atmosphere by soil respiration (Schlesinger et al., 2000). The upper 30 cm consists of about 50% of the total SOC located in the 0–100 cm soil profile (Batjes, 2014). Thus, most research about soil microorganisms in relation to C dynamics focus on up to 30 cm depth soil profile. Because of the discrepancy in natural processes, such as the quality and quantity of litter inputs and root exudates, soil microbial C use preference will be altered, and consequently affects soil C dynamics. Priming effect (PE) is the altered microbial metabolism of soil's indigenous C as a result of the input of an exogenous C source (Kuzyakov et al. 2000). The results could be positive, indicating more soil C storage was decomposed and released as CO_2, or negative, which may be due to the microbial properties and population shift. For example, the ratio of fungi and bacteria can affect soil C allocation because of their difference in C use patterns. Although there are models revealing that the shift of microbial community and activity alters soil C flow (Allison et al., 2010; Lipson et al., 2009), the crucial function of soil heterotrophic microorganisms, especially in regulating C flow is less clear. And also, a contentious debate is on how to determine the effect of soil microorganisms at the level up to ecosystem scale (Chapin et al., 1995). In conclusion, the essential role of microorganisms has been underestimated and only a limited number of studies have addressed the microbial feature regarding C dynamics and cycling in soil-plant-atmosphere continuum.

Besides the fact that microorganisms are the regulators of soil C flow and transformation, they also are responders to soil environmental conditions (Singh et al., 2010). Thus, soil microbiota as well as earthworms are the driving factors behind SOC decomposition and a sensitive indicator of soil health and quality (Lal, 2015).

When interpreting microorganism related C dynamics, the generally accepted and widely used relevant parameters include enzymatic activities, basal and induced respiration, MBC and microbial community composition (Lal, 2015). Most of the heterotrophic microorganisms are responsible for soil CO_2 emission. For example, when soil is subject to biological decomposition, it tends to become a net C source of GHG emissions (Lal, 2015). So examining soil microbiota is a promising way to assess soil health and fertility. Lal (2015) proposed that improving microbial activity and species diversity is essential to restore and improve soil quality, reducing risks of soil degradation, and mitigating the depletion of SOC and CO_2 release. But Nannipieri et al. (2003) argued that due to functional redundancy, the effect of microbial community diversity on C decomposition is questionable. Modern molecular technology and interdisciplinary approach are being used to explain particular microbial taxa in relation with soil environment variables. Singh et al. (2010) suggested the up-to-date supports from molecular techniques and bio-chemical tools contribute to a better understanding of the physiology and dynamics of microbiota. The information provided by modern technology is likely to increase our knowledge about the processes occurring inside the 'black box', i.e., the microbial functions in relation to C dynamics in soil. In studies examining the broad/large microbial groups, techniques such as cultivation (plating) and quantifying phospholipid fatty acid (PLFA) are used (Torsvik and Øvreås, 2002). Polymerase chain reaction (PCR)/quantitative polymerase chain reaction (qPCR), and fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), amplified rDNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP) and ribosomal intergenic spacer analysis (RISA) can also provide microbial structure and diversity information. The advantage of using a combination of the techniques is that it will provide an overview of the dominant soil microbiota and their functions. However, some microbial species may not be able to be accessed and detected (van Elsas and Boersma, 2011). Other techniques such as stable isotope probing and Bromodeoxyuridine(brdU) methods are suitable for detecting active communities *in situ* and microarrays can provide information on diversity at phylogenetic or functional levels (van Elsas and Boersma, 2011).

Hartemink et al. (2014) proposed C sequestration enhancement by improving carbon use efficiency (CUE) of organic matter input to soils. Microbial CUE is the ratio of soil C assimilated that is allocated in microbial biomass over uptake. Because the ratio represents the amount of C stored in microbial, therefore it can be used as an indicator of soil quality and also as an

indicator of net C sequestration through microbial processes (Rousk and Bååth, 2011; Sinsabaugh and Follstad Shah, 2012). So, with a higher microbial CUE value, the microbial anabolism will be greater, leading to more C retention. While a lower CUE value indicates higher microbial catabolism, resulting in the release of more soil C as CO_2. However, it is important to realize that higher C utilization of recalcitrant C by microorganisms can lead to increased release of CO_2. There have been a longer history and more experimental measurements on CUE of aquatic systems compared to soils and decomposing plant residues (Manzoni et al. 2012). Several approaches have been used to measure microbial CUE, resulting in variable results (Devêvre and Horwáth, 2000; Tiemann and Billings, 2011). However, there has been only limited research on the understanding of microbial regulation of soil C, especially in relation to microbial CUE, and subsequent sequestration of C and release of CO_2.

Soil provides a vital array of organic macromolecules (e.g., carbohydrates such as tannin and humus, protein and lipid), oxygen and water (Condron et al., 2010; Luis et al., 2005) to microbial activity and function. The variation of soil environment and stress is likely to alter soil microbiota diversity and function. Soil properties such as soil structure and cation exchange capacity (CEC) reflect soil aggregation, surface charge and bonding to nutrient ions and organic C (Tisdall, 1996). The elevated temperature resulting from greenhouse gas induced climate change can lead to more plant primary productivity and higher microbial activity. For example, models from Allison et al. (2010) and Carney et al. (2007), proved the direct interactions between microbial physiology and community composition and global warming (Singh et al., 2010). Other models revealed that the stimulation of soil CO_2 efflux in the early stage is only transitory due to the depletion of labile C pool (Eliasson et al., 2005; Kirschbaum, 2004). While Dinh et al. (2014) and Zogg et al. (1997) found microbial community shifts due to higher soil temperature, more research is required to demonstrate the direct linkages between microbial community composition and ecosystem processes. In short term, environmental stress such as drought is likely to cause changes in microbial physiological adaptation in terms of biomass and activity (Fterich et al., 2014; Lal, 2015), followed by microbial community shift (Schimel et al., 2007; Xu et al., 2017c). Last but not least, Singh et al. (2010) also suggested the need to consider the influence of abiotic and biotic factors on microbial processes controlling SOC.

1.3 Carbon sequestration in various land use systems

Land use alteration has been shown to influence soil condition, and consequently affecting soil microbiota and C use. During the past decades, a considerable body of information has

been accumulated on land use systems in relation to soil C storage (Gelaw et al., 2014; Sharma et al., 2014). For example, over-disturbed soil through cultivation and deforestation activities has been a major cause of terrestrial C depletion, and soil C stock can be increased through land-use change (Lal, 2004). Also, a long term monitoring over 26 years in Scotland demonstrated that renewed afforestation benefits C sequestration (Hargreaves et al., 2003). The anthropogenic management practices such as fertilization and deep plough in cropping land, are known to accelerate organic matter decomposition and C depletion (Singh et al., 2010). As for pastureland, the grazing intensity is a major factor which determines soil C losses (Dawson and Smith, 2007). There has been a debate and uncertainty on the role of microbial modulation of SOC in different land use systems, especially at global scale. In fact, microorganisms transform and transfer C because they 'assimilate' organic C as their energy source while respiring CO_2. Any changes in land use practices are likely to impact soil microbiota and subsequent C dynamics in soil. Singh et al. (2010) estimated that *ca*. 1–2 billion tonnes of C are released into atmospheric C pool each year due to changes in land use practices (predominantly tropical deforestation). Dawson and Smith (2007) indicated that any improvement in soil conditions is likely to favour microbial decomposition of C. For example, Rochette and Gregorich (1998) found microbial respiration increased by 2–3 times due to manure application. Other land management practices such as cultivation and liming have been shown to increase microbial decomposition of soil organic matter, thereby increasing dissolved organic C content in soil solution (Dawson and Smith, 2007).

1.4 Heavy metal toxicity and biochar remediation

1.4.1 Heavy metal influence microorganisms

Biotic and abiotic stress conditions in soil impact microorganisms, thereby affecting soil C dynamics (Singh et al., 2010). Heavy metal(loid)s toxicity, as one of the many environmental factors that affects soil microbiota, result from mining processes and smelting activities. Their impairment to ecosystem functions have raised global attention during the past century. Heavy metal(loid)s are among the most widespread contaminants present in groundwater and soils. As they are not degradable, they tend to bioaccumulate once they enter into organisms or ecosystems, making them difficult to remove from biota and ecosystems. Mercury, cadmium, lead, zinc, nickel and copper are the most common cationic metal pollutants whereas arsenic and selenium are the most common anionic metals (Olaniran et al., 2013). Although some metals such as iron and copper are involved in biochemical reactions, metals like cadmium, lead and silver have no biological role, yet are potentially toxic to microorganisms (Bruins et al., 2000). Giller et al. (1998) suggested that microorganisms are more sensitive to heavy

metal(loid)s than indigenous soil animals or plants. The toxic mechanism is mainly due to displacement or substitution of essential elements, either on extracellular enzymes or inside of microbial cells (Bruins et al., 2000; Nies, 1999), thereby consequently inhibiting enzyme function for metabolic processes. In addition, even the essential metal elements, when they reach a high concentration, can lead to adverse consequences, such as cell membranes and DNA structure damage, oxidative stress or enzyme dysfunction (Kachur et al., 1998). The decrease in microbial activity and function resulting from metal toxicity can affect microbial CUE, thereby regulating the microbial C assimilation and subsequent release of CO_2 (Bolan et al., 2015; Wang et al., 2007a). By developing metal resistance mechanisms including intra- and extra-cellular sequestration, bioprecipitation, chelation, transport efflux pumps, exclusion by permeability barrier and sensitivity reduction (Bruins et al., 2000; Carine et al., 2009; Haferburg and Kothe, 2007; Nies, 1999; Silver and Misra, 1988), microorganisms are able to adapt to environmental stress. There have been a limited number of studies that examined the underground soil microbial rehabilitation, evolvement, and their effect on the ecological system of the contaminated sites including mine areas. There is urgent need to examine the following aspects in relation to microbial modulation of soil C (Liao and Xie, 2007; Tordoff et al., 2000): (i) microbial responses to metal toxicity in terms of activity and diversity, (ii) heavy metal(loid)s toxicity processes and mechanisms, (iii) microbial regulation of soil C as impacted by heavy metal(loid)s. Because of the nature of this subject, the interpretation of the metal toxicity mechanisms on C dynamics are based on interdisciplinary knowledge, and a broad set of molecular techniques in the near future will contribute to discover the mystic underground world.

1.4.2 Biochar remediation

Biochar is produced anaerobically in an oxygen-restricted environment using C-based materials such as plant residues, composts, manures and solid wastes including biosolids. Traditionally, biochar is used for C sequestration in soil because of its long term stability in soils. Biochar has been extensively studied for its ability to enhance the nutrient level of soils (Schulz and Glaser, 2012) and plant growth (Sohi et al., 2010). Recently, there have been increasing interests in the potential value of biochar in contaminants sorption and their subsequent immobilization in relation to remediation of contaminated soil and groundwater resources. Biochar has a high surface area and favourable pore architecture characteristics enabling it to be very efficient in the retention of contaminants including metal(loid)s and organic pollutants in soils (Ok et al., 2015).

Beyond the sorbent nature and high C content of biochar, the effect of biochar amendments on microorganisms and the mechanisms behind the beneficial effects are not fully understood.

In the consideration of soil ecological sustainability, it is important to investigate the changes in microbial properties after biochar application. Some of the questions, which require urgent attention include: If biochar can reduce the availability of metal toxicity to organisms, are microbial properties directly linked to biochar amendment? Does the biochar-induced remediation of metal toxicity influence SOC decomposition? This thesis will begin with the determination of soil microbial C, followed by the relation of microbial CUE and environmental conditions, and finally with the presence of biochar. The results from this study can provide fundamental technology and supporting information to future research.

1.5 Thesis objectives

The overall aim of the thesis is to examine the microbial CUE in relation to C sequestration as impacted by land use changes and abiotic stress conditions. A set of experiments were designed to meet the specific objectives listed as follows:

- To examine different methodologies for measuring and determining soil microbial CUE
- To investigate the PE of organic amendments with varied C:N ratio in relation to microbial CUE
- To quantify microbial CUE in soils derived from various land use systems as impacted by organic C inputs
- To characterise the toxic effects of heavy metals on soil microbial properties in relation to CUE and soil organic decomposition
- To evaluate the heavy metal remediation function of biochar on soil microbiota and C decomposition in the context of microbial CUE.

1.6 Thesis structure

This thesis is divided into seven chapters (**Figure 1.1**), with the four experimental chapters on measurement of microbial processes under various parameters including the effect of land use systems, heavy metal bio-toxicity and biochar application to soil.

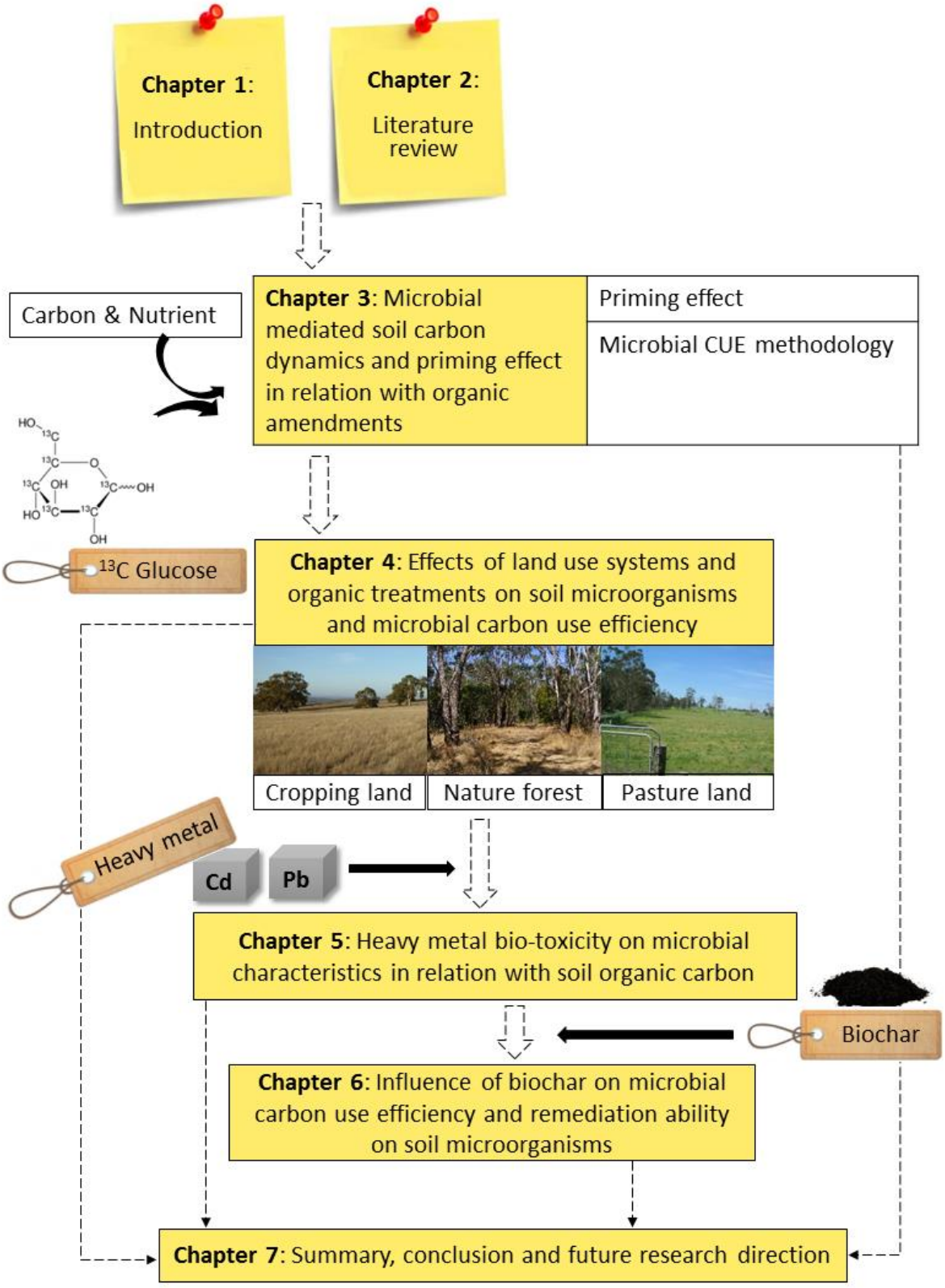

Figure 1. 1 Graphical representation of thesis outline and chapter layout.

Chapter 1 Introduction

Provides perspectives on soil microbial properties and community composition, terrestrial C dynamics and the influences of soil conditions, effect of heavy metal toxicity in soil C dynamics, and role of biochar on remediation and microbial processes, and also lists research objectives and thesis structure.

Chapter 2 Literature review

Includes the literatures covering: (i) microbial CUE and its measurements, (ii) PE in relation to microbial CUE, (iii) microbial mediation of soil C dynamics, (iv) heavy metal(loid)s toxicity in relation to microbial function and CUE, (v) biochar remediation function and its influences on microbial CUE. By addressing the research gaps, the review of literature highlights the need for the research in this thesis.

Chapter 3 Microbial mediated soil carbon dynamics and priming effect in relation with organic carbon and nutrient amendment

This chapter covers the various methods used to measure microbial CUE. These methods include isotope ^{13}C labelled technique to trace C allocation. Based on that, we can determine the microbial CUE measured as: (i) substrate C depletion, (ii) MBC variation, (iii) microbial PLFA amount variation, (iv) the ratio of biomass accumulation to the decrease of substrate C.

Chapter 4 Land use histories alter soil microbial community and preferences for degradation of different carbon sources

This chapter examines microbial properties and microbial CUE in three land use systems, coupled with three types of organic amendments (from easily accessed C to biochar). The purpose of this chapter is to reveal how land management or nutrient status alters microbial CUE. In this chapter, the PE of various organic matter sources in relation to microbial property and microbial CUEs will be both examined. Therefore, microbial C use preference will be revealed and determined.

Chapter 5 Microbial functional diversity and carbon use efficiency in soil as impacted by heavy metal contamination

This chapter investigates the influences of metal types and concentrations on soil microbiota. The hypothesis was that microbial community structure and activity change when exposed to metal toxicity. This is likely to impact SOC decomposition and CO_2 release.

Chapter 6 Effect of biochar on heavy metal toxicity and microbial carbon use efficiency in soil

Based on the results of experiments in chapter 6, the microbial properties and heavy metal availability after biochar application were examined, demonstrating biochar improved microbial function as well as reduced metal toxicity.

Chapter 7 Summary and conclusions

Summarizes findings and discussion based on this research, provides conclusions, and suggestions about future research directions. Based on the work in previous chapters, this chapter aims to highlight the C conundrum in the context of soil microbiota and set priorities for further research.

Chapter 2

LITERATURE REVIEW*

2.1 Introduction

The terrestrial biotic C accounts for *c.a.* 37.33% of total SOC content (up to 1 m belowground) (Guo and Gifford, 2002; Jobbágy and Jackson, 2000; Stockmann et al., 2013). As such, the mineralization of terrestrial C accelerate greenhouse gas (CO_2) accumulation in the atmosphere (Houghton, 2007; Lal, 2013). Terrestrial C sequestration can partially compensate CO_2 accumulation caused by anthropogenic activities, such as deforestation and extensive land management practices (1.6 Pg C per year, Lal, 2010), land-use changes (1.29 Pg C per year, Nakicenovic et al., 2000), fossil fuel combustion and industrial activities in general (8.0 Pg C per year, Lal, 2010). Photosynthesis and heterotrophic microbial respiration are the two major processes regulating terrestrial C balance. Therefore, soil C sink can be increased either by increasing organic C inputs or reducing breakdown of litter and soil organic matter (Luo et al., 2003). Jastrow et al. (2007) proposed the 'residence time' (RT) as a decisive measurement of soil C storage capacity. Soil microorganisms are the driving factors behind SOC dynamics and decomposition, and soil microbial properties (such as microbial activity, microbial biomass and microbial diversity) are recognized as sensitive indicators of soil health and quality (Nannipieri et al., 2003) (**Figure 2.1**). There are considerable gaps in understanding soil microbiota and their impact on the biological mechanisms regulating C exchange between atmospheric, terrestrial and aquatic reservoirs, and the way microbial communities interact with climate perturbation and environmental factors to affect soil C dynamics (Bardgett et al., 2008; Ganie et al., 2016; Gong et al., 2009). As such, ecosystem research that couples approaches ranging from macroscale to molecular scale interactions are necessary to understand the role of specific soil microorganisms and mechanisms contributing to C storage (King, 2011). In this chapter, the recent research and findings about microbially mediated soil C dynamics, especially under environmental parameter variation will be highlighted. The chapter addresses certain microorganism related C degradation features, such as priming effect (PE), and modern techniques for detecting microbiota and modelling strategy. Then,

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the chapter evaluates research focussing on how soil microbiota regulates C loading and organic matter decomposition, studying their implications for soil C sequestration and long-term management. Finally, it concludes by suggesting some future directions in these research areas.

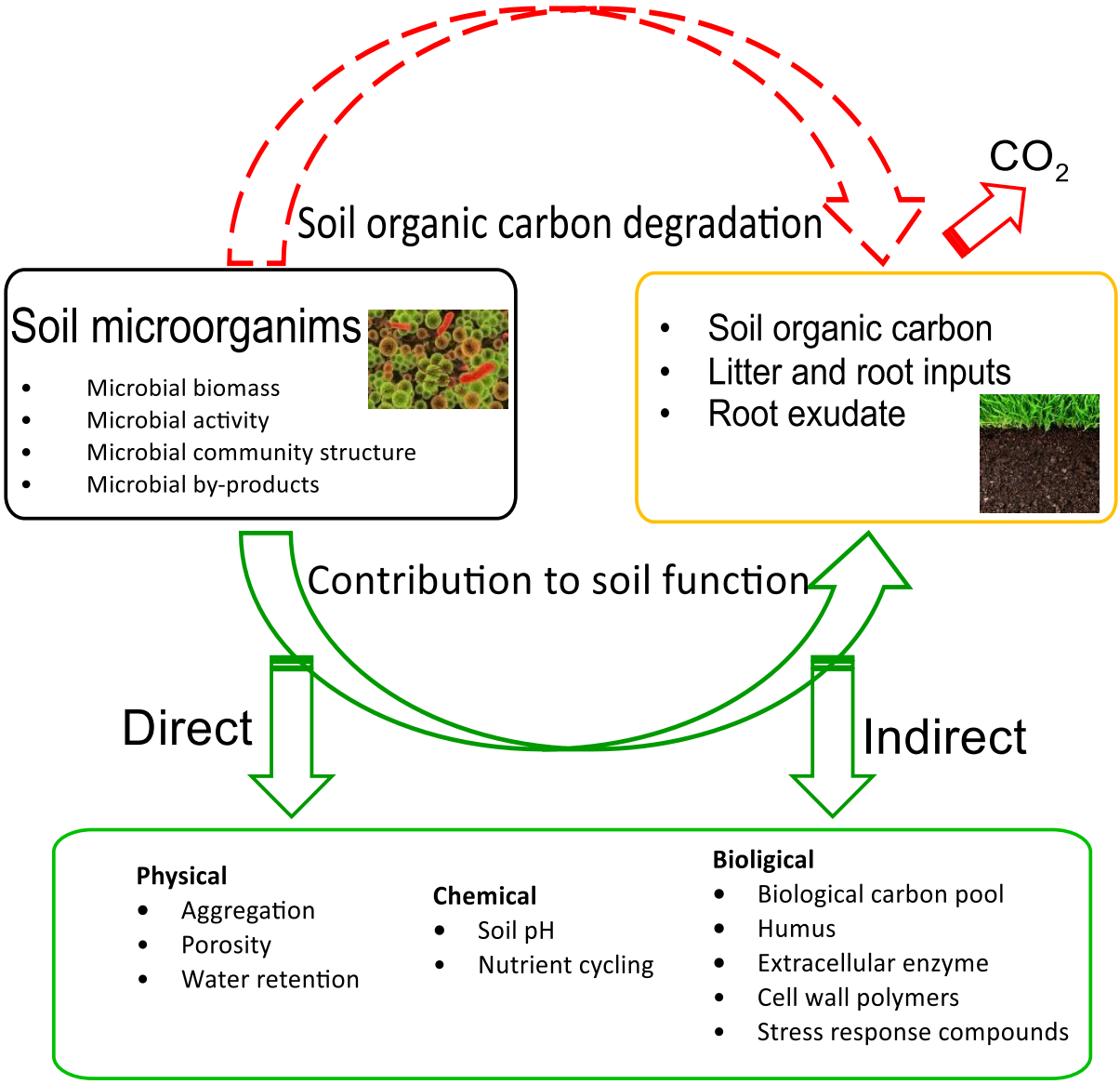


Figure 2. 1 Soil microorganisms in relation to soil carbon (im)mobilisation.

2.2 Soil microorganisms in relation to carbon cycling processes

2.2.1 Soil carbon pools and microbial accessibility

The soil C turnover rate after addition of litter or exogenous organic matter depends on several factors, including the chemical and physical properties of the materials, their capacity for adsorption on soil minerals and their suitability for microbial degradation (Kuzyakov et al., 2003). There are three major routes for plants to transfer organic C into soil systems: (i) above ground plant litter and leachates (i.e., dissolved organic C washed by infiltrating rainfall), (ii) below ground root exudates, and (iii) dead roots (Brimecombe et al., 2007; Six et al., 2006). Such materials originating from root cells and tissues, mucilages, volatiles, and soluble lysates and exudates (releasing from live and/or damaged cells) collectively form the rhizodeposits in due course of time (Dennis et al., 2010; Gougoulias et al., 2014). Although the relative magnitude of organic input is time and location specific, the C-containing compounds can be separated into two categories depending on their molecular structures. In the first category, there would be low molecular weight organics such as sugar, amino acids, sugar alcohol, organic acids and oligosaccharides, while the second category includes polymers like starch, lignin, cellulose and hemicellulose (Gougoulias et al., 2014). Low molecular weight organics can be directly used by microbes within seconds to hours, while high molecular weight compounds need to be hydrolysed before microbial take up (Jones and Darrah, 1994). As such, soil organic carbon (SOC) consists of a continuum of organic matter compounds ranging from labile to recalcitrant C pools. The various labile C fractions are key indicators of soil productivity and health. Generally, the labile C pool decreases right after entering into the soil due to diffusion and convection processes (Raynaud, 2010), and is more easily degraded by microbes, leading to short mean residential time (MRT) in the range of days/weeks/months (Cheng et al., 2007; Ogle et al., 2012). Labile C compounds provide energy to soil biota, and is thus rapidly mineralized, if not stabilized through interactions with clay mineral surfaces, or physically protected within soil aggregates (Krull et al., 2003; McLauchlan and Hobbie, 2004). The chemically recalcitrant C pool consists of less decomposable organic matter consisting of aromatic or aliphatic compounds. Although chemically recalcitrant, this pool is also degraded by the soil microbial biomass, its degradation occurs comparatively slowly, and a part of this recalcitrant pool may survive in soils for decades contributing to soil properties, such as cation exchange capacity (CEC) (Bot and Benites, 2005).

Microbial activity is highly related to the availability of fresh organic C inputs in the surface soil (Fang and Moncrieff, 2005; Ghiorse and Wilson, 1988) and in deeper soil horizons (Sanaullah et al., 2016). The microbial biomass carbon (MBC) pool includes the living MBC and the dead microbial biomass. Notably, the dead microorganisms become a labile C source to the living microorganisms, and the necromasses can represent a huge amount of C in soil. As such, microbes and microbial products contribute a large proportion to the stabilized organic matter pool (Miltner et al., 2012) with MRTs exceeding decades. It is well established that soil

environment (e.g., soil texture, pH, moisture, and temperature) can influence microbial turnover rates of SOC (Davidson et al. 1998; Fierer and Jackson 2006) (**Table 2.1**). Moreover, both soil physical properties (e.g., temperature and pore aeration) and organic C accessibility lead to different microbial C utilization patterns (Cotrufo et al., 2013). Ekschmitt et al. (2008) indicated that turnover rates of SOC depend not only on organic C concentration, but also on factors, such as substrate properties (e.g., substrate quality, composition and initial C:N:P ratio), organic C distribution in the soil matrix and activity, biomass and composition of soil biota.

Table 2. 1 Selected references on environmental factors and the influences on soil microorganisms

Factor of influence	Laboratory/Field conditions	Treatments	Microbial activity and composition response	References
Soil temperature	Laboratory incubation	Temperature control	Microbial respiration increased with temperature	(Wei et al., 2014)
	Laboratory column incubation	Temperature control	Emissions of CO_2 and CH_4 at 23 degrees C were an average of 2.4 and 6.6 times larger, respectively, than those at 10 degrees C	(Moore and Dalva, 1993)
	Laboratory chamber incubation	Temperature control	Microbial respiration increased with temperature up to 32 degrees C	(Fang and Moncrieff, 2005)
Water content	United states		Microbial communities consistently, exhibited the uniform distribution pattern regardless of soil water content	(Zhou et al., 2002)
	Laboratory column incubation	Water table position control	Static water table depths of 0, 10, 20, 40 and 60 cm, CO_2 emissions showed a positive, linear relation with depth, whereas CH_4 emissions revealed a negative, logarithmic relation with depth	(Moore and Dalva, 1993)
	Laboratory incubation	Drying-rewetting frequency	Microbial respiration decreased with drying-rewetting frequency	(Fierer and Schimel, 2003)

	Laboratory chamber incubation	Soil moisture control	Microbial respiration response to soil moisture content between 20 and 50 vol% is not so obvious	(Fang and Moncrieff, 2005)
Soil pH	United Kingdom	Amendments with lime for pH gradient	The composition of the bacterial communities was closely defined by soil pH, Fungal community composition was less strongly affected by pH than bacteria	(Rousk et al., 2009)
	United States		Soil pH as predictor of soil bacteria community, the overall phylogenetic diversity of the bacterial communities was at peak diversity in soils with near-neutral pH	(Lauber et al., 2008)
	United Kingdom	pH control	Archaeal amoA gene and transcript abundance decreased with increasing soil pH, while bacterial amoA gene abundance was generally lower and transcripts increased with increasing pH	(Nicol et al., 2008)
	Germany		Fungal/bacterial biomass index increased slightly with increasing pH, and microbial community was to a large extent determined on pH	(Bååth and Anderson, 2003)
Organic carbon content	United states		Microbial communities in low-carbon surface soils showed remarkably uniform distributions, and all species were equally abundant	(Zhou et al., 2002)
Soil heavy metal content	Laboratory incubation	Amendments with heavy metals	Heavy metal pollution severely decreases the functional diversity of the soil microbial community and impairs specific pathways of nutrient cycling	(Kandeler et al., 1996)

2.2.2 Soil properties in relation to microorganisms

Among many other physical (e.g., texture, structure, moisture content, aeration level) and chemical (e.g., pH, electrical conductivity (EC), clay contents and types) properties of soil, the CEC can reflect the overall soil fertility. It can interact with polycations, which can act as a bridge between soil aggregates and soil organic matter (Tisdall, 1996). Also, polyvalent cations, such as Ca^{2+}, Al^{3+} and Fe^{3+}, can lead to the formation of aggregates with organic C occluded in them. This interaction reduces the microbial degradation of SOC (**Figure 2.1**). It is well established that the negative surface charge on clay particles increases by increasing soil pH (Bronick and Lal 2005; Singh et al., 2017b). In variable charged soils, soil pH can significantly affect the adsorption of dissolved organic C as well as metals (Appel and Ma, 2002; Bolan et al., 1999; Singh et al., 2016; 2017a). Since pH affects activity and composition of microbial communities and also the charges of surface-reactive soil particles, changing soil pH may have a complex effect on microbial reaction and organic C turnover.

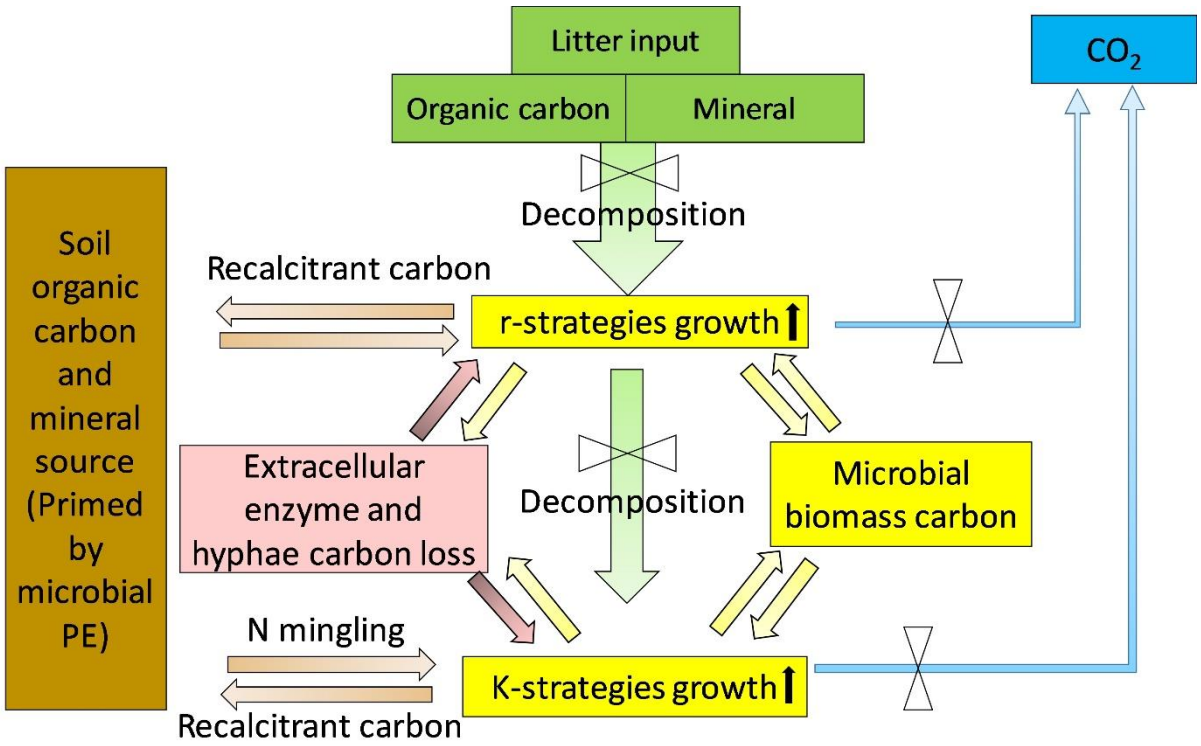


Figure 2. 2 Concept *r*- and *K*-strategy microorganisms mediating soil carbon dynamics. The thickness of and arrow of the arrow bars indicate the carbon flow volume and direction. The sum of extracellular enzyme and hyphae carbon loss and microbial biomass carbon are the carbon sequestrated by microorganisms.

2.2.3 Microbial adaptation to elevated temperature

Elevated temperature can stimulate microbial activity, and it is thus expected that SOC decreases upon global warming (Kirschbaum, 2004). However, this hypothesis was derived from laboratory incubation experiments with disturbed soil samples. Meanwhile, the higher CO_2 concentration may improve plant productivity and alter nutrient and organic matter inputs into the soil. Incorporation of microbial properties (composition, activity and biomass) into C dynamics models is a prerequisite for building up a theoretical framework to understand microbial mechanisms of mediating global C cycle. However, because of the variations in the ecosystems and models, as well as curve fitting techniques, feedback of soil microbiota to temperature is a major uncertainty (Davidson and Janssens, 2006; Knorr et al., 2005). The other problem is that the length of the experiment may blur the results and eliminate the microbial resilience in response to environmental stress. A hypothesis on microbial acclimation to global warming suggests that soil respiration (mainly microbial respiration) keeps dynamically shifting to the seasonal changes in temperature (Fenner et al., 2005). The initial stimulation of soil CO_2 efflux in the early stage could be only transitory due to the depletion of labile C pool (Eliasson et al., 2005; Kirschbaum, 2004). However, some responses of stable C pools could also be observed at this stage (Lefèvre et al., 2014; Leifeld and Fuhrer, 2005). Hartley et al. (2007) proposed that the substrate availability affects the response of microbial heterotrophic respiration to warming. Therefore, the heterogeneity of substrate C also needs to be considered when investigating microbial regulating soil C and simulating models. Fierer et al. (2005) reported that when the organic C content in litter declined, decomposition was more sensitive to temperature change. In another study, Knorr et al. (2005) demonstrated that non-labile organic C was more sensitive to temperature change than labile organic C. Interestingly, Pries et al. (2017) argued that there was no significant differentiation in microorganisms in terms of accessing different soil organic matter after two and half years of warming. This indicated the potential C release due to subsoil depletion in the background of increasing temperature. The composition of soil microbial communities may also change with temperature variation (Dinh et al., 2014; Zogg et al., 1997). Bardgett et al. (1999a) found a fast growth of Gram-positive (G+) bacteria in the first succession stages under an elevated atmospheric temperature, but no direct effect on slow growing microorganisms such as fungi and actinomycetes. Although the bibliography on soil heterotrophic respiration is extensive, only a few models include the overall response of soil microbial activity to climate change. In this context, Allison et al. (2010) calculated the carbon use efficiency (CUE), defined as the organic C incorporated into microbial biomass, to better simulate the process of SOC response to global warming. Attempts to quantify the effects of natural climate gradients on soil C and microbial activity based on laboratory and field experiments can often be vulnerable to uncertainty. This is because SOC pool consists of a variety of C compounds rather than having a uniform composition (Qafoku, 2015). Future modelling research should therefore address

microbial processes more explicitly, for example, by including microbial PE, enzymatic activities, microbial CUE, etc. under the changing climate to incorporate them into the C budget calculations.

2.2.4 Soil microbial activity in response to drought and rewetting cycle

Soil moisture deficit or surplus can cause soil physical and chemical changes, such as gas and solution diffusion, water retention and pore space alteration, and these changes can affect soil microbial properties. Microbial respiration may reach a plateau at an optimum moisture level because of the diffusion limitation of substrates with the consequently reduced substrate availability to microbes (Moyano et al., 2013). In addition, there may be a 'Birch Effect' occurring, which refers to the flush of soil CO_2 production because of a sudden rewetting after a long period of drought (Xiang et al., 2008). A long period of drought leads to inhibitory affect in soil microorganisms, while the rewetting increase microbial activity with sufficient organic accumulation during the whole drought period. Thus, there can be a close connection between increased water movement and simulated microbial activity/organic C depletion. Especially in the arid and semi-arid ecosystems, because of microbial sensitivity to water impulse, the stimulation of SOC decomposition by a sudden precipitation can lead to a cascade of responses (Carbone et al., 2011). Miller et al. (2005) reported that there was a 60% increase in CO_2 efflux due to a drought-rewetting event compared to soils at constant and optimal water content. In the models reported by Li et al. (2006) and Yuste et al. (2005), the rewetting pulse alone accounted for 10−14% of the annual CO_2 fluxes.

In order to understand how soil microorganisms respond to drought stress and rewetting, it is essential to study how the increased intracellular concentration of solutes may prevent microbial dehydration. Due to the existence of different C allocation strategies among microbial species (i.e., different CUE), bacteria tend to produce amino compounds such as proline, glutamine and glycine betaine (Csonka, 1989), while fungi generate more polyols such as glycerol, erythritol and mannitol (Witteveen and Visser, 1995). Fierer and Schimel (2003) proposed that the initial production of CO_2 after rewetting may be a result of the mineralization of cytoplasmic solutes, which maintains the equilibrium between intracellular and soil water potentials, following the depletion of easily available organic C. However, the physiological processes required for this phenomenon in bacteria and fungi can be energy intensive. Killham and Firestone (1984) estimated that osmotic stress can reduce more than half of microbial CUE compared to unstressed conditions. Multiple drought and rewetting cycles thus may strongly influence soil C flows. The magnitude of CO_2 release on rewetting varies between 7% and 20% of the size of microbial biomasses (Killham and Firstone, 1984). Major concerns

about the consequences of repeating drought followed by rewetting are: (i) sudden big flux of CO_2 emitting from soil, and (ii) uncertainty about the recovery of microbial biomasses following the rewetting (Fierer and Schimel, 2003). Schimel et al. (2007) found it difficult to conclude whether soil drought and rewetting slowed down or accelerated microbial decomposition of organic C, but suggested that this stress could shift the microbial allocation of degradable C substrates. This hypothesis was supported by the work of Xiang et al. (2008), who showed an increasing microbial use of substrates upon drying-wetting cycles related to physical release of protected C. In conclusion, there is a need for integration of soil drought-rewetting processes to both physiology and community composition of microorganisms as kinetic components regarding soil C dynamics and model development.

2.3 Microbial community composition and soil carbon dynamics

Because of the complex interactions among soil microorganisms and environmental factors, identification of microbial parameters that are primarily responsible for soil C dynamics is challenging. However, the succession of microbial communities and their structural change may be significantly related to the changes that occur during soil organic matter decomposition (Bai et al., 2016; Sanaullah et al., 2016). This may explain even more than 2/3 of soil C reactions (Louis et al., 2016). Fungi and bacteria are the two major groups of microorganisms responsible for soil C decomposition. These groups thus significantly relate with most of the soil quality parameters, and are responsible for more than 90% of the soil functions (McGuire and Treseder, 2010). However, there are significant differences between fungi and bacteria in terms of C use preferences. The general understanding is that, microbial C use is related to the ratio of soil bacterial:fungal (B:F) populations due to the difference in their C utilization abilities. Fungi favour acidic pH environment, while bacterial cells prefer neutral or slightly alkaline conditions (Ganie et al., 2016; Rousk et al., 2009). Compared to bacteria, fungi promote condensation type biochemical reactions, and can more easily grow on complex substrate sources, such as cellulose, tannin-protein and lignitic materials (Hanson et al., 2008). This leads to the trait differences of extra cellular enzymes they produce in order to decompose target C substrates. For example, fungi produce more phenoloxidases, laccases and peroxidases than bacteria. On the other hand, bacteria produce more lipases and cellulases than fungi (Jastrow et al., 2007). Although both, fungi and bacteria, can degrade saccharides, it has been proved that when the colonization of fresh litter happens, development of fungal population is more rapid than bacteria (Gessner and Chauvet, 1994; Hieber and Gessner, 2002). In addition, fungi may slightly modify the soil structure through

the production of glomalin or glomalin-related soil proteins that act as a ‘glue’ for soil aggregate formation (Rillig and Mummey, 2006). In addition to the habitat differences between fungal and bacterial species, Fierer et al. (2003) studied the relationship between C sources and microorganisms, and suggested that the dominant microbial species might change along the soil profiles due to variation in organic C availability. Ekschmitt et al. (2008) reported that fungi were located mostly in the upper 20 cm of soil profile, while bacterial habitats could be extended to more than 1.4 m soil depth. In addition, G+ bacteria and actinomycetes tend to inhabit deeper soil layers with limited C availability. Conversely, Gram-negative (G−) bacteria, fungi and protozoa have higher abundance in the surface soil. These differences consequently affect C allocation pathway and soil C dynamics. A model of Fontaine and Barot (2005) showed that the soil C pool is highly related to microbial community size and composition, and would continuously increase if nutrients were sufficient before reaching a threshold.

When soil microbiota face environmental disadvantage, drought for example, physiological modification is their primary short-term acclimation mechanism, while the shift of microbial community structure is generally the response to a long-term stress (Schimel et al., 2007). A significant research effort has been directed to understand microbial community composition shifts in relation to environmental stresses. There have been numerical results in terms of microbial species response to drought-rewetting cycles (Göransson et al., 2013). Fungi and bacteria generally have different preferences in decomposing various organic matter compounds, and play specific functional roles in the ecosystem. Soil moisture stress could result in different substrate depletion and nutrient utilization patterns by microorganisms. Schimel et al. (2007) found G+ bacteria and fungi more drought-tolerant than G− bacterial because of their enhanced biochemical ability of decompose SOC. Gordon et al. (2008) and Williams (2007) reported bacteria were more dominant in the rewetting cycle than fungi. However, there were reports indicating almost no difference among microbial groups due to rewetting (Butterly et al., 2009; Hamer et al., 2007). As such, drought stress might not always be related to the equilibrium of microbial community shift (Balser and Firestone, 2005). The magnitude of fungal activity is influenced by environmental parameters such as temperature and root development patterns of plants (Bell et al., 2009; Vandenkoornhuyse et al., 2002). Moreover, fungal function or community structure are both closely dependent on soil chemical parameters, such as moisture contents and C to mineral ratio. The variation of fungi population due to living condition changes will affect soil C degradation in return.

In addition, microorganisms find a natural balance between ecological selection and evolutionary adaptation in order to survive and bounce back from environmental stresses (Schimel et al., 2007). Categorized by ecological strategy, soil microbial community can be generalized into aggregated groups that share similar ecological function traits

(Blagodatskaya et al., 2004; Chen et al., 2016). *K*-strategies (oligotrops) have higher Microbial CUE yet slow growth rate in contrast to *r*-strategist that grow fast in rich substrate conditions with lower CUE. Blagodatskaya et al. (2004) suggested that microbial community shift to species with better stress tolerance, known as *K*-strategies rather than *r*-strategies with. However, there is limited research tried to explain drought-rewetting scenario with microbial *r*- and *K*-growth strategy. Moreover, the relative influence of drought-rewetting on microbial CUE are highly site dependent, especially in the context of changes in long term soil C pools (Balogh et al., 2011).

The investigation of soil micro-functions in relation to microbial community composition is more challenging under field conditions than laboratory-scale microcosm experiments because the former involve highly complex and unpredictable environmental factors. Modern molecular biology based techniques are powerful in investigating the complex microbial community compositions under such situations (**Table 2.2**). Techniques such as cultivation (plating) and PLFA analysis can be used to study the abundance of broad/large microbial groups (e.g., bacteria and fungi) (Torsvik and Øvreås, 2002). However, polymerase chain reaction (PCR)/quantitative polymerase chain reaction (qPCR) and genetic fingerprinting techniques, such as denaturant gradient gel electrophoresis (DGGE), amplified rDNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP) and ribosomal intergenic spacer analysis (RISA), should preferably be used to obtain precise and in-depth information of microbial community structures under complex soil environmental conditions. Moreover, a combination of two or more of these techniques provide an overview of the dominant soil microbiota. However, due to the limitation of specifically targeted microbial communities (< 1000), some microbial species may not be able to be accessed or detected (van Elsas and Boersma, 2011). Other techniques such as stable isotopic probing and brdU methods are suitable for detecting active communities *in situ*, and microarrays can provide information on diversity of microorganisms at phylogenetic or functional levels (van Elsas and Boersma, 2011). Molecular technique supports the study of microbial C use beyond the concept as organic decomposer. Since the particular functional genes may tend to cluster in the genome, and the microbial dispersal limitation may constrain microbial recolonization, bioengineering approaches by using modified bacteria have also been attempted to enhance CO_2–C sequestration in soils. Some promising bioengineering approaches include the formation of dolomite [$CaMg(CO_3)_2$] by genetically modified bacteria in certain hypersaline ecosystems (King, 2011), and incorporation of C into membranes of modified living microorganisms through small macrocyclic carbonate receptors (Brooks et al., 2006; Tossell, 2009). Whitman et al., (1998) predicted that 1.59×10^{31} bioengineered bacterial could potentially sequester around one Gt of C, which is about 12% of global annual anthropogenic

CO_2 emission.

Table 2. 2 Techniques to identify microbial community structure

Analysing communities approaches	Advantages	Current disadvantages	Reference
Plate counts	Fast, inexpensive.	Unculturable microorganisms not detected, bias towards fast growing individuals and fungal species with large quantities of spores.	(Kirk et al., 2004)
Community level physiological profiling (CLPP)/Sole-Carbon-Source Utilization (SCSU) Pattern	Fast, highly reproducible, relatively inexpensive, option of using bacterial, fungal plates or site specific carbon sources.	Only represents culturable fraction of community, favours fast growing organisms, only represents those organisms capable of utilizing available carbon sources and metabolic diversity instead of *in situ* diversity, sensitive to inoculum density.	(Kirk et al., 2004)
Cell membrane lipid fatty acids (Phospholipid fatty acids-PLFA)	Detect changes in microbial composition, direct extraction from soil, culturing of microorganisms is not required, distinguish broad taxonomic groups (such as gram - negative and –positive bacteria, actinomycetes, fungi etc. and some narrow taxa, such as methanotrophic bacteria).	Difficult in connecting specific microbial populations with ecosystem dynamics, more material is required when fungal spores are used, can be influenced and confused by external factors or other microorganisms	(Frostegård et al., 1993; Kirk et al., 2004; Schimel and Gulledge, 1998)
Substrate-use patterns and kinetics	Detect changes in bacteria composition in response to environment changes	Difficult in relating to *in situ* bacterial community function	(Kirk et al., 2004; Schimel and Gulledge, 1998)
Mol% Guanine plus Cytosine (GC content)	Quantitative techniques, not influenced by Polymerase Chain Reaction (PCR) biases, includes all DNA extracted and rare microbial member.	Requires large quantities of DNA, coarse level of resolution, dependent on lysing and extraction efficiency	(Kirk et al., 2004)
Nucleic acid re-association and	In situ specific study with total DNA extracted or RNA, not	Lack of sensitivity, sequences need to be in high copy number for	(Amann et al., 1995; Holmes et al., 1995;

hybridization technique	influenced by PCR biases.	detection, dependent on lysing and extraction efficiency	Schimel and Gulledge, 1998; Teske et al., 1996)
DNA microarrays and DNA hybridization	Same but more specified than nucleic acid hybridization, reproducible, thousands of genes can be analysed.	Only detect the most abundant species, need to culture organisms, only accurate in low diversity systems.	(Kirk et al., 2004)
Denaturing and Temperature Gradient Gel Electrophoresis (DGGE and TGGE)	Reliable, reproducible and repaid to specific genes, large number of samples can be analysed simultaneously.	Medium resolution, not provide present species identification, PCR biases, influenced by lying and extraction efficiency, and sampling, one band can represent more than one species (co-migration), only detects dominate species	(Kirk et al., 2004)
Single Strand Conformation Polymorphism (SSCP)	Same as DGGE/TGGE, no GC clamp or gradient.	PCR biases, some ssDNA cam form more than one stable conformation	(Kirk et al., 2004)
qPCR	Quantitative rRNA/rDNA ratios, detect active members of microbial community, can be used in a specific gene as well as a broad scale.	Influenced by extraction, lysing efficiency, and choice of restriction, type of *Taq* can increase variability	(Teske et al., 1996; Schimel and Gulledge, 1998)
Restriction Fragment Length Polymorphism (RFLP)	Detect structural changes in microbial community.	PCR biases, banding patterns often too complex	(Kirk et al., 2004)
Terminal Restrict Fragment Length Polymorphism (T-RFLP)	Simpler banding patterns than RFLP, reproducible, automated, large number of samples can be analysed, can compare microbial communities differences.	PCR biases, influenced by extraction, lysing efficiency, and choice of restriction, type of *Taq* can increase variability	(Kirk et al., 2004)
Ribosomal Intergenic Spacer Analysis (RISA)/Automated Ribosomal Intergenic Spacer Analysis (ARISA)/Amplified Ribosomal DNA Restriction	Highly reproducible community profiles.	PCR biases, required large of DNA (for RISA)	(Kirk et al., 2004)

Analysis (ARDRA)			
Pyrosequencing/ Metagenomics/	Reproducible, reliable, and rapid, large number of samples can be analysed to detect microbial species *in situ*.	Very expensive, datasets can be very big	(Jaenicke et al., 2011; Oikonomou et al., 2013; Wolcott et al., 2009)

The above discussions indicate that soil abiotic conditions, fresh organic matter additions and other anthropogenic interferences may directly or indirectly alter the microbial community composition with possible effects on C dynamics in soils. Anthropogenic disturbances such as tillage and grazing may trigger higher C loss from soils due to their influence on physical soil parameters and also the soil microbial community structure (Ingram et al., 2008; Jackson et al., 2003). Other factors, like fresh organic matter inputs, may also alter the microbial community structure by influencing the C utilization patterns (either indigenous or added C) of microorganisms. For instance, > 3.6 mg C g^{-1} as root exudates resulted in a negative PE in soils, and significantly shifted the B:F population ratio (de Graaff et al., 2010). Schutter and Dick (2001) also reported that microbial richness was positively related to the substrate utilization potentials of the soil microorganisms. Anderson et al. (2011), by using the Terminal Restriction Fragment Length Polymorphism (TRFLP) fingerprint techniques, found that certain microbial species that were related to recalcitrant C decomposition were promoted in the presence of biochar, which was followed by an indigenous organic C depletion. In another research investigating the effects of invading plant species, Yu et al. (2005) suggested that exotic weeds could replace the native plants by changing the native soil microbial community and their interactions with the plants. This ultimately would alter the C dynamics in soils too. In addition, environmental stresses caused by heavy metal(loid)s may lead to reduction in the microbial community diversity and activity in soils (Wang et al., 2007a). However, the attempt of using soil microflora and microbial activity as indicators of soil heavy metal(loid)s bioavailability should underscore the sensitivity of microorganisms to the concerned toxicity (Bolan et al., 2010). Despite the uncertainties in parameter calibrations and elicit assumptions, an increased number of researches have recently begun to incorporate microbial community shifts in modelling and simulation studies (Sinsabaugh et al., 2013). For example, in light of the concept of 'multiple C pools' models in Wu et al. (2013), they divided terrestrial C pools into eight parts, including two biotic C pools as surface microbial and soil microbial pools. This was in light of the fact that microbial population developed different features under different substrate (e.g., easily decomposable or recalcitrant C) availabilities and their decomposition

stages (e.g., residence age) (Berg and McClaugherty, 2008). However, Louis et al. (2016) suggested that a universal applicability and predication through these models may be challenging, mainly because: (i) fixed parameters in these models are suitable in certain time and space, and (ii) there are many discrepancies for species and functional diversity of microorganisms. In McGuire and Treseder (2010), they proposed several mechanisms that could influence the incorporation of microbial community diversity into model settings.

Although there has been a debate around the old microbiological citation “Everything is everywhere, but, the environment selects” from Baas Becking (1934) (de Wit and Bouvier, 2006), microbial diversity is still critical in terms of supporting soil stability and resilience ability (Griffiths and Philippot, 2013), and highly related to soil physiochemical properties, function, and consequently have influences on organic C decomposition (Baumann et al., 2013).

2.4 Priming effect

The PE of organic matter mineralization refers to the enhancement or retardation of organic matter decomposition as a result of the addition of fresh C substrates into the soil. Most of the previous studies reported on PE were laboratory incubation based experiments with significant manipulation of the real environmental conditions. Nevertheless, there is a slowly growing consensus regarding PE that it is a natural process, which is induced by a pulse or continuous input of fresh organic matter (Kuzyakov, 2010). Rhizosphere and detritusphere are considered as the hotspots for PE because these regions are more concentrated in labile C than bulk soils due to the occurrence of root exudates and leaf litters (Kuzyakov, 2010). Continuous organic matter inputs in these areas enable a higher microbial biomass and C turnover rate than in the bulk soil. Hütsch et al. (2002) reported that 64−86% of the ‘root-borne C’ was rapidly used by soil microbes, while 2−5% were incorporated to the soil matrix and protected from future degradation. It is commonly believed that fresh organic matter addition leads to two major phenomena: (i) enhancement of microbial activity, and (ii) uneven growth of microbial populations (Fontaine et al., 2003). Fresh organic matter inputs induce microbial community succession (Nottingham et al., 2009). Certain microbial species can be triggered by a dramatic increase in populations based on the characteristics of the fresh C supply, which indicate a strong C substrate preference for soil microbes (Cardon et al., 2001; Hagedorn et al., 2003; Nottingham et al., 2009). If the previously starving microbial species are able to use the new C supply, their population composition is supposed to experience significant growth. The *r*- and *K*-strategy theory provides information to understand the microbial succession with organic substrate alteration. Generally, the *r*-strategy microbes are more adapted to fresh and easily available organic compounds under nutrient favourable conditions, while the *K*-strategy

species are expected to decompose polymerized compounds at a late succession period (Blagodatskaya et al., 2009). The growth pattern difference is due to the different energy allocation strategy, leading to the C use discrepancy (**Figure 2.2**). The *r*-strategists prefer a population expansion, while *K*-strategists focus on extracellular enzyme production and defence from predation (Fontaine et al., 2003; Tate, 1995). The CO_2 release during the first 3 days after fresh organic amendment input was termed as the apparent PE, and this CO_2 flux indicated the accelerated microbial turnover rate or C pool alteration (Kuzyakov, 2010). The value of real PE was relatively lower than the apparent PE, which then increased with the incubation period and possessed a large portion of the total CO_2 flux in the later incubation stage (Blagodatsky et al., 2010; Kuzyakov, 2010). As such, soil microorganisms with fast growth rate, i.e., *r*-strategists, are the major response for the apparent PE, while the later stage microbes, i.e., *K*-strategists, produce real PE. Fontaine et al. (2003) suggested that the *K*-strategists play a more important role in maintaining nutrient availability and fertility of soils because their extensive growth of mycelia enables quick release of N from soil organic matter. Microorganisms regulate the direction and magnitude of PE, and the microbial community composition along with microbial abundance have essential influences on PE (Blagodatskaya and Kuzyakov, 2008). The biochemical properties of freshly applied organic matter alter the microbial population and biomass, consequently controlling the variation of PE and CO_2 production. Use of isotopically labelled C compounds to separate CO_2 released from added C source and native organic C is an essential technique to distinguish between real PE and apparent PE.

Most of the studies on PE showed a positive PE after fresh organic input, meaning an enhancement of SOC degradation (Cheng, 2009; Zhu et al., 2014). Fontaine et al. (2004) reported that soil humus was depleted by 174 mg C kg^{-1} because of cellulose application. As a result of positive PE, a decrease in soil mineral nutrient contents can be expected due to the stimulated microbial activity, which in turn may affect soil nutrient availability (Fontaine and Barot, 2005). However, negative PE and no obvious C mineralization were also observed in biochar amended soils (Liang et al., 2010; Zimmerman et al., 2011). The quantity of organic inputs may also mediate the PE direction. De Graaff et al. (2010) found that negative PE occurred at a higher liable C input rate (> 3.6 mg C g^{-1}), while positive PE took place at a lower C input rate (0.7 mg C g^{-1} soil). The characteristics of fresh organic materials, such as polymerization and C:N ratio (Brant et al., 2006; Khalil et al., 2005), and the inherent soil factors, like pH and soil type (Khalil et al., 2005), may have significant impacts on microbial population size and composition, and in turn on the magnitude of the PE. In addition, since specific enzymes are involved in the degradation of certain organic substrates, the enzyme type, quantity and activity in soils might significantly change with the congruent substrate

depletion processes (Fontaine et al., 2003). Fontaine et al. (2004) demonstrated that specific enzymes were produced with cellulose induced C decomposition, and this was the primary driver of positive PE in the system.

The plant species affect PE by changing the litter input quality and quantity, as well as root exudates and phenology (Pascault et al., 2013). An intensified PE was observed in an increased forest litter fall accompanied with rising temperature (Sayer et al., 2011), leading to significant SOC decomposition (Zhu and Cheng, 2011). Chen et al. (2014) suggested that the fresh organic C inputs served as a 'primer' for native C decomposition, while the PE was intensely regulated by mineral N supply. Crow et al. (2009) found a low PE and slow C turnover rates in soils with high nutrient availability. Similarly, Fontaine et al. (2011) suggested that microbes tend to decompose more indigenous soil organic matter and release more nutrients with a high PE performance in soils with limited nutrient availability. With the application of pyrolysed C, Lu et al. (2014) noticed a negative PE in a sandy loam soil. But Fang et al. (2015) suggested that the pyrolysis temperature may have a high impact of the microbial degradation ability, for instance, the biochar produced at over 550 °C led to a greater local C stabilization than those in lower temperature. However, no universal consensus on the magnitude of PE nor fully satisfied mechanisms to explain the process yet exist in the literature (Fontaine et al., 2011; Kuzyakov et al., 2000).

In conclusion, fresh organic matter application may alter the degradation of native organic matter and microbiota, causing soil PEs. However, the variety of litter quality and soil physicochemical properties could lead to different PE. Soil microorganisms is the regulator of PE. Microbial community composition and characterise, including metabolic activity and enzyme production, shifts depending on the fresh amendments. Although the 'hot spot' area, such as rhizosphere is the most active place for PE, the input affects soil organic matter in deeper layer along soil profile. Fontaine et al. (2007) observed an increase in deeper organic C decomposition after fresh organic C application to soils. Other environmental factors such as elevated soil temperature and frequently disturbed fields (i.e., tillage, drought, etc.) influence soil PE by controlling microbial functions. Future research attention should therefore be aiming at the following aspects in order to understand PE: (i) evaluating long term PE effect with pulse or continuous supply of fresh organic matter to encompass the timing and changes of C supply, (ii) incorporating PE with conditions in the real ecosystems, especially in a scenario of temperature, and (iii) upscale across ecosystems models. Integrated models can estimate global C turnover to evaluate directions and magnitude of C flow.

2.5 Incorporation of microbial parameters into soil carbon modelling

Carbon is constantly transformed between organic and inorganic forms among biotic and abiotic C reservoirs, keeping a continuous flow. Soil microorganisms are the driving force for SOC decomposition as well as biological C pool build-up in soils. These 'double roles' of microorganisms makes the interpretation of soil microbial functions and mechanisms influencing C dynamics really challenging. It is noteworthy that most of the current model settings are based on the concept to understand soil as a potential sink or source for atmosphere CO_2 (Manzoni and Porporato, 2009; Wieder et al., 2013). Since soil microbes are the main driving force for organic matter decomposition, including microbial communities into the models is necessary to improve the predictive capacity. However, incorporation of microorganisms and microbial features in the models also need to consider the feedback from environmental changes. Modelling the response of microorganisms to environmental changes is complicated due to the ecosystem diversity. This is one of the reasons why most of the models concerning C turnover and microorganisms are restricted to certain soil conditions or specific period (Lützow et al., 2006). To address that, long-term and large-scale field-based studies are required in the future. However, the challenge may still exist even if there is a 'one fit all' model because the model can smudge results from environmental factors as they also function as indirect influences. In addition, scaling up (both in time and space) is also important in terms of ecological modelling, uncertainties such as land histories and spatial variations must be considered (Luo et al., 2010). Singh et al. (2010) suggested that either reductionist approach, for individual taxa or communities in response of each environmental variable or multifactorial approach, for trophic interactions are both crucial in model interpreting ecological processes.

There are a variety of models regarding soil C dynamics including microorganisms as kinetic parameters. Miltner et al. (2012) developed a model based on the concept that the microbial residue was the source for the microbial biomass. This model explained and addressed soil microorganism not only as C decomposer, but also as a significant contributor to SOC formation. In addition, microbial species, especially fungi and bacteria represent most of the microbial functions in soil C degradation, and have been considered as important parameters in current models. Clemmensen et al. (2013) suggested the important role of fungi in the rhizosphere, and their model revealed that great contribution to a boreal forest from roots and roots-associated microorganisms. Resat et al. (2012) demonstrated that the bacterial community members are highly connected to microbial biochemical processes. Moreover, microbial function groups are also another important microbial parameter to be included while

modelling. Waring et al. (2013) suggested that the functional groups can replace the broad bacterial and fungal diversity in terms of their different influences on soil C degradation. For instance, microbial enzymes and extracellular polysaccharides are the initial approaches that link microbial C use and soil functions (Schimel and Schaeffer, 2012).

In conclusion, the incorporation of soil microbial parameters into global C dynamic modelling can be powerful because of the information they can provide. However, the uncertainty about the accuracy of the models is challenging (Schimel, 2013).

2.6 Conclusions and future research directions

The above discussions suggest that whilst environmental parameters (e.g., temperature, moisture, pH, CEC, soil nutrient contents) significantly affect soil microbial activity and community composition, interactions between fresh and stabilized organic matter also influence the soil microbial parameters.

SOC stability is largely influenced by the accessibility of the substrates to soil microbiota in different fractions. While modern molecular techniques (e.g., TGGE and 16sRNA) can be used to harness information on soil microbial taxa, the next step could be to answer how to link their functional and physiological capabilities with across the ecosystems and along environmental gradients. Manipulation of microorganisms using molecular and gene technologies have potentials to reveal the role of soil microorganism in terms of soil C sequestration, but significant future research is needed to understand the concerned mechanisms and also the risk of adding genetically modified microorganisms to the natural environment. Clearly, our fundamental knowledge of soil microorganisms is not qualified enough to give a mechanical explanation to present hitherto unknown microbes mediating soil C dynamics. Some progress in this regard can be made by conducting future research focussing on the following aspects:

- Introduction of soil measurement methods for a better understanding of soil functions in the perspective of microbiota, such as the function of soil structure as microbial habitat.
- Modelling soil micro-system for integrating ecosystem and environmental gradients in a global level.
- Understanding the functional implication of highly diverse microbial community and microbial succession stages in relation to C turnover.
- Use of molecular techniques and identification of C-cycle related microbial functional genes and taxa.

- Understanding enzyme types and activities in relation to soil organic matter decomposition in various microbe-plant systems.

Soil microorganisms are essential for the rejuvenation of C depleted soils. Microbiological models and research have already proved the ecological importance to protect microbial diversity and functions in terms of soil resilience and stability. However, there are still many questions remain to be answered. For instance,

- Would global climate change affect microorganisms due to their spatial distribution?
- What will be the long term effect of environmental stress on microorganisms?
- How far does the acclimation ability support microorganisms in enduring those stresses?
- Could soil microbiota be a reliable indicator in modelling or environmental assessment approaches?

Addressing the above research aspects will need interdisciplinary cooperation as well as modified measuring techniques. However, we hold high confidence for a better understanding of soil microbial features and their important functional roles in regulating the C dynamics, especially with the help of the emerging molecular analytical tools.

Chapter 3

MICROBIAL MEDIATED SOIL CARBON DYNAMICS AND PRIMING EFFECT IN RELATION WITH ORGANIC CARBON AND NUTRIENT AMENDMENT

3.1 Introduction

The Carbon (C) biogeochemical dynamic refers to the transformation and transfer of C among atmosphere, pedosphere, hydrosphere and biosphere of the earth. Soil is the largest C pool of the terrestrial ecosystem (Jobbágy and Jackson, 2000). During the past decades, the drastic increase of CO_2 accumulation in the atmospheric C pool is one of the consequences of C imbalance due to industrial revolution. In pedosphere, there are three major aboveground and belowground fresh organic C sources: (i) plant litter, (ii) root exudates, (iii) dead roots. Soils contain large amounts of microorganisms; for instance, In surface soils, there are over 10 billion microorganisms kg^{-1} soil and 2.5 t MBC ha^{-1} (Roper and Gupta et al., 2007). Compared to soil organic carbon (SOC) pool, the fresh organic C are less recalcitrant to microbial access and decomposition (Thiessen et al., 2013). Microorganisms regulate C movements by biological metabolic processes. The primary factor to categorise soil as a C sink rather than a C source, is that the decomposition rate by soil microorganisms should not overtake organic C input. The fresh organic supply influences soil microbial function as well as how they use indigenous C. Fontaine et al. (2011) proved that the fresh C stimulates the organic C pool depletion in deep soil layers. Ultimately, enhancing soil C sink for C remediation stems from two aspects: either increase in the organic C inputs or reduction in the breakdown of soil organic matter (Luo et al., 2003).

As a natural process, microbial PE refers to microbial C use alteration as a result of pulse or continuous C inputs of fresh organic matter (Kuzyakov, 2010). The increase of CO_2–C release after the addition of organic matter may result from the decomposition of additional CO_2 due to microbial turnover acceleration (Chen et al., 2014). This is generally accepted as apparent PE (aPE), while the real PE (rPE) is the depletion of native organic C induced by the organic matter decomposition (Kuzyakov, 2010). Up to date, studies about PE demonstrated that both increase and decrease of soil organic matter degradation exist, known as positive and negative priming (pPE and nPE), respectively (Garcia-Pausas et al., 2011; Guenet et al.,

2010a; Guenet et al., 2010b; Lehmann et al., 2011; Zimmerman et al., 2011). A pPE leads to over-mineralization of SOC, turning soil as C source. The nPE may attribute to the shift of microbial species from growing on original soil organic matter to freshly added organic matter (Guenet et al., 2010a). The mechanisms of PE are related to soil microbial function and microbial community dynamics (Blagodatskaya et al., 2008). Although there has been comprehensive experiments incorporated with isotopic labelled C to scrutinize PE (Kuzyakov, 2010), the conceptual understanding and mechanisms of PE remain unclear.

Of the key concern, the biochemical quality of the added fresh organic matter are critical in determining microbial C use (Pascault et al., 2013). One explanation is that the chemical property and quantity of organic inputs alter soil microbial community size and structure, and then C mineralization (Allison et al., 2008; Brant et al., 2006). Interestingly, Blagodatskaya et al. (2007) observed a low pPE with 48.7 mg C kg^{-1} soil, and later nPE when they increased the input amount to 4.87 g C kg^{-1} soil. The lack of nutrient support, such as N, may impede or even jeopardize the drastic microbial growth.

The microbial carbon use efficiency (CUE) is defined as the amount of organic C taken up by microorganisms and the resultant microbial biomass (Rousk and Bååth 2011; Sinsabaugh and Follstad Shah, 2012). It is also termed as microbial growth efficiency, C assimilation efficiency and gross microbial growth yield, and these terms are used interchangeably (Manzoni, 2012). Besides the informality of the terminology, various approaches have been used to measure CUE, resulting in variable results, which adds confusion to this concept (Frey et al., 2001; Tiemann and Billings, 2011). The different approaches of measuring microbial CUE result in the discrepancy of assumptions (Frey et al., 2001).

However, because of the diversity of microorganisms and complexity of microbial function, the mechanism of how soil microbes mediate SOC is still like a ‘black box’. A detailed characterization of the relation between soil microbial properties and SOC dynamics is essential for understanding the processes involved in C sequestration. We recognize that *ex situ* soil incubations may have inconsistency from those in ecosystems. However, the results obtained for the purpose of this chapter are to provide fundamental supporting data for the follow up measurements in the next three chapters.

3.2 Objectives

The overall objective is to examine the extent of regulation carried out by the heterotrophic microorganisms towards soil C dynamics, compare and understand microbial CUE measurements in other literatures. This chapter also intends to include the following specific objectives based on the designed experiments:

- To compare and evaluate three different approaches to measure microbial CUE.
- To investigate microbial growth strategy with the primed CO_2 evolved due to various exogenous C and nutrient amendments.
- To examine PE in relation with various levels of labile C and nitrogen (N) ratio, and interpret the difference between aPE and rPE, pPE and nPE.

3.3 Hypothesis

- The level of C:N ratio influences microbial activity, microbial biomass and consequently microbial CUE.
- By labelling different C sources with isotopic techniques, it is clearly to trace C flow and the destination, that is to say, either sequestrated in soil or released into atmosphere.

3.4 Experimental treatments and amendment characteristics

To test the above hypotheses and examine the degradation of ^{13}C labelled glucose, the experimental treatments and selected characteristics of the amendments used are summarised and presented in **Table 3.1**. Laboratory experiment included six treatments with triplicates.

Table 3. 1 The major experiment treatments and selected characteristics of the amendments

Treatment	Applied C as glucose (µg C g^{-1} dry soil)	Applied N as $(NH_4)_2SO_4$ (µg N g^{-1} dry soil)
Control	–	–
CL	25	–
CH	50	–
N	–	2.2
Optimum C:N ratio at 23	50	2.2
High C:N ratio at 50	50	1.0
Low C:N ratio at 10	50	5.0

Control as soil without amendments; CL: soil applied with low C level; CH: soil applied with high C level; N: soil applied with sole N; CN10: soil applied with C:N ratio at 10; CN23: soil applied with C:N ratio at 10; CN23; CN50: soil applied with C:N ratio at 10; CN50

3.5 Materials and methods

3.5.1 Soil collection and preparation

Surface soils (0–10 cm) were collected in Barossa Valley, South Australia (138°57'37''E, 34°27'48''S). The region is characterised by Mediterranean climate, with annual average high temperature of 22.3 °C and average low temperature of 12.2 °C, an average annual rainfall of 436.57 mm. After sampling, soils were air dried and sieved thoroughly (< 2 mm) for homogenization. Fine roots and other plant debris were carefully removed during processing. Soils were stored in 4 °C until further analysis. Prior to the experiment, the moisture content was adjusted to 50% of the water holding capacity (WHC) and pre-incubated at 25 °C, 28% room humidity for 7 days.

3.5.2 Soil physicochemical characterization

Soil physicochemical characteristics were analysed and the results are presented in **Table 3.2**. After mixed with water (soil:water ratio at 1:5) for 2 hours by an end-over-end equilibration and centrifuged, soil pH and EC were determined by a pH/conductivity meter of the upper solution. Soil texture was detected by the micro-pipette method from Miller and Miller (1987). Soil CEC and exchangeable cations were determined by summing the exchanged cations with Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) (Ciesielski et al., 1997; Hendershot and Duquette, 1986). Weighed 0.5 g soil was prepared for the purpose to detect total element. Firstly, soils and 5 mL of aqua regia (HNO_3:HCl ratio at 3:1) were put in Teflon digestion vessels and digested in a micro wave digestion oven. The suspension was decanted and filtered before being analysed by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) (McDowell, 2005). Soil total carbon (TC), total nitrogen (TN) and total organic carbon (TOC) were detected by dry combust technique with Leco C/N analyzer (Leco TruMac® CNS/NS). For TC and TN, 0.2 g oven dried soil was weighed and combusted at 1300 °C and 5 seconds Oxygen flow. The instrument was calibrated for every 10 detections with standard weights of Leco EDTA calibrator (containing 95.7 g N kg^{-1} and 410 g C kg^{-1}) (Wright and Bailey, 2001). As for TOC, we used the combustion method as described above with Leco C/N analyzer. In order to eliminate the influence of carbonate on TOC, two drops of H_2S were added onto soils before inserting samples into Leco C/N analyzer.

Table 3. 2 Selected properties of sample soil

Soil property	Values
Soil pH	6.26
Electrical conductivity (mS cm^{-1})	27.53
Cation exchange capacity	32.71
Total organic carbon (%)	2.29
Total nitrogen (%)	0.14
C:N ratio	16.36
Potassium (%)	0.059
Calcium (%)	0.139
Copper (%)	0.890
Iron (%)	0.142
Zinc (%)	0.819
Phosphorus (%)	0.013
Sodium (%)	0.023
Sulphate (%)	0.037
Manganese (%)	2.850

3.5.3 Isotopic glucose addition and microbial respiration

Fifty kg air-dried soils were universally mixed with ^{13}C labelled glucose (CO_2, 99.9 atom% ^{13}C). The ^{13}C labelled glucose was diluted with unlabelled glucose, while N was applied to the soil as $(NH_4)_2SO_4$ to make the C:N ratio at: (i) low as 10:1, (ii) optimized as 23:1, and (iii) high as 50:1. The different level of C:N treatments were used to stimulate sufficient PE reaction (Chen et al., 2014; Conde et al., 2005). The C:N treatments were evenly applied across the soil surface using a syringe. Soils treated solely with deionized water were prepared and physically mixed as control samples. All the treatments were tightly sealed and incubated in dark at 25 °C for 30 days (Brant et al., 2006). Soil moisture was adjusted to 60% WHC before sealing and incubation.

The CO_2 efflux was monitored during the 720 h incubation with the goal to determine PE and distinguish the intensive and slow phase of decomposition (Chen et al., 2014; Werth and Kuzyakov, 2010). Gas probes were used for sampling at 6, 12, 24, 36, 48, 72, 96, 120, 144, 168, 192, 216, 312, 480, 720 h, then followed by destructive sampling for microbial biomass

carbon (MBC) (Chen et al., 2014; Werth and Kuzyakov, 2010). CO_2 gas was sampled by 1 mL syringes, and injected into 5 mL glass bottles with rubber lid. For the purpose of rinsing the syringe, injection and suction was carried out every time before taking the samples. $\delta^{13}C$ values of CO_2 were analysed by mass spectrometry (Nu Instruments, Nu Horizon) in line with a Nu Instruments GasPrep.

Separate jars were set to measure the total CO_2–C content. Ten g soils were prepared in Schott bottles for measuring microbial respiration with the same incubation status as described above. Three blank Schott bottles were set as control. A 20 mL open-top Schott bottle containing 10 mL of 0.05 M NaOH was used to trap evolved CO_2 from microbial respiration. Then the 20 mL bottle was decanted and rinsed with deionized water three times. All the solution was added into an Erlenmeyer flask for titration. After adding 5 mL of 0.5 M $BaCl_2$ solution, CO_3^{2-} was precipitated as $BaCO_3$. The amount of evolved CO_2 was measured with back-titration of unreacted NaOH using 0.03 M HCl in the presence of a phenolphthalein indicator until the colourless end point was reached. Microbial respiration was calculated using the following equation (**Eq. 3.1**):

$$MR = \{MWCO_2(V_b - V_s) \times M \times 1000\}/(DW \times T \times 2) \qquad \text{Eq. 3.1}$$

where *MR* is the microbial respiration (mg CO_2–C kg^{-1} soil hr^{-1}), *MWCO₂* is the Molecular weight of CO_2, V_b is the volume of HCl for control titration, V_s is the volume of HCl for sample titration, *M* is the concentration of HCl, *DW* is the dry weight of the soil, *T* is the time of incubation, and *2* is the factor that accounts for the fact that two OH^- are consumed by one CO_2.

3.5.4 Total and ^{13}C labelled microbial biomass carbon

The determination of MBC was based on the fumigation-extraction method in Vance et al. (1987). To the extracted microbial biomass, 10 g (dry wt.) soils were set in a vacuum desiccator with 50 mL of ethanol free chloroform. The desiccator was tightly sealed and pumped until chloroform was vaporized. The whole incubation needs to be in dark for 48 hrs. Another set of un-fumigated soils was prepared as well. After 48 hrs, both the fumigated and non-fumigated soils were mixed with 0.5 M K_2SO_4 at a solid:solution ratio of 1:4. After shaking in an end-over-end equilibration for an hour, samples were centrifuged and filtered through Whatman #40 filter papers. The C content was analysed by a Leco CN analyzer (Leco TruMac® CNS/NS) at 1100 °C. MBC was calculated using equation **Eq. 3.2**:

$$MBC = E_c/K_c \qquad \text{Eq. 3.2}$$

where, *MBC* stands for MBC (mg C kg^{-1} soil), E_c stands for the value=(C content extracted from fumigated soils – C content extracted from non-fumigated soils), and K_c stands for the

conversion factor (0.45) from chloroform flush C values into MBC (Anderson and Domsch, 1989).

To detect the $\delta^{13}C$ incorporated into microbial biomass, K_2SO_4 extractions were pipetted into acetone-rinsed glass vials (Brant et al., 2006), and frozen dried for 48 hrs. Then, the samples were freeze-dried and analysed by mass spectrometry (Nu Instruments, Nu Horizon) in line with a Nu Instruments GasPrep.

3.5.5 Microbial Phospholipid fatty acid analysis

Microbial phospholipid fatty acid (PLFA) patterns were used to estimate the relative abundance of Gram positive (G+) and negative (G−) bacteria, fungi and actinomycetes (Marschner et al., 2003). Microbial PLFAs were extracted following the method of Frostegård et al. (1993) and Bossio et al. (1998). In brief, 5 g freeze-dried soils were extracted with one-phase extraction solvent. The one phase solvent was a mixture of chloroform, methanol and citrate buffer (1:2:0.8, *v/v/v*), while the citrate buffer was made from citric acid and sodium citrate (3:1, *v/v*) with pH at 3.6 (Bligh and Dyer, 1959). After equilibrating with an end-over-end shaker and centrifuging twice, the upper solution was decanted into non-transparent vials, vortexed before standing over nights. Removed up layer as the solution layered. Dried the left liquid with pure N_2 gas at 32 °C. The thin solid phase extraction left in vials was re-dissolved with chloroform and transferred with 1 mL chloroform into separation phase extraction (SPE) column. To set up the column, 0.5 g of silica was packed, then followed with chloroform conditioning by three times chloroform flow. The transferring included three steps: chloroform (5 mL), acetone (5 mL) and methanol (5 mL). Discarded the first two leaching liquids, or kept them separately if future analysis was needed. Dried the final leaching solution with continuous N_2 flow at 32 °C. Then added 0.5 mL of 1:1 (*v/v*) of methanol:toluene and 0.5 mL of 0.2 M methanolic KOH (by dissolving 0.28 g KOH in 25 mL of methanol). The PLFAs were converted into fatty acid methyl esters (FAMEs) with mild alkaline methanolysis. The samples were incubated at 37 °C for 30 minutes, and then cooled to room temperature. One mL deionized water, 0.15 mL 1 M acetic acid and 1 mL hexane were added, vortexed for 30 seconds and centrifuged to separate the solution into two layers. The upper layer was carefully decanted with a pipette into GC vials. This procedure was repeated and finally the solution was dried with continuous N_2 flow. The samples were stored at −20 °C in total dark before further analysis. An internal standard (methyl nonadecanoate, C19:0) (10 ng) was added to all samples as a quality control measure. The FAMEs were analysed by gas chromatography-mass spectrometry (GC-MS) (Model 7890B/5977B, Agilent Technologies Ltd., USA; AxION iQT with Cold EI Source, Perkin Elmer, USA). An RTX-5MS fused silica capillary column (60 m, 250 µm × 0.25 µm film thickness) (Supelco, Sigma-Aldrich, Australia) was used. Sample

(1 µL) was injected in splitless mode with an injector temperature of 250 °C, and helium carrier gas at a constant flow rate of 1.4 mL min^{-1}. The temperature program was set as follows: column temperature initially at 60 °C for 1 min, then increased to 180 °C at a rate of 12 °C min^{-1}, then increased to 300 °C at a rate of 4 °C min^{-1} and kept at 300 °C for 4 min. Electron energy in the detector was set to 70 eV. Data was acquired in scan mode from 50 to 400 Da at 3 scans per second. Quantification was conducted against a Supelco 37 standard mixture (Supelco, Bellefonte, PA), and the C19:0 internal standard with a 6 point linearity curve analysed in triplicate ($r^2 \geq 0.98$ for each component). Each PLFA peak was identified by comparing the respective retention time and by their mass spectra. The isomers not included in the standard mix were quantified against the relative response factor for C16:0, and were individually identified by their mass spectra from a Cold EI TOF scanning analysis conducted on a Perkin Elmer AxION iQT instrument. The specific microbial species were identified by the signature PLFAs listed in **Table 3.3**.

Table 3. 3 Biomarker phospholipid fatty acid (PLFA) used to characterize microbial communities in the experimental soils (Frostegård et al.,1993; Zelles, 1999)

Microbial group	Biomarker PLFAs
Gram− bacteria	C16:1ω7c
Gram+ bacteria	iC15:0, aC15:0, C15:0, iC16:0, iC17:0, aC17:0, C:170
Actinobacteria	10MeC16:0, 10MeC17:0, 10MeC18:0
Fungi	C18:2ω6c, C18:1ω9c

3.5.6 Calculations

3.5.6.1 Isotopic carbon calculation

The absolute primed C was calculated based on the difference between C content in CO_2 evolved from soils with amendments and evolved from soils unamended (**Eq. 3.3**). The identification of CO_2 evolved from glucose was based on the added ^{13}C labelled glucose.

$$PE_C = (C\text{–}CO_2^t - C\text{–}CO_2^g) - C\text{–}CO_2^c \quad \text{Eq. 3.3}$$

The microbial priming effect (PE), expressed as the percentage of primed CO_2–C as calculated in **Eq. 3.3** to CO_2–C evolved from the control:

$$PE_{\%} = \{(C\text{–}CO_2^t - C\text{–}CO_2^g) - C\text{–}CO_2^c\}/C\text{–}CO_2^c \times 100 \quad \text{Eq. 3.4}$$

where PE_C is primed soil CO^2–C, expressed as µg C–CO_2 g^{-1} soil, $PE_{\%}$ is the relatively intensity of PE, $C–CO_2^t$ is the TC content of CO_2 evolved from soils amended with substrate applications, $C–CO_2^g$ is the unlabelled C content in CO_2 evolved from glucose, $C–CO_2^c$ is the unlabelled C content in CO_2 evolved from soils without substrate addition.

The $\delta^{13}C$ value of MBC was calculated as **Eq. 3.5** and **Eq. 3.6**

$$\delta^{13}C_{MBC} = (\delta^{13}C_f - \delta^{13}C_{nf})/K_c \quad \text{Eq. 3.5}$$

$$C_{MB} = C_{MB}^t - C_{MB}^g \quad \text{Eq. 3.6}$$

where $\delta^{13}C_{MBC}$ is the $\delta^{13}C$ derived from microbial biomass, $\delta^{13}C_f$ and $\delta^{13}C_{nf}$ are the $\delta^{13}C$ values in fumigated and non-fumigated soil samples, respectively, C_{MB} is the microbial C from soil, C_{MB}^t is the total MBC, C_{MB}^t is the C from glucose.

3.5.6.2 Microbial carbon use efficiency

Microbial CUE, for four different approaches was estimated as in **Eq. 3.7**–**3.10** (Frey et al., 2001).

$$C_s = (dS_c - \Sigma CO_2–C)/(dS_c) \quad \text{Eq. 3.7}$$

$$C_m = \Delta M_c/(\Delta M_c + \Sigma CO_2–C) \quad \text{Eq. 3.8}$$

$$C_p = \Sigma(\Delta PLFA \times mf)/dS_c \quad \text{Eq. 3.9}$$

$$C_r = \Delta M_c/dS_c \quad \text{Eq. 3.10}$$

where C_s is microbial CUE measured as substrate depletion, dS_c is the decrease of substrate C, $\Sigma CO_2–C$ is the cumulative C as microbial respiration, C_m is CUE measured as microbial biomass variation, ΔM_c is the change of MBC, C_p is the CUE measured based on PLFA, $\Delta PLFA$ is the change of microbial PLFA, mf is the conversion factor (Bailey et al., 2002) to convert PLFA to biomass C, $\Sigma(\Delta PLFA \times mf)$ is the sum of PLFAs from various microbial species, S_c is the total amount of substrate C added, C_r is the CUE of the ratio of biomass accumulation to the decrease of substrate C.

3.5.7 Statistical analysis

Shapiro-Wilk test and homogeneity of variance were used for normal distribution of all data with Levene statistics. Significant differences among treatments were tested using one factor ANOVA followed by the least significant difference (LSD) test. The effect of C:N ratio on microbial properties were assessed by one factor ANOVA. PLFA data were analysed with principal component analysis (PCA) to elucidate major variation and covariation both for

individual PLFA and microbial species implying varimax rotation. All these statistical analysis were performed in SPSS version 23.0 (SPSS Inc., Chicago, USA) with the significant differences accepted at $p < 0.05$.

3.6 Results and discussion

3.6.1 Priming effect in relation to microbial growth strategies

Induced by organic and nutrient amendment, cumulative PE dynamics during the incubation period are presented in **Figure 3.1**. The cumulative PE differed significantly ($p < 0.05$) among treatments. Glucose with low or no nutrient amendments lead to higher cumulative PE (as indicated by the dash line with solid dots and solid line with square, respectively). Organic C amendments coupled N at ratio of 23 and 10 also showed increased positive PE, though their values are lower. Similar results were observed by Köster et al. (2011). This suggested that the fresh organic matter promoted microorganisms to use soil organic matter. In our research, the cumulative PE increased during the whole incubation period. Because 14 days incubation is shorter than the microbial turnover, which is about 21–75 days (Blagodatskaya et al., 2009). So most of the increased CO_2 emission was likely from SOM rather than microbial biomass.

We found positive PE as induced by fresh organic input (**Figure 3.1**). More importantly, same in our research, both high- and low-N availabilities could induce the PE (Chen et al., 2014). The highest total PE by the end of incubation was 454.30 % in C:N ratio at 50, while the lowest is 151.96% in ratio at 10 (apart from sole N treatment). This is a good demonstration that the fresh organic matter in the presence of mineral nutrient amendments impact microbial mineralization of indigenous C, especially the C:N ratio that is strongly related to microbial community response to organic amendments. Kuzyakov and Cheng (2004) also concluded that the quality of root exudates influence microbial mobilization and immobilization ((im)mobilization) processes, hence alter soil C flow.

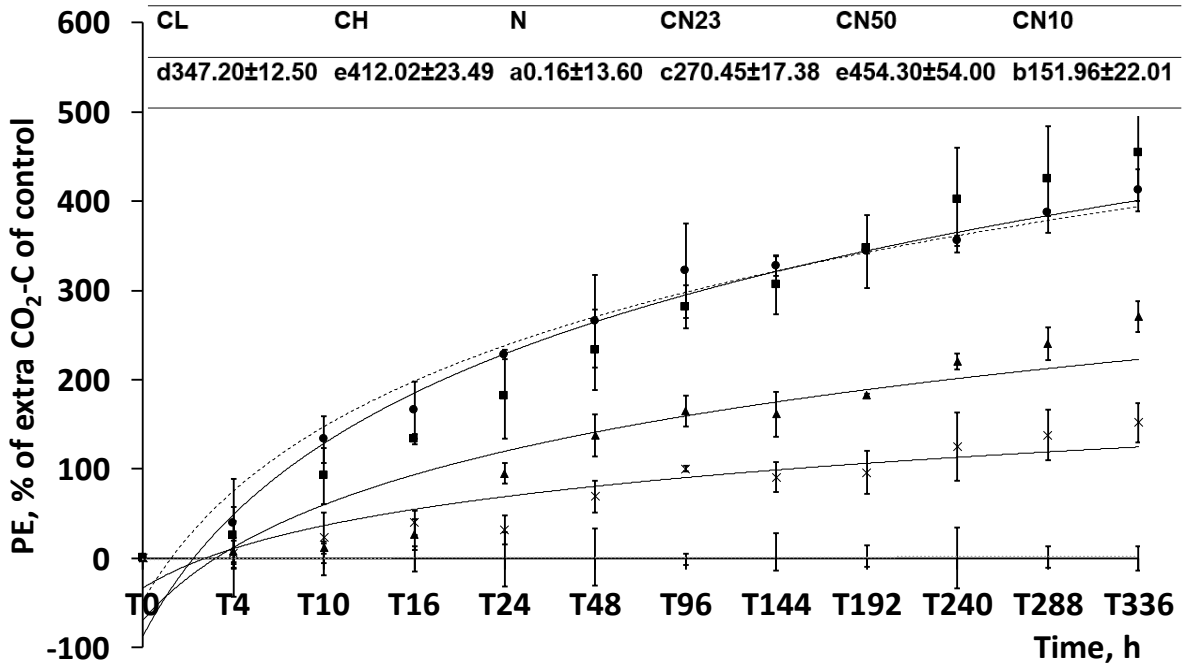


Figure 3.1 Cumulative unlabelled CO_2–C efflux rate of control as priming effect (PE is calculated as in **Eq. 3.4**). Inset table shows priming effect value at the end of incubation for the 6 treatments. Different letters indicate significant differences ($p < 0.05$). Carbon as low (CL) and high glucose amounts (CH), only nitrogen (N) or only carbon (C), and carbon:nitrogen ratio at low (CN10), medium (CN23) and high (CN50). Bars show standard errors of the means (n = 3). Standard errors are not shown when less than the symbol size.

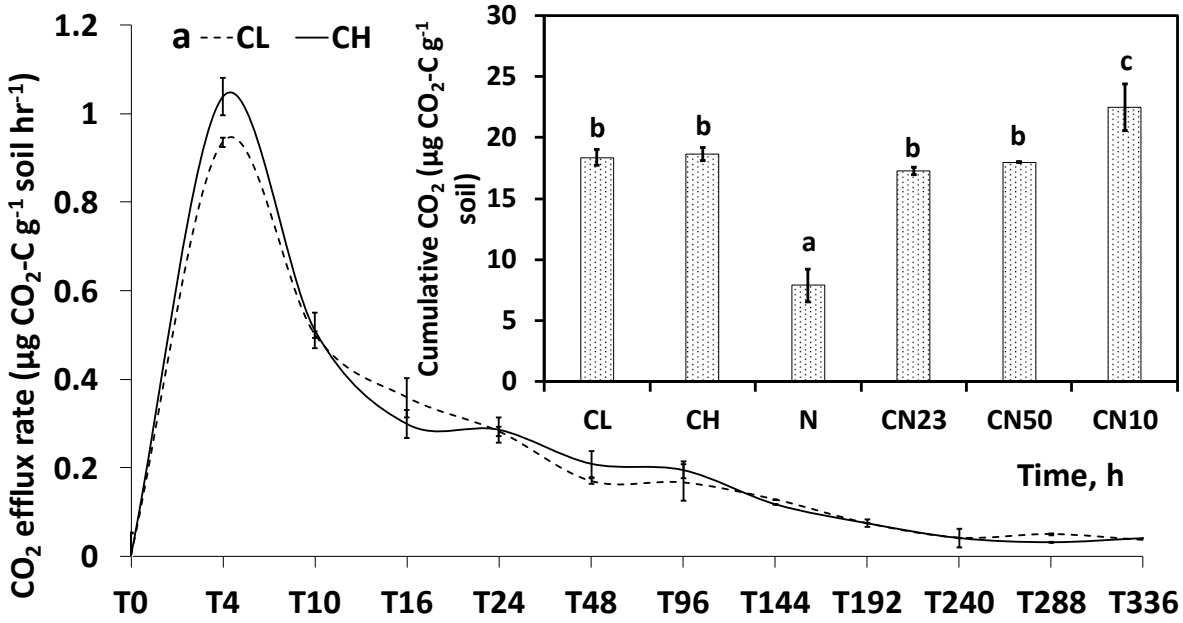
The disparate response to organic amendments appear to be from the interference of mineral N. It is not a surprise to notice the stimulated microbial population and the initial loss of C after C and N application. Hence, the highest PE is in the C:N ratio at 50, rather than with only C amendments or higher C:N ratio. This finding concurs with the results of some previous research by Fontaine et al. (2011), Xu et al. (2017a) and Xu et al. (2016b). In conclusion, the organic amendments served as a primer for the growth of microorganisms. The PE intensity depends on the property of soil native microbiota as well as the incorporated fresh organic matter. The impact on microbiota was based on the quality, in our search, the C:N ratio. The stimulated microorganisms were then regulating the duration and intensity magnitude of PE.

The argument about PE always lead to the direction that it is a short-term phenomenon and are negligible in the ecosystem level. However, not only did Kuzyakov et al. (2010) argue that the priming is a natural phenomenon, but Fontaine et al. (2011) also pointed out that PE persists several months after the fresh added organic matter decomposed and contributed to C sequestration underground. As for our research, the amended fresh organic matter with higher N content facilitates a reduction in PE intensity. The consideration of the proportion of C and nutrient in an added organic matter may have very different consequences regarding terrestrial C storage. However, this research omits the possible PE discrepancy among

different ecosystems. Therefore, future research can include more varieties of soil properties or considering plant-soil mosaic/patch/mesocosm ecosystem.

3.6.2 Microbial respiration response to carbon and nitrogen amendments

This research showed similar microbial respiration rate across different scale treatments. Microbial respiration peaked their highest values then dropped gradually afterwards (**Figure 3.2 a and b**) due to labile C depletion. Similar result was in Xu et al. (2016b) and Zhang et al. (2008). The highest microbial activity after 4 hours' incubation was C:N ratio at 50, followed by the solely C addition, C:N ratio at 10 and 23, lowest value was in sole mineral N amendments. Although microbial respiration also increased after 4 hours incubation with solo mineral N input, yet the respiration rate was lower compared to other treatments with organic C input (**Figure 3.2b**). The possible explanation was that the soils used in this experiment were low in nutrient content (**Table 3.2**). The application of N was beneficial to microbes, which was suppressed by the previous nutrient deficiency. In addition, we referred the optimum C:N ratio at 23 based on previous studies, but the microbial activity did not get significantly higher in this treatment.



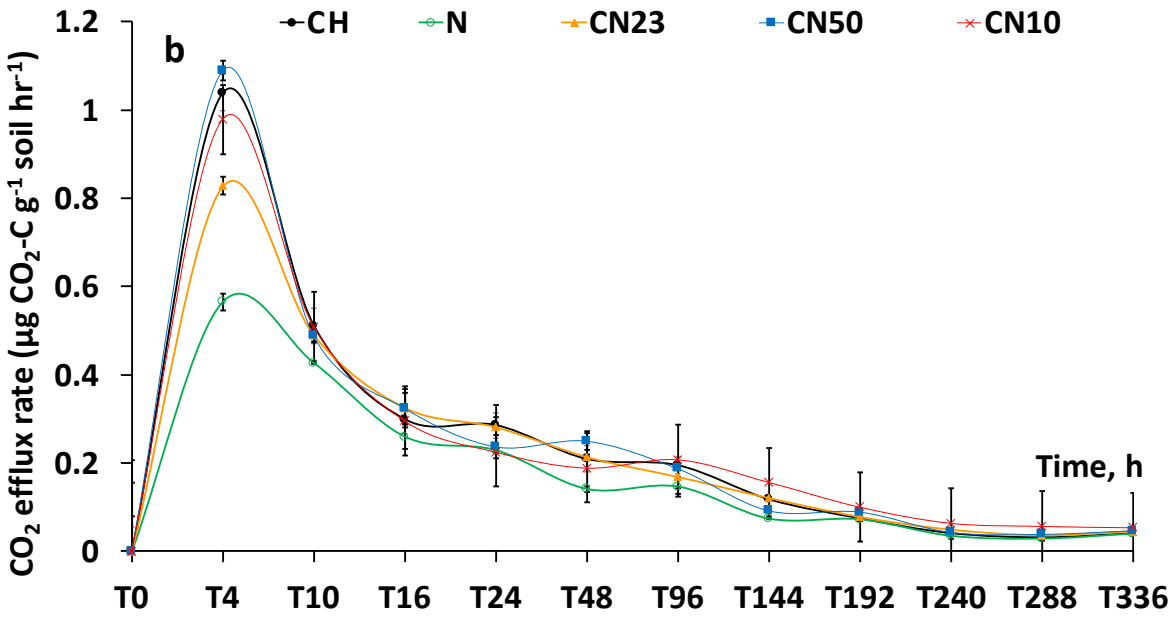


Figure 3. 2 Dynamics of total CO2 efflux rate in different treatments: (**a**) soil applied with low organic C (CL) and high organic C (CH), and (**b**) soil applied only with organic C (C) or mineral N (N), and soil with C:N ratio at 23 (CN23), 50 (CN50) and 10 (CN50), respectively, **inset** in (**a**) showed cumulative microbial respiration values by the end of incubation among the different treatments. Different letters in the same column indicate significant differences ($p < 0.05$). Bars show standard errors of the means (n = 3). Standard errors are not shown when less than the symbol size.

The labile C input increased the total microbial activity, and the higher the concentration the more CO_2 was released (**Figure 3.2a**). The cumulative C released as CO_2 was 85.72 µg C g^{-1} dry soil in control, 183.66 and 186.40 µg C g^{-1} dry soil with low and high glucose addition, respectively. There also was significant difference of total respired CO_2–C ($p < 0.05$) as affected by C:N ratio in amendments (presented in **Figure 3.2a inset**). They were 172.69, 179.85 and 224.96 µg C g^{-1} dry soil in samples with C:N ratio at 23, 50 and 10 respectively. Rousk and Bååth (2011) asserted that the highest cumulative respiration was observed without N addition. In our research, the relatively higher CO_2–C releases were also in soil samples with lower N but higher glucose amendment.

While there is no significant difference in total respired CO_2–C among CL, CH, CN23 and CN50. There is significant difference of the glucose derived C ($p < 0.05$, blank bar in **Figure 3.3**). The largest two values were in sole C application (77.58 µg C g^{-1} dry soil) and C:N ratio at 10 (70.15 µg C g^{-1} dry soil). As discussed in PE, the relatively high C input triggered the microbial growth initially. However, the discrepancy of glucose derived C was due to the quality of amendment, in this research it is C:N ratio. On the other hand, although the largest total CO_2–C emission was from CN10, most of the CO_2–C was SOC derived (174.24 µg C g^{-1} dry

soil) rather than the added glucose (50.72 µg C g^{-1} dry soil). The largest SOC–C was in sample with C:N ratio at 10. The other values were 124.17 and 108.81 µg C g^{-1} dry soil in solo low glucose and solo high glucose amendments, respectively. And 132.57 and 109.69 µg C g^{-1} dry soil with amendments of C:N ratio at 23 and 50, respectively. The CO_2–C production was 78.85 µg C g^{-1} dry soil in solo N amended soil. To sum up, with the same glucose addition, the low nutrient supply driven microbes utilize more indigenous C, leading to higher C depletion (shown in **Figure 3.3** with treatments of CH and CN50).

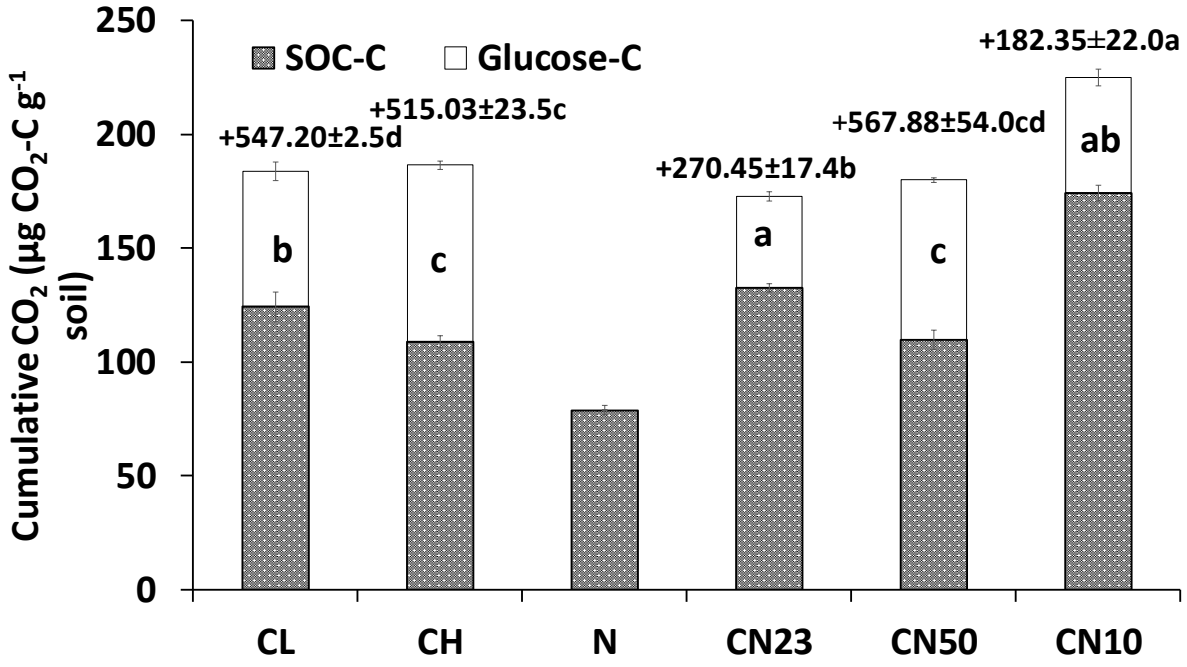


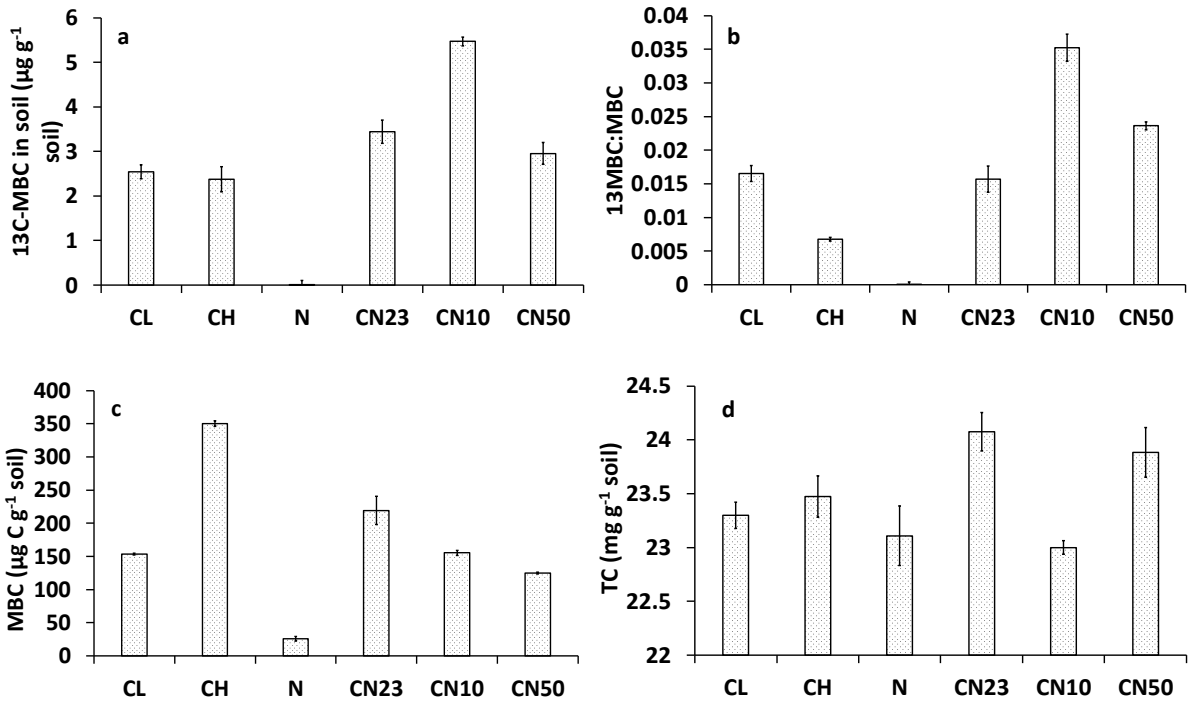
Figure 3. 3 Cumulative CO_2 released from SOC (grey bar) and CO_2 from organic amendment (blank), and priming effect data (above the bars ± means, n = 4) at the end of incubation period. Both bars show standard errors of the means (n = 3). Bars containing the same letter are not significantly different ($p < 0.05$). Different letters inside of bars indicate significant differences ($p < 0.05$). Small letters for SOC derived CO_2–C and priming effect. Capital letters for glucose derived CO_2–C. Treatments as control: soil applied only with water, CL and CH: soil applied with glucose at low and high quality, respectively; N: soil applied only with mineral nitrogen; CN23, CN50 and CN10: carbon:nitrogen ratio at 23, 50 and 10, respectively.

3.6.3 Microbial biomass and soil carbon storage

3.6.3.1 Microbial biomass carbon and nitrogen

The largest value of glucose-derived C in microbial biomass was from the CN10 treatment (5.47 µg C g^{-1} dry soil as in **Figure 3.4a**), but there was no significant difference among the

other four treatments of CL, CH, CN23 and CN50. This indicated a relatively higher glucose–C amount was incorporated into microbial biomass compared to other treatments, notably within treatment CH, CN23 and CN50 with the same glucose addition. Fresh organic addition improves microbial biomass (Bardgett et al., 1999b). But the source of microbial C could be varied, depending on soil microbiota and their utility preference. In this research, the highest MBC (350.24 µg C g^{-1} dry soil) and lowest 13MBC:MBC ratio (0.0068) were both in CH treatment (**Figure 3.4b and c**). This demonstrated that the high glucose amendment increased microbial biomass at the cost of indigenous SOC depletion. However, the treatment with relatively higher N (CN10) supply presented a high 13MBC:MBC ratio, although the total MBC was only 155.26 µg C g^{-1} dry soil. The microbial C was 25.66 µg C g^{-1} dry soil in solo N amendment. Compton et al. (2004) observed reduced microbial abundance with N fertilized soil. Fontaine et al. (2011) also noticed significant difference between soils applied with low and high N application: with the low N application, the speed of soil stable C depletion was faster than the C sequestration. The microbial biomass N content was the largest (57.74 µg N g^{-1} dry soil) (**Figure 3.4e**) while the lowest (0.44) (**Figure 3.4g**) in solo N application. Not surprisingly, the solo glucose amendment had a significant high C:N ratio (5.04) in microorganisms ($p < 0.05$) (**Figure 3.4g**).



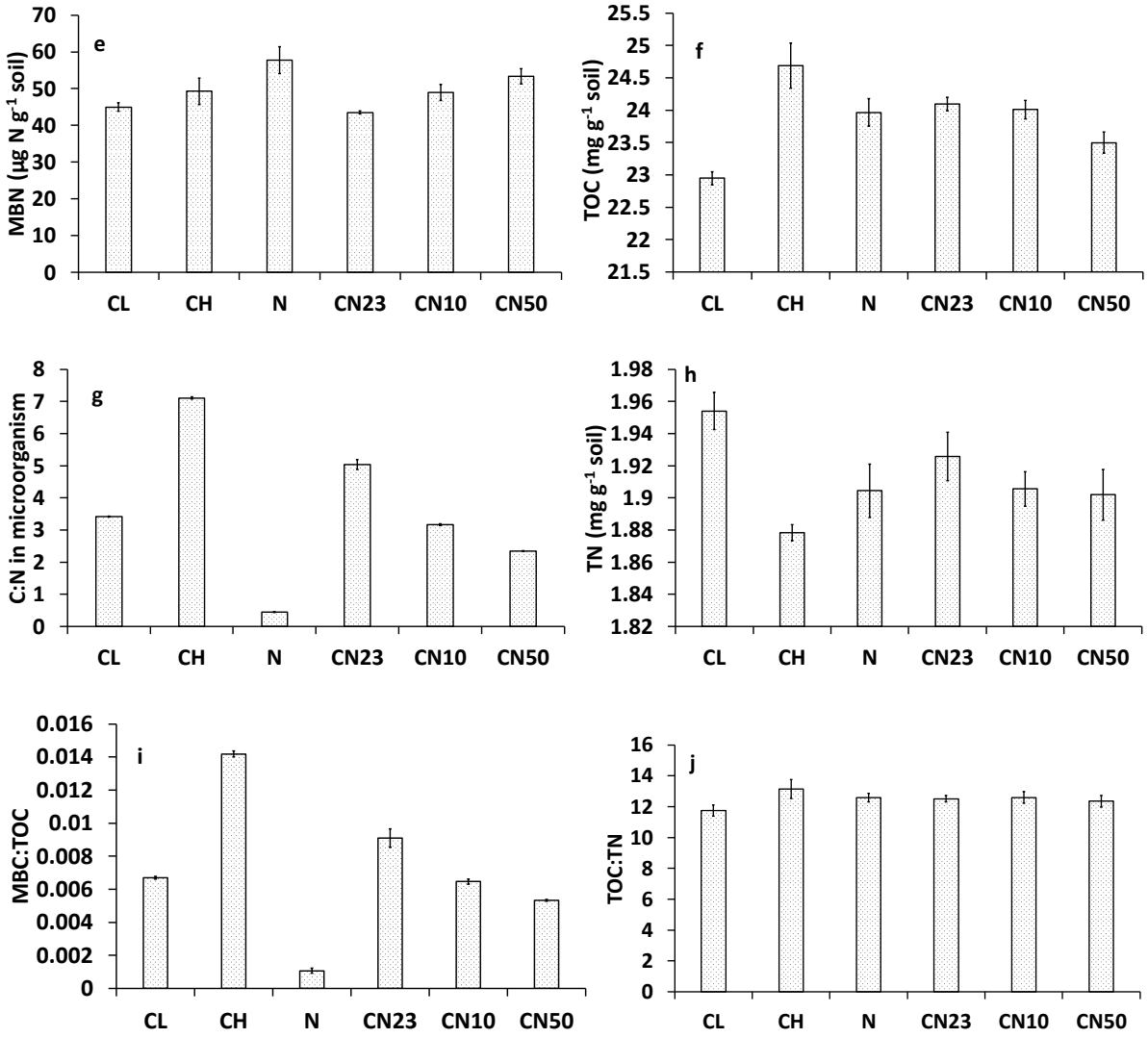


Figure 3. 4 Carbon and nitrogen contents in microbial biomass and soil among the different treatments. Treatments include control: soil applied only with water, CL and CH: soil applied with glucose at low and high quality, respectively; N: soil applied only with mineral nitrogen; CN23, CN50 and CN10: carbon:nitrogen ratio at 23, 50 and 10, respectively. Microbial biomass carbon as MBC, microbial biomass nitrogen as MBN, δ13C in microbial biomass carbon as 13C-MBC, total carbon as TC, total organic carbon as TOC, total nitrogen as TN. Bars show standard errors of the means (n = 3).

3.6.3.2 Soil carbon and nitrogen storage

The highest TC value was in CN23 treatment (24.08 mg C g^{-1} dry soil) (**Figure 3.4d**) while the largest TOC value was in CH due to glucose input (24.69 mg C g^{-1} dry soil) (**Figure 3.4f**). The TN was significantly low in CH treatment (1.88 mg N g^{-1} dry soil) (**Figure 3.4h**). After the stimulation of fresh organic amendments on microorganisms, especially microbial species such as fungi, were tended to acquire N from soil organic matter in nutrient limited condition, causing a low N soil. This 'mining theory' explained why there was lower TN with higher glucose addition (Fontaine et al., 2011). Nutrient deficiency of litter could intensify PE and

promote C degradation. The possible reason may be due to the co-mineralization of exogenous by fast-growing microbial species (Xu et al., 2017a). The MBC:TOC ratio varied significantly ($p < 0.05$) among samples at the ending of incubation (**Figure 3.4i**). The smallest was in solo N addition while the largest was in solo high glucose input. The latter was ~13 times larger than the former. Fontaine et al. (2004) argued that there was N sequestration in the high nutrient treatment, but it was not observed in this research. In spite of the various ratio of organic C and mineral N addition, the TOC:TN ratio by the end of incubation did not show too much difference (**Figure 3.4j**). The TOC:TN ratio was in CL at 11.74 while the maximum was 13.14 in CH.

3.6.4 Microbial community regulated soil carbon dynamics

Microbial community composition showed various percentage among different treatments (**Figure 3.5**). Compared to fungi, a general concept is that bacteria are tended to mineralize labile organic material and have relatively higher C:N ratio (Hodge et al., 2000). However, both fungi and bacteria will seek natural N source when C:N reaches over 30:1 (Hodge et al., 2000). The relation between microbial species and C degradation are controversial. Fungi and G+ bacteria may both serve important roles in new litter C degradation. But PE may be controlled by fungi as suggested by Fontaine et al. (2004) and Garcia-Pausas et al. (2011) or by G− bacteria instead of fungi in Bastida et al. (2013). Also suggested by Xu et al. (2016b), they found that the N addition could be beneficial for fungi, especially yeast fungi and so called sugar fungi, they are the cause for the increased CO_2–C release and litter decomposition. While Bastida et al. (2013) suggested that the G− bacteria have more influence on the initial glucose decomposition than G+ bacteria. In our research, the two highest PEs were in CH and CN50. Bacteria were the dominant species in both the treatments, 39.93 and 33.72%, respectively. But followed by actinomycetes (26.26%) in CH treatment while fungi (26.90%) in CN50. Fungi did not have a big percentage in CH, only 15.91%. In one year study on litter decomposition by Xu et al. (2017a), they found that actinomycetes were important in regulating the CO_2 emissions sourced from litter after the addition of fresh organic amendments.

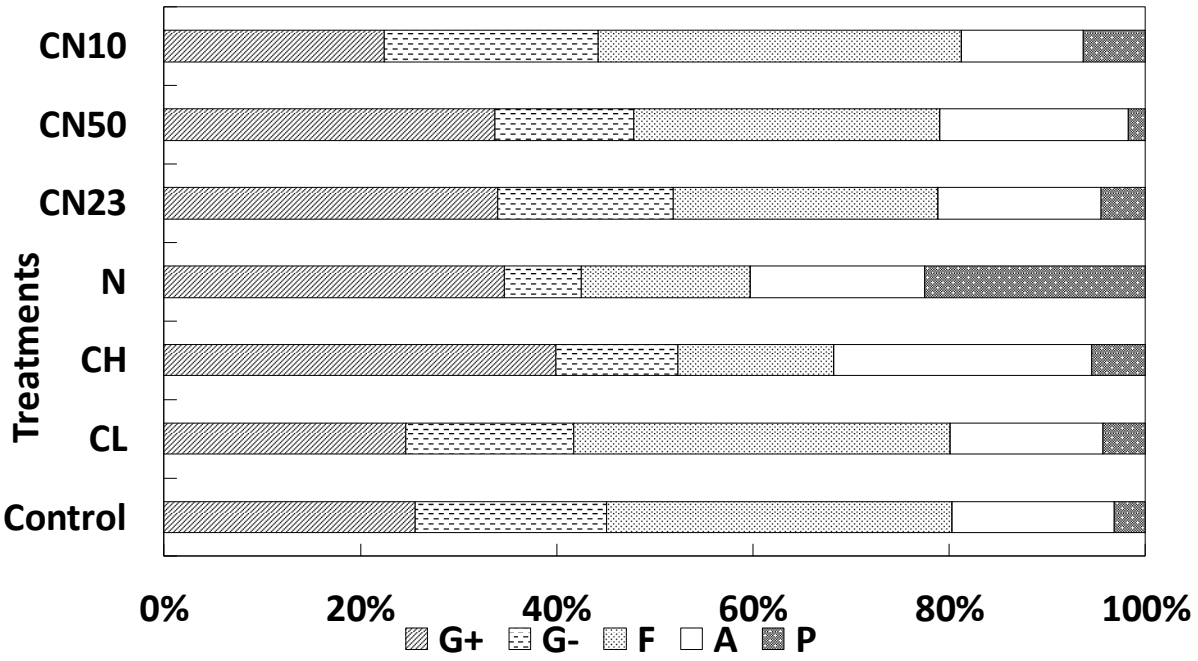


Figure 3. 5 Proportion of fatty acids represent four microbial species (%): Gram-positive bacteria, Gram-negative bacteria, fungi and actinomycetes. Treatments include control: soil applied only with water, CL and CH: soil applied with glucose at low and high quality, respectively; N: soil applied only with mineral nitrogen; CN23, CN50 and CN10: carbon:nitrogen ratio at 23, 50 and 10, respectively.

The properties of the added fresh organic matter are closely related to soil microbial community composition (Xu et al., 2016a; Xu et al., 2017a). In this research, the fresh labile glucose amendments contribute to the initial growth of G+ bacteria. Similar results were found in Zhang et al. (2008) and Garcia-Pausas et al. (2011). Because the added labile C was much over MBC, the native microorganisms used the fresh C instead of SOC at the beginning. Only after less labile C exit in the later stage, did the microorganisms begin to utilize the native SOC. That is to say, the occurrence of PE is based on two primes: (i) the stimulation of microbial growth (mainly by added fresh amendments), (ii) the depletion of energy and nutrient rich amendments come afterwards. Rousk and Bååth (2011) observed increase and decrease of fungi with the organic amendments and no changes of bacteria with N applications. Therefore the bacteria, especially the G+ bacteria regulated the organic C decomposition, then followed by the alteration of microbiota due to C:N ratio. In treatments CN23, CN10 and CN50, fungi were the second dominant species after G+ bacteria (26.90, 31.16 and 36.95% in CN23, CN10 and CN50, respectively). The possible reason is that the N addition favoured the growth of fungi. However, fungi also showed a large percentage in CL treatment (38.32%), but not in CH (15.91%). Mille-Lindblom and Tranvik (2003) and Rousk and Bååth (2011) demonstrated the presence of bacteria inhibited fungal growth. Scientists have long debated that fungi are more efficient than bacteria in terms of assimilating substrate, especially in low nutrient

substrate conditions. With the limited initial organic C addition, fungi are likely to utilize recalcitrant C and out-grow other microbes, attributing to PE.

Microbial species and abundance were expressed by the concentration of PLFAs. The previous data about PLFAs distribution along with N availability were inconsistent. Certain PLFA biomarkers are accompanied with PE and ecological stage (Zhang et al., 2008). We found that total PLFA abundance and community composition increased due to energy and nutrient inputs, yet the changes differed significantly ($p < 0.05$) among species and treatments (**Figure 3.6a–h**). For G+ bacteria, the significantly high ($p < 0.05$) abundance occurred in high glucose treatment, 7.59 μg g^{-1} dry soil (**Figure 3.6a**). As for G− Bacterial, only sole N amendments showed significantly ($p < 0.05$) lowest amount (1.31 μg g^{-1} dry soil) and CN50 with the highest value (0.04 μg g^{-1} dry soil) (**Figure 3.6b**). The discrepancy for G+ and G− bacteria were noticed by other researchers as well. Rousk and Bååth (2011) noticed a promoted bacterial growth in soils applied with barley straw, but not in soils applied with N, indicating that the bacteria are more sensitive to organic C than mineral N (**Figure 3.6b**). Rousk and Bååth (2011) also confirmed the significant low fungal growth rates with high N application. Similar with our findings, the fungi population was relatively low in N addition (0.025 μg g^{-1} dry soil) (**Figure 3.6c**). The significantly high ($p < 0.05$) quantity of actinomycetes in CH treatments (0.049 μg g^{-1} dry soil) indicated their preference in utility of labile organic C (**Figure 3.6d**). The protozoan population was significantly low ($p < 0.05$) in high glucose treatment (**Figure 3.6e**). Bardgett et al. (1999b) noticed the total PLFAs were highest in low N nutrient sites. Similar results were found in our research, the lowest or without N amendment (with the same amount of glucose added) had the highest total PLFA values (6.96 and 9.40 μg g^{-1} dry soil for CN50 and CH, respectively) (**Figure 3.6f**). The supply of N could reduce the fungal abundance and fungi:bacterial (F:B) ratio, while increase the G+:G− bacteria ratio (**Fig 3.6g and h**). These results were confirmed by Peacock et al. (2001) and Denef et al. (2007). In addition, the PLFAs listed showed that they differed significantly based on the quality of organic and mineral addition ($p < 0.05$) (**Table 3.4**). Some PLFA biomarkers such as C15:0, C17:0, C16:1, C16:1ω7c, C16:1ω7t and C18:3ω3 have not been detected, so they will not be included in the PCA analysis (**Table 3.5**). However, for the detectable PLFAs, iC15:0, aC15:0, C16:0, iC17:0 and aC17:0 had the largest mol percentage in high glucose addition. But not all the bacterial PLFA biomarkers had the highest mol percentage in high glucose amended treatments. For the three actinomycetes biomarkers, 10MeC16:0, 10MeC17:0 and 10MeC18:0 the largest percentage were in CH, CH/N and CH, respectively. The different pattern may provide more detailed information regarding soil microbiota assimilating and regulating C dynamics.

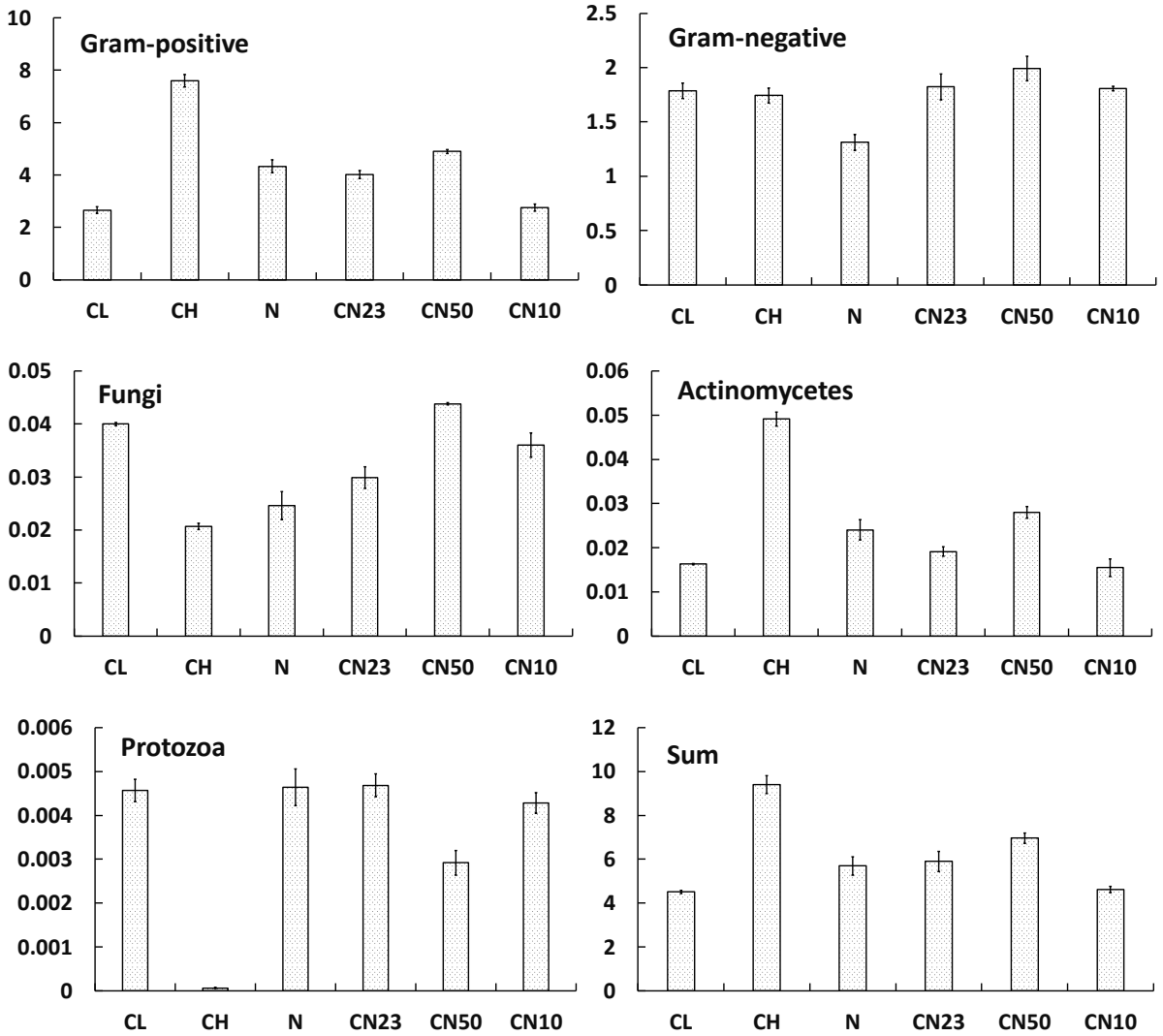


Figure 3. 6 Soil microbial community characteristics, based on lipid biomarker (PLFAs), at the ending of incubation period. Treatments include: CL and CH: soil applied with glucose at low and high quality, respectively; N: soil applied only with mineral nitrogen; CN23, CN50 and CN10: carbon:nitrogen ratio at 23, 50 and 10, respectively.

Considering the discrepancy of soil microbiota variation with previous studies, this research suggested that the reaction to organic/mineral amendments may be related to the microbial *r*- and *K*-strategy. The increase of microbial species is not necessarily following the same pattern. But, there was an initial bloom of *r*-strategies as stimulated by fresh organic matter (the PE in CN23, CN10 and CN50) even without N supplement (CH). Then following the depletion of labile C and the micro-community, *K*-strategies outcompeted *r*-strategies in subsequent stages of SOC degradation after the initial labial C became exhausted and began to use the recalcitrant C source left by *r*-strategies (Chen et al., 2014). This indicated that *K*-strategists are more competitive in decomposing substrates with low N availability. Fontaine et al. (2011) suggested that fungi are the main contributors to PE and 'microbial mining' in a long term. That is why most of the real positive PE occur after the apparent PE. There was evidence showing the community dynamics discrimination along the time (Fontaine et al., 2004).

Table 3. 4 Average mole percentages of individual PLFAs at the ending of incubation. Data displayed as mol% (mean ± SE, n = 3) of PLFA

PLFAs	Control	CL	CH	N	N23	N50	N10
iC15:0	6.9 ± 0.0	5.6 ± 0.4	11.9 ± 0.1	8.9 ± 0.8	8.4 ± 0.3	8.3 ± 0.1	6.0 ± 0.9
aC15:0	3.3 ± 0.0	2.6 ± 0.4	4.7 ± 0.1	3.8 ± 0.3	3.7 ± 0.2	3.7 ± 0.1	2.9 ± 0.4
C15:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
iC16:0	1.9 ± 0.0	1.9 ± 0.2	4.5 ± 0.1	3.3 ± 0.2	2.8 ± 0.4	2.7 ± 0.1	1.9 ± 0.7
C16:0	7.0 ± 0.4	7.7 ± 1.0	14.6 ± 0.3	9.9 ± 1.8	11.9 ± 1.2	11.6 ± 0.6	6.3 ± 1.2
iC17:0	3.4 ± 0.1	3.8 ± 0.1	5.1 ± 0.0	4.4 ± 0.1	3.8 ± 0.4	4.0 ± 0.1	2.9 ± 0.7
aC17:0	3.0 ± 0.0	3.1 ± 0.2	5.4 ± 0.0	4.3 ± 0.1	3.4 ± 0.3	3.5 ± 0.1	2.4 ± 0.8
C17:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C16:1ω7c	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C16:1ω7t	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C18:1ω9c	19.6 ± 0.5	17.1 ± 0.8	10.5 ± 0.0	7.8 ± 1.3	17.9 ± 3.0	14.1 ± 1.0	21.8 ± 6.2
C18:2ω6c	13.6 ± 0.6	18.5 ± 1.0	2.8 ± 0.7	8.8 ± 1.5	6.9 ± 1.0	14.3 ± 1.3	12.4 ± 1.0
C18:3ω3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C18:1ω9t	21.6 ± 0.5	19.8 ± 0.7	11.5 ± 0.1	8.4 ± 1.4	20.0 ± 3.9	16.9 ± 1.2	24.5 ± 6.0
10MeC16:0	5.1 ± 0.0	4.7 ± 0.1	6.0 ± 0.1	4.6 ± 0.4b	4.5 ± 0.3b	5.3 ± 0.1	3.5 ± 0.6
10MeC17:0	1.0 ± 0.0	0.7 ± 0.1	1.3 ± 0.0	1.3 ± 0.1b	0.9 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
10MeC18:0	10.4 ± 0.2	10.1 ± 0.7	21.4 ± 0.2	11.9 ± 2.0	11.2 ± 0.5	13.1 ± 1.1	8.3 ± 0.7
C20:3ω6c	2.8 ± 1.6	4.2 ± 1.4	0.0 ± 0.0	22.4 ± 1.9	4.2 ± 0.9b	1.8 ± 1.0	6.3 ± 1.6
C20:3ω3c	0.4 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0

Control as soil without amendments; CL: soil applied with low C level; CH: soil applied with high C level; N: soil applied with sole N; CN10: soil applied with C:N ratio at 10; CN23: soil applied with C:N ratio at 10; CN23; CN50: soil applied with C:N ratio at 10; CN50.

The PLFA data and microbial species were analysed with PCA and found that the G+ and G− bacteria, fungi increased more as affected by the combination of C and N addition (**Figure 3.7**). Principal components 1 and 2 loading score revealed the contribution from individual PLFA. It showed that iC16:0 had the highest loading score followed by aC17:0 and 10MeC18:0 (**Table 3.5**). All of the PLFA biomarkers had positive relation with component 1, which possessed 93.08% explanation ability.

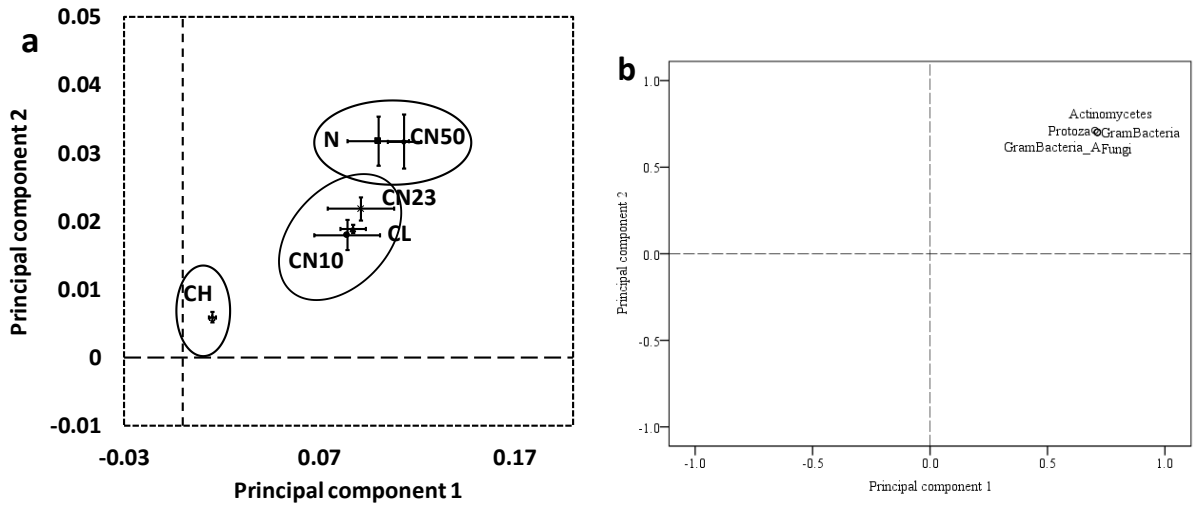


Figure 3. 7 (**a**): Principal component analysis (PCA) score plot of the two first principal components of the microbial species data set, providing a map of how the C:N treatment sets related to each other, (**b**): shows loading values for the first two principal components of the individual microbial species. The specie located far from plot origin is less influential. Figure also shows all of the microorganisms are closely positively correlated.

Table 3. 5 Principal component analysis (PCA) loading plot of the first two principal components of the individual PLFAs from the PLFA data set

	Principal component 1	Principal component 2
iC15:0	0.07701	0.00298
aC15:0	0.07697	0.00051
iC16:0	0.07708	0.00979
C16:0	0.07696	0.00007
iC17:0	0.07699	0.00117
aC17:0	0.07706	0.00616
C18:1ω9c	0.07687	-0.00513
C18:2ω6c	0.07687	-0.00511
C18:1ω9t	0.07685	-0.00617
10MeC16:0	0.07695	-0.00101
10MeC17:0	0.07699	0.00142
10MeC18:0	0.07706	0.00903
C20:3ω6c	0.00000	0.99951
C20:3ω3c	0.07667	-0.01373

*The loading score for each individual PLFA shows which PLFAs are influential (the contribution to component 1 and 2)

3.6.5 Microbial carbon use efficiency

The competition for energy and nutrients among microbial species lead to the variation of microbial CUE. The disproportionate balance between microbial growth and activity indicated the necessity of determining microbial CUE (**Eq. 3.7–3.10**). Six et al. (2006) listed the available data in different habitats. However, the limited knowledge of controlling factors and the scarcity of reports in soils slow our understanding of interpreting microbial mediating soil C dynamics. The microbial CUE varies considerably, with reports of values ranging from 1% to 85% (del Giorgio and Cole, 1998; Payne and Wiebe, 1978). This research used four approaches to measure CUE (**Table 3.6**). Microbial CUE based on ratio of biomass depletion to the decrease of substrate C had the lowest values, while the CUE calculated based on substrate depletion are general higher. It is due to the fact that some microbial by-products such as enzyme and hyphae could not be included as the effective assimilated C portion. The conversion factor to transfer PLFAs into microbial C varies and is difficulty to conclude in a universal number.

Except C_p (the highest use efficiency was in high C addition of 0.55), the highest microbial CUEs were from CN23 treatment in C_s (0.48), C_m (0.40) and C_r (0.35). There were increased TC and TOC in CN23 treatment. This indicates the optimum C:N ratio for microorganism ratio contributing to C sequestration by microorganisms. The smallest CUEs were 0.32 of C_s (CN10), 0.07 of C_m (CH) and 0.04 of C_r (CH). The high glucose increased both of microbial activity and biomass, yet lowered the C efficiency. The microbial CUE approach based on the accumulation of MBC will be used later in the following chapters of this thesis.

Table 3. 6 Microbial carbon use efficiency based on various approaches. Data shows mean ± SE (n = 4)

Treatment	C_s	C_m	C_p	C_r
CL	0.44 ± 0.07	0.21 ± 0.05	0.20 ± 0.02	0.15 ± 0.01
CH	0.43 ± 0.01	0.07 ± 0.01	0.55 ± 0.03	0.04 ± 0.01
CN23	0.48 ± 0.10	0.40 ± 0.03	0.30 ± 0.03	0.35 ± 0.05
CN50	0.43 ± 0.07	0.22 ± 0.04	0.39 ± 0.04	0.17 ± 0.02
CN10	0.32 ± 0.09	0.09 ± 0.02	0.20 ± 0.01	0.07 ± 0.02

Control as soil without amendments; CL: soil applied with low C level; CH: soil applied with high C level; N: soil applied with sole N; CN10: soil applied with C:N ratio at 10; CN23: soil applied with C:N ratio at 10; CN23; CN50: soil applied with C:N ratio at 10; CN50

3.7 Conclusions and recommendations

- This research indicated that the organic residues coupled with mineral input were closely related to soil microbial properties and their influence on soil C. Initially, the labile C source stimulated the microbial activity and abundance, resulting in positive PE and native SOC depletion. Changing the proportion of exogenous organic C and mineral source for microbial use resulted in varied magnitude of PE and microbial C use.
- Microorganisms played a key role in regulating soil C dynamics. Microbial species variation depended on joint environmental influences and the accessibility of different SOC pools. The *r*- and *K*-strategies theory coupled with 'microbial mining' theory could help us to understand soil PE. The *r*-strategies *are benefited by the labile organic source. Because of the more 'tolerance features' for K*-strategies, they are important in translocation and transformation with recalcitrant and indigenous C sources.
- The optimum C:N ratio at 23 was beneficial to microbial growth and to the highest C sequestration potential. Both PE and microbial C assimilation were high in C:N ratio.

Considering the added amount of fresh organic matter, they are not contradictory. Depending on the research purpose, by adjusting the quality of fresh organic matter, research can ‘manipulate’ microbial responses and their utility preferences of soil C.

- Future research may include the long-time detection of the shift of microbial community composition along various ecosystems. Because the PE is a natural process, *in-situ* experiments under different land systems will be beneficial to understand the processes of litter decomposition.

Chapter 4

LAND USE HISTORIES ALTER SOIL MICROBIAL COMMUNITY AND PREFERENCES FOR DEGRADATION OF DIFFERENT CARBON SOURCES

4.1 Introduction

Land use management modifies soil environment, and consequently affects soil carbon (C) decomposition (Bandyopadhyay and Lal, 2014; Meng et al., 2014). For instance, afforestation or reforestation will have an essential influence on soil C stock, because of more storage of C. In agricultural land management, practices such as liming, tillage and cultivation are highly associated with soil fertility and microbiota function. Organic matter input including straw (known as mulch) and biochar increases organic C quantity and improves soil quality. Some organic amendments are more accessible by soil microorganisms, leading to indigenous C depletion. Based on the accessibility to soil microorganisms, organic matters can be divided into: (i) Relatively labile organic matter (malic acid, glucose), (ii) Relatively stable organic matter, (iii) Recalcitrant organic matter (charcoal). Microbial C decomposition is much slower in biochar compared to that from labile organic input (Bolan et al., 2012). However, biochar also contains a small portion of labile C that can be utilized by soil microorganisms (Farrell et al., 2013). On the other hand, biochar addition not only influence terrestrial C sequestration, but also soil health and nutrient cycling (Nelissen et al., 2012). Besides organic applications, including animal faeces, pasture land is also impacted by grazing animal stock. Unlike highly managed lands such as agriculture or pasture, the natural forest land had less interruption and fertilizer application (de Barros Ferraz et al., 2009). McGrath et al. (2001) suggested that the land use types are closely related to soil C sequestration ability. Soil microorganisms are the main driving force in terms of soil organic carbon (SOC) decomposition (Bardgett et al., 1999b; Carney and Matson, 2005). The microbial properties such as respiration rate, biomass, enzyme efficiency varied along with soil environment variations, leading to differentiation in soil C mobilization and immobilization ((im)mobilization) features. This mediation of underground C dynamics impacts soil fertility in return. Microbial carbon use efficiency (CUE) is an indicator for the C utility from the microbial perspective (**Chapter 3**). It is the C assimilated as microbial biomass proportional to the C exploited by microorganisms (Manzoni et al., 2012).

The interpretation of microbial CUE can be used as soil microbial controlled C dynamics and C sequestration ability (Frey et al., 2008; Geyer et al., 2016). Besides microbial properties, microbial community composition is also affected by land management (Brussaard et al., 1990; Reganold et al., 1993), leading them to be a promising indicator while evaluating soil conditions (Mazzetto et al., 2016). De Vries et al. (2006) suggested a more sustainable ecosystem with a fungi dominated soil. The detection of phospholipid fatty acids (PLFAs) has been an efficient technique to identify microbial community based on **Chapter 3**. Because phospholipids degrade once cell death, PLFAs represent the living or active component of microbial community (Bardgett et al., 1999a; Frostegård et al., 1996), indicating the microbial structure in real time.

So far, land use systems have been incorporated into models that predict terrestrial C dynamics. It is likely that land use histories affect soil microbiota and their regulation on soil C dynamics. Research that relates land use systems to microbial C use is crucial in current times, especially the deficiency of information regarding agriculture, pasture and forest land systems. Given that the organic amendments have been observed showing significantly divergent effects on SOC degradation (Farrell et al., 2015), there is a practical need to investigate the variation one step further than the discrepancy in different land histories, and the mechanisms linked to microbial community.

4.2 Objectives

The overall objective was to examine microbial CUE in different land use systems with regard to fresh organic addition. The specific objectives are listed as following:

- To evaluate the land use systems influences on soil microbiota and microbial regulating soil C dynamics
- To compare microbial C use ability with different types of organic sources based on the C lability.
- To interpolate the relation between microbial CUE and community composition alteration with the influences of organic amendments and land uses.

4.3 Hypothesis

- Microbial properties and C use preferences vary under different land use histories.
- Fresh Organic C amendment alters microbial community composition, and consequently changes microbial CUE.

- Microbial community composition is an essential indicator for land use systems and partly explains the mechanisms of terrestrial C dynamics.

4.4 Materials and methods

4.4.1 Soil sampling and site record

The three most common land use systems were selected in South Australia. They were cropping land, pastureland and natural forest. The purpose was to compare two representative land use systems that are constantly affected by anthropogenic managements to a less interrupted soil system (**Plate 4.1**). Records from the local land owners showed different managements of these three lands (**Table 4.1**). In brief, three surface soils (0–10 cm) from the same region were collected from Barossa Valley, South Australia. The region is characterised by Mediterranean climate, with annual average high temperature of 22.3 °C and average low temperature of 12.2 °C, the average annual rainfall ranged from 257 to 691 mm during the past 8 years. Soil types of all of these three land use systems are Sodosol (based on Australian soil classification, http://www.asris.csiro.au/). The cropping area (CS, 34°27'38''S 138°57'7''E) was a rotation-cultivated land with wheat and barley for approximately 10 years. Fertilizers were applied to the cropping soils as part of anthropogenic management activities. The annual yield for wheat or barley ranged from 1.5 to 9.1 t ha^{-1} per year ith 9.5% of protein content in wheat and 9.2% of protein content in barley. Direct drill as tillage was employed for the past 8 years. Plant residues were retained in fields as organic amendments. The pasture soil (PS, 34°22'38''S 139°0'32''E) is a land used for grazing mainly sheep and cows, only was re-sown with balansa clover (Frontier cultivar) at a seeding rate of 14.3 kg ha^{-1} in 2011, including monoammonium phosphate (MAP) fertiliser at 62 kg ha^{-1}. The natural forest (NF, 34°27'48''S 138°57'47''E) is about 4 hectares in area, and without anthropogenic disturbance for almost 20 years. Prior to that, it has never been cultivated or had any fertiliser applied.

Plate 4. 1 Soil sampling location landscape in the three land use systems of South Australia: (**a**) Cropping land, (**b**) Pasture land, (**c**) Natural forest.

Table 4. 1 Land management records and soil information of sampling sites

Cropping Land

Crop	N applied (kg ha^{-1})	P applied (kg ha^{-1})	Land management	Stubble management	Yield (t ha^{-1})
Wheat/Barely	88.67	16.33	Direct drill	Retained	ca. 5.25 (Wheat) 5 (Barely)

Pasture Land

Phosphorus (mg kg^{-1} soil)	P buffer index	Optimum P level	Potassium (mg kg^{-1} soil)	Sulphur (mg kg^{-1} soil)	Organic C (%)	pH (water)	pH ($CaCl_2$)	Salinity (mS cm^{-1})	Nitrate N (mg kg^{-1} soil)	Ammonium N (mg kg^{-1} soil)	Al ($CaCl_2$, mg kg^{-1} soil)
43	61.3	30	365	10	1.5	7.0	6.7	0.247	53	7	<0.2

Natural Forest

Land management	Annual rainfall (mm)	GSR rainfall (mm)
Untouched	ca. 448.17	ca. 335.67

4.4.2 Physicochemical characterization of soil and organic amendments

Before analysis, soils were air dried and sieved thoroughly (< 2 mm) for homogenization, fine roots and other plant debris were carefully removed during processing. Soils were analysed for pH, EC and elemental composition following the methods described as follows: Soil pH and EC were measured in a 1:5 soil:water ratio (Rayment and Lyons, 2011). The total C (TC) and total nitrogen (TN) in soil, wheat straw and biochar were determined by combustion using Leco C/N analyzer (Leco TruMac® CNS/NS) after being acidified with H_2SO_3 to remove carbonates. For TN and organic C, 0.2 g samples were weighed and combusted at 1300 °C. Oxygen flow was 5 seconds. Instruction was calibrated every 10 detection with standard weights of Leco EDTA calibrator (containing 95.7 g N kg^{-1} and 410 g C kg^{-1}) (Wright and Bailey, 2001). Other physicochemical properties of the soils and amendments were determined using the analytical methods outlined in Rayment and Lyons (2011) and listed in **Table 4.2**. Soil textures were detected by the micro-pipette method from Miller and Miller (1987). Soil CEC and exchangeable cations were determined by summing the exchanged cations (Ciesielski et al., 1997; Hendershot and Duquette, 1986). To determine the total element, soil and biochar samples were mixed with 5mL of aqua regia (HNO_3:HCl ratio at 3:1) and digested in a micro wave digestion oven, then Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES, Agilent 7900, USA) was used for detection (McDowell, 2005) (**Table 4.3 and 4.4**). Glucose, wheat straw and biochar were spread on to soil then evenly mixed to achieve the same C addition quantity. Soils were stored at 4 °C until further analysis.

Table 4. 2 Selected soil properties

Site	pH_{H2O}	EC (mS cm^{-1})	Clay (wt. %)	Silt (wt. %)	Sand (wt. %)	CEC (c mol (+) kg^{-1})
Cropping soil	6.43	34.60	44.23	21.11	34.66	33.75
Pasture soil	6.88	106.53	37.83	18.20	43.97	27.88
Natural forest	6.26	27.53	32.71	25.73	41.56	20.17

Table 4. 3 Soil element information

Site	P (g kg^{-1})	K (g kg^{-1})	Fe (g kg^{-1})	Ca (g kg^{-1})	Mg (g kg^{-1})	Na (g kg^{-1})	S (g kg^{-1})	Mn (mg kg^{-1})	Cu (mg kg^{-1})	Zn (mg kg^{-1})
Cropping soil	0.37	1.94	3.18	2.14	1.47	0.24	0.78	58.57	23.61	17.52
Pasture soil	0.46	2.30	1.78	3.44	1.42	0.11	0.29	265.43	14.51	20.47
Natural forest	0.13	0.59	1.42	1.39	0.46	0.23	0.37	28.50	8.90	8.19

Table 4. 4 Selected biochar properties

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

The biochar used for the experiment was from Macadamia nut shell and produced at 465 °C (pyrolysis temperature) with limited oxygen present. The detailed methods for detection of pH and EC of biochar are described as follows: Biochar water mixture (1:10 ratio, *dw/v*) was shaken in an end-over-end shaker for 1 h, and analyzed for pH and EC using smartCHEM-LAB Laboratory Analyzer (Thangarajan et al., 2018). To extract the dissolved organic C content, biochar was mixed with deionized water (1:10 ratio, *dw/v*), shaken (3 hours in a horizontal shaker) and centrifuged (3000 g for 20 min). Physicochemical properties of the biochar are detailed in **Table 4.4**. Biochar SEM (**Figure 1a**) images and FTIR analysis (**Figure 1b**) show pore structure and element information of biochar used in this research.

4.4.3 Microbial respiration monitoring during incubation

Prior to the experiment, the moisture content was adjusted to 50% of the water holding capacity (WHC) and pre-incubated at 25 °C, 28% room humidity for 7 days for microbial activity to recover. Microbial respiration was measured by detecting headspace CO_2 concentrations using a Servomex 1450 infra-red gas analyzer (Servomex, UK) over the duration of incubation (Setia et al., 2011). In each sealed Mason jar, 10 g (dry-weight equivalent) soil samples were placed with a tube containing deionized water to maintain a water-saturated atmosphere. The samples were incubated in the dark during the whole 31 days incubation time, and several measurements were taken periodically. Water was replenished on a weight basis every 3 days during the incubation period. Calibration was undertaken every time before each measurement for correcting CO_2 concentration. An initial measurement of the headspace CO_2 concentration was immediately taken after sealing the jars to compare with CO_2 concentration at the end of each measurement period. Jars were opened to refresh the air with a fan after each CO_2 measurement. Microbial respiration rate was presented as the CO_2–C (µg) released per g soil per hour as in the following equation:

$$Y = \frac{A \times 1000 \times Atomic\ weight\ of\ C}{V_{std} \times Time\ (hours) \times Mass\ of\ soil\ (g)} \qquad \text{Eq. 4.1}$$

where Y is mg CO_2–C g^{-1} soil $hour^{-1}$, $A = A_1 - A_0$ ($A_1 = CO_2$ (mL) evolved from soil during time T_1 and $A_0 = CO_2$ (mL) evolved during time T_0), V_{std} is the volume of 1 mol of CO_2 at given temperature calculated from ideal gas law (volume of 1 mol of CO_2 at 15 °C and 1 atmospheric pressure is 24465.3 mL). The cumulative respiration values over the 31 days incubation period were also calculated.

4.4.4 Microbial biomass and carbon use calculation

The microbial biomass carbon (MBC) was measured prior to start and periodically after incubation began, following the chloroform-fumigation extraction method (Vance et al., 1987). The purpose was to quantify MBC accumulation along incubation. In brief, three replicates of 10 g (dry-weight equivalent) of incubated soil samples were fumigated in a desiccator with ethanol-free $CHCl_3$, other non-fumigated samples were kept in dark at the same room temperature. Both of the two treatments were mixed with 0.5 M K_2SO_4 prior to be put in an end-over-end shaker. The extract was analysed for organic C content using a total organic C (TOC) analyzer (Shimadzu: TOC-LCSH). The MBC was measured as in **Eq. 4.2**

$$Microbial\ biomass\ carbon\ (mg/kg)\ = E_c/K_c \qquad \text{Eq. 4.2}$$

where, E_c is the difference of K_2SO_4 extractable–C content between fumigated and non-fumigated soil samples (mg/kg), K_c is 0.45 (factor of organic C extraction efficiency).

Based on the reasons and conclusions listed in **Chapter 3**, we decided to make the calculation uniform for microbial CUE based on the cumulative of microbial biomass (**Eq. 4.3**)

$$C_m = \Delta M_c + \sum CO_2\text{–}C \qquad \text{Eq. 4.3}$$

where C_m is microbial C use measured as microbial biomass variation, ΔM_c is the change of MBC, ΣCO_2–C is the cumulative C as microbial respiration.

4.4.5 Microbial PLFA analysis

Microbial PLFAs were extracted following the method of Frostegård et al. (1993, as modified by Bossio and Scow, 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. Then a solid phase extraction column (0.5 g silica packed with chloroform condition) was set up in order to separate PLFA fractions (**Plate 4.2**). PLFAs were converted into fatty acid methyl esters (FAMEs) with mild alkaline methanolysis as descripted in **Chapter 3**.

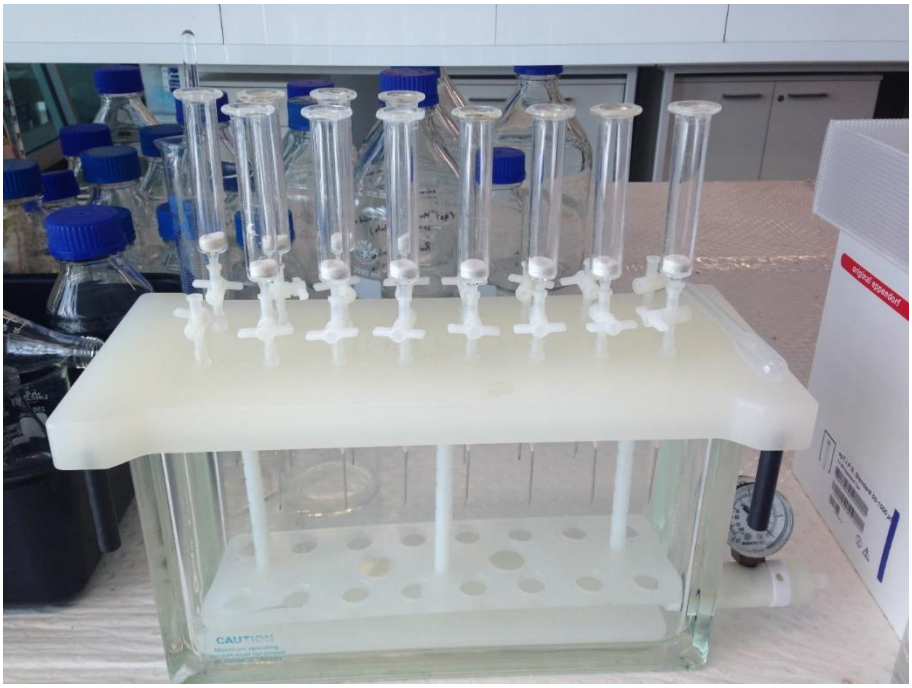


Plate 4. 2 Set up of solid phase extraction column for different PLFA fractions separation.

Methylnonadecanoate (19:0) was added to each samples as an internal standard. Supelco 37 standard mixture (supelco, Bellefonte, Pa.) was used as standard to compare with retention times of each PLFA peak. Electron energy in electron impact will be 70 eV.

FAMEs were analysed by GC-MS (HP 5973) with an SP-2560 fused silica capillary column (75 m, 180 µm × 0.14 µm film thickness) (Supelco, Sigma-Aldrich, Australia). Helium was used as carrier gas. Temperature program was set as follows: injector temperature 250 °C, initial at 140 °C for 5 min, increase 140 °C to final temperature of 260 °C at a rate of 4 °C min^{-1}, then keep 260 °C for 15 min.

4.4.6 Statistic analysis

All treatments were in triplicates. Shapiro-Wilk test and homogeneity of variance were used for normal distribution of all data with Levene statistic. The effects of land use systems and organic amendments on microbial properties and microbial CUE were assessed by one factor ANOVA followed by the least significant difference test. All these statistical analysis were performed in SPSS version 23.0 (SPSS Inc., Chicago, USA) with the significant differences accepted at $p < 0.05$.

4.5 Results and discussion

4.5.1 Soil properties, total organic carbon and nitrogen in different land uses

Sampling sites were selected based on the same soil order and similar soil texture, pH and other properties (**Table 4.2**). Soils were slightly acidic. The highest pH_{H2O} value was in pasture soil (6.88). Soil EC in pasture soil (106.53 mS cm^{-1}) was much higher than in cropping land (34.60 mS cm^{-1}) and natural forest (27.53 mS cm^{-1}). The three soils are all slit loam, with slightly clay, slit and sand content differentiation among land uses. Compared to grazing and natural forest soil, cropping soil had higher level of clay content with a value of 44.23%. The largest CEC was in cropping soil (33.75 c mol (+) kg^{-1}), then followed by pasture soil and natural forest. Kizilkaya et al. (2010) and Cookson et al. (2007) also demonstrated that the natural forest had lower clay and pH value than in adjacent cultivated soils due to the water erosion. Based on the anthropogenic management, soil properties varie among land use practices (Jaiyeoba et al., 2003); this variation consequently impacts soil aeration for microbiota.

The element content showed discrepancy among these three land use systems (**Table 4.3**). Because cropping and pasture soils were both modulated by anthropogenic amendments, our result showed cropping and grazing soils shared more similarities compared to natural forest soil, while most of the elements were low in natural forest soils. For instance, cultivated and pasture had similar Ca, K and Zn concentration, P quantity was 3.54 times higher in pasture soil than in natural forest, while 2.11 times for S in cropping land compared to natural soil. However, there were also exception elements, such as Mg, which was 4.54 times higher in pasture than in cropping soil, and 2.69 times higher S content in cropping than in pasture soils. Litter input from trees can be beneficial for nutrient cycling in in a long run and kept a balance of soil elements (Jose et al., 2000).

Soil total organic nitrogen (TON) content was significantly different ($p < 0.05$) among land use systems (**Table 4.5**). In spite of the organic amendments and fertilizers, natural forest land use (22.94 mg kg^{-1} soil) had the largest TON content, while the cropping land had the lowest C value (13.15 mg kg^{-1} soil). By comparison the TON stock between cropping and pasture soils, our results noticed that the cultivation had lower TOC values than the latter. Previous research also confirmed the results that cultivated land had the lowest TON (Jaiyeoba et al., 2003; Lobe et al., 2001). Qiao et al. (2015) suggested that the continuous harvest is one of the major reasons. Microbial metabolism on SOC is reduced due to lack of fresh organic input (Guillaume et al., 2016). Related to our results, we assumed that the C accumulation in natural forest attributes to less anthropogenic interference, such as fertilizer application, and higher microbial activity ascribes to less soil disturbance, because the soil C degradation is highly

related to microorganisms. Notably, the TN content was significantly ($p < 0.05$) different among the three land use systems, yet the highest value was in pasture (2.01 mg kg^{-1} soil), followed by natural forest (1.41 mg kg^{-1} soil) and cropping land (1.20 mg kg^{-1} soil). It is not surprising that cultivation depletes soil N, what results in the significant lowest TN value. The same results were found in Zhang et al. (2016). The relatively high TN in pastureland may be due to the anthropogenic amendments without constant plant rotation. The highest C:N ratio was in forest with the value at 16.31, followed by cropping soil at 10.97 and pasture soil at 8.68. Land use alters soil physicochemical properties, modifying living conditions for soil microbiota (Mazzetto et al., 2016). Both SOC and N content differed significantly among land use systems, just as other soil physicochemical properties (**Table 4.6**). Soil physicochemical characteristics may have essential influence on soil microbiota and their C utility patterns, as they are the microbial habitat basic index. In order to examine soil microbiota and microbial C use under different land use systems, the microbial properties were also investigated. The independent values that affect soil microbiota could be subjected as less as possible. However, it is challenging considering soil is a complex admixture containing interactions of air, liquid and solid phases.

Table 4. 5 Soil total organic carbon (TOC), total nitrogen (TN) content and C:N ratio in the three land use systems. Data shows mean ± SE (n = 4)

Site	TOC(g kg^{-1} soil)	TN (g kg^{-1} soil)	C/N ratio
Cropping soil	13.15±0.38	1.20±0.03	10.97±0.18
Pasture soil	17.36±1.26	2.01±0.03	8.68±0.69
Natural forest	22.94±1.22	1.41±0.02	16.31±0.72

Table 4. 6 One factor ANOVA (land use) and significance of differences of soils chemical and biochemical properties (Tukey test, $p < 0.05$, n = 4)

	TOC	TN	C/N	pH_{H2O}	EC	CEC
ANOVA	*	*	*	*	*	*
Tukey test						
Cropping land	a	a	b	b	a	c
Pasture land	b	c	a	c	b	b
Natural forest	c	b	c	a	a	a

Different letters (a-c) indicates significant differences. Electrical conductivity (EC), cation exchange capacity (CEC), total organic carbon (TOC), total nitrogen (TN)

4.5.2 Microbial activity variation with organic amendments under different land uses

4.5.2.1 Basil respiration variation as affected by land use histories

Previous research on microbial respiration based on land uses are mainly targeting *in situ* variation (Arevalo et al., 2010; Fan et al., 2015). However, influences such as temperature and rain can add complications to the results. In this research, microbial respiration dynamics along the entire 31 days period was measured. The cumulative microbial respiration was higher in natural forest soil while it was the lowest in cropping soil all along the whole incubation period (**Figure 4.1**). To investigate the relation between microbial biomass and respiration, microbial biomass on day 1, 3, 7, 14, 21 and by the end of incubation were also measured (**Figure 4.1** and **Figure 4.2**). Notably, the largest biomass was in pasture soil instead of natural forest, indicating there was higher microbial biomass in natural forest. Cropping land had the lowest microbial biomass. More detailed analysis and discussion will be presented in **4.5.3.1**. Not surprisingly, the microbial biomass amount increased along the incubation period. Several management factors, such as plant shading and tillage, could result in differentiation of microbial respiration capacity (Franzluebbers et al., 1995; Kizilkaya et al., 2010). With *in situ* detection Iqbal et al. (2010) and Liang et al. (2007) assumed it is the tillage practice in cultivated land that stimulated microbial activity. But Bini et al. (2013), Arevalo et al. (2010) and Kizilkaya et al. (2010) noticed a lower microbial respiration accumulation in agriculture land than forestland. The higher microbial activity can be attributed to the resilient ability of the microorganisms after 'dormancy'. Mazzetto et al. (2016) argued that the microbial attributes is less in agriculture land due to anthropogenic management. The constant anthropogenic interference weakens soil recovery ability in cropping and pasture lands. In a study of volcanic soils in Chili, Dube et al. (2009) proved that soil pH controlled microbial respiration. Because of the alkaline feature of macadamia biochar used in this research, we believed that the modification of soil pH also played an indirect role on microbial activity.

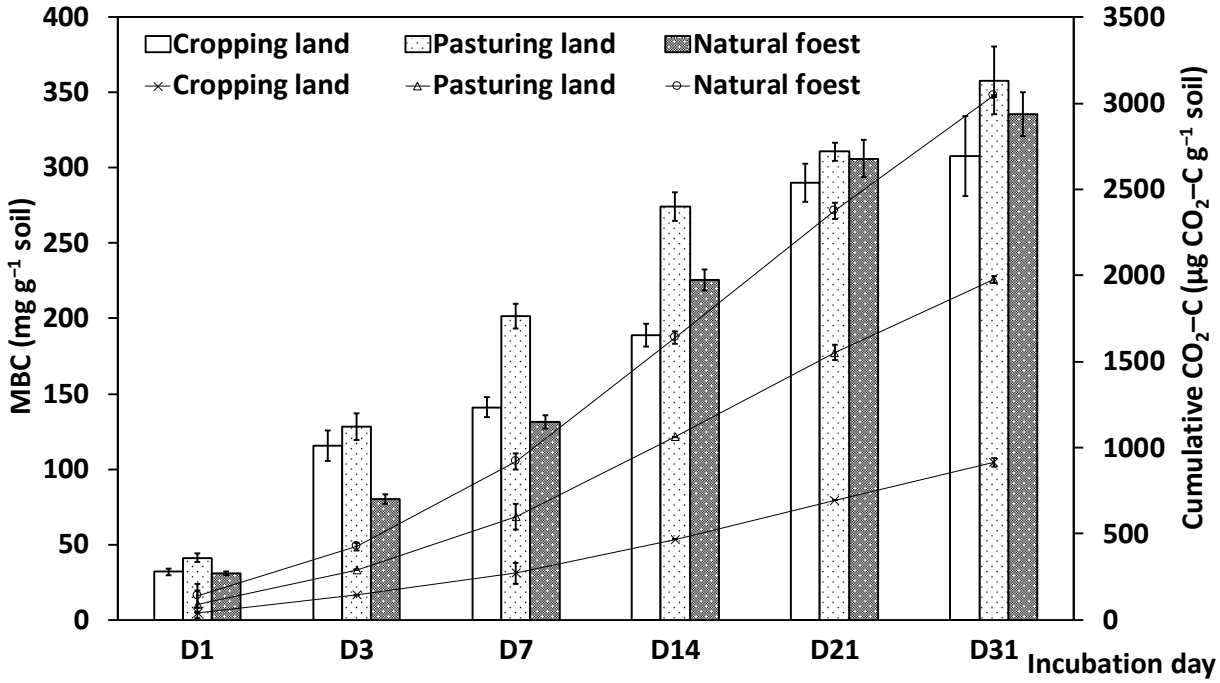


Figure 4. 1 Basal commutative microbial respiration CO_2–C during incubation period and microbial biomass carbon results as on day 1, 3, 7, 14, 21 and 31. Data expressed as mean ± SE (n = 4).

4.5.2.2 Induced microbial respiration influenced by carbon availability of amendments

To investigate if there was differentiation in microbial C use preferences based on land systems, we added glucose, wheat straw and biochar in all of the three land use soils. Li et al. (2017) suggested that the microbial respired CO_2 flux was influenced by the labile C and N addition more than by the temperature. The three organic C amendments distinguishably varied in C availabilities. Biochar have been considered as a recalcitrant C sources due to the pyrolysis process (Bolan et al., 2012). The physicochemical traits of the biochar used in this research are listed in **Table 4.4**. The wheat straw we used in this research had 45% C and 0.38% N content, the C:N ratio was 118.43, much larger than biochar (with C:N ratio at 99.13). To achieve the same C input, we measured and calculated before applying organic amendments.

With the C amendments, microbial respiration dynamics showed different patterns among the three land uses (**Figure 4.2**). In cropping land with glucose, microbial activity increased after 5 days incubation, leading to a significant different ($p < 0.05$) peak on day 6. After reaching the peak value, the microbial activity decreased to 13.72 µg CO_2–C g^{-1} soil h^{-1} on day 7. By adding glucose, microbial respiration rate peaked on day 6 and day 4 for cropping and pasture land, respectively. However, the stimulation by adding straw and biochar were not the same

as with glucose. For instance, the largest respired CO_2–C in cropping soil was reached on day 6 at 33.24 μg g^{-1} soil h^{-1} by adding glucose, about 11 and 27 times compared with straw (2.95 μg g^{-1} soil h^{-1}) and biochar (1.21 μg g^{-1} soil h^{-1}) treatments, respectively. Both glucose and wheat straw significantly ($p < 0.05$) promoted microbial activity, yet the promotion weakened after the 19 days incubation. There were similarities shared between cropping and pasture lands. In both soils, microbial activity did not significantly ($p > 0.05$) differ between biochar and control soil. In pasture soil, the glucose addition stimulated CO_2–C release to 28.31 μg g^{-1} soil h^{-1} on day 4, about 2.8 and 9 times more than straw (10.21 μg g^{-1} soil h^{-1}) and biochar (3.11 μg g^{-1} soil h^{-1}) applied soils, respectively. Not surprisingly, glucose addition soils showed the highest overall CO_2 production, followed by straw and biochar. Microbial respiration rate dropped significantly ($p < 0.05$) to 20.38 CO_2–C μg^{-1} g^{-1} soil h^{-1} the following day with glucose addition. However, if compare to the same C amendment, the stimulation of microbial activity in cropping soils rose more dramatically than in pasture soil. Compared to day 1, CO_2–C on day 6 increased by 79.6% in cropping soil, while 54.7% on day 4 in pasture soil. In natural soil, organic C inputs lead to a CO_2 production peak on day 4 at 12.86 μg g^{-1} soil h^{-1}, 20.38 and 15.45 μg g^{-1} soil h^{-1} less than the peaks in cropping soil and pasture soil, respectively. Cumulative microbial respiration at the end of 31 days incubation was significantly ($p < 0.05$) increased by glucose and wheat straw in all of the three land uses (**Figure 4.3**). Compared to control soils, biochar amended soil did not show any significant difference ($p > 0.05$). The order for overall CO_2–C release also was glucose > wheat straw > biochar. Although the stimulated microbial activity due to fresh organic input could be short-term effects, generally, organic amendment is beneficial to promote microbial activity (Zimmermann and Frey, 2002). But the availability of labile C content was the determine factor (Cross and Sohi, 2011).

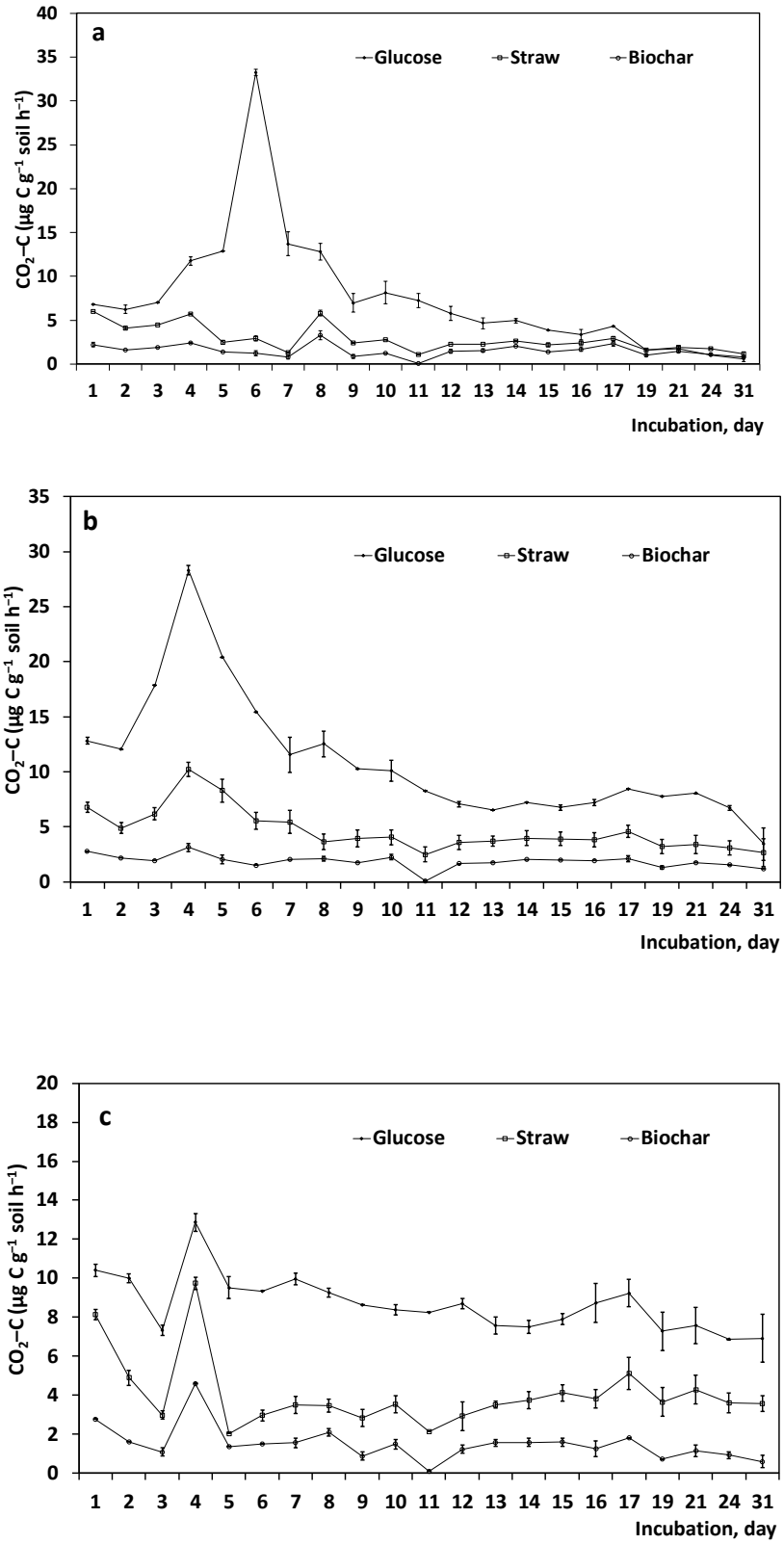


Figure 4. 2 Fresh organic carbon induced microbial respiration rate along the incubation period in cropping soil (**a**), pasture soil (**b**) and natural forest (**c**). Data expressed as mean ± SE (n = 4).

Most of the previous studies related to C source have been focused on the alteration of microbial community. Generally, labile C source, such as glucose, favours fast growing microbes. The following depletion of initial fresh C might lead to the microbial priming effect (PE) of native C or the shift to slow the growth of microbes. There is also competition for soil nutrients, such as N and phosphorus among microbial species. The influence of C availability amendment on soil microorganisms will be discussed further in section **4.5.4**.

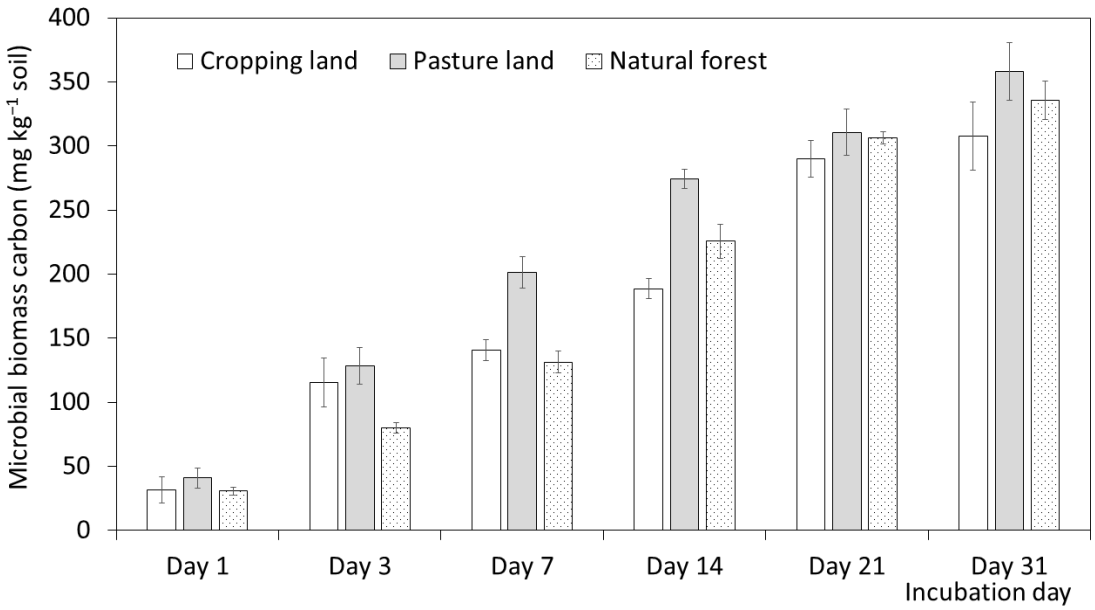


Figure 4. 3 Microbial biomass carbon in three land use systems during 31 days incubation. Data expressed as mean ± SE (n = 4).

4.5.3 Microbial biomass carbon and carbon use as affected by organic source and land use

4.5.3.1 Microbial biomass abundance in different land use histories

Fresh organic inputs varies among land use systems (Degens et al., 2000; Moscatelli et al., 2007), and consequently affect soil microbiota. For instance, there is a greater proportion of organic recalcitrant complexes coming from tree canopy in natural forest compared to cropping and pasture soils (Chantigny, 2003). In this research, the largest MBC value was in pasture soil along the incubation period (**Figure 4.2**). In pasture soils, MBC was 41.17 mg kg^{-1} soil at the beginning of incubation and 357.98 mg kg^{-1} soil by the end of the incubation. The values were larger by 9.41 and 50.01 mg kg^{-1} soil compared to cropping soils. And 10.45 and 22.31 mg kg^{-1} soil compared to natural forest. These results confirmed with a study on the Tibetan Plateau, where both SOC and microbial biomass decreased with the land use conversion from pasture to cropping land (Qiao et al., 2015). But the microbial biomass may

reduce along increased grazing intensity (Bardgett et al., 1995). We suggested that the pasture land supported soil microbiota population lays the two facts that: (i) less disturbance compared to cultivated land, and (ii) greater organic and nutrient return to soils as manure (Moscatelli et al., 2007). The cropping land had temporarily larger biomass (by 1.04 mg kg^{-1} soil) at the beginning of incubation while significantly ($p < 0.05$) dropped behind (by 27.77 mg kg^{-1} soil) natural forest. Dube et al. (2009) noticed there was less microbial C in natural forest surface soil than in agriculture system, and the negative relation between soil pH and biomass. Bini et al. (2013) demonstrated the agriculture land had the highest microbial biomass, while Kizilkaya et al. (2010) and Fang et al. (2014) demonstrated that both microbial biomass and natural forest had relatively larger microbial biomass. Considering the fresh C inputs in cultivated land, our results did not show the highest microbial biomass in cropping land use. Kizilkaya et al. (2010) noticed the lowest C_{min}:C_{org} ratio in agriculture land. We suggested that the constant changing of above ground land cover and interference lead to deterioration of soil microbiota and their resilience ability. Besides the organic and fertilizer supply to agriculture land, Mazzetto et al. (2016) assumed soil C stock also plays an important role in regulating microbial abundance. To explore more about the relation between soil C and microbiota, we added three types of organic sources based on their C availability.

4.5.3.2 Organic carbon alter microbial biomass under different land use systems

The fresh organic amendment led to varied microbial population reactions in different land uses. (**Figure 4.3**). The generally accepted concept is that fresh organic amendments promote microbial activity and biomass (Marinari et al., 2006; Moscatelli et al., 2007). In this research, the wheat straw significantly ($p < 0.05$) increased microbial biomass by the end of the incubation period. The increased values were 480.30, 603.40 and 290.76 mg kg^{-1} soil in cropping, pasture and natural forest soils, respectively. Pietri and Brookes (2009) noticed wheat straw addition had distinguishable benefits to total microbial biomass. They suggested that this is due to the ninhydrin N followed straw application that favoured soil microbiota. In cropping soils, microbial biomass increased by 278.98 and 328.32 mg kg^{-1} soil with biochar and glucose addition, respectively, and reached to 360.08 and 310.74 mg kg^{-1} soil by the end of incubation. In pasture soils, MBC increased by 316.20 and 528.76 mg kg^{-1} soil with biochar and glucose addition, respectively, and reached to 357.37 and 569.93 mg kg^{-1} soil by the end of incubation. While in natural forest soils, biochar application increased biomass by 193.52 and 408.88 mg kg^{-1} soil with biochar and glucose addition, respectively, and reached up to 224.23 and 439.60 mg kg^{-1} soil by the end of incubation. There was a discrepancy about the organic amendments' influence on microbial biomass in different land use systems. We

suggested that the land-specific microorganisms showed unique responses to the same fresh amendment. In addition, there was a greater microbial respiration rate with glucose application (**Figure 4.1**), yet the lowest microbial biomass content (**Figure 4.2**). On the contrast, biochar amendment increased microbial biomass production while not significantly (p > 0.05) increasing microbial respiration.

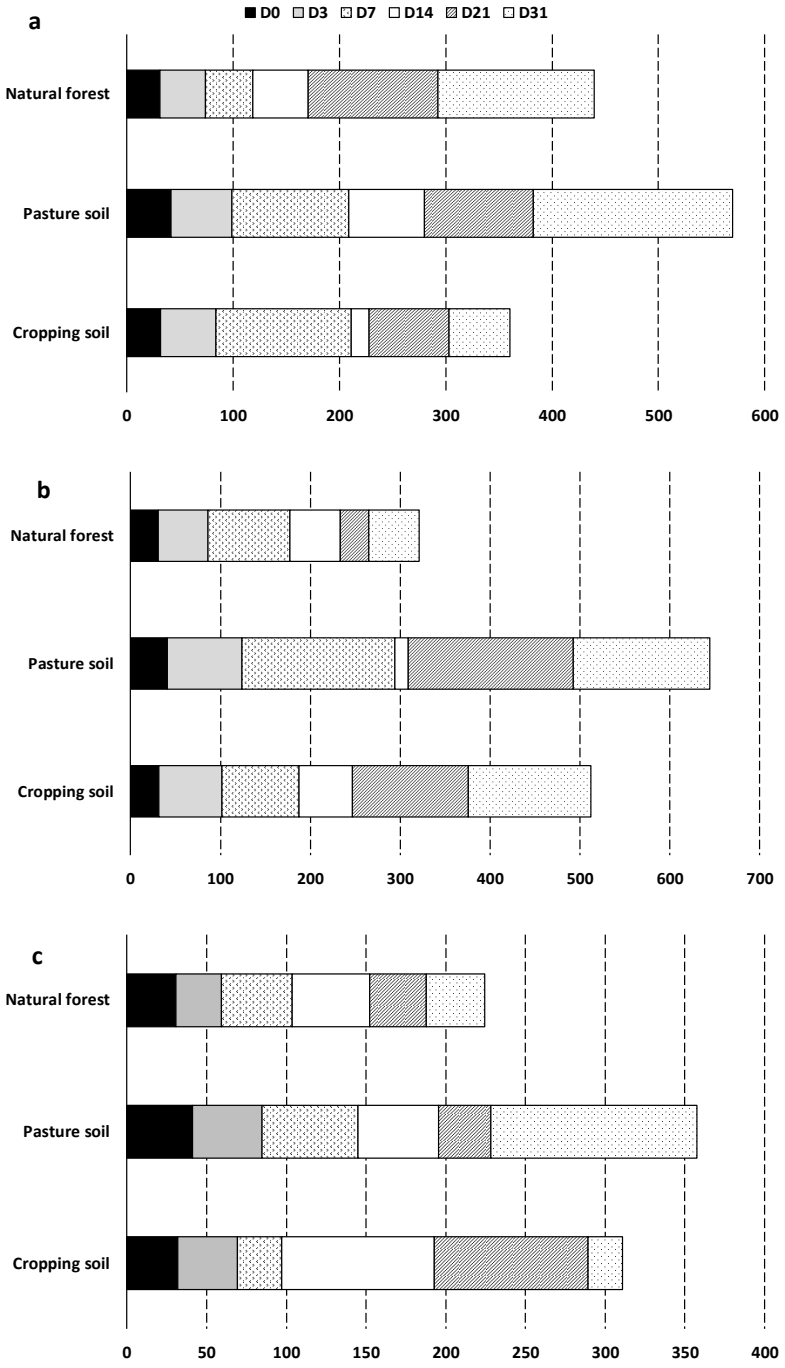


Figure 4. 4 Microbial biomass carbon (MBC) variation as affected by organic amendments under different land use systems: (**a**) glucose, (**b**) wheat straw, and (**c**) biochar, during 31 days incubation.

4.5.3.3 Carbon immobilization as regulated by soil microorganisms

In spite of the importance, the link between soil microbial C use and land use history are among the least understood subjects. Land use changes resulted in loss of surface organic C content (Arevalo et al., 2010), and generally the conversion from natural area to cultivation land lead to less SOC stock. Guillaume et al. (2016) demonstrated that soil C losses have a strong relation with soil biological decrease. Soil microorganisms were the driving force for the C degradation in a subtropical planation and their activity slowed down when lacking fresh organic input. Fresh organic C stimulated both microbial respiration and biomass (**Figure 4.2** and **4.3**), yet differently based on different land use histories. The concept of microbial CUE revealed the microbial sequestrated C (**Table 4.7**). There was not significant microbial C variation ($p > 0.05$) among land use. Along with incubation, the microbial CUE decreased less in biochar applied soils compared to control, or glucose and straw. For instance, in control soils, microbial CUE decreased by 0.15, 0.17 and 0.16 in cropping, pasture and natural forest soils, respectively. But with biochar, the differences were 0.01, 0.02 and 0.02, in cropping, pasture and natural soils, respectively. This indicates that the biochar enables a more stable soil microbial system. Although there were slight differences among land uses, by the end of incubation, the microbial CUE was 0.38, 0.37 and 0.36 in cropping, pasture and natural forest soils, respectively. The low nutrient availability in the forest and anthropogenic interferences may both affect microorganisms. However, there is a significant ($p < 0.05$) difference among organic amendments. In cropping soils, microbial CUE was 0.22 and 0.01 larger with biochar applications than with glucose and wheat straw, respectively. In pasture soils, the biochar increased microbial CUE by 0.20 and 0.05 compared to glucose and straw, respectively. In natural area soils, the differences were 0.18 and 0.13 between biochar with glucose and wheat straw application. There was greater microbial C use with biochar application compared to glucose and wheat straw. Organic inputs induced larger CO_2 flux, and recalcitrant C sources, such as biochar, are beneficial to native C stabilization (Cross and Sohi, 2011).

Table 4. 7 Microbial carbon use efficiency (CUE) with different organic carbon addition in three land use systems. Data showed means ± SE (n = 4). Different letters in one column indicate significant ($p < 0.05$) difference among treatment in one land use

	Incubation period 1	Incubation period 2	Incubation period 3	Incubation period 4	Incubation period 5
Cropping land					
Control	0.53 ± 0.04c	0.44 ± 0.03b	0.40 ± 0.07d	0.43 ± 0.04b	0.38 ± 0.06b
Glucose	0.19 ± 0.08a	0.14 ± 0.00a	0.10 ± 0.02a	0.12 ± 0.00a	0.14 ± 0.01a
Wheat straw	0.30 ± 0.08ab	0.33 ± 0.01b	0.28 ± 0.06b	0.32 ± 0.11b	0.35 ± 0.03b
Biochar	0.37 ± 0.05b	0.33 ± 0.10b	0.38 ± 0.03c	0.40 ± 0.02b	0.36 ± 0.07b
Pasture land					
Control	0.54 ± 0.07c	0.49 ± 0.02c	0.44 ± 0.00c	0.39 ± 0.02c	0.37 ± 0.01c
Glucose	0.11 ± 0.01a	0.11 ± 0.00a	0.10 ± 0.01a	0.11 ± 0.02a	0.14 ± 0.00a
Wheat straw	0.29 ± 0.03b	0.31 ± 0.04b	0.24 ± 0.07b	0.28 ± 0.01b	0.29 ± 0.02b
Biochar	0.36 ± 0.09b	0.36 ± 0.04b	0.33 ± 0.04b	0.29 ± 0.04b	0.34 ± 0.05bc
Natural forest					
Control	0.42 ± 0.01a	0.39 ± 0.02c	0.40 ± 0.01d	0.40 ± 0.01d	0.36 ± 0.05c
Glucose	0.12 ± 0.03b	0.10 ± 0.02a	0.08 ± 0.01a	0.11 ± 0.02a	0.12 ± 0.03a
Wheat straw	0.24 ± 0.01c	0.27 ± 0.01b	0.23 ± 0.02b	0.19 ± 0.04b	0.17 ± 0.03a
Biochar	0.32 ± 0.04d	0.30 ± 0.06b	0.31 ± 0.02c	0.30 ± 0.05c	0.30 ± 0.02b

4.5.4 Microbial community composition patterns in relation to organic amendments and land use systems

4.5.4.1 Microbial community composition patterns under different land use

There have been controversial results regarding land use histories and influences on soil microbiota. The microbial community compositions along land use systems were investigated, with the attempt to examine their relation and influence on microbial C use (**Figure 4.4**). The microbial community composition from the three land uses were detected and found that microbial total PLFA was significantly ($p < 0.05$) low in natural forest (91.74 nmol g^{-1} dry soil). This was consistent with the microbial biomass content as shown in **Figure 4.1**. Pasture land had a slightly larger total PLFA content (94.27 nmol g^{-1} dry soil) compared to cropping soil (93.58 nmol g^{-1} dry soil). Fungi population in natural forest was significantly ($p < 0.05$) larger than in cropping and pasture land by 28.75 and 50.07% respectively. The Gram-positive (G+) bacteria PLFA amounts were high in cropping and pasture land uses. Compared to natural forest, cropping and pasture land uses increased by 32.25 and 32.57%, respectively. The Gram-negative (G−) bacteria population was the lowest in cropping land (22.63 nmol g^{-1} dry soil) and highest in pasture land (29.75 nmol g^{-1} dry soil). There was discrepancy in microbial population among land use histories. The ratio of G+:G− bacteria revealed that cropping land use systems (2.15) favoured G+ bacteria compared to natural forest (1.20, **Figure 4.5**). Fertilizer application stimulated bacteria population growth, while repressed fungi spices (Lovell et al., 1995). Compared to bacteria, fungi are significantly ($p < 0.05$) dominating in natural area. Similar results were found in Lauber et al. (2008). The ratios were 0.31 and 0.52 in cropping and natural soils respectively. Compared to fungi that had greater C assimilation efficiency, bacteria required higher C:N ration and was more C demanding (Islam et al., 2000; Moscatelli et al., 2007). In addition, fungi are more sensitive to environmental stress and disturbance (Bardgett et al., 2005), and soil pH may also affect fungi and bacterial population. Pietri and Brookes (2009) noticed there was more fungi abundance at low pH.

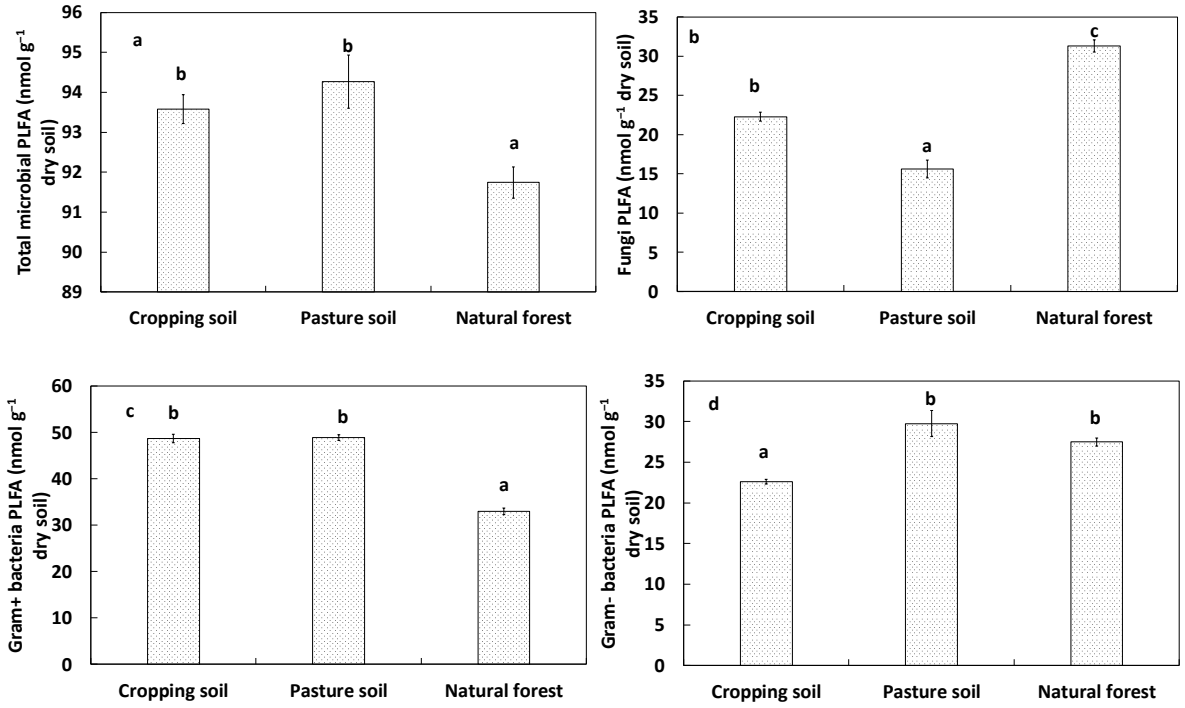


Figure 4. 5 Soil total microbial PLFA (**a**), fungi PLFA (**b**), Gram+ bacteria PLFA (**c**), and Gram- bacteria PLFA (**d**) under different land use systems. Values are mean ± SE (n = 3). Different letters above bars indicate significant different among land uses.

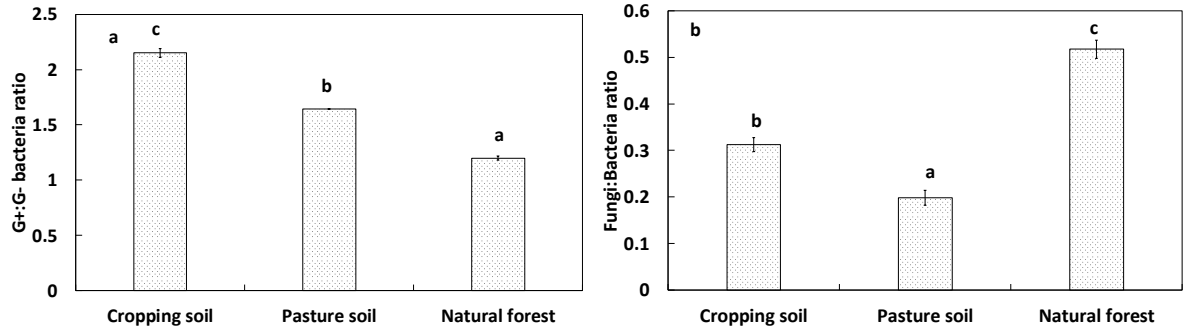


Figure 4. 6 Soil Gram-positive: Gram-negative (G+:G−) bacteria ratio (**a**) and Fungi:Bacteria ratio (**b**) under different land use histories. Values are mean ± SE (n = 3). Different letters above bars indicate significant different among land uses.

Microbial community population shifts by the end incubation period (**Figure 4.6 and 4.7**). Compared to the beginning of incubation, microbial total PLFA no longer showed a significant ($p > 0.05$) difference among land uses. G+ bacteria population was still dominant in cropping land, excessed pasture land and natural forest by 74.93 and 64.24%, respectively. So, in cropping soil, G+ bacteria amount was more than pasture land and natural forest by 56.87 and 59.71%, respectively. Fungi population decreased in natural forest along the incubation. The values of G+, negative bacteria and fungi were 65.07, 28.06 and 26.21 nmol g^{-1} dry soil, respectively. This suggested that the influences of land managements reduced along with incubation, and microbial community shifted to adapt the depletion of SOC and nutrient. Our research supported the theory that the microbial community composition, especially G+ bacteria and fungi population response to spatial variation.

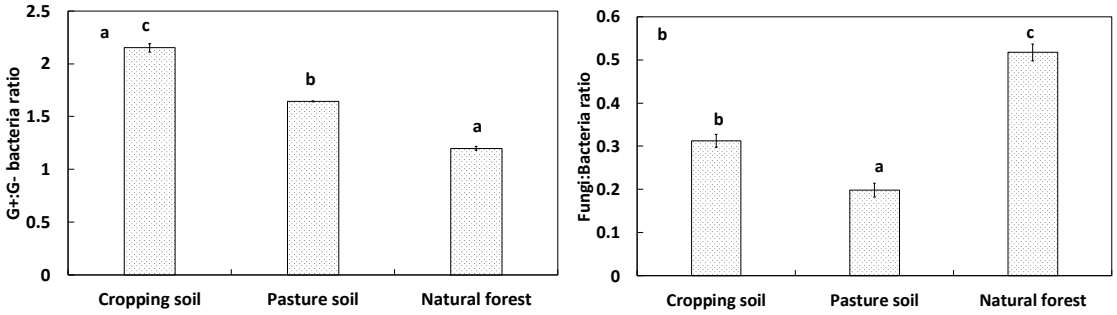


Figure 4. 7 Soil Gram-positive: Gram-negative (G+:G−) bacteria ratio (**a**) and Fungi:Bacteria ratio (**b**) under different land use histories. Values are mean ± SE (n = 3). Different letters above bars indicate significant different among land uses.

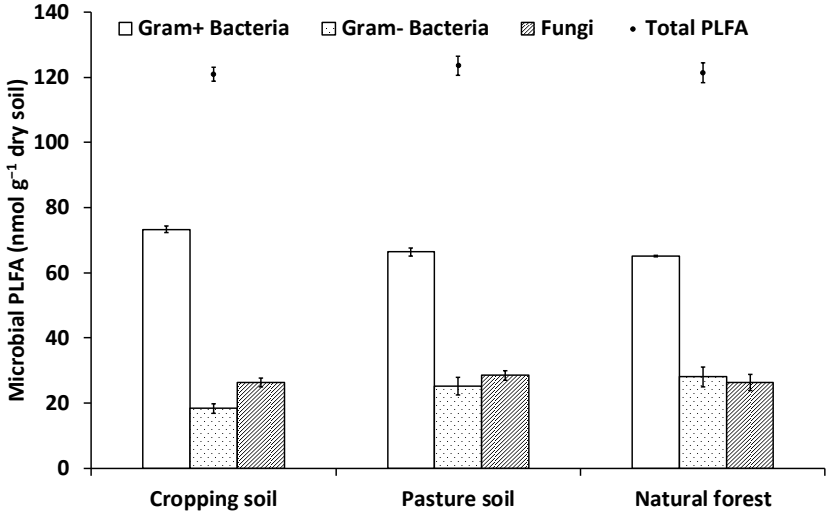


Figure 4. 8 Content of total microbial PLFA (close symbol), fungi, Gram positive and Gram negative bacteria PLFA (bars) under different land use histories by the end of incubation period. Data are mean ± SE (n = 3).

4.5.4.2 Microbial community shift with organic application

By adding fresh organic C, microbial community population varied (**Figure 4.8**). Especially bacteria and fungi showed very different responses to fresh organic C inputs. The labile C inputs, such as glucose addition, can accelerate indigenous C degradation by modifying microbial community composition. The labile substrate tended to favour microbes that assimilate and grow fast right after fresh addition, while the less labile C source shifted the microbial community to slow growing microbes (Blagodatskaya et al., 2009; Wright and Hobbie, 1966). Then this alteration of microbial population might affect the ingenious C mineralization process and speed as influenced by microorganism (Blagodatskaya et al., 2009). Fungi are better in decomposition of relatively more complex organics, such as lignin and polyphenol (Dix and Webster, 1995; Mutabaruka et al., 2007). We noticed significant ($p < 0.05$) increase of fungi population with biochar and wheat straw application compared to glucose comparison in all of the three land use systems. Mutabaruka et al. (2007) also noticed condensed tannin complexes favoured fungal community, increasing F:B ratio in agriculture land. Fungal PLFA value increased by 48.89, 75.31 and 60.0% in cropping, pasture and natural soils, respectively. While for G+ bacteria, PLFA value decreased by 26.93, 16.64 and 1.96% in cropping, pasture and natural forest, respectively. The largest value of total PLFA was in pasture soil with wheat straw application (210.40 nmol g^{-1} dry soil), followed by pasture soil with biochar (179.43 nmol g^{-1} dry soil). Our results confirmed with previous research. The adaptation of complex organic C inputs, such as straw and biochar, led to a higher fungal dominated microbial biomass. With biochar application, the F/B ratios were 0.73, 1.10 and 1.02 in cropping, pasture and natural forest, respectively. While glucose application led to F:B ratios at 0.27 (cropping land), 0.29 (pasture land) and 0.47 (natural forest). The heterogeneous C sources favour higher fungi population, and consequently a healthier and higher resilient soil system (Nsabimana et al., 2004). The highest G+:G− bacterial ratio was in natural forest with glucose application at value of 6.05, while the lowest was in pasture soil with biochar application. The G+ bacteria grew better with labile C sources than G− bacteria. And the impact varied among land uses.

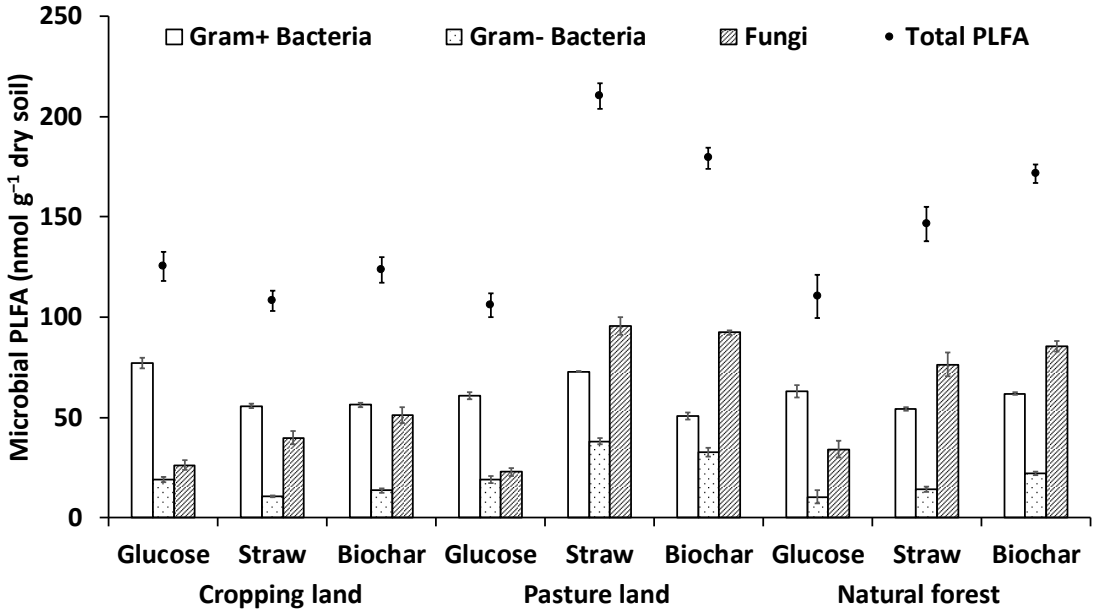


Figure 4. 9 Content of total microbial PLFA (close symbol), fungi, Gram-positive and Gram-negative bacteria PLFA (bars) as affected by different organic amendments under three land use histories by the end of incubation period. Data are mean ± SE (n = 3).

4.5.4.3 The interpretation of microbial community composition on carbon (im)mobilization

Soil microbiota can improve soil function and stability (Kizilkaya et al., 2010). De Graaff et al. (2010) suggested the availability and frequency of root and litter inputs affected soil microbiota, even small changes could alter microbial community and their decomposition process. This research demonstrated a larger fungi population and F:B ratio with wheat straw and biochar application, while there was a relatively greater microbial CUE with biochar amendment. The increase of fungi species may be responsible for the microbial C immobilization in different land uses. Unlike the constant decrease of microbial CUE along incubation in soils without any amendments, the biochar applied soil had the most stable microbial CUE values (**Table 4.7**). In a long term, this is essential for keeping soil C balance. As a result of land use alteration, a fungal-based underground food society showed more resistance ability than bacterial-based ones, and more importantly, less soil C and N loss (de Vries et al., 2006). This shows that the fresh organic amendments had a stronger impact on microbial community changes than land use (Jangid et al., 2008).

4.6 Conclusions and recommendations

- Land use histories lead to soil physiochemical and microbial properties variations. The natural forest showed the largest organic C stock while relatively low soil elements content. Microbial activity and biomass both reduced because of the constant disturbance in cropping land. We also suggested that anthropogenic land management, especially fertilizer application, favours bacteria community growth, while fungi are more dominant in natural land systems.
- The fresh organic amendments had more influences on microbial CUE compared to land use systems. The addition of labile C source stimulated microbial activity, while complex C sources such as wheat straw and biochar promoted microbial CUE. When an easily degradable C source is added, it stimulated an explosive growth of G+ bacteria, while biochar favoured fungi population growth.
- The PLFA analysis method supported better characterization of changes that occur with different management schemes and organic amendments. Therefore, microbial community composition is a promising indicator to monitor natural ecosystem conversion and consequently the evaluation of ecosystem service change.
- The length of organic inputs influences microbial population alteration along time. Future research may focus on the mechanism of microbial community alteration by CUE differences with long-term observations.

Chapter 5

MICROBIAL FUNCTIONAL DIVERSITY AND CARBON USE EFFICIENCY IN SOIL AS IMPACTED BY HEAVY METAL CONTAMINATION

5.1 Introduction

Soil is heterogeneous, containing physical, chemical and biological components, which determine soil functions and ecosystem services (Doran and Parkin, 1996). Soil is also a major reservoir of organic carbon (C), can reconcile nutrient balance, and provide habitats and supports for living organisms (Lehmann and Kleber, 2015). It can also be a large sink for organic and inorganic contaminants, such as the accumulation of heavy metal(loid)s (Seshadri et al., 2015). Both natural and anthropogenic processes can lead to the release of heavy metal(loid)s into the ecosystem (Khan et al., 2010; Margesin et al., 2011). Because heavy metal(loid)s are non-degradable by soil biochemical reactions, they are identified with high potential for bioaccumulation in living organisms (Adriano et al., 2004). The biohazard feature of metals has captured scientific community's attention, leading to increasing public awareness and research regarding the metal toxicity 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 regulate the central part of the soil C decomposition and nutrient dynamics (Blagodatskaya and Kuzyakov, 2013). Microbial carbon use efficiency (CUE) is defined as the amount of C that is incorporated into the microbial biomass to that being utilized by microbes for biomass production (Sinsabaugh et al., 2013). The microbial CUE parameter has been incorporated into a number of soil biogeochemistry models (Allison et al., 2010; Manzoni et al., 2012), and considered as an essential factor to reveal microbial controls on soil organic carbon (SOC) degradation (Geyer et al., 2016). Microbial population is highly sensitive to soil environmental changes and stresses, leading to microbial CUE variation along with the differentiation of environmental parameters (Frey et al., 2001). While some heavy metal(loid)s serve as micro-nutrients and are necessary for maintaining biological functions of microorganisms (Diels et al., 2002), excessive quantities of heavy metal(loid)s may lead to biotoxicity, inhibit microbial

activity and alter the community composition (Khan et al., 2017). The interaction between soil microorganisms and heavy metal(loid)s can also affect metal condition, leading to metal mobilization, such as dissolution, leaching and redox transformation, or immobilization such as organic-metal binding and precipitation (Gadd, 2004).

Brookes (1995) suggested that soil microorganisms could be a promising indicator in monitoring the soil fertility and health condition. Previous research has focussed on the adverse impacts of heavy metal(loid)s on soil microorganisms and their buffering mechanisms to control the environmental fates of metals (Gupta et al., 2016). The identification of phospholipid fatty acids (PLFAs) has been introduced as a relatively effective way to determine specific components of the soil microbial community, therefore providing an insight to the microbial community structure (Bååth and Anderson, 2003; Frostegård et al., 1993). Total PLFA and PLFA signatures can be useful parameters when related to risk assessment of soil heavy metals and their environmental regulation (Gans et al., 2005), as well as broader soil health indicators (Griffiths et al., 2001). Lenart and Wolny-Koladka (2013) suggested that the PLFA approach could reveal more critical information of the negative effects of metal contamination because it would provide microbial community structure alteration due to metal toxicity, which help to interpret microbial CUE data. Numerous studies earlier focussed on the physiochemical interactions between heavy metal(loid)s and soil components (e.g., organic matter, clay minerals, oxidic particles) (Adriano et al., 2004), but little attention was given on the specific and systemic elucidation of heavy metal(loid) impacts on soil microbiota, and most importantly, alteration of microbial C use in soil. A thorough understanding of the relationship between heavy metal(loid)s and microbial properties, including activity and community composition, will contribute to the interpretation of microbial C use patterns under metal contaminations.

5.2 Objectives

The overall objective was to evaluate microbial C use alteration under metal stress, by measuring microbial properties: activity, CUE and community composition. The specific objectives are as follows:

- To determine the effect of metal type, concentration and incubation period on soil microbial properties.
- To examine microbial CUE, and consequently C dynamics alteration under the metal stress.
- To estimate the value of microbial community composition, such as diversity and bacteria:fungi ratio, and consequently regulating soil C fate.

5.3 Hypothesis

- Metal biotoxicity would vary depending on the metal types and contamination levels.
- Microbial respiration is suppressed due to metal toxicity thereby reducing microbial CUE and decreasing biomass production in metal contaminated soils.
- Microbial species compositions show discrepancies under different metal contaminations.
- Microbial CUE can be a promising indicator when evaluating soils with heavy metal contamination.

5.4 Materials and methods

5.4.1 Soil preparation and spiking

The location, description and soil properties of sampling soils were presented in **Chapter 3 and Table 3.2**. A fresh soil sample was collected form the Barossa Valley region, South Australia (138°57'37''E, 34°27'48''S). The region was identified as Mediterranean climate with the major soil type as Sodosol. 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., 2017b). After collection, the soil was processed by removing all fine roots and debris, air-drying, homogenising and passing through < 2 mm sieve. The soil was stored for 7 days at 4 °C until further analysis. Soil pH and EC were determined in 1:5 (*w/v*) soil suspension in deionized water with a pH/conductivity meter (smartCHEM-LAB Laboratory Analyzer, TPS, Pty Ltd., 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^+, then followed by the determination of the concentration of NH_4^+ (Ross and Ketterings 1995) on a Continuous Flow Analyzer (San^{++}, Skalar Analytical B.V., Netherlands). Soil total organic carbon (TOC) and total nitrogen (TN) were measured by Leco C/N Analyzer (Leco TruMac® CNS/NS Analyzer, LECO Corporation, Japan). In brief, 0.2 g soil samples were weighed and combusted at 1300 °C with an O_2 flow for 5 s. In order to 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 = 6.26) in nature with EC value of 27.53 mS cm^{-1}, and CEC value of 32.71 cmol (p^+) kg^{-1}. The soil was silty loam in texture, containing 26, 42 and 20% of clay, silt and sand, respectively. The soil was adjusted to 50% of the water holding capacity (WHC), incubated at 25 °C, 28% relative humidity for a week before conducting the microbiological analysis.

The soil used for this chapter was spiked with low and high levels of $Cd(NO_3)$ and $Pb(NO_3)$. They were added into soils both separately and in combination. Control soil portions were similarly amended with KNO_3 to compensate for the amount of nitrate added to the polluted soil. Briefly, metal solutions were sprinkled evenly on the soil, which was spread on a polyethylene sheet. To achieve homogenization, soils were stirred and mixed thoroughly on an end-over-end shaker. Soils were then air-dried, and passed through a 2 mm-sieve again. The final concentrations of metals in the spiked soils for each treatment are listed in **Table 5.1**. All the experiments were conducted in triplicate.

Table 5. 1 Soil spiking rate and final metal concentrations. Mean ± SE, n = 3

Sample	Cd (mg kg^{-1} soil)	Pb (mg kg^{-1} soil)	Cd concertation (mg kg^{-1} soil)	Pb concentration (mg kg^{-1} soil)	Cd recovery rate (%)	Pb recovery rate (%)
Control	–	–	–	–	–	–
CL	25	–	23.18	–	92 ± 3	–
CH	50	–	41.65	–	83 ± 7	–
PL	–	2500	–	2270.0	–	90 ± 5
PH	–	5000	–	4605.0	–	92 ± 1
CPL	25	2500	22.64	2227.8	90 ± 3	89 ± 4
CPH	50	5000	43.85	4687.5	87 ± 4	93 ± 2

Control as uncontaminated soil; CL: soil applied with low Cd level; CH: soil applied with high Cd level; PL: soil applied with low Pb level; PH: soil applied with high Pb level; CPL: soil applied with low Cd + Pb level; CPH: soil applied with high Cd + Pb level

5.4.2 Potential and bio-availability of heavy metals

Bioavailable 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 bioavailable and potentially available metals were measured on day 7 and again at the end of the incubation (day 49). The extracts were filtered through 0.45 µm syringe filter before analysis by inductively coupled plasma mass spectrometry (ICP-MS) instrument (ICP-MS, Agilent 7900, Agilent Technologies Ltd., USA). Non-contaminated soils (without Cd and Pb contaminated) were set up as control. The treatments and their abbreviates are as follows: un-contaminated 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.

5.4.3 Microbial properties

The microbial activity of soils was monitored by measuring the basal respiration (CO_2 evolution) from the samples. In brief, 10 g soil samples were incubated in Schott bottles at 25 °C and 28% relative humidity, in dark for a 49 days incubation period. Open-top 20 mL vials containing 10 mL of 0.05 M NaOH solution were used to trap the evolved CO_2 within the sealed Schott bottles. The NaOH was transferred into an Erlenmeyer flask by rinsing with deionised water three times. Five mL of 0.5 M $BaCl_2$ solution was added to ensure the evolved and trapped CO_2 precipitated as $BaCO_3$. The collected alkaline aliquot was then titrated against 0.03 M HCl in the presence of phenolphthalein indicator. Ten mL of freshly prepared alkali was replaced every time. Sampling was 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. 5.1**:

$$MR = \{MWCO_2(V_b - V_s) \times M \times 1000\}/(DW \times T \times 2) \quad \text{Eq. 5.1}$$

where, *MR* is the microbial respiration (mg CO_2–C kg^{-1} soil h^{-1}), *MWCO₂* is the molecular weight of CO_2, V_b is the volume of HCl for the blank titration, V_s is the volume of HCl for the sample titration, *M* is the concentration of HCl, *DW* is the dry weight of the soil, *T* is the time of incubation, and *2* is the factor that accounts for the fact that two OH^- are consumed by one CO_2.

The amount of microbial biomass carbon (MBC) was measured by the method of Vance et al. (1987). In brief, 10 g dry weight equivalent soil was placed in 50 mL beakers in the presence of 50 mL ethanol-free chloroform in a vacuum desiccator. The desiccator was pumped until chloroform was vaporized. It was then sealed and placed in dark for 48 h incubation. 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. Carbon content in the filtrates was analysed by a TOC Analyzer (TOC-LCSH, Shimadzu Corporation, Japan). MBC was calculated using **Eq. 5.2**:

$$MBC = E_c \,/\, K_c \quad \text{Eq. 5.2}$$

where, *MBC* stands for microbial biomass carbon (MBC, mg C kg^{-1} soil), E_c stands for the value=(C extracted from fumigated soils − C extracted from non-fumigated soils), and K_c

stands for the conversion factor (0.45) from chloroform flush C values into MBC (Anderson and Domsch, 1989).

The following **Eq. 5.3** was used to calculate the microbial CUE, which is based on the microbial biomass accumulation during the incubation.

$$C_m = \Delta M_c / (\Delta M_c + \sum CO_2\text{–}C \quad \text{Eq. 5.3}$$

where, C_m is microbial CUE measured as microbial biomass variation, ΔM_c is the change of MBC, ΣCO_2–C is the cumulative C as microbial respiration.

The PLFAs were used as biomarkers for determining certain microbial species 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. The citrate buffer in the one phase solvent was made from citric acid and sodium citrate (3:1, *v/v*) adjusted to pH = 3.6 (Bligh and Dyer, 1959). After equilibrating on an end-over-end shaker for 2 h, the suspension was centrifuged twice at 4500 rpm for 30 min. The upper solution was decanted into non-transparent vials, and vortexed before standing over night. Then the solution changed into two separate layers due to density differences. The upper layer was removed and discarded. 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. Each transfer step was repeated three times. The solid phase extraction column was set up by adding 0.5 g silica in the glass columns. In order to condition it, the silica in the SPE column was leached with chloroform at 1 mL three times. The final leaching solution was collected in glass tubes and then dried using continuous N_2 flow at 32 °C. After drying, 0.5 mL of 1:1 (*v/v*) of methanol:toluene and 0.5 mL of 0.2 M methanolic KOH in the glass tube were added. The PLFAs were converted into fatty acid methyl ester (FAMEs) with mild alkaline methanolysis at 37 °C for 30 min. After cooling the samples back to room temperature, 1 mL deionized water, 0.15 mL 1 M acetic acid and 1 mL hexane were added, then the mixture was vortexed for 30 s and centrifuged to separate the solution into two layers. The upper layer was transferred into GC vials by pipette. Finally, the samples were dried under N_2 and stored at −20 °C in the dark before further analysis.

The 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, USA) with an RTX-5MS fused silica capillary column (60 m, 250 µm × 0.25 µm film thickness) (Supelco, Sigma-Aldrich, Australia). The setup of GC-MS parameters was presented in section 3.3.5, **Chapter 3**. 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 G− bacteria were as: C16:1ω7c, C16:1ω9c; biomarkers for 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 C18:2ω6c, C18:1ω9c (Frostegård et al., 1993; Zelles, 1999).

5.4.4 Statistic analysis

Shaprio-Wilk test was used to determine normal distribution, and the homogeneity of variance test was also employed. One factor ANOVA was used to test the significant differences among treatments. The LSD test was used and the significant differences accepted at $p < 0.05$. The PCA was used for microbial PLFA data to elucidate the major variation and covariation both for individual PLFA and microbial species using varimax rotation. All these statistical analysis were performed in SPSS version 23.0 (SPSS Inc., Chicago, USA).

5.5 Results and discussion

5.5.1 Soil physicochemical characteristics

5.5.1.1 Soil pH and electrical conductivity

Soil physiochemical characteristics were determined to reveal the variation on microbial living conditions under metal contamination. Soil pH increased from 5.51 to 6.02 in the uncontaminated soil from Day 7 to 49 (**Figure 5.1a**). After 7 and 49 days incubation, the lowest pH values were both in the high concentration co-contaminated (CPH) soils, at 5.09 and 4.94, respectively. Soil pH slightly increased in samples spiked with Cd although not significant, whilst it decreased in Pb spiked samples, also with Cd and Pb co-contamination. 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. Soil pH is a critical factor in determining metal mobility in soils, and even the forms and toxicity of some metals, such as Cd (Bolan et al., 2014; Dai et al., 2017). For instance, with increased soil pH, the heavy metal bioavailability reduced (Houben et al., 2013). However, there are few research can prove that metal contamination affect soil pH. In this research, there was no significant correlation ($p > 0.05$) between heavy metal concentrations and soil pH values. Also in Deng et al. (2015), they did not find any correlation between metal contamination and pH. However, in Lenart and Wolny-

Koladka (2013), they demonstrated the pH differentiation had limited effects on microbial abundance with the presence of heavy metal contamination, while in Chodak et al. (2013), they argured that soil pH is the determine factor for bacterial community. We noticed slightly discrepancy of pH values among treatments after 7 and 49 days incubation. This differentiation might need to consider the biochemical reaction, which highly influenced by soil microbiota (Philippot et al., 2013). As a matter of fact, according to, the microbial regulated soil process, such as element cycle and energy dynamics, also modified by soil properties that also influenced by metal (Giller et al., 2009).

The changes of soil electrical conductivity (EC) were shown in **Figure 5.1b.** Compared to control, after 7 days incubation, the lowest EC value (10.16 mS cm^{-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, 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 with different metal concentrations.

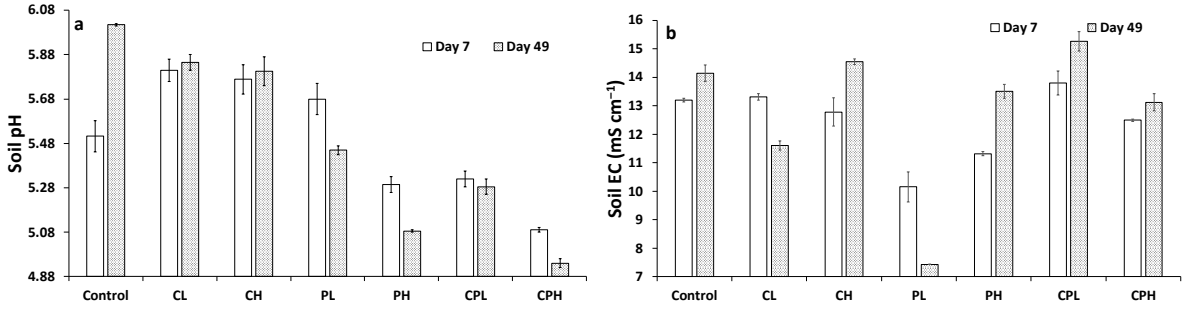


Figure 5. 1 Soil pH and EC response in Cd and Pb contaminated soils; (a) soil pH, (b) soil EC. Values show means ± SE. Control as un-spiked soil, CL and CH as soils spiked with low and high Cd concentration, PL and PH as soils spiked with low and high Pb concentration, CPL and CPH as soils spiked with low and high Cd + Pb concentration.

Soil EC values are ascribed to soluble salts, such as Ca^{2+} and Na^{+}, in soil concentration in soil. When the relatively soluble metals reach certain high amount, soil EC will increase. Some research demonstrated that soil EC affects metal bioavailability (Salimi et al., 2012). In this research, the non-significant correlation between soil EC values and Cd/Pb addition suggested soil EC doesn't seem to be effected by the type and concentration of the metal contamination.

5.5.1.2 Soil total organic carbon and nitrogen content

For the C content, we measured the TOC and TN contents after 49 during incubation (**Table 5.2**). There was no significant ($p > 0.05$) correlation between organic C and nitrogen contents

with total heavy metal concentrations in this study. After 49 days incubation, CL and PL had the lowest SOC 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 SOC had the lowest value in CPH, while the greatest value in the uncontaminated soil (8.92 in control).

Table 5. 2 Soil organic carbon, nitrogen, C:N ratio and Cmin:Corg ratio in different metal contaminated soils by the end of incubation period. Means ± SE (n = 3)

Sample	Soil organic C (%)	Soil nitrogen (%)	C:N	C_{min}:C_{org}
Control	2.73 ± 0.08	0.27 ± 0.003	10.21 ± 0.23	8.92 ± 0.22
CL	2.84 ± 0.05	0.23 ± 0.003	12.53 ± 0.36	4.03 ± 0.17
CH	2.68 ± 0.01	0.25 ± 0.002	10.57 ± 0.05	3.72 ± 0.66
PL	2.64 ± 0.00	0.22 ± 0.002	11.83 ± 0.14	3.50 ± 0.30
PH	2.59 ± 0.04	0.23 ± 0.005	11.34 ± 0.27	3.09 ± 0.15
CPL	2.46 ± 0.02	0.25 ± 0.005	9.77 ± 0.45	3.44 ± 0.36
CPH	2.67 ± 0.05	0.23 ± 0.004	11.42 ± 0.30	2.49 ± 0.11

Control as uncontaminated soil; CL: soil applied with low Cd level; CH: soil applied with high Cd level; PL: soil applied with low Pb level; PH: soil applied with high Pb level; CPL: soil applied with low Cd + Pb level; CPH: soil applied with high Cd + Pb level

The salt-metals combination will be dissolved in soil solutions as ions, which are ascribed to electrolytes, the formation of organo-metal complex can lead to the reduction of metal mobility (Bolan and Thiagarajan, 2001). However, the TOC content in soil is highly mediated by active microorganisms and their function (Blagodatskaya and Kuzyakov, 2013). In addition, microbial processing of organic C and mineral nitrogen could be affected by metal toxicity. The inhibition of microbial population due to metal toxicity might lead to higher mineral nitrogen concentration. The low value for the ratio of MBC to SOC in CPH may be ascribed to the decrease in the formation of microbial biomass and also the accumulation of soil TC. Khan et al. (2010) and Zhang et al. (2008) noticed that microbial biomass decreased significantly with increasing metal concentrations. This indicated that the microbial biomass content was repressed in metal polluted soils. The effect of metal types and/or concentrations on soil microorganisms will be further discussed in section **5.5.3**.

5.5.2 Potential and bioavailable heavy metal concertation

Except CL, the bioavailable Cd and Pb decreased with the incubation period (**Figure 5.2**). By the end of incubation (49 days), the bioavailability of Cd significantly ($p < 0.05$) decreased by 59% (CH), 58% (CPL) and 58% (CPH) compared to those after 7 days (**Figure 5.2a**). In comparison between day 7 and 49 days of Pb bioavailability, it reduced by 100, 37, 23 and 30% in PL, PH, CPL and CPH, respectively (**Figure 5.2b**). In spite of the same initial input rates, the Cd bioavailability was significantly ($p < 0.05$) 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 in day 7 and 49, respectively). Also, Pb bioavailability was significantly ($p < 0.05$) higher in Cd-Pb co-spiked 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 (**Figure 5.2 c**). The reduction was even more remarkable in CPL (from 16.04 to 3.44 mg kg^{-1} soil) and CPH (from 48.18 to 12.44 mg kg^{-1} soil) (**Figure 5.2c**). Compared to the concentration on day 7, the potentially bioavailable Pb increased in all treatments by 22, 26, 66 and 45% in PL, PH, CPL and CPH, respectively on day 49 (**Figure 5.2d**). Compared to the single Cd polluted soils, potentially available Cd content 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 difference disappeared on day 49.

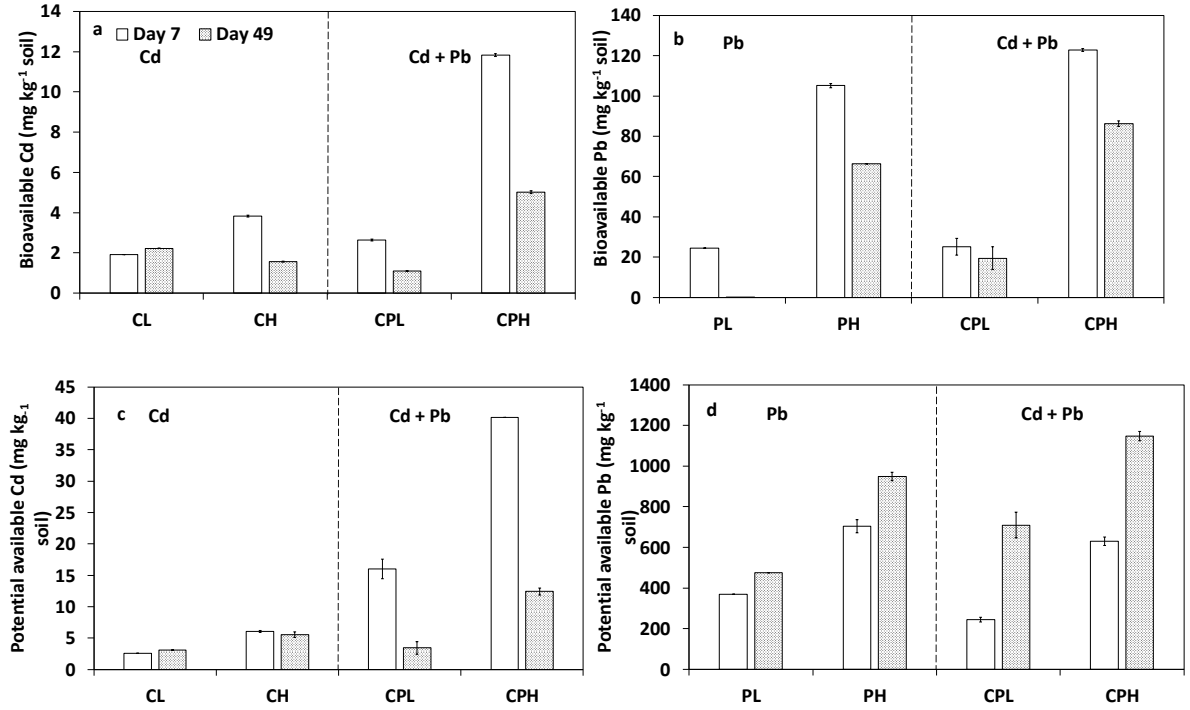


Figure 5. 2 Bioavailable Cd (**a**) and Pb (**b**), potential Cd (**c**) and Pb (**d**) concentrations in different treatments. Data are displayed as means, bars indicate SE (n = 3). Control as uncontaminated soil; CL: soil applied with low Cd level; CH: soil applied with high Pb level; CPL: soil applied with low Cd + Pb level; CPH: soil applied with high Cd + Pb level.

Although there has been debate about the definition and determination of metal bioavailability and the biotoxicity also depends on bio-process and tolerance (Kim et al., 2015), it was introduced in this research as a quantity monitoring parameters for the variation of metals in soils. Also, Chen et al. (2014) suggested the concentration of metal bioavailability can potentially modulate soil biochemical process. In the current research, the metal bioavailability concentration was significantly higher in co-contaminated soils than single-metal-contaminated soils. This indicated that the metal bioavailability may increase in the co-contaminated samples with high concentrations. In spite of the possibility that there was relatively higher metal concentration in co-contaminated samples than the latter, previous research has proved that soil organic matters can serve as adsorbents to reduce metal bioavailability and mobility (Bolan et al., 2014). So the competition among metals for the adsorbent sites could result in the greater metal bioavailability in co-polluted soils than a single metal-contaminated soil. As for soil organisms, bioavailable metals influence microbial activity and C utilization patterns (Wang et al., 2007b), and consequently affect the metal-organic-complex formation and metal release in turn. In addition, some soil microorganisms are especially sensitive to metal pollutants, making them effective indictors in terms of environmental assessment (Vig et al., 2003). In the current study, there was significant (p <

0.05) negative correlation between metal bioavailability and microbial abundance and biomass C formation. Therefore, the biotoxicity had negative influences on soil microorganisms, and consequently affect soil C dynamics. The bioavailable metal concentration decreased in the end compared to one week incubation. Lu et al. (2005) investigated the residence time effects on soil metal transformation and found that the bioavailability of metals decreased with incubation. They suggested that the binding between organic matter and metal was the major reason for the reduced easily extractable fractions. The reduced bioavailability may lead to less biotoxicity and affect soil microorganisms at the end of incubation.

Because of the hyperaccumulaion property of heavy metals, the metal potential availability was measured for a better assessment of the metal concentration during the period of incubation. The co-contamination did not influence potentially available Pb concentration like in the case of Cd-polluted samples. Generally, the relative mobility of Cd is larger than Pb in soils (Kim et al., 2015). The differences between potentially available Cd and Pb contents in soils could be attributed to their relative mobility in soils. However, Khan et al. (2010) noticed that the toxicity of metals to soil microorganisms was more related to metal concentrations than metal types. In this research, the potential availability significantly affected by the spiked metal concentration (p > 0.05), and also showed slightly negative relation to microbial abundance and biomass. Although there has been numeric research on potential and bio-availability metals in soil, in the current research, both of the two parameters revealed the access possibility and toxicity level to soil microorganisms, showing adverse effects on their function. Compared to metal potential availability, microbial properties were more affected by the metal bioavailable concentration.

5.5.3 Effect of heavy metal pollution on microbial carbon use

5.5.3.1 Heavy metal toxicity on microbial activity

Microbial basal respiration rate and total respired CO_2 were analysed to determine the soil microbial activity under metal stress (**Figure 5.3 and Figure 5.4**). By the end of incubation, total respired CO_2–C differed distinguishably among different metal treatments (**Figure 5.3**). The microbial respiration declined gradually from day 1, over the incubation period for all the treatments (**Figure 5.3**). However, the respiration rate in uncontaminated soil (grey line) was higher than those in the contaminated soil (color lines) all along the incubation period. The largest respiration rate was in the uncontaminated soil (1.27 μg CO_2–C g^{-1} soil h^{-1}), and it stayed greater than the polluted soils over the whole incubation period. The respiration rates in metal-spiked soils followed the order on day 1 was CPH < CH < CPL < PL < CL < PH (0.29,

0.31, 0.37, 0.58, 0.66 and 0.75 µg CO_2–C g^{-1} soil h^{-1} in the respective treatments. As for the accumulated microbial respiration, after 49 days of incubation, the largest cumulative CO_2–C production was in the control soil (351.41 µg CO_2–C g^{-1} soil) (**Figure 5.4**). The cumulative CO_2–C productions were not significantly different ($p > 0.05$) between PL and PH, but there was significant difference ($p < 0.05$) between CL and CH. At low metal concentrations, the cumulative microbial respiration was decreased by 43, 47 and 68% in CL, PL and CPL spiked soils, respectively, when in comparison to the control. While at the relative higher metal-spiked treatment, the decreases were 78, 55 and 77% in CH, PH and CPH, respectively (compared to control). The lowest accumulated released CO_2–C value was in CH (77. 52 µg CO_2–C g^{-1} soil), followed by CPH (79.88 µg CO_2–C g^{-1} soil) by the end of incubation period.

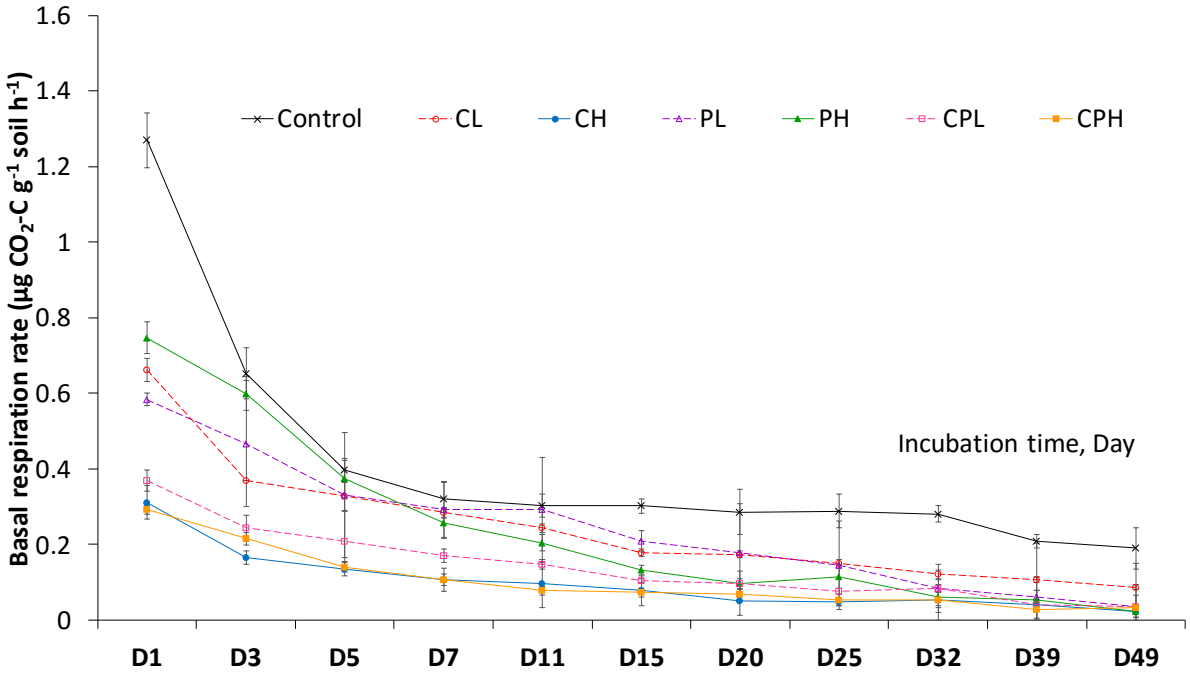


Figure 5. 3 Microbial respiration rate variation with different heavy metal contamination. Data are displayed as means, bars indicate SE (n = 3). Control as uncontaminated soil; CL: soil applied with low Cd level; CH: soil applied with high Cd level; PL: soil applied with low Pb level; PH: soil applied with high Pb level; CPL: soil applied with low Cd + Pb level; CPH: soil applied with high Cd + Pb level.

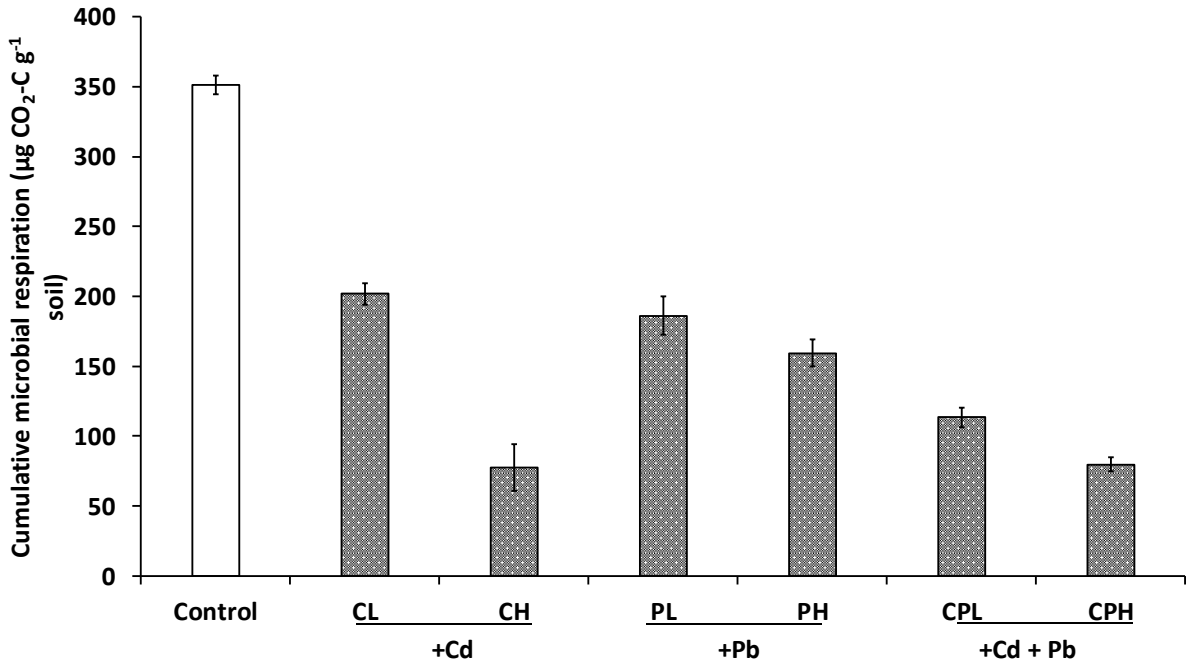


Figure 5. 4 Effect of heavy metal contamination on cumulative microbial respired CO_2-C. Data are displayed as means, bars indicate SE (n = 3). Control as uncontaminated soil; CL: soil applied with low Cd level; CH: soil applied with high Cd level; PL: soil applied applied with low Pb level; PH: soil applied with high Pb level; CPL: soil applied with low Cd + Pb level; CPH: soil applied with high Cd + Pb level.

A number of studies have shown that environmental stress induced by heavy metals could lead to inhibitory effects on microbial activity (Chen et al., 2014; Dai et al., 2017; Epelde et al., 2009). The microbial activity in control soil was significantly higher than in the contaminated soils, with the decrease of metal bioavailable concentration in this research, there was no significant difference at the end of incubation. It is not surprising to have suppressed microbial activity due to metal pollution in this research. The reduction was not only about the total released CO_2–C, but also in the microbial respiration rate. The respiration inhibition was ascribed to the metal biotoxicity, which had negative influences on microbial enzyme process, cell function and nuclei formation (Jiang et al., 2010; Tchounwou et al., 2012). Therefore, the metal lead to a 'disabled' soil microbial community, which had a lower function activity. However, Kaplan et al. (2014) suggested when contaminated soil was applied with 0.1 g glucose, the differences of induced microbial respiration became not significant, indicating the metal pollution did not reduce the potential mineralization ability of soil microbiota. Interestingly, some research found increased microbial activity under metal stress (Bhattacharyya et al., 2008; Renella et al., 2004). This might be related to the energy re-location from biomass formation to cell maintenance when microorganisms facing stress (Killham, 1994), leading to a resulted as elevated energy demand of microbial species in order to survive under undesirable living conditions (Lu et al., 2013; Zhang et al., 2010). In addition, some microbial

species show tolerance to heavy metals (Fließbach et al., 1994; Giller et al., 2009). This also may be an explanation for discrepancy in the results of microbial activity in metal contaminated soils among literatures.

5.5.3.2 Microbial biomass carbon

The MBC formation was inhibited by metal toxicity (**Figure 5.5**). Values of MBC in contaminated soils were significantly lower ($p < 0.05$) than un-spiked soil both on day 7 and 49. The biomass values in CL was 7% higher than CH, in PL was 13% higher than PH, and in CPL was 22% high than CPH. The results were in consistence with those in Abaye et al. (2005), Li et al. (2008) and Yao et al. (2003). They all reported decreased microbial biomass with metal pollution. After 7 days 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, in comparison to the un-spiked soil MBC values were 53, 59, 62, 67, 65 and 73% less in CL, CH, PL, PH, CPL and CPH, respectively. However, along the incubation period, MBC values increased at the end of incubation compared to those on day 7. The largest increase was in the uncontaminated soil, from 187.40 to 243.86 mg kg^{-1} soil. Both microbial respired CO_2–C (as discussed in section **5.5.3.1**) and biomass C decreased due to metal toxicity, we will investigate soil C dynamics by introducing microbial CUE in section **5.5.3.3**.

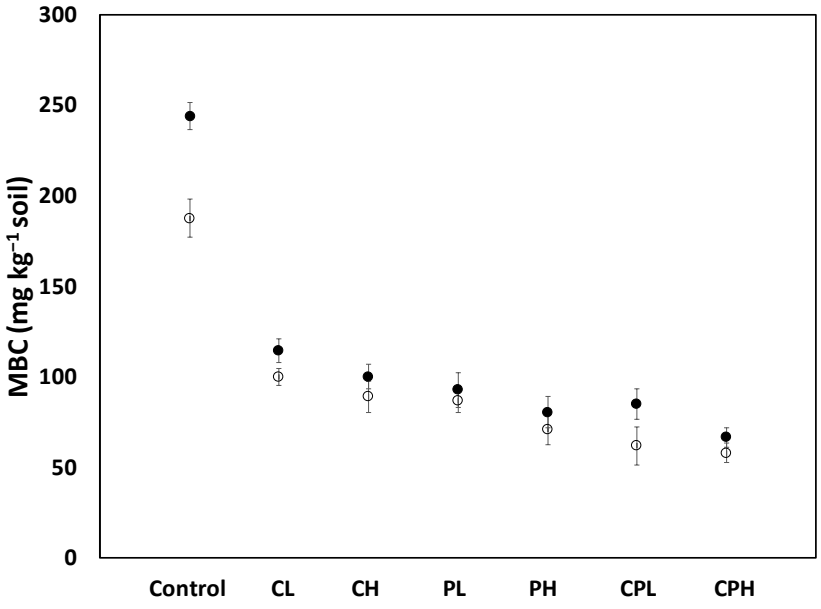


Figure 5. 5 Microbial biomass carbon in different treatment soils. Data are displayed as means, bars indicate SE (n = 3). Control as uncontaminated soil; CL: soil applied with low Cd level; CH: soil applied with high Cd level; PL: soil applied with low Pb level; PH: soil applied with high Pb level; CPL: soil applied with low Cd + Pb level; CPH: soil applied with high Cd + Pb level.

Soil microbial biomass, which is an essential part of SOC pool, simultaneously regulate soil elements transformation and energy flow， even in contaminated soils (Dai et al., 2003). A number of documented researches proved decreased microbial biomass due to metal-pollution (Papa et al., 2010; Wang et al., 2007b; Zhang et al. 2010). Compared to the prompt response of microbial activity to metals, microbial biomass might be less affected (Knight et al., 1997). This maybe because the 'time lag' for the changes in microbial population, similarly with microbial CUE and community composition.

5.5.3.3 Microbial carbon use efficiency in metal spiked soils

The difference in biomass is connected to microbial metabolism. Therefore, in order to understand the microbial C sequestration, the microbial CUE was calculated, compared different microbial CUE approach based on the preliminary experiment in **Chapter 3** and measured CUE based on microbial biomass accumulation. Similarly, compared to day 7, microbial CUE values decreased by the end of incubation. The highest CUE was found in the uncontained soil, 0.59 on day and 0.35 on day 49. The smallest CUE values for the two measurements were both found in CPH, 0.08 and 0.12 on day 7 and 49, respectively (**Table 5.3**). Co-contamination with Cd and Pb contributed to a greater microbial-driven C degradation than the single metal contamination, and the extent of the effect increased with increasing metal concentration. Although Cd might exert a larger biotoxicity level due to its higher mobility (Neethu et al., 2015), microbial CUE did not differ significantly ($p > 0.05$) between single Cd and single Pb spiked soils in this study.

Table 5. 3 Effect of heavy metal toxicity on microbial carbon use efficiency (CUE). Date shown as means ± SE (n = 3)

Microbial CUE	Day 7	Day
Control	0.59 ± 0.03	0.35 ± 0.02
Cd Low	0.41 ± 0.01	0.23 ± 0.02
Cd High	0.55 ± 0.02	0.36 ± 0.03
Pb Low	0.32 ± 0.02	0.17 ± 0.02
Pb High	0.16 ± 0.02	0.13 ± 0.02
Cd + Pb + High	0.14 ± 0.03	0.21 ± 0.02
Cd + Pd + Low	0.08 ± 0.01	0.12 ± 0.01

Heavy metal biotoxicity evolves due to displacement or substitution of essential elements by toxic elements either in the extracellular enzymes or even in nuclear proteins of microorganisms, damaging cell membrane or DNA structure (Gupta et al., 2016; Kachur et al., 1998; Tchounwou et al., 2012). The formation of microbial biomass was suppressed while metal ions excessed in contaminated soils, impeding the microbial population multiply. However, this did not mean less organic C mineralization, rather, there might be more CO_2–C was released per biomass. Therefore, back to the question: if both microbial respiration and biomass decreased under metal stress, how about the microbial C use? Chen et al. (2014) found reduced microbial activity, yet increased metabolic quotients. As contrary, the current research demonstrated heavy metal exerted inhibitory influences on microbial CUE, This is in consistence with Liu et al. (2012). They also reported decreased microbial CUE in metal polluted soils. Although Liu et al. (2012) used microbial quotient as the indicator for microbial CUE, they demonstrated a relatively more CO_2–C was released at the precursor that with the same microbial biomass in soil. Gupta et al. (2016) proposed several metal toxicity mechanisms in microorganisms, including protein denaturation, cell membrane disruption, transcription inhibition, inhibition of cell division and enzyme activity. Depending on metal types and concentration, metals can have inhibitory or even toxic effect on soil microbial populations (Mudhoo and Kumar, 2013). As a result, soil microorganisms may show metabolic dysfunction or community alteration, resulting in lower microbial reproduction, yet relatively higher energy consumption. Apparently, the microbial respiration was suppressed suggesting the metal's instant inhibitory effect on microbial bio-process, while the microbial CUE decrease indicated the possible consumption that: soil microorganisms demanded more energy for repair and maintenance under stress (Fließbach et al., 1994).

On the other hand, microbial processes, including metal bioleaching, biosorption and bio-precipitation, may also contribute to metal immobilization and bio-translocation (Gadd, 2004). Certain microorganisms might even develop adaptation and tolerance to metal toxicity by altering their CUE in long term (Khan et al., 2010; Zhang et al., 2010). Gupta et al., (2015) summarized the potential possibility for microorganisms as bioadsorbents. However, they also pointed out the non-selectively to particular metal ion while using microorganisms as adsorbents. In the current research, there was no significant microbial CUE among soils solely spiked with Cd and Pb, the biotoxicity level may be more related to the metal concentration rather than the metal type. Considering the discussion above, microbial CUE might be used as sensitive indicators of heavy metal contamination in soils.

5.5.4 Microbial community composition features altered by heavy metal

5.5.4.1 Total microbial PLFA and microbial PLFA profile

Each specific microbial species abundance was presented in **Table 5.4**. After 49 days incubation, the uncontaminated soil had the largest biomarker values (fungi: 5.10 nmol g^{-1} soil, actinomycetes: 104.92 nmol g^{-1} soil, G+ bacteria: 89.44 nmol g^{-1} soil, and G− bacteria: 1.65 nmol g^{-1} soil). On day 49, the smallest fungal value was 1.42 nmol g^{-1} in PH, while 31.60 and 0.54 nmol g^{-1} soil for G+ and G− bacteria, which both in CPH. The soils that spiked with both Cd and Pb had the smallest actinomycetes values on day 49. The actinomycetes in uncontained soils over-number CPL and CPH by 65 and 62%, respectively. After 49 days, microbial population increased, yet the uncontained soils had the largest values either at the beginning and end of incubation (On day 7, microbial biomarkers was 1.69, 43.16, 37.42 and 0.42 nmol g^{-1} soil for fungi and actinomycetes, G+ and -negative bacteria, respectively). The smallest fungi (1.10 nmol g^{-1} soil), G+ (25.38 nmol g^{-1} soil) and G− (0.25 nmol g^{-1} soil) populations were all in CPH by the end of incubation period. In the comparison of day 7 and day 49, the ratio of G+:G− and B:F values both decreased.

Table 5. 4 Comparison of Gram-positive bacteria (G+ bacteria), Gram negative bacteria (G− bacteria), fungi and actinomycetes as obtained through respective 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 bacteria and fungi

Sample	Gram+ 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

Control as uncontaminated soil; CL: soil applied with low Cd level; CH: soil applied with high Cd level; PL: soil applied with low Pb level; PH: soil applied with high Pb level; CPL: soil applied with low Cd + Pb level; CPH: soil applied with high Cd + Pb level

Compared to uncontaminated soils, all microbial species decreased in metal-polluted soils. However, the variation among microbial population varied, which showed by the ratio of G+:G− bacteria and B:F. At the end of incubation (day 49), the largest G+:G− ratio was in PL (64.42), followed by PH and CPL, while the lowest in CH (36.87). As for the ratio of B:F, the lowest B:F value was in uncontaminated soil at 17.87. The ratio of B:F was significantly ($p < 0.05$) decreased under metal contamination. The ratio of B:F values decreased from 22.44 to 17.87 in uncontaminated soils.

The results from PCA of the PLFA signatures showed change in the microbial PLFA pattern with metal toxicity. This indicated a shift in the microbial community structure that strongly impacted by heavy metal treatments (**Figure 5.6-5.8**). The first two components accounted for 96.65 and 1.05% for day 49 analysis, while 94.87 and 1.81% for day 7 analysis. In addition, certain biomarkers for bacteria and fungi were suppressed under metal-pollution (**Figure 5.9** and **Table 5.4**). **Figure 5.9a** revealed that on day 49, biomarkers for Gram-postive (G+) and negative (G−) bacteria (aC17:0, iC17:0, aC15:0, iC16:0, C16:0, iC15:0, C15:0) and fungi biomarker (C18:1ω9c) were generally more positively positioned on PC1 and negatively on PC2 values, while actinomycetes biomarkers was (10MeC16:0, 10MeC18:0 and 10MeC17:0) were posited along on the PC2 positive axis, especially 10MeC16:0 was remarkably positioned on the negative value on PC1. This indicated by the end of incubation, soil microbial population were being driven more by G+ and G− bacteria, and then fungi to some extent. However, at the beginning of incubation, microbial signature profile significantly ($p < 0.05$) showed different patterns from that on day 49 (**Figure 5.9b**). Still, positive values of PC1 were generally assigned to bacteria. The most remarkable difference was biomarker Gr+ bacteria bio-signature (iC17:0) and fungi bio-signature (C18:1ω9c), which was PC1 positive, PC2 negative on day 49, but in PC1 negative, PC2 positive on day 7. This may suggest that the negative effect of heavy metal treatments. The contribution of fungi and one certain G+ bacteria PLFA reduced to the overall microbial community shifted due to heavy metal contamination. In addition, the biomarker of G− bacteria did not show significant change, while the one of actinomycetes biomarker.

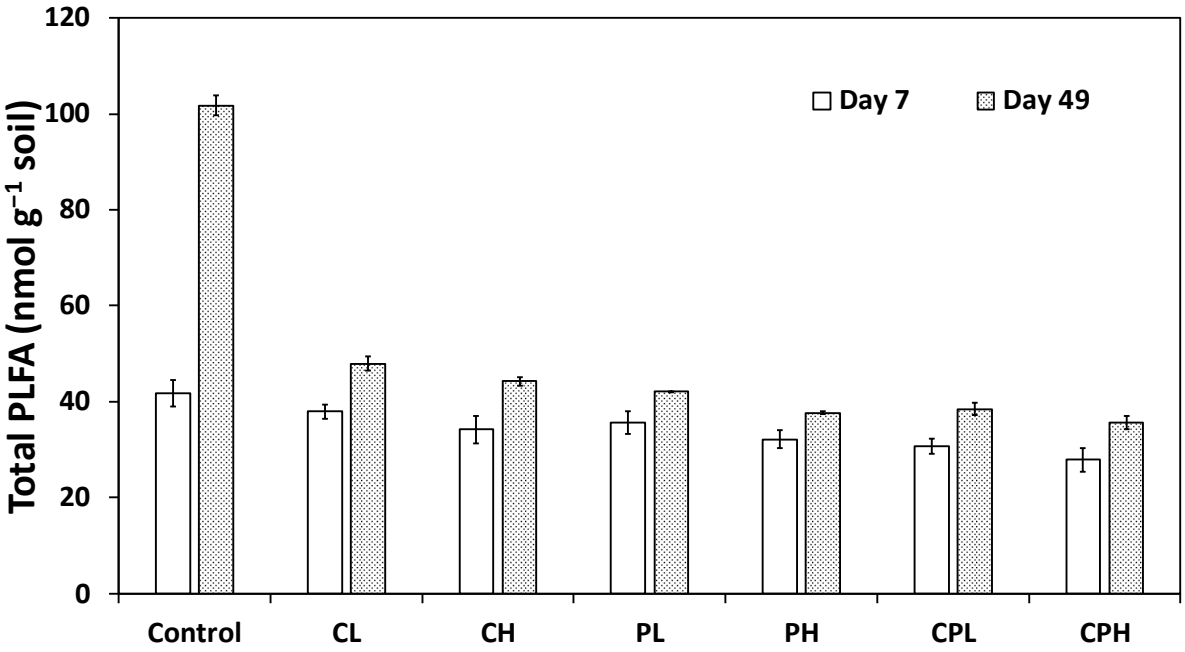


Figure 5. 6 Total microbial PLFA content with different heavy metal contamination. Data are displayed as means, bars indicate SE (n = 3). Control as uncontaminated soil; CL: soil applied with low Cd level; CH: soil applied with high Cd level; PL: soil applied with low Pb level; PH: soil applied with high Pb level; CPL: soil applied with low Cd + Pb level; CPH: soil applied with high Cd + Pb level.

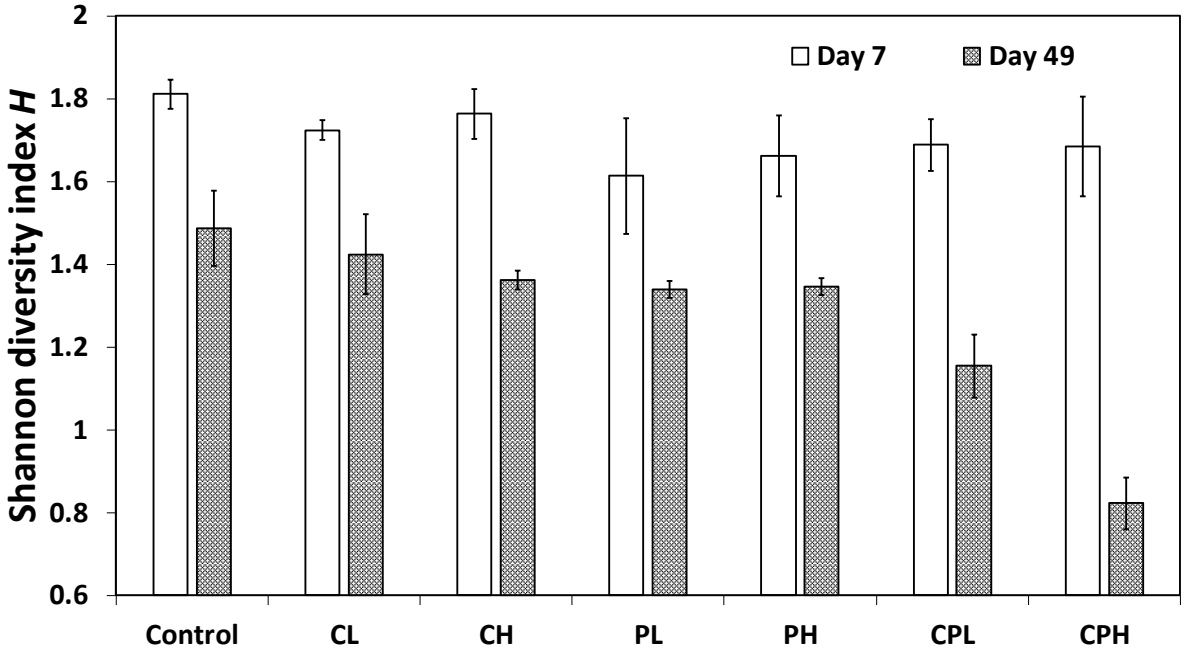


Figure 5. 7 The diversity index H rooted in microbial PLFA profile with different heavy metal contamination. Data are displayed as means, bars indicate SE (n = 3). Control as uncontaminated soil; CL: soil applied with low Cd level; CH: soil applied with high Cd level; PL: soil applied with low Pb level; PH: soil applied with high Pb level; CPL: soil applied with low Cd + Pb level; CPH: soil applied with high Cd + Pb level.

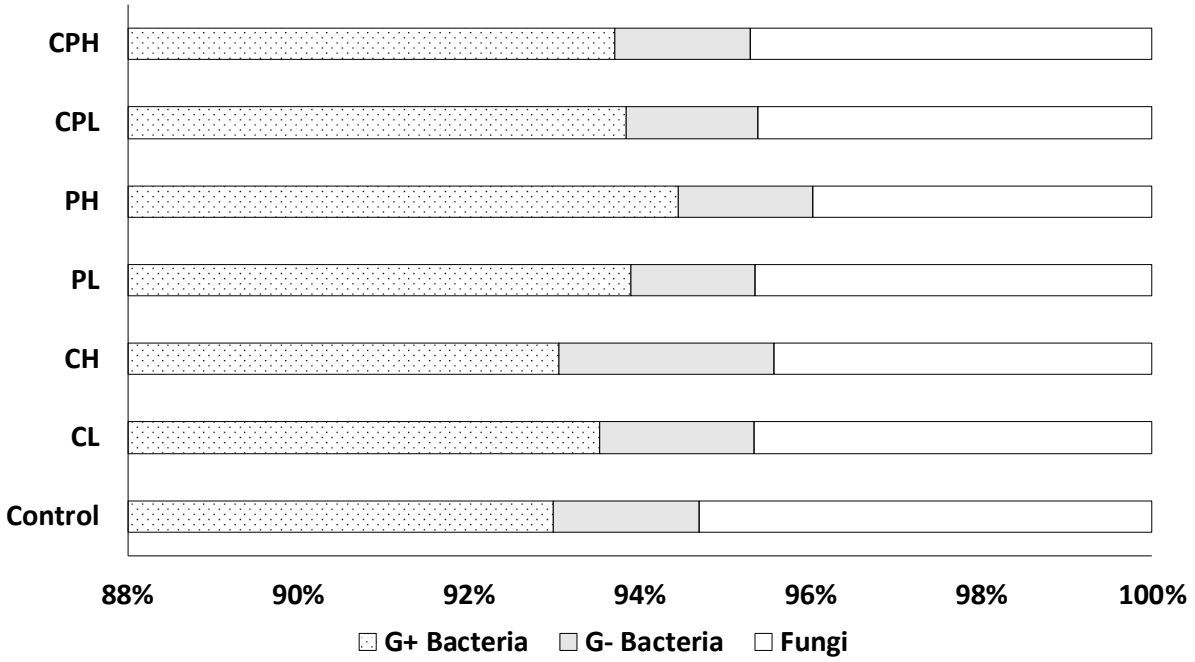


Figure 5. 8 Proportion of fatty acids representing five microbial species (%). Control as uncontaminated soil; CL: soil applied with low Cd level; CH: soil applied with high Cd level; PL: soil applied with low Pb level; PH: soil applied with high Pb level; CPL: soil applied with low Cd + Pb level; CPH: soil applied with high Cd + Pb level.

The principal component score plot based on PLFA biomarkers in metal stress showed the distinguish difference among treatments (**Figure 5.9**). With the two principal components, which together explained 96.68% of the total PLFA variance, the difference position of the metal polluted soils in the first two components explained that the great differentiation of PLFA patterns under metal pollution, and the influences are slightly related to metal types and toxicity level (Jiang et al., 2010). The uncontaminated soil was distinctly different from the contaminated soils. While among the contaminated soils, those spiked with one type of heavy metal (CL, CH, PL and PH) had relatively similar PLFA patterns separating from the soils for CPL and CPH.

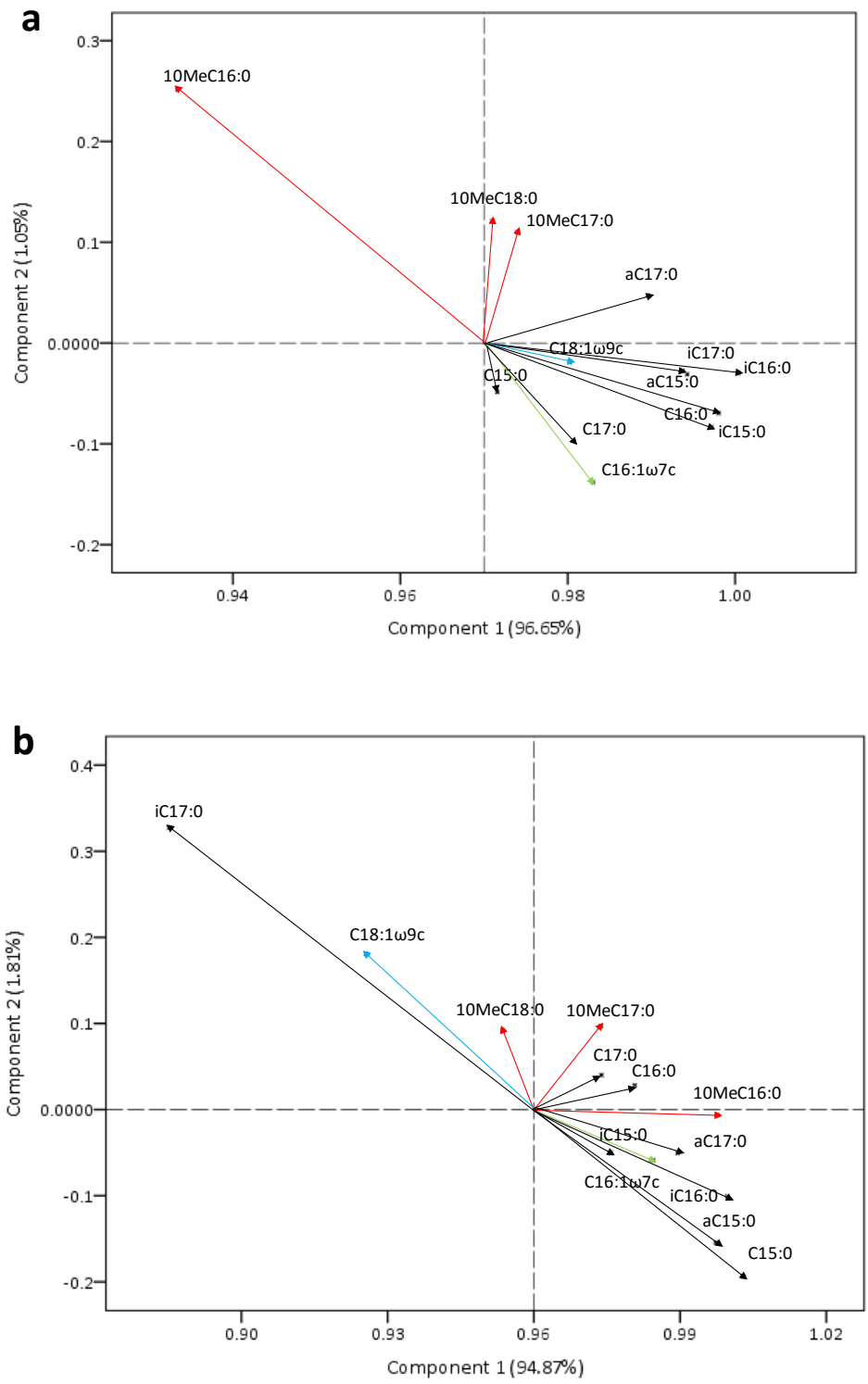


Figure 5.9 Plot of principal component analysis (PCA) showing individual PLFA loading score variation based on phospholipid fatty acid (PLFA) patterns after 49 (upper Figure) and 7 day (lower Figure) incubation. Control as uncontaminated soil; CL: soil applied with low Cd level; CH: soil applied with high Cd level; PL: soil applied with low Pb level; PH: soil applied with high Pb level; CPL: soil applied with low Cd + Pb level; CPH: soil applied with high Cd + Pb level.

5.5.4.2 Microbial population and carbon use

In the current research, microbial PLFA fingerprint provided the information of microbial community composition under metal stress. The total microbial PLFA decreased in heavy metal contaminated soils, and the CPH had more negative influences compared to un-contaminated and single pollution soils (**Table 5.5**). Similar results were found in Azarbad et al. (2013).

Table 5. 5 Comparison of mean values for selected indicator phospholipid fatty acids from soil samples after 7 days and 49 days incubation

Sample	iC15:0	aC15:0	C15:0	iC16:0	C16:1 ω7t	C16:0	iC17:0	aC17:0	C17:0	C16:1 ω 7c	C18:2 ω 6c	C18:1 ω 9c	10MeC16:0	10MeC17:0	10MeC18:0
Day 7															
Control	8.95	3.87	0.52	24.54	0.00	40.98	4.07	5.88	0.63	1.65	0.10	5.00	2.31	1.10	2.11
CL	4.05	1.72	0.28	10.92	0.00	21.67	1.68	2.48	0.36	0.83	0.17	1.98	0.54	0.43	0.77
CH	3.93	1.54	0.28	10.54	0.00	18.27	1.73	2.60	0.26	1.06	0.07	1.80	0.81	0.42	0.92
PL	3.36	1.57	0.27	9.45	0.00	19.48	1.29	2.08	0.23	0.59	0.10	1.76	0.84	0.33	0.69
PH	2.71	1.32	0.19	9.14	0.00	16.82	1.01	2.33	0.23	0.56	0.02	1.40	0.88	0.42	0.64
CPL	2.84	1.42	0.19	8.63	0.00	17.65	1.02	2.37	0.25	0.57	0.11	1.58	0.79	0.31	0.67
CPH	2.76	1.21	0.20	8.47	0.00	15.66	0.86	2.16	0.24	0.54	0.06	1.52	0.73	0.35	0.77
Day 49															
Control	2.89	1.29	0.24	10.34	0.00	17.95	2.06	2.42	0.24	0.42	0.00	1.69	0.76	0.47	0.97
CL	2.80	1.09	0.21	7.30	0.00	19.15	1.80	1.79	0.27	0.34	0.00	1.60	0.62	0.34	0.65
CH	2.17	0.86	0.19	7.05	0.00	17.22	1.41	1.85	0.22	0.31	0.00	1.19	0.59	0.36	0.81
PL	1.45	0.67	0.17	7.25	0.00	18.50	1.88	2.02	0.23	0.32	0.00	1.53	0.61	0.35	0.67
PH	1.66	0.93	0.19	7.47	0.00	16.04	0.69	1.93	0.22	0.32	0.00	1.18	0.56	0.29	0.63
CPL	1.79	0.92	0.21	7.12	0.00	14.83	0.75	1.83	0.20	0.35	0.00	1.23	0.55	0.29	0.62
CPH	1.37	0.73	0.17	6.38	0.00	14.25	0.58	1.72	0.18	0.25	0.00	1.10	0.47	0.25	0.39

However, different microbial species showed variation discrepancy (Azarbad et al., 2013). After 49 days incubation, microbial PLFA patterns changed among different contamination. Chodak et al., (2013) and Desai et al., (2009) both found decreased microbial diversity in metal contaminated soils. Some microbial species may have higher metal toxicity tolerance level and developed mechanical tolerance, and consequently grow better than other species, leading to microbial diversity loss (Ahmad et al., 2005). Aoyama and Tanaka (2013) also noticed the shift in microbial community composition.

Bacteria and fungi comprise the majority of soil MBC and have important functional roles in relation to SOC degradation (Rinnan and Bååth, 2009; Six et al., 2006). A fungi domain soil contributes to organic matter accumulation (Six et al., 2006). However, compared to bacteria, fungi may be more sensitive to an environmental toxic stress (Liu et al., 2012). In the current research, fungal population was significantly suppressed in metal spiked soils (**Table 5.4**). Similar results (increased B:F in contaminated soil compared to uncontaminated soil) were found in Aoyama and Tanaka (2013) and Liu et al. (2012). These results could address that fungi had more sensibility under metal stress than bacteria as we mentioned in the last section. On the other hand, bacteria population have been proved higher tolerance compared to fungi.

The shifts of microbial PLFA composition regulate C use pattern alteration, and consequently have implications for soil and ecosystem function (Liu et al., 2012; Schimel et al., 2007). Compared to bacteria, fungi have lower C:N component and C turnover rate (Six et al., 2006). Shift of fungal and bacteria abundance can lead to C use differentiation (Liu et al., 2012). Liu et al. (2011) demonstrated increased F:B ratio supporting greater SOC accumulation in rice paddy soil. In another research by Liu et al. (2012), they demonstrated that the heavy metal pollution altered fungal and bacterial population, therefore had a strong potential influences on soil C degradation. In this research, fungal population and fungal PLFAs showed greater decrease under metal contaminated soils than bacteria. Fernández-Calviño et al. (2010) demonstrated bacterial PLFA biomarkers increased even in high metal soils. The current research demonstrated the variation of microbial PLFA profile along the incubation, indicating the metal influence on certain biomarkers. Hinojosa et al. (2005) demonstrated the common negatively correlated with metals were: 16:1ω5c, 17:1 ω 8c, 18:1 ω 9c, 18:1ω6c, i14:0, i18:0, 18:0 3OH, 16:0 10Me, 18:0 10Me, a15:1, i16:1, and 18Me18:1ω7c. While in this research, the most affected PLFA biomarkers were: C18:1ω9c, iC17:0, C17:0, aC17:0, C16:0 and 10MeC16:0. However, even with the PCA analysis, it is not conclusive evidence that one single environmental factor is the cause because soil physicochemical properties are inter-related (Fernández-Calviño et al., 2010). Chodak et al., (2013) suggested that soil pH was the dominant influence on microbial community composition, while the metal toxic influence was relatively low. However, unlike the tested soil in our research (pH = 6.26), and Müller et al.

(2001) (pH = 6.9–7.2) or Desai et al. (2009) (pH = 7.9–8.3), the pH range in their samples was 3.4–5.6. Such low pH lead to a dramatic influence even if there was a tiny pH variation. As suggested previoulsly, the potential influence of soil pH leads to the variation of metal mobility, and consequently the difference of metal toxicity to soil microorganisms. The metal pollution length also plays an important role (Jiang et al., 2010). Fernández-Calviño et al. (2010) concluded that the effect of heavy metal pollution could lead to discrepancy of microbial community change depending on the study period.

The shifts in microbial community maybe is a compenzation in a long term, yet also at a cost at soil function and service ability decrease. The microbial community showed relative higher C allocation to energy demanding as determined by microbial CUE. This was in consistence with other research results in Liao and Xie (2007) and Yang et al. (2016). Reduced fungi population and F:B ratio was also found reduced under metal stress in the research. The shift of microbial community composition might partial explained the decreased microbial CUE.

The loading plot (**Figure 5.10**) demonstrated the effect of metal pollution on microbial community structure, leading to the un-contaminated soils were discrete from those contaminated soils. The variation of microbial PLFA profile at the end of incubation compared to the beginning indicated metal pollution had affected certain microbial PLFAs. Also, soils with different metal treatments had differentiation in terms of microbial community profile. There was significant microbial population difference between contaminated and uncontaminated soils, and soils spiked with different metal concentration and type. In this research, the higher metal concentration had more suppressive influence on microbial PLFAs, leading to smaller microbial population. Similar results were observed by Khan et al. (2010). They also found metal inhibited microbial activity and altered population shifts, particularly in higher metal concentrations. They also noticed that the co-pollution of Cd and Pb had greater negative influences on microbial enzyme activities. This current research showed the combination of Cd and Pb contamination had more influences on microbial profile, yet it might due to the co-contaminated soils had larger metal concentration compared to the single metal polluted soils. Caliz et al. (2012) suggested metal toxicity of Cd to soil microbiota is slightly larger than Pb. Other metals, such as Cu, may have even toxic influences on fungi population as it is used as fungicide (Fernández-Calviño et al., 2010).

5.6 Conclusions and recommendations

- In the current research, soil properties, including pH and EC varied during the 49 days incubation. Also, the bioavailable and potential available metal concentration changed. These variations are influenced by a combination of bio-chemical interactions.

- There is general acceptance that heavy metal(loid)s were biotoxic to soil microorganisms. Due to metal contamination, the current study demonstrated both microbial activity and biomass reduced with a set of variation in soil parameters such as pH, EC, organic C, nitrogen and heavy metal concentration including bioavailability.
- In accordance with our hypothesis, microbial C use preference was suppressed due to higher energy requirement under metal stress.
- The determination of microbial community composition by biomarkers such as microbial PLFA could address the different responses of individual species targeting certain metal stress.
- Therefore, with the interpretation of PLFAs as microbial biomarkers, it may be postulated that microbial CUE provide direct and precious information on soil C biochemical dynamic under metal pollution.
- Microbial community composition varied with different metal concentration and type. Heavy metal toxicity had greater negative influence on fungi population than other microbial species, leading to reduced F/B ratio because of metal toxicity. As described previously, PLFA profile showed discrepancy among treatments. Metal biotoxicity to microbial C use preference and microbiota abundance are more affected by metal concentration, while microbial PLFA biomarkers and community population were influenced both by metal concentration and metal type.
- The metal toxicity induced microbial community shifts in relation to C dynamics need further investigation, especially the C use mechanisms for specific microbial species. Future research and reports on metal contamination could investigate the different microbial responses due to incubation length, especially include the possibility of microbial adaption due to community shifts.
- As conclusion, the potential impact on soil C dynamics by altered soil microbiota would be a critical and important issue as part of environmental assessment within metal polluted area.

Chapter 6

EFFECT OF BIOCHAR ON HEAVY METAL TOXICITY AND MICROBIAL CARBON USE EFFICIENCY IN SOIL*

6.1 Introduction

Biochar is increasingly being acknowledged as a potentially effective material to sequester terrestrial carbon (C), while at the same time improving microbial habitat in soil (Quilliam et al., 2013; Lehmann et al., 2011). Biochar can play an important role in the biogeochemical cycling of C and other elements in soils (Bolan et al., 2012; Kuzyakov et al., 2009). In addition to improving soil fertility and water holding capacity (Paetsch et al., 2017), applications of biochar have attracted a rising attention due to the possibility of heavy metal remediation in contaminated soils (Rees et al., 2014). Dominance of oxygen-containing functional groups in the highly porous structure of biochar makes the material suitable for the adsorption of a range of contaminants including heavy metals (Bolan et al., 2014; Mandal et al., 2017).

Heavy metal(loid)s are among the most toxic and widespread contaminants in our environment because of their persistent nature and high bioaccumulation potential. Some metal elements (e.g., Fe, Zn, Cu, Mn) are involved in many biochemical reactions, but metals like Cd, Pb and Ag have no biological role. They are rather potentially toxic to micro- and macro- organisms (Bruins et al., 2000). The key mechanism of metal toxicity to microorganisms evolves due to the displacement or substitution of essential elements by toxic elements either in the extracellular enzymes or even in nuclear proteins, which consequently may lead to enzyme synthesis inhibition and metabolic process dysfunction (Baumann et al., 2013; Tchounwou et al., 2012). Additionally, when present at a high concentration, even the essential metal elements may lead to adverse consequences (e.g., damage to cell membranes and DNA structure and oxidative stress) (Kachur et al., 1998; Tchounwou et al., 2012). Therefore, toxic levels of heavy metal(loid)s may give rise to the deterioration of soil

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microbial populations and their metabolic activities through denaturing the protein structure and impairing cell membrane functions (Jiang et al., 2010).

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

Biochar is reported to recover microbial activities in metal contaminated soils (Yang et al., 2016). Such improvement in microbial activities could be attributed to: (i) enhancement of soil physiochemical properties (increase of soil aeration, moisture content and pH), (ii) immediate supplement of soil C pools, especially the recalcitrant pool, (iii) supply of nutrients, and (iv) modification of microbial habitat and ecological niche (Jones et al., 2011). Soil microbiota and their C utilization preferences could be significantly altered by biochar amendments (Farrell et al., 2013; 2015). However, due to the complexity of soil and ecosystem diversity, there is a lack of understanding about biochar modulated microbial responses in metal polluted environments (Pan et al., 2016). Microbial carbon use efficiency (CUE) is defined as the conversion of organic C assimilated into the microbial biomass in the net C sequestration process (Rousk and Bååth, 2011). Different approaches of microbial CUE measurement and interpretation of results may involve some discrepant assumptions (Frey et al., 2001), but it can be used as a reference for the microbial C utility preference in soils (Blagodatskaya et al., 2014; Sinsabaugh et al., 2013). Some microbial species, especially fungi often positively respond to biochar addition (O'Neill et al., 2009; Warnock et al., 2007). However, metabolic features of the assimilated C in fungi and bacteria are different, which can potentially distinguish between the preferences of SOC decomposition patterns, and also the specific functional roles of respective microorganisms. The phospholipid fatty acid (PLFA) profiles of microorganisms can be a useful chemotaxonomic biomarker to interpret the microbial community composition and C utilization differences in response to biochar addition to soils under metal stress (Birk et al., 2009).

To our knowledge, limited information is available in the literature on how soil microbial parameters, especially microbial population react to metal pollution in the presence of biochar (Ahmad et al., 2016). The current study not only quantified microbial C use patterns (as

measured by the percentage of microbial biomass formation over substrate C uptake), but also coupled those information with microbial community compositions.

6.2 Objectives

The overall objective was to evaluate biochar remediation ability in relation with microbial CUE under metal contaminated soils. Specific objectives include

- To examine the parameter variation of metal contaminated soil with biochar application.
- To explore and explain the relation between microbial CUE and community profile, with metal and biochar present.
- To demonstrate the remediation ability of biochar in metal-polluted soils targeting enhancement of microbial parameters.
- To determine the influence of biochar on microbial community composition change in heavy metal contaminated soils.

6.3 Hypothesis

- Heavy metal pollution has negative impact on microbial community and inhibitory on microbial activity and biomass formation.
- Biochar application reduces metal bioavailability, improves both soil function and microbial properties.
- In metal contaminated soils, biochar provides C and minerals, reduces metal toxicity, which are beneficial to soil microbial community, and microbial CUE increase.
- Microbial community composition varied with biochar under different metal contaminations, which are essential in terms of microbial C use patters.

6.4 Materials and methods

6.4.1 Soil sampling and preparation

A natural surface soil (0–10 cm) sample was collected from the Barossa Valley region, South Australia (138°57’37’’E, 34°27’48’’S). The region is characterised by Mediterranean climate, with an average summer temperature range of 26–29 °C (daytime) and 12–14 °C (night time), and winter range of 12–16 °C (daytime) and 3–6 °C (night time). The Barossa region receives an average annual rainfall of 437 mm and the soil pattern is extremely variable with the chief

soils are Sodosol (Australia soil taxonomy). The soil was classified as silty loam (USDA textural classification).

After sampling, the soil was homogenised and sieved (< 2 mm). Fine roots and other plant debris were carefully removed during the processing steps. Prior to the experiment, the soil moisture content was adjusted to 50% (weight basis) of the water holding capacity (WHC), and pre-incubated at 25 °C, 28% relative humidity for 7 days in order to recover the microbial activity. The biochar sample used in this study was prepared from macadamia nutshell by pyrolysing the feedstock slowly at 465 °C under O_2-limited environment, as described by Khan et al. (2014).

6.4.2 Soil and biochar characterization

Soil and biochar pH values in 1:5 (*w/v*) suspensions of deionised water following equilibration on an end-over-end shaker for 2 h were determined by a pH/conductivity meter (smartCHEM-LAB Laboratory Analyzer, VWR International Pty Ltd., Australia). Soil texture was determined by the micro-pipette method (Miller and Miller, 1987). The CEC of the soil was determined by first saturating the exchange sites (positive charges) with NH_4^+, then extracting and analysing the exchanged NH_4^+ on a Continuous Flow Analyzer (San ++, Skalar Analytical B.V., The Netherlands). For the total elemental analysis, soil and biochar samples were mixed with 5 mL of aqua-regia (HNO_3:HCl at 1:3 *v/v*), and digested in a micro-wave digestion oven (MARSXpress, CEM Corporation, USA). The digested samples were decanted and filtered before analysing elements on an Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Agilent 7900, Agilent Technologies Ltd., USA). The total nitrogen (TN) and total carbon (TC) in soil and biochar samples were determined by dry combustion technique using a Leco C/N Analyzer (Leco TruMac® CNS/NS Analyzer, LECO Corporation). Soil samples (0.2 g) were weighed and combusted at 1300 °C with an O_2 flow for 5 sec. The instrument was calibrated at every 10 samples by analysing standard weights of Leco EDTA reference material (containing 95.7 g N kg^{-1} and 410 g C kg^{-1}). Soil and biochar physico-chemical characteristics are presented in **Table 6.1**.

Table 6. 1 Selected properties of sample soil and macadamia nutshell biochar

Soil property	pH	EC(mS cm^{-1})	CEC (cmol (+) kg^{-1})	C (%)	N (%)	C:N	Clay (Wt. %)	Silt (Wt. %)	Sand (Wt. %)			
	6.26	27.53	32.71	2.29	0.14	16.36	25.73	41.56	20.17			
	Ca (g kg^{-1})	Cu (mg kg^{-1})	Fe (g kg^{-1})	K (g kg^{-1})	Mg (g kg^{-1})	Mn (mg kg^{-1})	P (g kg^{-1})	Zn (mg kg^{-1})	Na (g kg^{-1})	S (g kg^{-1})		
	1.39	8.90	1.42	0.59	0.46	28.50	0.13	8.19	0.23	0.37		
Biochar	pH	EC (mS cm^{-1})	Pyrolysis temperature (^{0}C)	C (%)	N (%)	P (%)	K (%)	S (%)	C:N	DOC (g Kg^{-1})	Specific surface area (m^2 g^{-1})	Pore volume (ml g^{-1})
	10.29	0.17	465	74.72	0.66	0.09	1.02	0.05	113.21	0.55	202.49	0.0085

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

6.4.3 Soil spiking, biochar amendment and incubation experiment

In the present study, the experimental soil was spiked with 50 and 5000 mg kg^{-1} of Cd^{2+} and Pb^{2+}, respectively. These concentrations were chosen to reflect a contamination level above the sensitivity threshold of the respective metals in order to detect responses of microbial C use patterns under metal stresses in the soil (Sobolev and Begonia 2008; Smolders et al., 2009).Two concentrations of $Cd(NO_3)$ and $Pb(NO_3)$ were mixed with the soil separately, and also in combination (**Table 6.1**). Briefly, metal solutions were sprinkled evenly on the soil spread on a polyethylene sheet. To achieve homogenization, soils were then stirred and mixed thoroughly on an end-over-end shaker. Soils were then air-dried, and passed through a 2 mm-sieve again. The final concentrations of metals in the spiked soils and abbreviations for each treatment are listed in **Table 6.2**.

Table 6. 2 Soil spiking rate and final metal concentrations. Mean ± SE, n = 3

Sample	Cd (mg kg^{-1} soil)	Pb (mg kg^{-1} soil)	Biochar (%)	Glucose (%)	Cd concertation (mg kg^{-1} soil)	Pb concentration (mg kg^{-1} soil)	Cd recovery rate (%)	Pb recovery rate (%)
Control	–	–		–	–	–	–	–
CB	50	–	5		41.65	–	83.3 ± 7.5	
CG	50	–		16	41.65	–	83.3 ± 7.5	–
PB	–	5000	5		–	4605	–	92.1 ± 1.8
PG	–	5000		16	–	4605		92.1 ± 1.8
CPB	50	5000	5	–	43.85	4687.5	87.7 ± 4.6	93.75 ± 2.5
CPG	50	5000	–	16	43.85	4687.5	87.7 ± 4.6	93.75 ± 2.5
B	–	–	5	–	–	–	–	–

Control as uncontaminated soil; CB: Cd-contaminated soil applied with biochar; CG: Cd-contaminated soil applied with glucose; PB: Pd-contaminated soil applied with biochar; Pd-contaminated soil applied with glucose; CPB: Cd- and Pb-contaminated soil applied with biochar; CPG: Cd- and Pb-contaminated soil applied with glucose; uncontaminated soil applied with biochar

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

6.4.4 Bioavailability of heavy metals

Bioavailable heavy metal concentrations were measured by extracting the biochar/glucose-amended and unamended soils with 0.01 M $CaCl_2$ solution (1:10 *w/v*) for 60 min reaction time (Sparks et al., 1996). The extracts were filtered through 0.45 µm syringe filter before analysing the metal elements on an ICP-MS instrument (ICP-MS, Agilent 7900, Agilent Technologies Ltd., USA).

6.4.5 Microbial properties

6.4.5.1 Microbial activity

The microbial activity of soils was monitored by measuring the rate of CO_2 evolution from the samples. Sealed soil microcosms (10 g) in Schott bottles having different treatments as stated above were incubated for 49 days in dark at 25 °C and 60% WHC. Three blank Schott bottles without any soil were set as controls. A 20 mL open-top vial containing 10 mL of 0.05 M NaOH solution was used to trap the evolved CO_2 within the sealed Schott bottles. Periodically, the alkali was decanted into an Erlenmeyer flask and rinsed with deionised water three times. The small vial was replaced with 10 mL of fresh alkali every time. The collected alkaline aliquot was then titrated against 0.03 M HCl in the presence of phenolphthalein indicator following the addition of 5 mL of 0.5 M $BaCl_2$. The amount of evolved CO_2 was thus measured, and the microbial respiration was calculated using (**Eq. 6.1**):

$$MR = \{MWCO_2(V_b - V_s) \times M \times 1000\}/(DW \times T \times 2) \quad \text{Eq. 6.1}$$

where, *MR* is the microbial respiration (mg CO_2−C kg^{-1} soil h^{-1}), $MWCO_2$ is the molecular weight of CO_2, V_b is the volume of HCl for the blank titration, V_s is the volume of HCl for the sample titration, *M* is the concentration of HCl, *DW* is the dry weight of the soil, *T* is the time

of incubation, and *2* is the factor that accounts for the fact that two OH^- are consumed by one CO_2.

6.4.5.2 Microbial biomass carbon

The measurement of microbial biomass carbon (MBC) was determined by the fumigation-extraction method (Vance et al., 1987). Soils (10 g, dry weight basis) were placed in 50 mL beakers within a vacuum desiccator containing 50 mL of ethanol-free chloroform. The desiccator was tightly sealed and pumped until chloroform was vaporised. Soils were thus incubated in chloroform vapour for 48 h within the desiccator. Another set of un-fumigated soils were maintained simultaneously. After 48 h, both the fumigated and un-fumigated soils were mixed with 40 mL of 0.5 M K_2SO_4, and shaken on an end-over-end shaker for 1 h. Samples were then centrifuged and filtered through Whatman #40 filter papers. The content of C in the filtrates was analysed by a total organic carbon analyzer (TOC-LCSH, Shimadzu Corporation, Japan). MBC was calculated using **Eq. 6.2**:

$$MBC = E_c/K_c \qquad \text{Eq. 6.2}$$

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

The microbial CUE was estimated following **Eq. 6.3**.

$$M_C = \Delta MBC/(\Delta MBC + \Sigma CO_2{-}C) \qquad \text{Eq. 6.3}$$

where, M_C is microbial CUE measured as microbial biomass variation, ΔMBC is the change of MBC, $\Sigma CO_2{-}C$ is cumulative CO_2−C as microbial respiration.

6.4.5.3 Microbial community composition

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

In brief, soil samples were first freeze-dried at −45 °C and at less than 1 millibar (0.8 mbar) pressure. Then, freeze dried soils (5 g) were extracted with one-phase extraction solvent. The one-phase solvent was a mixture of chloroform, methanol and citrate buffer (1:2:0.8, *v/v/v*), while the citrate buffer was made of citric acid and sodium citrate (3:1, *v/v*) with pH adjusted

at 3.6 (Bligh and Dyer, 1959). After shaking on an end-over-end shaker, the mixture was centrifuged twice at 4500 rpm for 30 min. The supernatant was decanted into a non-transparent vial, and vortexed before standing overnight. The upper layer of the standing liquid was removed, and the remaining bottom portion was dried under N_2 flow at 32 °C. Following drying, the thin solid phase left at the bottom of the vial was re-dissolved in chloroform (1 mL), and transferred into SPE column for extraction. To set up the column, 0.5 g of silica was packed, followed by conditioning with chloroform thrice (3 mL). Then, the sample transfer in the SPE column included three steps: chloroform (5 mL), acetone (5 mL) and methanol (5 mL). The final leaching solution was dried with continuous N_2 flow at 32 °C. To the pellet obtained, 0.5 mL of 1:1 (*v/v*) of methanol:toluene and 0.5 mL of 0.2 M methanolic KOH (by dissolving 0.28 g KOH in 25 mL of methanol) were added. The mixture was incubated at 37 °C for 30 min, and then cooled to room temperature. The PLFAs were thus converted into fatty acids methyl esters (FAMEs) with mild alkaline methanolysis. Following incubation, 1 mL of deionised water, 0.15 mL of 1 M acetic acid, and 1 mL of hexane were added to the mixture, vortexed for 30 sec, and centrifuged at 4500 rpm for 30 min to separate the solution into two layers. The upper layer was carefully transferred into a Gas Chromatography (GC) vial with a pipette. This separation procedure was repeated twice with the addition of fresh extractants. Finally, the extract was concentrated by continuous N_2 flow, and stored at −20 °C in total darkness before further analysis.

An internal standard (methyl nonadecanoate, C19:0) (10 ng) was added to all samples as a quality control measure. The FAMEs were analysed by GC-MS (Model 7890B/5977B, Agilent Technologies Ltd., USA; AxION iQT with Cold EI Source, Perkin Elmer, USA). A RTX-5MS fused silica capillary column (60 m, 250 µm × 0.25 µm film thickness) (Supelco, Sigma-Aldrich, Australia) was used. Sample (1 µL) was injected in splitless mode with an injector temperature of 250 °C, and helium carrier gas at a constant flow rate of 1.4 mL min^{-1}. The temperature program was set as follows: column temperature initially at 60 °C for 1 min, then increased to 180 °C at a rate of 12 °C min^{-1}, then increased to 300 °C at a rate of 4 °C min^{-1} and kept at 300 °C for 4 min. Electron energy in the detector was set 70 eV. Data was acquired in scan mode from 50 to 400Da at 3 scans per second. Quantification was conducted against a Supelco 37 standard mixture (Supelco, Bellefonte, PA), and the C19:0 internal standard with a 6 point linearity curve analysed in triplicate ($r^2 \geq 0.98$ for each component). Each PLFA peak was identified by comparing the respective retention time and by their mass spectra. The isomers not included in the standard mix were quantified against the relative response factor for C16:0, and were individually identified by their mass spectra from a Cold EI TOF scanning analysis conducted on a Perkin Elmer AxION iQT instrument. The specific microbial species were identified by the signature PLFAs listed in **Table 6.3**.

Table 6. 3 Phospholipid fatty acid (PLFA) biomarkers used to characterise microbial communities in the experimental soils

Microbial group	Biomarker PLFAs
Gram-negative bacteria	C16:1ω7c
Gram-positive bacteria	iC15:0, aC15:0, C15:0, iC16:0, iC17:0, aC17:0, C17:0
Actinobacteria	10MeC16:0, 10MeC17:0, 10MeC18:0
Fungi	C18:2ω6c, C18:1ω9c

6.4.6 Statistical analysis

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

6.5 Results and discussion

6.5.1 Influence of biochar on heavy metal availability

6.5.1.1 Influence of biochar-induced pH increase on heavy metal availability

The soil used in this study was slightly acidic in reaction (pH = 6.26). The pH of the macadamia nutshell biochar was 10.29 (**Table 6.1**). The pH of the biochar-amended and unamended soils was analysed 7 and 49 days after incubation. The pH value was found to be increased significantly ($p < 0.01$) throughout the incubation period as a result of biochar addition (**Figure 6.1**). For example, the pH increased by 0.3 and 0.1 units after 7 days of incubation in soils spiked with Cd and Pb, respectively, while it increased by 0.3 units in soils spiked with both metals. The soil pH did not show any significant drop at the end of 49 days of experimental period. Acidic soils lead to a higher metal biotoxicity risk and subsequent C depletion than alkaline soils (Bolan et al., 2014; Dai et al., 2017; Sheng et al., 2016). Soil pH is also critical

in determining the various forms of Cd and their toxicities to certain microorganisms (Bolan et al., 2014). The naturally-released Pb in mining deposits are less mobile, but they may become more soluble and mobile if soils are moderately acidic (John and Leventhal, 1995). The increase in pH values in this study, although small but potentially significant, was brought about by biochar addition, and we speculate this may reduce the mobility and availability of metals to soil microorganisms (Rees et al., 2014). Liang et al. (2014) also noticed a rise in soil pH value due to biochar addition, and they suggested that the pH variation could cause a shift in the soil microbial population, such as bacteria and fungi. The alkaline feature of formed metal oxides, hydroxides and carbonates admixed with the biochar during the pyrolysis process might have increased the soil pH (Novak et al., 2009).

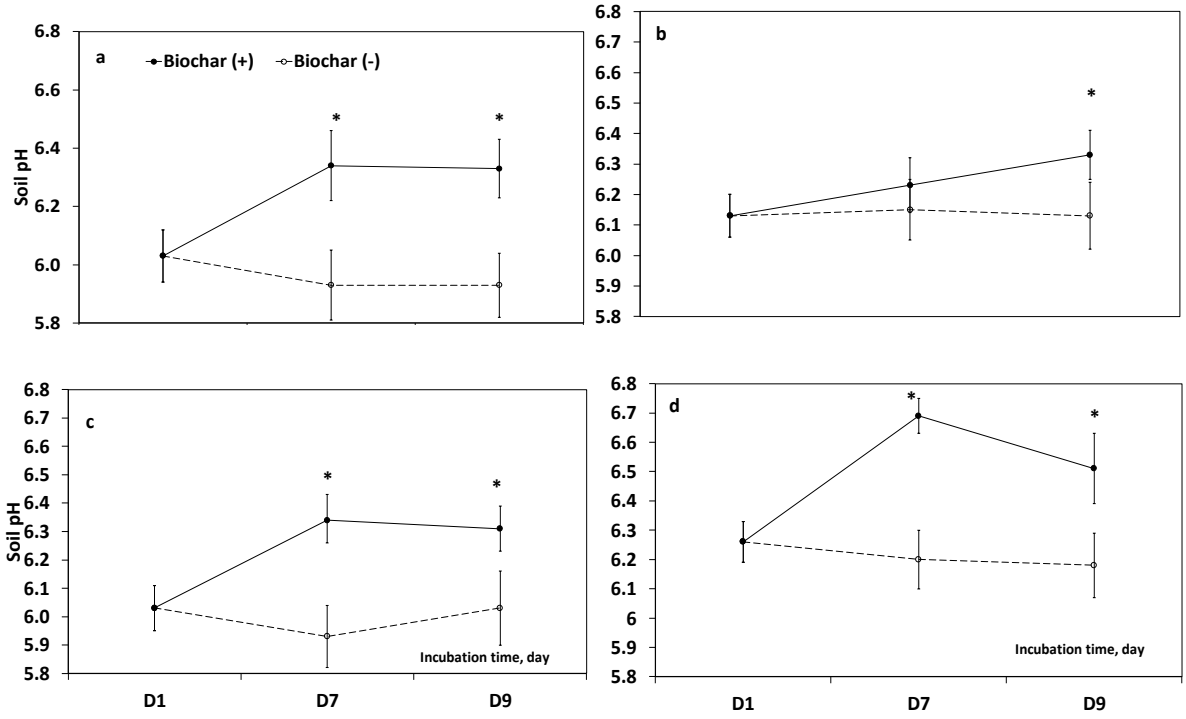


Figure 6. 1 Soil pH responses to biochar and glucose amendments in different types of metal contaminated soils; (**a**) soil with Cd, (**b**) soil with Pb, (**c**) soil with combined Cd and Pb, (**d**) soil without any heavy metal. Black dots indicates treatments with biochar application, transparent dot indicates treatments without biochar. Values show means ± SE. * indicates significant difference between glucose and biochar amendments ($p < 0.01$).

6.5.1.2. Heavy metal immobilization by biochar

Bioavailability is critical in the determination of accessibility and toxicity of metals to soil microorganisms (Wang et al., 2007a). In this study, both Cd and Pb bioavailabilities were significantly ($p < 0.01$) reduced due to the biochar amendment, but not in the glucose-amended soils (**Figure 6.2a and b)**. Metal remediation ability of biochar based on the elevated

soil pH theory may be envisaged through the following two mechanisms: (i) elevated pH may contribute to metal (co-)precipitation with carbonates, and (ii) it may increase the net negative charges that favour the formation of metal-organic complexes. In the current study, the elevation of soil pH due to biochar addition was up to 0.3 units, which might have imparted only a small effect on metal immobilization. The SEM images and elemental analysis clearly showed that heavy metal ions clustered or spread on the surface and pores of the biochar (**Figure 6.3a, b and c**). Additionally, a highly porous structure of the biochar sample was observed as a result of the pyrolysis process. The porous structure of biochar could reduce the metal mobility and bioavailability (Puga et al., 2015). However, Han et al. (2013) pointed out that the metal adsorption was not solely ascribed to biochar pore structure. The adsorption of metal ions by biochar through its surface hydroxyl, carboxyl, and phenolic functional groups (−OH, −COOH or C−OH) (**Figure 6.4**) might have imparted a more prominent effect. The FTIR spectra show a variety of oxygen-containing functional groups which were negatively charged. Strong bands at 1400 cm^{-1} and 875 cm^{-1} presented C=O and aromatic C=C groups, respectively (Abdel-Fattah et al., 2015; Wang and Griffiths, 1985). Although the functional groups composition could be affected by the parent feedstock and pyrolysis temperature during biochar production (Hossain et al., 2011), the spectral features correlated well with the elemental analysis of the material (**Figure 6.3c**), showing a relatively high C content originating from the organic parent material (macadamia nutshell) (Chia et al., 2012).

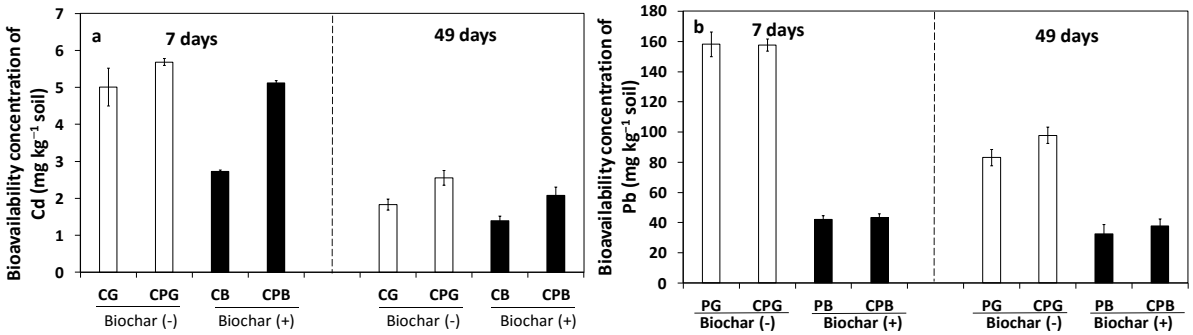
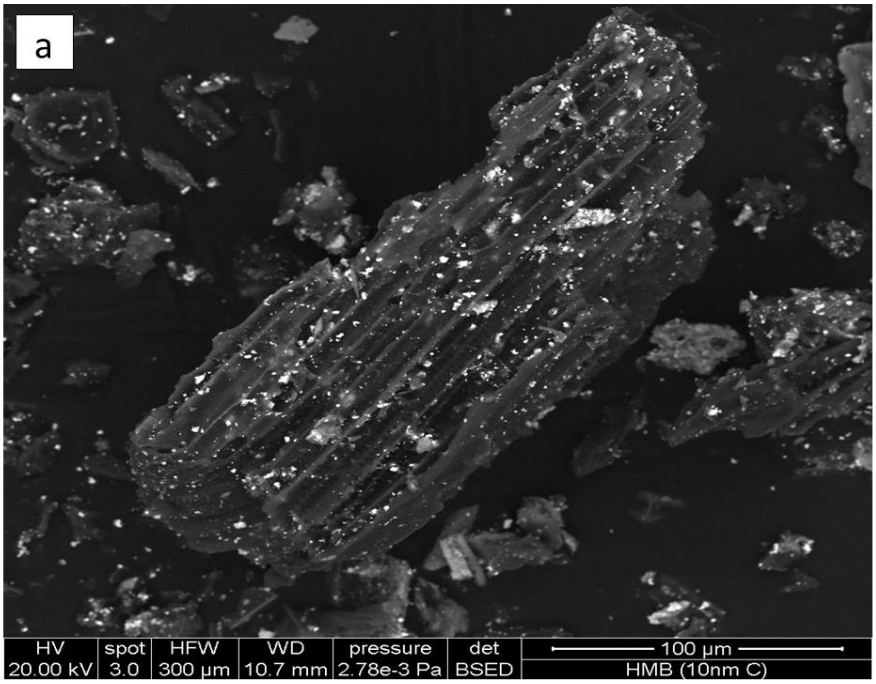


Figure 6. 2 Bioavailable Cd (**a**) and Pb (**b**) concentrations in different treatments. Data are displayed as means, bars indicate SE (n = 3). CB: soil applied with Cd + biochar; CG: soil applied with Cd + glucose; PB: soil applied with Pb + biochar; PG: soil applied with Pb + glucose; CPB: soil applied with Cd + Pb + biochar; CPG: soil applied with Cd + Pb + glucose.

The aging effect of metal immobilization was also observed in the present study. In comparison to the initial 7 days of incubation, both bioavailable Cd and Pb concentrations were decreased at the end of incubation (49 days) (**Figure 6.2a and b**). At that stage, bioavailabilities of Cd were 1.83 and 2.55 mg kg^{-1} dry soil in glucose-amended Cd-spiked soil

(CG) and glucose-amended Cd-Pb-spiked soil (CPG), respectively, while these values were significantly ($p < 0.01$) lower in respective biochar-amended soils (1.40 and 2.08 mg kg^{-1} dry soil in biochar-amended Cd-spiked soil (CB) and biochar-amended Cd-Pb-spiked soil (CPB), respectively). The bioavailable concentrations of Pb were 83.05 and 97.86 mg kg^{-1} dry soil in glucose-amended Pb-spiked (PG) and glucose-amended Cd-Pb-spiked soil (CPG), respectively, while these values were reduced to 32.46 and 37.78 mg kg^{-1} dry soil in the respective biochar-amended soils (PB and CPB). The bioavailable metal concentrations were decreased remarkably, indicating that biochar application reduced the metal mobility. Investigations have shown that the specific morphology and chemical features may support the metal sorption potential of biochar (Igalavithana et al., 2017). In addition, a discrepancy was observed in Cd and Pb bioavailability decline patterns due to biochar application. Bioavailability of Cd was decreased by 49% and 59% in CB and CPB, respectively (**Figure 6.2a**), while that of Pb was decreased by 23% and 13% in PB and CPB, respectively (**Figure 6.2b**). The decrease of Cd bioavailability was larger than Pb, which interestingly was consistent with the slightly greater pH rise in Cd-spiked soil than the Pb-spiked soil. The rapid adsorption of metals to biochar functional groups during the incubation period might have attributed to their decreased mobilities in biochar-amended soils (Houben et al., 2013). The incubation duration (ageing) was essential for the formation of effective adsorption bonds between biochar surfaces and metal ions. In addition, soil type and clay content could often play an important role in metal immobilization by biochar. For example, Shen et al. (2016) suggested that the biochar-amended clayey soils was not satisfactory for adsorption of Pb. Therefore, the effect might become more prominent in a light-textured soil as used in the present study.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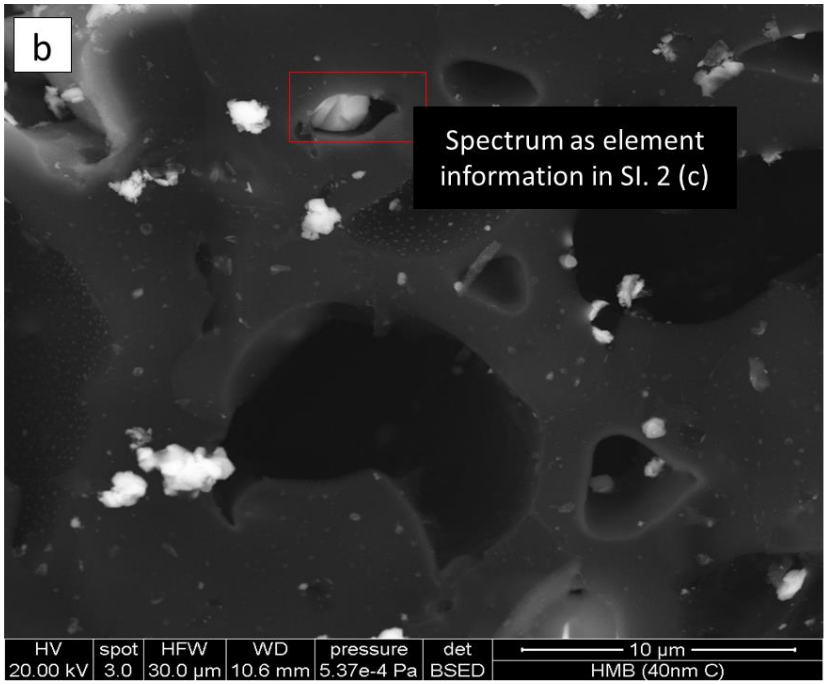
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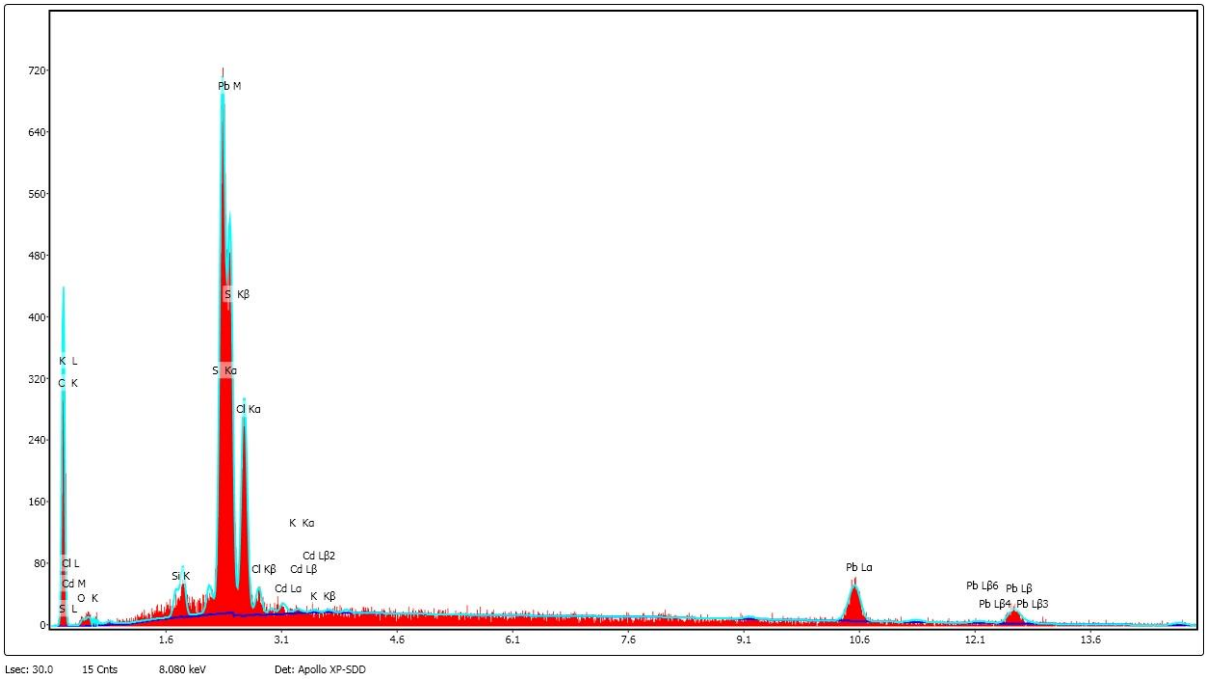

Figure 6. 3 Morphological and surface chemical characteristics of macadamia nutshell biochar, including scanning electron micrographs (SEM) (**a, b**) and energy dispersive spectrum (**c**).

The specific surface area of the biochar sample was 202.49 m^2 g^{-1}. This feature of biochar along with its highly porous structure (**Figure 6.3a and b**) supported the existence of large quantity of organic functional groups on the surface (**Figure 6.3c and Figure 6.4**), and consequently their electrostatic as well as specific interactions with metal cations (**Figure 6.4**). The metal adsorption ability can vary depending upon the properties of biochar as affected by the pyrolysis conditions and feedstock sources (Park et al., 2011; Uchimiya et al., 2011). Results of the current study also showed that the bioavailability of metals were slightly higher (p > 0.05) when Cd and Pb coexisted in the system than the single metal-spiked soil. The bioavailability of Cd was 1.40 mg kg^{-1} dry soil in CB against 2.08 mg kg^{-1} dry soil in CPB (0.68 mg kg^{-1} difference), while the bioavailability of Pb was 32.46 mg kg^{-1} dry soil in PB and 37.78 mg kg^{-1} dry soil in CPB (5.32 mg kg^{-1} difference). This might be due to the competition among metal cations for the adsorption sites on biochar surfaces. Moreover, Rees et al. (2014) demonstrated that the metal adsorption to organic materials may be partially irreversible with multiple and element-dependent mechanisms, which could imply that biochar might play a more prominent role in a long-term soil remediation approach.

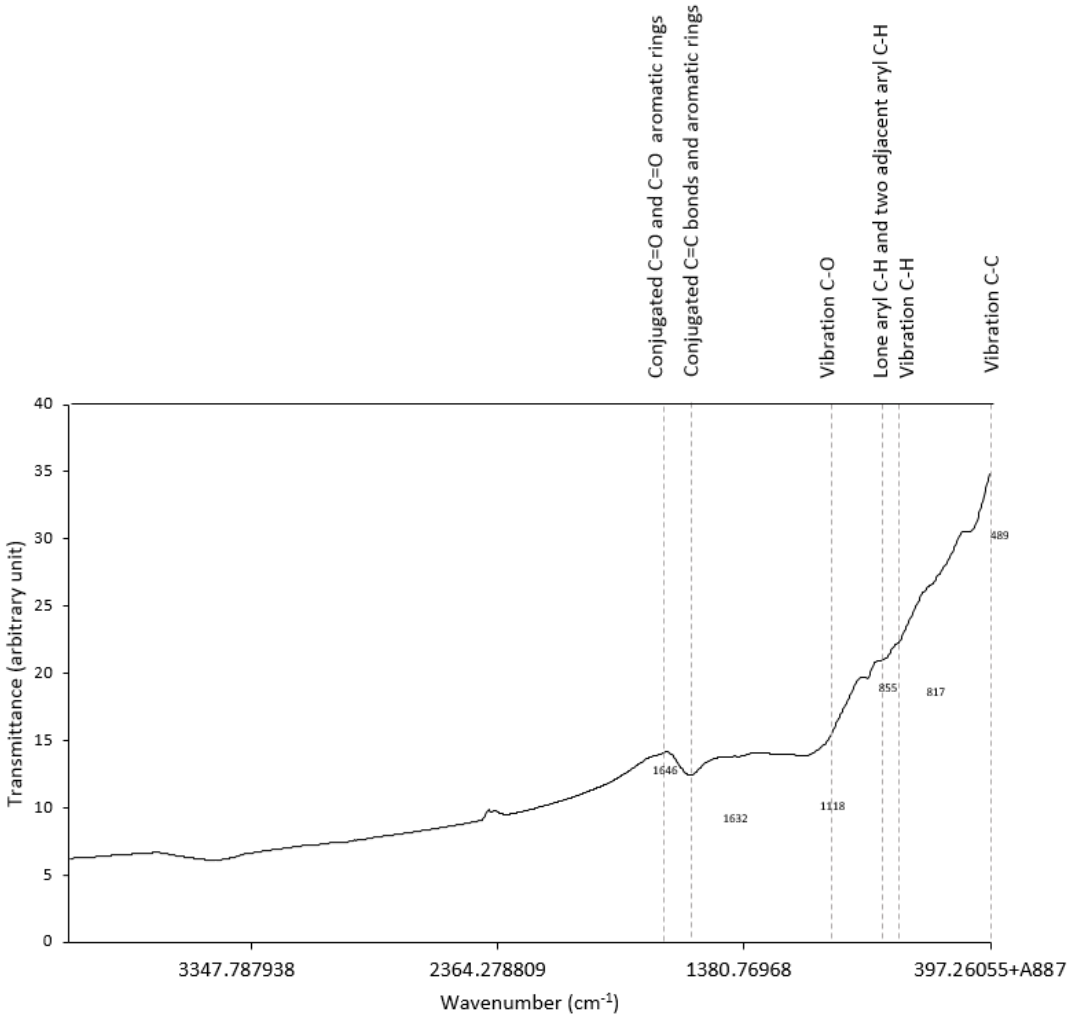

Figure 6. 4 Fourier transformed infrared (FTIR) spectrum showed the functional group of the macadamia nutshell biochar used in this research.

6.5.2. Influence of biochar on soil microbiota under metal stress

6.5.2.1. Microbial activity

In all cases, the microbial activity was gradually decreased after the peak value on day 1 (**Figure 6.5a**). Despite the patterns of respiration were similar irrespective of the treatments, the cumulative respiration dropped significantly ($p < 0.01$) in metal spiked soils in comparison to un-spiked soils (**Figure 6.5b**). This demonstrated that the metal toxicity caused a reduction of the soil microbial activity. Compared to the control soil, microbial respiration rate was significantly ($p < 0.01$) stimulated immediately after biochar addition (**Figure 6.5a**). The respiration rates in uncontaminated soils with biochar amendment were 1.78 µg CO_2−C g^{-1} dry soil h^{-1} on day 1, and 1.11 µg CO_2−C g^{-1} dry soil h^{-1} on day 3. These values were greater

than the control soil (uncontaminated and without biochar) on the respective days (1.27 µg CO_2−C g^{-1} dry soil h^{-1} on day 1, and 0.65 µg CO_2−C g^{-1} dry soil h^{-1} on day 3). Afterwards, the respiration rate decreased likely because of the depletion of labile organic C supply. The respiration rate in the control soil was slightly higher than biochar-amended soils on day 25 (p > 0.05), and this trend continued until the end of incubation. The cumulative microbially respired CO_2−C values at the end of incubation were 351.41 and 379.56 µg CO_2−C g^{-1} dry soil in the control and biochar-amended soils, respectively (**Figure 6.5b**). The difference in cumulative CO_2−C release between them (28.15 µg CO_2−C g^{-1} dry soil) is consistent with most of the previous reports that biochar addition could increase the microbial activity and CO_2−C liberation from soils (Jones et al., 2011). The stimulation of soil microbial activity resulted from biochar in uncontaminated soils could be attributed to the higher organic C content and supplement of base nutrient elements (primarily Ca, Mg, K and Na) (Houben et al., 2013; Novak et al., 2009).

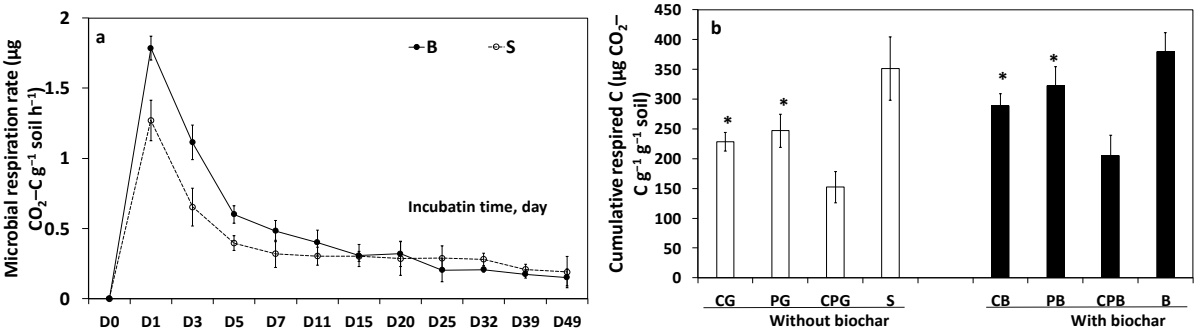

Figure 6. 5 Microbial respiration rate (**a**) and cumulative CO_2–C respired (**b**) in different treatment soils. Data are displayed as means, bars indicate SE (n = 3), * indicates significant difference between glucose and biochar amendments (p < 0.01). S: control soil without any amendment; B: soil applied with biochar; CB: soil applied with Cd + biochar; CG: soil applied with Cd + glucose; PB: soil applied with Pb + biochar; PG: soil applied with Pb + glucose; CPB: soil applied with Cd + Pb + biochar; CPG: soil applied with Cd + Pb + glucose.

The least microbial cumulative respiration was observed in Cd and Pb co-contaminated soils (**Figure 6.5b**). Compared to either Cd or Pb spiked soils, the cumulative microbial respiration was reduced significantly (p < 0.01) in the co-contaminated soils, but the effect did not differ significantly (p > 0.05) between Cd and Pb. Nwuche and Ugoji (2008) also noticed that the combination of Zn and Cu amplified the negative influence on soil microbial activity. As expected, biochar addition was found beneficial to improve the microbial activity. Soil respiration was increased by 26% (from 152.21 to 204.58 µg CO_2−C g^{-1} dry soil) due to biochar application compared to un-spiked control soil. The CO_2−C amount due to biochar addition increased by 21% and 23% in Cd and Pb spiked soils, respectively (18.66 and 33.65 µg CO_2−C g^{-1} dry soil with glucose and biochar amendment, respectively). There were similar patterns of microbial activities in the Cd and Pb singly spiked soils, meaning the different metal

types did not significantly affect microbial respiration rate in this study. The respiration values were slightly higher in Pb-spiked soils than Cd-spiked soils, but not significantly ($p > 0.05$). Some previous reports, however, indicated that the level of biotoxicity of Cd was larger than that of Pb to soil microorganisms at an equal molar concentration because Cd was more bioaccessible than Pb owing to dissimilar solubilities of the respective metal salts (Neethu et al., 2015). Microbially respired CO_2−C values in the contaminated soils were increased by 8 and 10% in glucose and biochar treatments, respectively. It was noteworthy that the biochar-amended contaminated soils respired a higher amount of CO_2−C than the glucose-amended contaminated soils despite the fact that both the treatment groups received an equal amount of C at the beginning of the experiment and C in glucose was more easily mineralisable than that in biochar. This again confirmed that biochar imparted a metal remediation effect on microorganisms in the contaminated soils. This study thus demonstrated that the improvement of microbial activity was not only due to the organic C supplied by biochar, but also due to its metal remediation ability.

6.5.2.2. Microbial biomass carbon

Compared to the control sample, MBC values were significantly ($p < 0.01$) increased in biochar-amended uncontaminated soils (**Figure 6.6**). The values were 243.86 and 421.77 mg C kg^{-1} dry soil in the control and biochar-amended soils, respectively, indicating a 42% increase. The MBC values in the metal-spiked soils were significantly ($p < 0.01$) lower than the control soil due to the possible metal toxicity. A reduced MBC value due to heavy metal toxicity of soils was also observed in numerous previous studies (Abaye et al., 2005; Li et al., 2008).

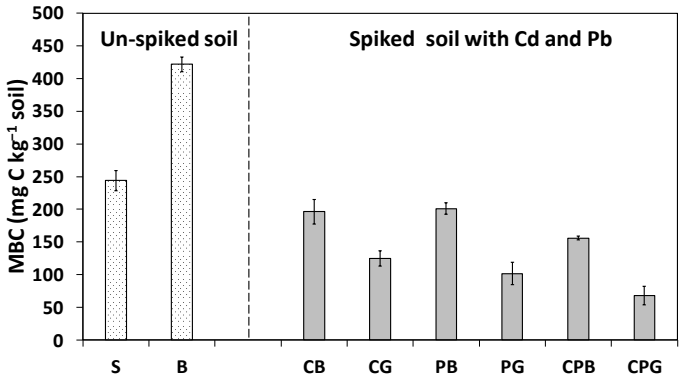

Figure 6. 6 Microbial biomass carbon in different treatment soils. Data are displayed as means, bars indicate SE (n = 3). S: control soil without any amendment; B: soil applied with biochar; CB: soil applied with Cd + biochar; CG: soil applied with Cd + glucose; PB: soil applied with Pb + biochar; PG: soil applied with Pb + glucose; CPB: soil applied with Cd + Pb + biochar; CPG: soil applied with Cd + Pb + glucose.

The MBC values were 124.60, 101.55 and 68.02 mg C kg^{-1} dry soil in glucose amended Cd, Pb and Cd + Pb spiked soils, respectively. These values in biochar-amended contaminated soils were significantly ($p < 0.01$) increased (37, 50 and 56% in Cd, Pb and Cd + Pb spiked soils), demonstrating that the metal toxicity inhibited the microbial respiration as well as MBC formation. However, the MBC value did not show any significant ($p > 0.05$) difference between the metal types. Nwuche and Ugoji (2008) noticed that the combination of Cu and Zn pollution had a lower MBC content than the individual metal. However, the current study did not indicate any significant difference in MBC due to the metal types, or in single or metal co-contaminated situations.

6.5.2.3. Microbial community composition

Total microbial PLFA was decreased due to metal biotoxicity, while it was increased with biochar application (**Table 6.4**). The toxicity of heavy metals had significant ($p < 0.01$) negative influence on the PLFA abundance. Total PLFA contents were decreased by 27%, 21% and 34% in unamended Cd, Pb and Cd + Pb spiked soils, respectively. Similar results were reported earlier (Oliveira and Pampulha, 2006). Total PLFAs increased from 101.75 nmol g^{-1} dry soil in the control soil to 122.22 nmol g^{-1} dry soil with uncontaminated biochar-amended soil. The increased PLFA concentration due to biochar amendments could be attributed to the increased C and nutrient availabilities as well as the alleviation of metal toxicity. The highly porous structure of biochar could also provide a congenial habitat niche for soil microorganisms (Dai et al., 2017; Quilliam et al. 2013).

Table 6. 4 Comparison of Gram-positive bacteria (G+ bacteria), Gram negative bacteria (G− bacteria), fungi and actinomycetes as obtained through respective 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 bacteria and fungi. Mean values followed by the same letter are not significant according to ANOVA ($p > 0.05$)

		Glucose applied*			**Biochar applied**			
	Soil	Soil + Cd	Soil + Pb	Soil + Cd + Pb	Soil	Soil + Cd	Soil + Pb	Soil + Cd + Pb
G+ bacteria	26.84 ± 3.68a	35.05 ± 2.21b	39.72 ± 3.19c	32.49 ± 2.26ab	51.21 ± 1.79d	39.72 ± 2.04c	43.28 ± 1.73c	33.24 ± 2.20b
G− bacteria	20.56 ± 1.90ab	26.44 ± 2.09b	27.73 ± 2.09b	23.77 ± 2.10b	18.33 ± 2.06a	22.41 ± 2.25ab	21.52 ± 2.08ab	22.15 ± 2.28ab
Fungi	36.97 ± 1.03c	8.20 ± 1.07a	8.48 ± 0.90a	6.45 ± 1.10a	45.33 ± 0.97d	26.23 ± 1.00b	27.78 ± 1.06b	24.34 ± 0.98a
Actinomycetes	17.38 ± 0.67d	1.01 ± 0.22a	1.07 ± 0.48a	1.24 ± 0.78a	7.35 ± 0.29c	1.29 ± 0.33a	3.69 ± 0.31b	0.54 ± 0.33a
Microbial species feature								
G+:G− ratio	1.31 ± 0.19a	1.33 ± 0.11a	1.43 ± 0.15a	1.37 ± 0.11a	2.79 ± 0.09d	1.77 ± 0.09b	2.01 ± 0.08c	1.50 ± 0.10a
B:F ratio	1.28 ± 0.54a	7.50 ± 0.40b	7.95 ± 0.59bc	8.72 ± 0.40c	1.53 ± 0.40a	2.37 ± 0.47a	2.33 ± 0.36a	2.28 ± 0.48a
Total PLFA (nmol g^{-1} dry soil)	101.75 ± 17.44bc	70.70 ± 11.00ab	77.01 ± 10.96ab	63.95 ± 10.10a	122.22 ± 17.46c	89.64 ± 13.75a	96.28 ± 11.47b	80.27 ± 12.81ab
Diversity (*H'*, Shannon's)	2.14 ± 0.04c	1.13 ± 0.13b	1.34 ± 0.17b	0.84 ± 0.09a	2.55 ± 0.23d	1.44 ± 0.11b	1.62 ± 0.10bc	0.92 ± 0.17a

Control as uncontaminated soil; CB: Cd-contaminated soil applied with biochar; CG: Cd-contaminated soil applied with glucose; PB: Pd-contaminated soil applied with biochar; Pd-contaminated soil applied with glucose; CPB: Cd- and Pb-contaminated soil applied with biochar; CPG: Cd- and Pb-contaminated soil applied with glucose; uncontaminated soil applied with biochar

The microbial community composition varied among the treatments (**Figure 6.7**). A detailed microbial marker concentration presented as individual PLFA data was presented in **Table 6.5**. The B:F ratio in uncontaminated soil was 1.28 without biochar addition, and 1.53 with biochar addition (**Table 6.4**). Compared to the uncontaminated soil amended with biochar, B:F ratio was significantly higher ($p < 0.01$) in metal-contaminated soils, indicating that heavy metal toxicity could result in the variation of different microbial species. Generally, fungi are more sensitive to environmental stress than bacteria (Lu et al., 2015). A more distinct drop of fungi abundance compared to bacteria was also observed in the current study. The uncontaminated soil applied with biochar had the largest fungi (45.33 nmol g^{-1} dry soil) and Gram-positive (G+) bacteria (51.21 nmol g^{-1} dry soil) abundances. Therefore, fungal species were favoured by organic amendments such as biochar. The high C and nutrient contents of biochar could support the fungal species that usually have a relatively lower C:N ratio in their cell composition. Surprisingly, the largest Gram-negative (G−) bacterial abundance (27.73 nmol g^{-1} dry soil) was shown in Pb-spiked soil without biochar amendment. Certain G− bacterial species show resilience to metal stresses, and even exhibit capability of metal bioremediation in contaminated sites (Kang et al., 2016).

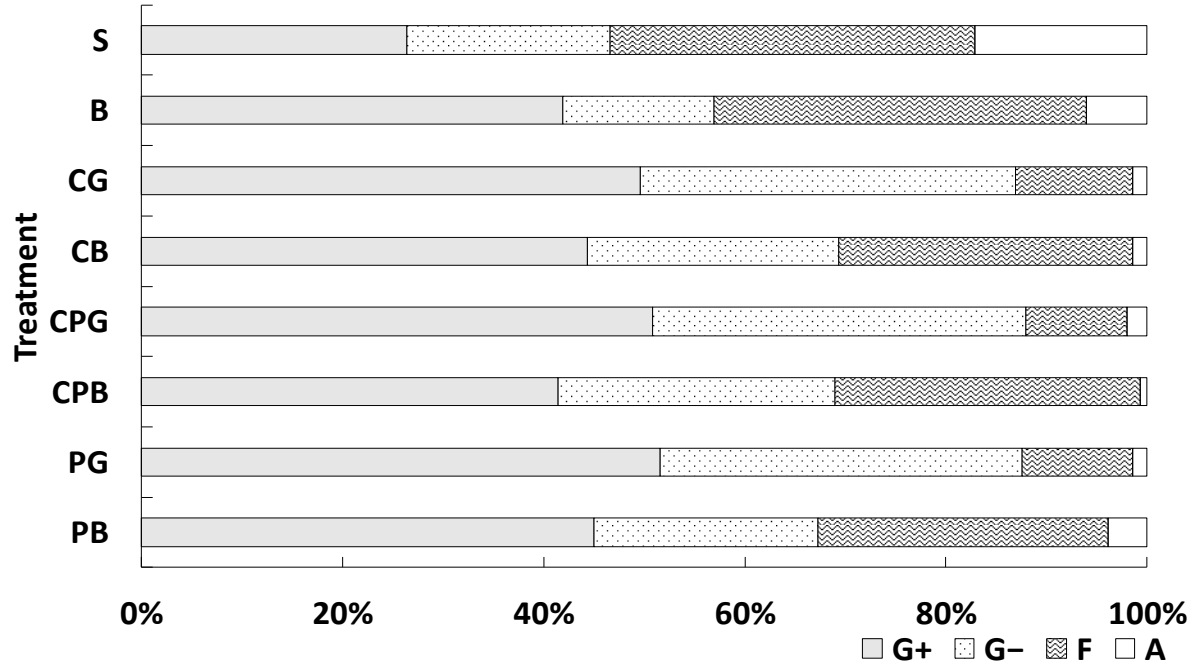

Figure 6. 7 Proportion of fatty acids representing five microbial species (%). G+: Gram-positive bacteria; G-: Gram-negative bacteria; F: fungi; A: actinomycetes; S: control soil without any amendment; B: soil applied with biochar; CB: soil applied with Cd + biochar; CG: soil applied with Cd + glucose; PB: soil applied with Pb + biochar; PG: soil applied with Pb + glucose; CPB: soil applied with Cd + Pb + biochar; CPG: soil applied with Cd + Pb + glucose.

Table 6. 5 Detected microbial PLFA data under different treatments after 49 days of incubation (nmol g^{-1} soil)

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

Control as uncontaminated soil; CB: Cd-contaminated soil applied with biochar; CG: Cd-contaminated soil applied with glucose; PB: Pd-contaminated soil applied with biochar; Pd-contaminated soil applied with glucose; CPB: Cd- and Pb-contaminated soil applied with biochar; CPG: Cd- and Pb-contaminated soil applied with glucose; uncontaminated soil applied with biochar

Sheng et al. (2016) suggested that the G+:G− bacteria is a promising indicator for predicting C sequestration in soils. A higher G+:G− ratio may lead to a positive soil C depletion (Sheng et al., 2016). In the current study, G+ bacteria showed a higher tolerance to metal pollution than G− bacteria and fungi. An increased G+ bacteria population with a decreased fungi population was also earlier reported with increasing metal concentrations (Aoyama and Tanaka, 2013). In addition, the G+:G− ratio was increased by biochar addition, meaning that biochar would favour the G+ bacteria more than the G− bacteria to grow in a heavy metal contaminated soil. The fungal abundance was increased significantly ($p < 0.01$) because of biochar addition, while it was negatively affected by metal toxicity. In spite of the fact that an elevated soil pH should support the bacterial population more than fungi, the C and nutrients supplied by biochar might favour fungi to grow better than bacteria (Liang et al., 2014). Biochar was reported to alter soil microbial community composition, and a fungi dominated soil might lead to a higher resistance and resilience when facing environmental stresses (Paz-Ferreiro

et al., 2015). In this study, however, compared to the bacterial groups, metal toxicity induced much severe inhibition of the fungal populations. Such difference in microbial species was in consistence with earlier reports (Deng et al., 2015; Hinojosa et al., 2005). Biochar in the present study had more prominent positive effect on fungi than bacteria. Chen et al. (2013) also noticed a microbial community composition shift after biochar addition to soils, and fungi communities were benefited more than bacteria.

There was no consistent pattern of metal types that influenced the soil microbiota, but a slightly lower population of G+, G− bacteria, fungi and actinomycetes was noticed in Cd-spiked soils than Pb-spiked soils. There were controversial reports on metal toxicity variations when the metals were present singly or in combination. The competition for adsorption sites by metal cations could modify the respective metal bioavailability to soil microorganisms (Bur et al., 2012).

A change in the microbial PLFA pattern with metal toxicity and biochar-mediated remediation indicated a shift in the microbial community structure. This was shown by the PLFA patterns from different treatments (PCA, **Figure 6.8**). The first axis, which accounted for 80% of the variation in the PLFA data, separated different treatments. In a reasonable agreement with the hypothesis that biochar addition would modulate the soil microbial community under heavy metal stress, the community composition after biochar-mediated remediation showed a certain distance from those without biochar amendment, and the individual components remained close to each other. In addition, uncontaminated soils with biochar addition were separated to the far on the right in the PC analysis, and thus had a different PLFA pattern than the metal-spiked soils. The glucose- and biochar-amended uncontaminated soils were grouped together, indicating that they had similar PLFA patterns. It has been shown recently that the effect of biochar on the soil microbiome is modulated by time and site (Thies et al., 2015). Therefore, further investigations on biochar parameters and monitoring the duration effect are necessary for interpreting the microbial population variation.

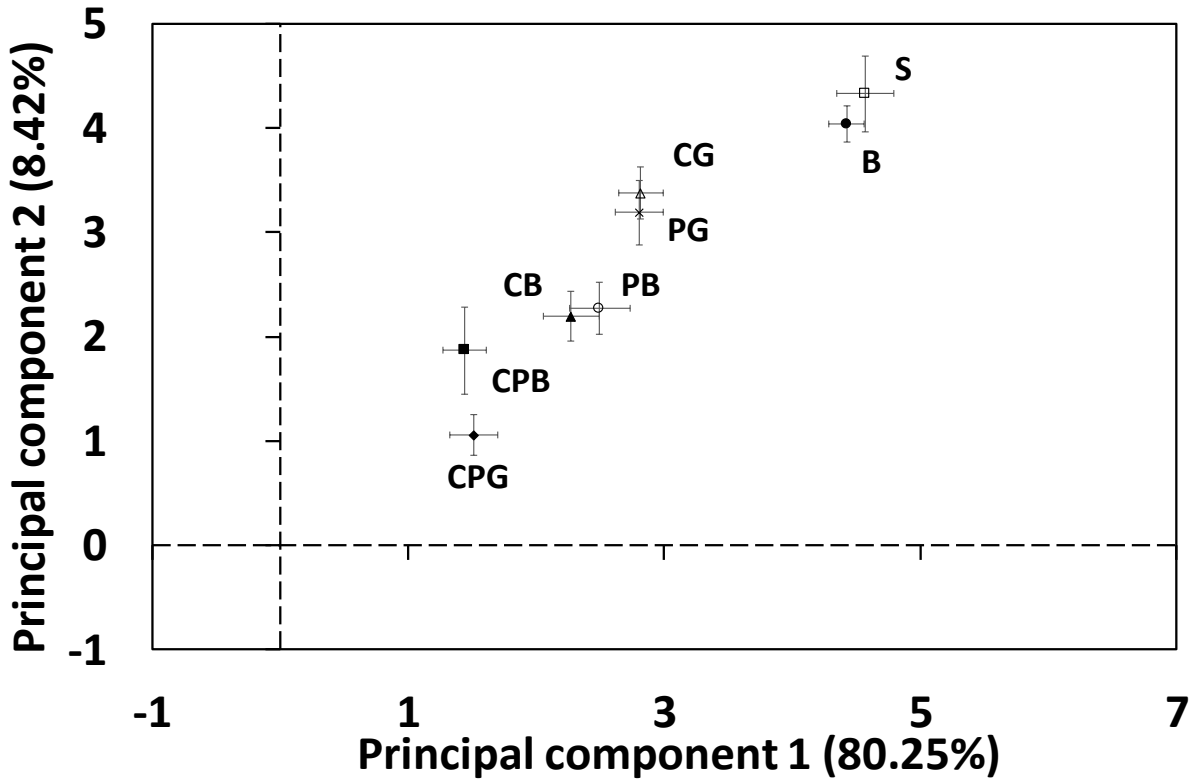

Figure 6. 8 Score plot of principal component analysis (PCA) showing treatment variation based on phospholipid fatty acid (PLFA) patterns. S: control soil without any amendment; B: soil applied with biochar; CB: soil applied with Cd + biochar; CG: soil applied with Cd + glucose; PB: soil applied with Pb + biochar; PG: soil applied with Pb + glucose; CPB: soil applied with Cd + Pb + biochar; CPG: soil applied with Cd + Pb + glucose.

6.5.3. Influence of biochar on soil and microbial carbon

6.5.3.1. Soil organic carbon and nutrient pool

Soil TOC was measured at the end of 49 days incubation. Results showed that TOC value decreased in the control soil as the microbial mineralization increased (**Table 6.4**). With the addition of biochar, the SOC amount increased, contributing to heavy metal immobilization. The formation of metal-organic complexes on biochar surfaces could contribute to the increased metal retention in biochar-amended soils (Bolan et al., 2014). Biochar addition significantly ($p < 0.05$) increased the soil C stock by 7% compared to unamended control soil. However, SOC content was also high in glucose-amended soils even under metal contamination. Because of the metal induced inhibition of microbial activity, TOC content was slightly higher in the contaminated soils than uncontaminated soils. Although exactly similar quantity of C was added to soils in the form of glucose and biochar, SOC patterns in those soils after incubation were largely varied. With glucose addition, TOC was increased by 6, 6 and 14% in CG, PG and CPG, while with biochar addition it was increased by 17, 15 and 21%

in CB, PB and CPB, respectively. This could be attributed to the dominance of microbially resistant organic carbon (OC) in biochar as well as the metal remediation capability of the material following its application to soils.

The TN value was the lowest in singly metal-spiked biochar amended soils, indicating an acceleration of native N depletion. New organic C addition might have caused soil microbial populations to deplete the native N, which is also known as the ‘mining theory’ (Tian et al., 2016). As a consequence, the C:N ratio was significantly ($p < 0.05$) higher in singly metal-spiked biochar amended soils than unamended soils (**Table 6.6**). There was also a discrepancy in C:N ratios among glucose and biochar treatments, ranging from 25.8 to 29.1 in glucose treatments, while 30.67 to 31.03 in biochar treatments.

Table 6. 6 Comparison of total organic carbon (TOC), total nitrogen (TN), and ratio of C:N in soils. Means ± SE (n = 3)

		Glucose applied*			**Biochar applied**			
	Control	CG	PG	CPG	B	CB	PB	CPB
TOC (g kg^{-1} soil)	27.68 ± 0.68	29.42 ± 0.98	29.36 ± 1.24	32.12 ± 1.88	33.22 ± 0.92	33.51 ± 0.91	32.71 ± 1.04	34.96 ± 1.12
TN (g kg^{-1} soil)	1.18 ± 0.05	1.14 ± 0.08	1.01 ± 0.04	1.15 ± 0.08	0.91 ± 0.03	1.08 ± 0.06	1.01 ± 0.02	1.14 ± 0.10
C:N	23.53 ± 1.23	25.81 ± 1.75	29.07 ± 1.41	27.93 ± 0.71	36.51 ± 1.39	31.03 ± 1.38	32.39 ± 1.58	30.67 ± 1.24

*Glucose applied at the same carbon loading rate as biochar. Control as uncontaminated soil; CB: Cd-contaminated soil applied with biochar; CG: Cd-contaminated soil applied with glucose; PB: Pd-contaminated soil applied with biochar; Pd-contaminated soil applied with glucose; CPB: Cd- and Pb-contaminated soil applied with biochar; CPG: Cd- and Pb-contaminated soil applied with glucose; uncontaminated soil applied with biochar

Table 6. 7 Effect of heavy metal toxicity on microbial carbon use efficiency. Means ± SE (n = 3) of total PLFA, PLFA diversity, ratio of Gram-positive and Gram-negative bacteria, ratio of bacteria and fungi. Mean values followed by the same letter are not significant according to ANOVA ($p > 0.05$)

	Glucose applied*				**Biochar applied**			
	Soil	Soil + Cd	Soil + Pb	Soil + Cd + Pb	Soil	Soil + Cd	Soil + Pb	Soil + Cd + Pb
Microbial CUE	0.41 ± 0.02b	0.35 ± 0.04b	0.29 ± 0.02a	0.31 ± 0.03a	0.53 ± 0.01d	0.40 ± 0.01b	0.38 ± 0.02b	0.43 ± 0.01c
	Biochar applied: Glucose applied				Heavy metal: Biochar			
	CB:CG	PB:PG	CPB:CPG		CB:B	PB:B	CPB:B	
CUE ratio	1.15 ± 0.06	1.32 ± 0.05	1.40 ± 0.01		0.77 ± 0.12	0.73 ± 0.10	0.82 ± 0.04	

6.5.3.2. Microbial carbon use efficiency in soil

Microbial CUE represents the ratio of C assimilated in microbial biomass over uptake, which is an indicator of net C sequestration by soil microorganisms. In this study, both microbial respiration and biomass C were significantly ($p < 0.01$) reduced in heavy metal contaminated soils. The microbial CUE was also reduced in a similar manner (**Table 6.7**). Microbial CUE values in metal contaminated soils were 0.35, 0.29 and 0.31 in Cd, Pb and Cd + Pb spiked soils, respectively, while it was 0.41 in uncontaminated soils. The inhibition of microbial activity and proliferation due to metal biotoxicity was reported in many studies (Liao et al., 2005; Sobolev and Begonia, 2008). Biochar addition however was able to increase both microbial respiration and biomass C in soils even under heavy metal stress. Due to biochar application, microbial CUE was increased by 0.05, 0.09 and 0.12 units in Cd, Pb and Cd + Pb spiked soils, respectively. This indicated that a higher portion of assimilated C was incorporated into the microorganisms rather than it was released as CO_2 (Chen et al., 2017; Lehmann et al., 2011).

In spite of the same C amount added to soil with biochar and glucose, CUE ratios of biochar:glucose in Cd, Pb and Cd-Pb-spiked soils were all larger than 1 (1.15, 1.32 and 1.40, respectively) (**Table 6.7**). Compared to labile C source, such as glucose, a higher C sequestration by microbiota was noticed in biochar-amended soils. Unlike biochar, glucose induced a larger microbial respiration, but smaller C sequestration. The CUE ratios in biochar-amended contaminated and uncontaminated soils were all less than 1. It indicated that more CO_2−C was released in the metal contaminated soils than the healthy soils by producing a similar amount of biomasses. The metal toxicity led to less CUE by microorganisms, and consequently less C sequestration ability in polluted soils.

Microbial CUE needs to take microbial community composition into account because the differentiation of microbial species may contribute to MBC or CO_2 release, and also may slow down the population turnover rates of fungi (Six et al., 2006). The alteration of microbial community structure could modify the C dynamics, and consequently might lead to either depletion or sequestration of terrestrial C (Compant et al., 2010; Malcolm et al., 2009). In this study, the heavy metal toxicity had a more negative effect on fungi than bacteria, and bacteria tended to release more CO_2 to form the same amount of biomass. Due to biochar application, the abundance of fungal species was increased by 2, 60, 62 and 67% in uncontaminated, Cd-spiked, Pb-spiked and Cd-Pb-spiked soils, respectively (**Figure 6.7**). The assimilated C was likely incorporated into microbes and their secondary metabolites instead of being released as CO_2, and consequently contributing to increased CUE. Modulation with biochar thus reduced the metal biotoxicity and altered the microbial community composition, and consequently improved the microbial CUE. The microbial community shift might have occurred as the results of biochar modulation (Cross and Sohi, 2011).

6.6 Conclusions and recommendations

- The present study demonstrated that biochar contributed to soil pH increase, metal bioavailability reduction, and consequently heavy metal immobilization.
- The SEM images, EDX elemental analysis and IR spectra suggested binding of metals by biochar and thereby potentially reducing their mobility in soils. However, there is a need to examine the long term stability of metal immobilization in soils through biochar application and the underlying chemical interactions.
- This study also provided evidence that biochar improved the microbial CUE by modulating heavy metal stresses in contaminated soils. Biochar application increased the microbial activity, microbial biomass, and benefitted certain microbial populations,

such as G+ bacteria and fungi, which were otherwise repressed under heavy metal stresses.

- Microbial community populations were also shifted in response to metal stresses and biochar modulation. Biotoxicity from heavy metals affected the soil C metabolism by inhibiting the microbial activity. Biochar amendment increased both microbial respiration and biomass, but most importantly it imparted positive influences on microbial CUE, thereby improving microbial C assimilation rate.
- However, the biochar-modulated C sequestration in metal contaminated soils might lead to a native N mining phenomenon. Future research is needed to investigate the long-term shift of microbial populations under similar scenarios by monitoring the microorganisms' C source preferences.

Chapter 7

SUMMARY AND CONCLUSIONS

7.1 Research Concept

The overall concept including the principal research components and the processes covered in this research are shown in **Figure 7.1**. Soil microorganisms can change dynamically based on alternating soil conditions resulting from anthrophonic disturbances such as cultivation, nutrient inputs (including organic amendments) and soil contamination. Fresh organic amendments are one of the major carbon (C) sources for soil micro-communities. Organic amendments such as glucose, wheat straw, and biochar show different responses by soil microorganisms depending on C and N contents and nature of organic amendments (Francioli et al., 2016; Lundquist et al., 1999; Treonis et al., 2010). This research demonstrated the bacterial:fungi (B:F) ratio altered due to the organic amendments, and the effect varied with the properties of amendments. Also, the research validated that microbial reactions to the organic additions are influenced by land use histories.

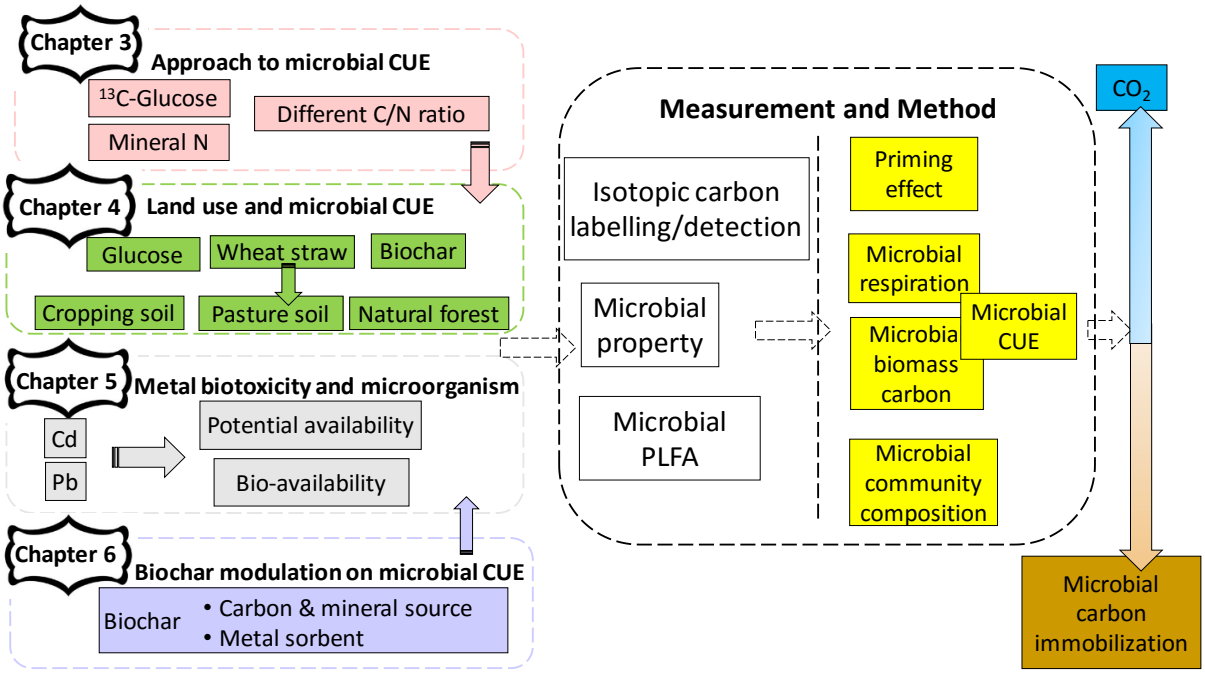

Figure 7. 1 Conceptual diagram containing the principal research components covered and processes addressed in various research chapters (**Chapter 3-6**) in this thesis.

Environmental stress conditions, such as heavy metal toxicity inhibits microbial activity, microbial biomass assimilation, and alters microbial community composition. Notably, they repress the assimilation of soil organic carbon (SOC) by microorganisms, resulting in increased CO_2–C release. This is attributed to the alteration of microbial community composition, thereby resulting in changes to C use preference. Soil microorganisms regulate soil C mobilization and immobilization ((im)mobilization) and dynamics in return. As organic remediation strategy, biochar addition supports soil microbiota by providing energy (organic C) and nutrients (mineral element), and reduces metal mobility. Eventually, the improvement in soil living conditions contributes to the increased microbial carbon use efficiency (CUE). In this research, the major outcomes and conclusions are based on various laboratory investigations on soil microbial properties and C use patterns in relation to environmental factors, and will be summarized in the following section (**Section 7.2**).

7.2 Research components and processes involved

7.2.1 Microbial mediation of soil carbon dynamics

Microbial decomposition of SOC is the key process for soil organic matter degradation and the consequent release of CO_2 into the atmospheric C reservoir. The scope of the literature review includes comprehensive studies investigating the role of soil microorganisms in C dynamics together with environmental influences, such as soil properties and fresh organic matter inputs (**Chapter 2**). Traditional methods to evaluate soil microorganisms' functional and genomic diversity include the determination of microbial respiration, microbial biomass metabolic quotient and plating techniques, while modern technology introduces methods, such as phospholipid fatty acids (PLFAs) and ribosomal intergenic spacer analysis. However, the information is not complete if only microbial activity or biomass C are measured. For instance, results in the present study demonstrated that: (i) microbial CUE values were also affected by the ratio of carbon:nitrogen (C:N) of the fresh amendments (**Chapter 3**), and (ii) both microbial activity and biomass increased with glucose application, but microbial CUE decreased (**Chapter 4**). In **Chapter 3**, the concept of microbial CUE involving four approaches by using ^{13}C labelled glucose to trace C flow was evaluated. There were discrepancies among the four approaches, even between the two approaches that are based on microbial biomass (i.e., C_m and C_p). Because there is no unified concept and determination of microbial CUE, depending on the aim of the study, the microbial CUE should be measured accordingly.

The PE is also an essential microbial process in relation to soil C dynamics. The isotopic C used in this research is an efficient method to separate apparent and real priming effects (PEs).

Generally, fresh C amendments promote microbial activity and stimulate positive PE. But the PE level and intensity are related to the ratio of organic C and mineral nitrogen (N) (**Chapter 3**), which is the amount and quality of the added organic amendments (Chowdhury et al., 2014b). The primed CO_2 release was higher in glucose alone treatments and C:N at 50 while the organic C amendments with higher N content reduced PE intensity, demonstrating the absence of the stimulation of PE by N input. Fontaine et al. (2011) suggested the concept of 'microbial mineral mining' of microorganisms due to the fresh organic addition which may help to understand this phenomenon: soil microorganisms are tended to 'mining' indigenous organic matter when nutrient availability is low, leading to the destruction of soil organic matter; yet less organic matter decomposition occurs when nutrients are abundant. In addition, the shifts of microbial community may play an important role in terms of indigenous C depletion and added-C transferring (Fontaine et al., 2003). Firstly, the dominated *r*-strategies (which are stimulated by the labile C source) may be overtaken by *K*-strategies in the later stage of organic amendments (Chen et al., 2014); on the other hand, the litter-associated fungi may bio-relocate C into soil macroaggregates and subsequently contribute to soil organic matter formation (Frey et al., 2003).

7.2.2 Microbial carbon use efficiency-Effect of organic amendments

Of the environment factors in relation to soil microbial properties and function, both the amount and nature of fresh organic amendments play an essential role in the alteration soil microbiota. Both microbial activity and biomass were stimulated by fresh organic C amendments (**Chapter 4**). Therefore, fresh organic C results in positive soil PEs and mineralization of indigenous C and N. In addition, the addition of mineral N sources significantly increased the microbial biomass N and mineral N concentration in soils.

However, the properties of amendments, such as C lability and C:N ratio, alter the magnitude of the influences on microbial C use patterns. For instance: (i) results in Chapter 3 indicated that microbial CUE was the highest at the C:N ratio of 23 when organic amendments were tested at three C:N ratios (10, 23 and 50), and (ii) data in Chapter 4 indicated that the stimulation of microbial respiration was less intense with the addition of biochar, which has higher recalcitrant C content and lower C:N ratio, than with the addition of labile C (glucose) (**Chapter 4**). Because it is difficult to evaluate microbial C use with both stimulated biomass and respired C, the measurement of microbial CUE is necessary. Not surprisingly, biochar treated soils showed higher microbial CUE than glucose (**Chapter 4**). Thus, microbial C use preferences are highly related to the quality of exogenous organic amendments. This will have implications on the study of soil PE and also soil management.

7.2.3 Microbial carbon use efficiency-Effect of land use

The influence of land use histories on microbial CUE result from both direct input of C sources and indirect effects on soil properties. Firstly, land use results in the alterations of soil physiochemical and microbial properties. Anthropogenic disturbance and soil supplement lead to major effects on indigenous microbial community function and composition, and consequently alters their C use patterns. While bacterial species showed larger percentage within the microbial community with land management and fertilizer application, fungi were the dominant species in natural forest systems.

The microbial activity and biomass also showed significant differences among land use systems. The microbial biomass carbon (MBC) content in the tested soils followed: pasture soil > cropping soil > natural forest, while the cumulative microbial respiration followed: natural forest > pasture soil > cropping soil. Compared to natural forest, both the cropping and pasture soils are at high disturbance and organic inputs rate, however, they differed in terms of the nature and frequency of disturbance (for example, cultivation), as well as the quality and quantity of the organic inputs. The land management practices in cropping land like tillage and irrigation affect soil aeration, while grazing intensity attributes to the alteration of soil bulk density (Lauber et al., 2008). Unlike cropping soils that are supplied with inorganic NPK fertilizers and cropping residues, pasture lands are enriched with excreta from the grazing livestock. Moreover, the vegetation in the different land use affects soil microbiota due to the alteration in root exudates and rhizosphere conditions. This is likely to result in the alteration of microbial habitat and energy/nutrient supporting patterns. Also, the addition of C sources showed different pattern in microbial respiration rate among land use systems. Although the microbial CUE did not show significant difference among the tested land use soils, there was variation in microbial CUE pattern during the incubation period in different land uses. The microbial CUE fell less in natural forest compared to the systems with greater disturbance (cropping and pasture lands).

7.2.4 Metal pollution and biochar remediation in relation to soil microorganisms

It has been reported that heavy metals are biotoxic to soil microbiota (Hinojosa et al., 2005). The application of biochar to metal polluted soils may have several benefits due to the following reasons: (i) biochar contain high amounts of organic C and mineral elements, and (ii) with the features of negatively charged functional groups and highly porous structure, biochar may serve as sorbents for heavy metals (Bolan et al., 2014). In this research, soils were spiked with Cd(II) and Pb(II). Both microbial activity and biomass C were suppressed

(**Chapter 5**), and the inhibitory effect was more obvious at higher metal concentration, yet there was no significant difference between metal types. Microbial CUE was significantly low in metal contaminated soils, leading to less C assimilation by microbes. As proposed in one of the hypotheses of **Chapter 5,** biochar addition to metal contamination soils showed remediation ability by improving microbial CUE. By the end of incubation, heavy metal bioavailability reduced while soil pH, microbial activity and biomass all increased in biochar-amended soils. However, the biochar-modulated microbial C use in metal contaminated soils might lead to a native N mining phenomenon (**Chapter 6**). Soil microbial property and C use preference showed that environmental stress conditions, such as heavy metals, have negative influence on microbial sequestration of soil C. In summary, the un-favoured living conditions cause soil microorganisms to convert their energy from anabolism to catabolism. This living strategy shift leads to higher organic C transforming into CO_2–C that is released into the atmosphere.

7.2.5 Microbial community composition and the interpretation on carbon use alteration

The examination of PLFA in order to determine microbial community composition possibly helps to explain the alteration in microbial functions as affected by the change in soil conditions (Frostegård et al., 2011). The hypothesis was that the variation of microbial CUE under a variety of soil conditions are related to the microbial population shifts. The abundance of microbial community, the ratio of Gram+ and Gram− bacteria (G+:G− bacteria) and the ratio of bacteria and fungi (B:F) provide essential information to address microbial CUE. In the current research, microbial community composition differed among amendments and land use histories. For instance, labile organic C source input was beneficial to Gram+(G+) bacteria population, while biochar promoted fungi population. Because the fungi produce hyphae and are relatively slow-growing species, they may be more responsible for the later stage of PE (Fontaine et al., 2004; Garcia-Pausas et al., 2011). The current research work also demonstrated that fungi had a larger population than bacteria in the natural forest systems. Compared to fungi, that had greater C assimilation efficiency, bacteria required higher C:N ratio from soil sources, consequently was more C demanding (Islam et al., 2000; Moscatelli et al., 2007). These microbial features indicate that: (i) microbial population succession due to substrate C depletion as indicated by the shift of microbial community composition during incubation in our study, and (ii) a stable soil condition in relation to soil organic matter status with less C and N degradation in a fungi dominant soil microbial environment (Nsabimana et al., 2004).

Although all the microbial populations decreased under metal stress, Bardgett et al. (2005) suggested that fungi are more sensitive to environmental stress and disturbance than bacteria. Similarly, results in the present research also indicated that fungi population was significantly ($p < 0.05$) suppressed in the metal contaminated soils. However, biochar modulated metal toxicity and increased microbial community population. Also, the decreased fungi population due to metal stress was mitigated with biochar addition, showing a more prominent positive effect on fungi than on bacteria. The total PLFA and microbial diversity are both reduced under metal stress. This highlights that the biotoxicity of metal not only reduced the reproduction of soil microorganisms, but also narrowed population diversity, leading to a more fragile soil micro-ecosystem. Biochar alleviated the metal biotoxicity on soil microbial community, promoting total PLFA and microbial diversity. In the current research, there was a lower microbial CUE and suppressed fungi population in metal contaminated soils (**Chapter 5**); the altered microbial community indicated that there was a consequent influence on microbial C use. In addition, the application of biochar is not only beneficial by providing C and metal adsorption sites, the lower C:N ratio and mineral elements but also favours fungi population, leading to a higher microbial CUE (**Chapter 4**) and a microbial community with higher resilience and stability.

7.3 Application of this research

This research has addressed the essential role of microorganisms in regulating C (im)mobilization processes in soil. This research focused on PE, land use histories, heavy metal biotoxicity and biochar-induced metal remediation, aiming to reveal the link between microbial function and soil C dynamics. As we have discussed, organic amendment inputs are inevitable to soil, either from a natural process of plant litter addition or anthropogenic supplement through compost and manure addition. Thus, their influences on soil microbiota are critical in controlling C and nutrient dynamics in soil.

This research determined the magnitude of PE under different C and nutrient additions, and compared various approaches of measuring microbial CUE. Depending on the C:N ratio of organic amendments, soil microorganisms can alter CUE and subsequent C immobilization through microbial assimilation. The comparison of soils from three land uses (cropping, pasture and nature forest) showed the variation of soil physiochemical characteristics as well as microbial community composition, providing important information about soil microbiota characteristics in various land use systems. Both of the organic amendments and land managements influenced microbial population and CUE. From the environmental perspective, a healthy ecosystem should have higher microbial CUE and diverse microbial population. A

higher microbial CUE of exogenous C input leads to a higher microbial C assimilation and less release of CO_2, resulting in greater stabilization of C in soil through microbial biomass. Whereas a higher microbial CUE of endogenous C sources may lead to the release of CO_2 from these sources, resulting in less C stabilization of C from these sources. Thus, the land use practices will lead to alterations in soil microbiology in relation to both microbial function and composition leading to long-term changes in C assimilation and stabilization in soil.

As a step further, heavy metal (Cd and Pb) contamination was included as one of the environmental stress conditions and biochar as a remediation method for metal contamination. Results in the current research confirmed that the microbial activity and CUE reduced with metal biotoxicity, but restored with biochar modulation of metal contamination. The current research highlighted the biochar remediation mechanism and the importance to microbial CUE. Biochar amendment contributes to the increase of soil physicochemical properties such as pH, C and nutrient content, reduces heavy metal bioavailability, stimulates microbial activity and CUE, promotes microbial population and diversity.

The current study enable farmers and researchers to understand the role of microbial CUE and microbial community population as promising indicators for soil function and health assessment. With the insight into microbial community composition, the possible explanation on the relation between microbiota and soil C use has been provided, and thereby providing suggestions for future research and implications for land use management in the perspective of microbial function.

7.4 Future research

This research has addressed the important role of microorganisms regulating soil C dynamics, and carried out detailed investigation into mechanisms underpinning their relationship with varies soil management and environmental conditions. Any environmental changes or pollution can be critical to soil C dynamics by affecting microbial composition and function. However, due to diverse and complex features of soil ecosystems and the limitation on technology and knowledge, the research gaps still exist and will continue to exist. The research work based on this project suggests a number of areas that require further investigations in the future, which include:

- Future research should investigate the microbial community succession in a long-term and field-based perspective. The land use practices and soil amendment inputs are site specific and continue to change with time. With these environmental influences, soil microorganisms are in constant change along with C biogeochemical processes and dynamics in soil.

- The C and mineral element content along with the morphology of biochar vary depending on the following factors: (i) feedstock material (plant based or animal based material), and (ii) production conditions (pyrolysis temperature, aerobic conditions). The effects of biochar input on soil microbiota and remediation ability can vary based on the properties. Therefore, a range of biochar materials should be characterized and assessed for microbial composition and function in relation to CUE and subsequent C stabilization in soil.
- Rhizosphere is highly related to soil microorganisms, which has been recognized as a belowground 'hotspot'. The litter inputs and PE both are natural processes, and root turnover and exudates support native microorganisms while controlling their C use patterns (Kuzyakov and Cheng, 2004). The rhizosphere effects on C dynamics in relation to microbial CUE and C stabilization need to be examined using isotopic (^{13}C and ^{14}C) pulse labelling techniques (i.e., trace C allocation within plant-soil system, Chowdhury et al., 2014a).
- With the continuous change of microbial function in relation to C (im)mobilization, it is necessary to address the importance of microbial PE (Kuzyakov, 2010). While there have been a number of studies on the microbial response to fresh organic amendment inputs, future research should focus on: (i) the implications to the belowground C dynamics, and (ii) microbial mechanisms in terms of controlling C (im)mobilization process.
- Modern molecular technologies, such as polymerase chain reaction, denaturant gradient gel electrophoresis and amplified rDNA restriction analysis, can provide greater in-depth information about molecular mechanisms of microbial stabilization of C than the traditional microbial measurements. The C stability within mineral-organic association can be detected by Fe *K*-edge ESAFS spectroscopy (Yang et al., 2016), the microbial growth strategies can be determined with the support of PCR-DGGE (Chen et al., Milcu et al., 2011), and the chemical composition of organic C can be traced with isotopic labelled C and analysed by CPMAS NMR ^{13}C and by FTIR spectroscopy (Fontaine et al., 2007). However, there is a need to clearly identify the purpose before incorporation of these technologies, because there are knowledge gaps about up-grading microbial function into ecosystem level.
- In this research, C and mineral sources have been introduced and quantified in relation with microbial PE. However, there are other important soil, plant and environmental parameters that could affect the direction and magnitude of PE. Future research should consider soil properties (such as moisture and soil type), environmental conditions

(such as temperature and spatial-temporal variation in relation to microbial CUE) and subsequent C stabilization in soil (Kuzyakov et al., 2010).

- To date, soil microorganisms and microbial CUE have been incorporated into several models to interpret C biochemical dynamics (Manzoni and Porporato, 2009). Although this research did not include the modelling approach, it is a necessity that new models should not only include soil microorganisms and microbial community composition, but also microbial dynamics along with the continuous variation of environmental parameters.

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