Biochar affects soil organic matter cycling and microbial functions but does not alter microbial community structure in a paddy soil

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HIGHLIGHTS

- BC addition increased total soil C and POC for 47.4–50.4% and 63.7–74.6%, respectively;
- Addition of BC altered the microbial community structure in conjunction with NPK fertilization;
- BC addition increased microbial utilization of amino acids and amines.

GRAPHICAL ABSTRACT
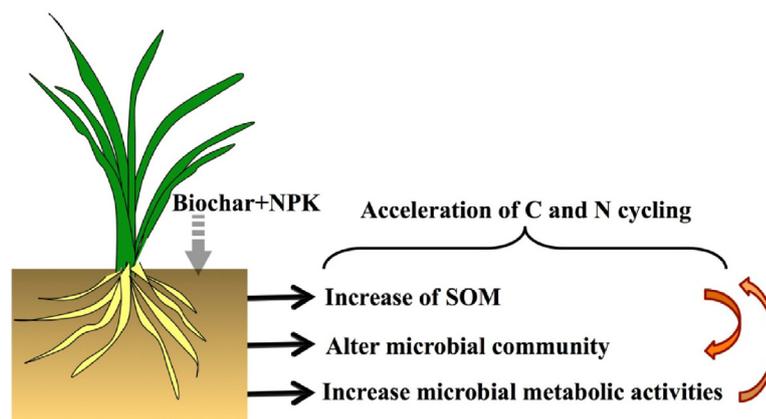
ABSTRACT

The application of biochar (BC) in conjunction with mineral fertilizers is one of the most promising management practices recommended to improve soil quality. However, the interactive mechanisms of BC and mineral fertilizer addition affecting microbial communities and functions associated with soil organic matter (SOM) cycling are poorly understood. We investigated the SOM in physical and chemical fractions, microbial community structure (using phospholipid fatty acid analysis, PLFA) and functions (by analyzing enzymes involved in C and N cycling and Biolog) in a 6-year field experiment with BC and NPK amendment. BC application increased total soil C and particulate organic C for 47.4–50.4% and 63.7–74.6%, respectively. The effects of BC on the microbial community and C-cycling enzymes were dependent on fertilization. Addition of BC alone did not change the microbial community compared with the control, but altered the microbial community structure in conjunction with NPK fertilization. SOM fractions accounted for 55% of the variance in the PLFA-related microbial community structure. The particulate organic N explained the largest variation in the microbial community structure. Microbial metabolic activity strongly increased after BC addition, particularly the utilization of amino acids and amines due to an

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Enzymes
Microbial function

increase in the activity of proteolytic (L-leucine aminopeptidase) enzymes. These results indicate that microorganisms start to mine N from the SOM to compensate for high C:N ratios after BC application, which consequently accelerate cycling of stable N. Concluding, BC in combination with NPK fertilizer application strongly affected microbial community composition and functions, which consequently influenced SOM cycling.

1. Introduction

Microorganisms mediate many processes, including soil organic matter (SOM) cycling and carbon (C) sequestration (Balser and Firestone, 2005; Clemmensen et al., 2013). The addition of biochar (BC) to soil is likely to feedback on ecosystem SOM and nutrient cycling by affecting the composition and function of microorganisms (Lehmann et al., 2011). However, the mechanisms by which BC application affects SOM dynamics via microorganisms remain unclear (Gul et al., 2015).

The addition of BC to soil can increase the SOM level (Lehmann et al., 2006), affect C cycling (Bolan et al., 2012; Farrell et al., 2015; Liang et al., 2010), accelerate nitrogen (N) dynamics (Nelissen et al., 2012) or in some cases, even reduce organic N turnover (Prommer et al., 2014). The changes in the total SOM due to management practices are gradual because SOM is a heterogeneous mixture that contains numerous compounds varying degradability and turnover rates (Stevenson, 1994). Therefore, studying individual SOM fractions separated by physical and chemical methods is a more informative practice, especially for the study of nutrient cycling (von Lützow et al., 2007). SOM fractions have been suggested as more sensitive indicators of the effects of management practices and soil disturbance than the total SOM (Haynes, 2005). The addition of BC to soil can differentially affect individual SOM fractions. For example, BC amendment increased the dissolved organic C content (DOC), microbial biomass C and light fraction organic C compared with un-amended soil (Demisie et al., 2014; Lin et al., 2012). Specifically, the microbial biomass was up to 43–125% higher in BC-rich soils than in BC-poor adjacent soils during 532 days of incubation (Liang et al., 2010). On the contrary, BC amendment did not affect the DOC and dissolved organic N (DON) in one field study (Jones et al., 2012), whereas it decreased the DOC concentration (Prommer et al., 2014) in another field study. These contradicting positive and negative effects of BC application on SOM fractions may be attributed to the specific processes governing C and N cycling under specific management practices. These processes vary with climate, crop rotation, fertilization and soil biology characteristics and consequently result in inconsistent SOM dynamics and transformation (Demisie et al., 2014; Jones et al., 2012; Lin et al., 2012; Liang et al., 2010; Prommer et al., 2014).

Microorganisms are actively involved in SOM dynamics and transformation in terms of mineralization and formation (Bowles et al., 2014; Ng et al., 2014). After BC addition, greater decomposition of soil SOC was observed, accompanied by a higher microbial activity (Wardle et al., 2008) or changes in the microbial community (Farrell et al., 2013). On the other hand, BC addition suppressed SOC decomposition with altered microbial community structure by increasing Gram-positive bacteria in a 30-day incubation study (Lu et al., 2014). The addition of BC altered C sources use patterns of microorganisms, perhaps via changes in the microbial population (Pietikäinen et al., 2000). All these studies suggested that SOM cycling might be influenced via microorganisms after BC addition. A number of studies reported changes in the microbial community after BC addition that were attributed to the physico-chemical properties of BC (e.g., aeration, sorption), as well as BC-induced changes in soil properties such as pH (Gul et al., 2015; Jindo et al., 2012; Rillig et al., 2010; Xu et al., 2014). However, information on microbial communities associated with soil SOM dynamics related to BC amendment is particularly limited (Gul et al., 2015). Furthermore, the majority of BC studies have relied on short-time incubation experiments (Farrell et al., 2013; Pietikäinen et al., 2000; Xu et al., 2014). Long-term field trials examining organic C- and N-related effects

of BC on microorganisms still remain to be studied. As such studies will help us understand the role of environmental factors in controlling biochar-induced changes in soil chemical and biological properties.

In field studies, N is an important nutrient for maintaining crop yield. Because BC is generally low in inorganic N, it is usually applied in conjunction with mineral fertilizer (Chen et al., 2013). In fact, the combination of BC and mineral fertilizers has been shown to synergistically affect crop yield and growth in field studies (Atkinson et al., 2010; Lehmann et al., 2003; Steiner et al., 2007). In addition to the possible effects of BC on microorganisms described above, the combination of fertilizer and BC may also affect microorganisms in field studies (via direct mineral nutrient input or indirect crop effects). However, whether BC amendment affects microorganisms and SOM alone or synergistically with fertilizer remains unclear in field studies. Thus, long-term field trials examining the effects of BC and fertilizer on microbial community structure and SOM cycling are urgently needed.

We hypothesized that (1) BC and mineral fertilizer synergistically affect microbial community structure and activities because fertilization will directly and indirectly decrease possible nutrient limitation after BC addition; and (2) these changes in microbial community composition and function will alter SOM cycling. To test our hypothesis, we studied the effects of BC and mineral fertilizer on SOM and its fractions, microbial community structure (using phospholipid fatty acid analysis, PLFA) and functions (by analyzing enzymes involved in C and N cycling and Biolog) in a long-term field study.

2. Materials and methods

2.1. Study site and field experiment

A 6-year field study of a rice paddy was started in 2009 at Qianyanzhou ecological station in southern China (26°44′N and 115°03′E), which belongs to the Chinese Academy of Science (CAS). The regional climate is classified as warm and humid with a mean annual temperature of 18 °C and precipitation of 1470 mm. The soil has a heavy loam texture of 22.8% sand, 61.4% silt and 15.7% clay. The basic soil characteristics at the start of the field experiment were as follows: 6.6 g kg^{-1} SOC content, 0.7 g kg^{-1} TN content, 33.9 mg kg^{-1} Olsen-P, and a soil pH of 4.9.

Four treatments were laid out in a randomized complete block design in triplicate: control (CK), compound fertilizer (NPK) application, BC application (BC), and combined NPK fertilizer and BC application (BC-NPK). Each plot was 9 m^2 in size. The compound chemical fertilizer application rate was 750 kg ha^{-1}, which was equivalent to 112.5 kg N ha^{-1}, 49.1 kg P ha^{-1} and 93.4 K kg ha^{-1}. The BC was applied at 6 t ha^{-1} per year. The applied 6 t ha^{-1} per year corresponded to twice of the amount of rice biomass that could be produced in the field. The BC used in this experiment was made from pine wood via pyrolysis at a temperature of 450 °C for 8 h in a closed container under oxygen-limited conditions. The BC was crushed to particle sizes smaller than 2 mm before application to the soils. The BC contained 653 g C kg^{-1} and 3.9 g N kg^{-1}.

2.2. Soil sampling

After the rice harvest in autumn 2014, soil was sampled from each plot by collecting 5 randomly selected cores (0–20 cm deep), which were mixed to yield one composite sample per plot. The samples were

then stored in airtight polypropylene bags, placed in a cooler box at approximately 4 °C and transported to the laboratory. The leaves, roots and rock fragments were carefully removed, and the remaining soil samples were divided into several subsamples. Subsamples for microbial metabolic function, enzymes activities and dissolved organic matter (DOM) concentration analyses were stored at 4 °C for no longer than one week before finishing the analyses. Subsamples for SOM and particulate organic matter (POM) analyses were air dried at room temperature. The subsamples for the microbial community analysis were stored at −80 °C.

2.3. Soil SOM fractions analysis

The total C and total N content in the soil was measured by dry combustion using a Vario Max CN elemental analyzer (Elementar, Germany).

The C and N concentrations in the DOM were measured following the method of Jones and Willett (2006). Eight grams of dry-weight-equivalent fresh soil was extracted with 40 ml of 0.05 mol L^{-1} K$_2$SO$_4$ (soil/solution ratio 1:4) for 1 h. The extract was then passed through a 0.45-μm membrane filter and analyzed for C and total dissolved N using a Multi 3100 N/C TOC analyzer (Analytik Jena, Germany). The concentrations of NH$_4^+$ and NO$_3^-$ in the subsamples were analyzed using an auto-analyzer (TRAACS-2000, BRAN + LUEBBE, Germany); the DON was determined by calculating as the difference between the total dissolved N and the combined NH$_4^+$ and NO$_3^-$ content.

The particulate organic C and particulate organic N (POC and PON) content were determined using the method described by Cambardella and Elliott (1992). Twenty grams of air-dried soil (<2 mm) was dispersed in 100 ml of sodium hexametaphosphate ((NaPO$_3$)$_6$) (5 g L^{-1}). The sample was then first shaken by hand for 10 min, followed by shaking on a reciprocating shaker (180 rpm min^{-1}) for 18 h. The soil suspension was poured over a 53-μm sieve using a flow of distilled water. All material remaining on the sieve, which was defined as the particulate organic matter (POM), was washed into a dry dish, oven dried at 65 °C, weighed, ball-milled and analyzed for C and N by dry combustion using a Vario Max CN elemental analyzer (Elementar, Germany).

2.4. Soil microbial community structure analysis

The microbial community was determined using a PLFA analysis described by Frostegård et al. (1991) with modifications. The fatty acids were extracted with 8 g of dry-weight-equivalent fresh soil using a one-phase extraction mixture containing chloroform:methanol:phosphate buffer. The amounts of fatty acid methyl esters (FAMEs) were analyzed on a GC–MS (TRACE GC Ultra ISQ). The individual compounds were identified by comparing their relative retention times to commercially available 37 FAMEs (FAME 37 47885-U, Supelco, Inc.) and a mixture of 26 bacterial FAMEs (BAME 26 47080-U, Supelco, Inc.). The individual compounds were quantified based on an internal standard.

We divided 4 microbial groups: Gram-positive (i14:0, i15:0, a15:0, i16:0, a16:0, i17:0), Gram-negative (16:1Ω7c, 18:1Ω7c, cy17:0, cy19:0), fungi (18:2 Ω6,9c) and actinomycetes (10Me17:0, 10Me18:0) based on previous studies (Bossio and Scow, 1998; Frostegård et al., 1993; Zelles, 1997; Zelles, 1999).

2.5. Soil microbial metabolic function analysis

The microbial metabolic function was characterized using the Biolog Eco-plate, which contained three replicate sets of 31 substrates (Garland and Mills, 1991). These substrates are associated with plant root exudates and can be divided into six groups: carbohydrates, amino acids, carboxylic acids, amines, polymers and miscellaneous (Preston-Mafham et al., 2002). Though it represents certain metabolic functions in the Micro-Plates, Biolog Micro-Plate breathprint reflects diversity of carbon-oxidation pathways, and therefore functional diversity

of soil microbial communities (Preston-Mafham et al., 2002). Briefly, 10 g of fresh soil was added to 90 ml of sterilized NaCl solution (0.85%) and shaken at 200 rpm min^{-1} for 30 min. Ten-fold serial dilutions were prepared, and each well of the Biolog Eco-plates was inoculated with 150 μL of the suspension. The plates were incubated at 30 °C for 10 days, and the color development was assessed by measuring the absorbance every 24 h with an automated plate reader (VMAX, Molecular Devices, Crawley, UK) at a wavelength of 590 nm.

The 72 h absorbance values were used to calculate the average well color development (AWCD), which indicates the microbial metabolic activity. The AWCD was determined as follows (Garland and Mills, 1991):

$$AWCD = \sum (C_i - r)/31$$

where C_i is the absorbance of each well, and r is the comparable absorbance of the control well (water in control well). Negative (C$_i$-r) values were set to zero.

The 72 h absorbance values were also analyzed to calculate the catabolic diversity (Shannon–Weiner diversity index, H′) (Zak et al., 1994). The microbial community functional diversity indicated by the Shannon–Weiner diversity index was calculated as follows:

$$H' = \sum p_i \ln p_i$$

where $p_i = (C_i\text{-}r)/\sum (C_i\text{-}r)$.

2.6. Soil enzyme assays

The activities of five enzymes involved in C and N cycling in the soil were measured: β-1,4-glucosidase (βG), β-1,4-xylosidase (βX), cellobiohydrolase (CBH), β-1,4-N-acetylglucosaminidase (NAG) and L-leucine aminopeptidase (LAP). The following enzyme substrates, which were based on 4-methylumbelliferone (MUF), were used to assess the enzymatic activities: 4-MUB-β-D-glucoside for βG, 4-MUB-β-D-xyloside for βX, 4-MUB-N-acetyl-β-D-glucosaminide for NAG, 4-MUB-β-D-cellobioside for CBH and L-leucine-7-amino-4-methylcoumarin for LAP (Saiya-Cork et al., 2002).

The soil (1 g) was suspended in 125 ml of 50 mM acetate buffer (pH 5.0). The suspensions were stirred using a magnetic stir plate to ensure thorough mixing. A subsample of the soil suspension (0.2 ml) was dispensed into 96-well microplates, and 8 replicate wells were employed per sample per-assay. Fifty microliters of 200 mM substrate solution was also added to each sample well. The microplate was incubated in the dark at 20 °C for 4 h, and 10 μl of 1.0 M NaOH was then added to the microplate to stop the reaction before measuring the fluorescence using a microplate fluorometer (Synergy H4, BioTek) with 365 nm excitation and 450 nm emission filters. Replicate blank, negative control and quench standard wells were also measured. The enzyme activities were calculated according to German et al. (2011) and are expressed as nmol of MUF released per g soil and hour (nmol g^{-1} h^{-1}).

2.7. Statistical analysis

The PLFAs patterns were subjected to a principal component analysis (PCA). The mole percent of individual fatty acids was used in PLFAs pattern analysis according to Bossio and Scow (1998) and Bowles et al. (2014). A permutational multivariate analysis of variance (PERMANOVA) revealed the effect of experimental treatments on the overall microbial community structure (Anderson, 2005). The Monte-Carlo test was performed to test the significance of the effects of all measured SOM fractions on the community structure using Canoco software. These SOM fractions included SOC, TN, DOC, DON, POC and PON.

The SOM content, enzyme activity and metabolic function data were analyzed using a two-way ANOVA with SAS (SAS Inc. 1996). A two-way

analysis of variance (ANOVA) was also used to examine the contribution (%) of BC and fertilization effects on the soil SOM content, enzyme activities, abundance of microbial groups and microbial metabolic functions. Differences were considered significant at $p < 0.05$, with a separation of mean values by a least significant difference (LSD) test.

3. Results

3.1. Soil C and N fractions

The application of BC showed dominant effects on the content of all SOM fractions except PON (Table 1). The total soil C content increased by 48.9% after BC amendment (BC, BC-NPK) ($p < 0.05$, Fig. 1 and Table 1). The DOC and DON concentrations were 18.7–41.0% higher after BC-NPK application compared with CK or NPK application alone ($p < 0.05$, Fig. 1 and Table 1). The POC content was 2.7–3.9 times higher after BC and BC-NPK applications than after CK or NPK application alone ($p < 0.05$, Fig. 1 and Table 1). Although the POC was mainly affected by BC application, the PON was primarily affected by fertilizer application (up to 62.5% variation) (Table 1). Specifically, the PON content was approximately 1.4 times higher after fertilizer application (NPK, BC-NPK) compared to the CK treatment ($p < 0.05$, Fig. 1 and Table 1). Although the C and N fractions were consistently higher in either the BC or the BC-NPK paddy than in the CK paddy, none of the measured SOM fractions significantly differed between BC and BC-NPK treatments ($p > 0.05$, Fig. 1 and Table 1).

3.2. Microbial community structure

Fertilizer application significantly affected the abundance of microbial groups and the patterns of the microbial community (Table 1, Fig. 2 and Fig. 3A). The total PLFA abundance increased by 26.1–49.3% after fertilizer application (NPK, BC-NPK) ($p < 0.05$; Table 1 and Fig. 2). NPK application (NPK, BC-NPK) also increased the abundance of bacterial communities, specifically 38.2% and 13.9% higher G (−) PLFAs than

CK and BC, respectively ($p < 0.05$; Table 1 and Fig. 2). Fungi and actinomycetes were 43.9–70.4% and 21.2–64.7% more abundant, respectively, after NPK fertilizer application (NPK, BC-NPK) than without fertilizer amendment (CK, BC) ($p < 0.05$; Table 1 and Fig. 2). The PCA plot showed that the PLFA composition after fertilization (NPK, BC-NPK) significantly differed from that of the unfertilized treatments (CK, BC) (PERMANOVA test; Fig. 3A). Also the soil microbial community was significantly differed between NPK and BC-NPK treatments (PERMANOVA test; Fig. 3A). The PC1 and PC2 components accounted for 44.3% and 26.6% of the total variance, respectively (Fig. 3A).

The correlations between microbial community structure and SOM fractions (analyzed by the RDA plot) indicated that the RD1 and RD2 components accounted for 24.6% and 21.7% of the total variance (Fig. 3B). All SOM fractions explained 55% of the variance in the PLFA-related structure of the microbial community (Monte Carlo permutation test). Among all SOM fractions, the PON explained the largest significant variation in the community structure (18%) ($p = 0.04$).

3.3. Enzymes activities

Fertilizer application more significantly affected the βG and βX activities than BC amendment, accounting for approximately 80% of the variation (Table 1). The βG and βx activities for the BC-NPK and NPK treatments were 1.5 and 2.1 times higher than for the CK and BC treatments, respectively ($p < 0.05$; Table 1 and Fig. 4). Fertilization also increased the NAG activity, especially in combination with BC amendment ($p < 0.05$; Table 1 and Fig. 4). Similarly, the CBH activity was mainly increased by NPK fertilization (51.9%) (Table 1). The lowest CBH activity was observed after the application of only BC ($p < 0.05$; Table 1 and Fig. 4).

The LAP activity significantly differed from that of C-cycling enzymes, showing a different response of the N cycle to BC addition compared to the enzymes responsible for C. The application of BC contributed 70% of the variation in LAP activity, whereas the

Table 1
Results of two-way ANOVA analyses on the effects of biochar and NPK fertilization on soil chemical and biological parameters.

Source of variation/factors	Biochar		Fertilization		Interaction	
	F value	Contribution (%)[a]	F value	Contribution (%)	F value	Contribution (%)
SOM fractions						
SOC (g C kg^{-1})	**88.1**	88.2	0.01	0.02	0.13	0.14
DOC (mg C kg^{-1})	**8.89**	49.1	1.51	5.50	0.64	3.50
DON (mg N kg^{-1})	**17.8**	64.3	1.51	5.50	0.63	2.30
POC (g C kg^{-1})	**95.5**	92.8	0.21	0.20	0.89	0.86
PON (g N kg^{-1})	0.77	3.70	**13.1**	62.5	0.96	4.56
Microbial groups (nmol g^{-1})						
Total PLFAs	4.78	5.54	**66.9**	77.5	**8.06**	9.34
Bacterial PLFAs	4.49	4.10	**88.9**	81.2	**9.48**	8.66
Fungi PLFAs	4.82	8.99	**40.9**	75.4	0.58	1.09
G(+) PLFAs	0.98	0.64	**133.2**	87.7	**10.4**	6.86
G(−) PLFAs	3.7	11.8	**19.4**	61.9	2.09	6.65
Actinomycetes PLFAs	2.3	10.0	**10.5**	45.7	3.08	13.2
Soil enzymes (nmol g^{-1} h^{-1})						
βG	0.27	0.7	**31.5**	79.9	1.25	3.19
βX	0.36	0.68	**42.7**	80.7	3.25	6.14
CBH	**19.1**	17.8	**55.9**	51.9	**22.3**	20.8
NAG	0.9	2.33	**17.4**	45.2	**12.7**	33
LAP	**85.4**	70.0	**24**	19.6	**6.08**	4.98
Metabolic function						
AWCD	**14.2**	43.2	3.86	11.8	5.73	17.4
Amino acids	**33.6**	35.8	1.27	3.69	1.55	0.15
Amines	**59.6**	34.8	0.12	3.3	14.1	19.7

Significant contribution at $p < 0.05$ or $p < 0.01$ is shown in bold.
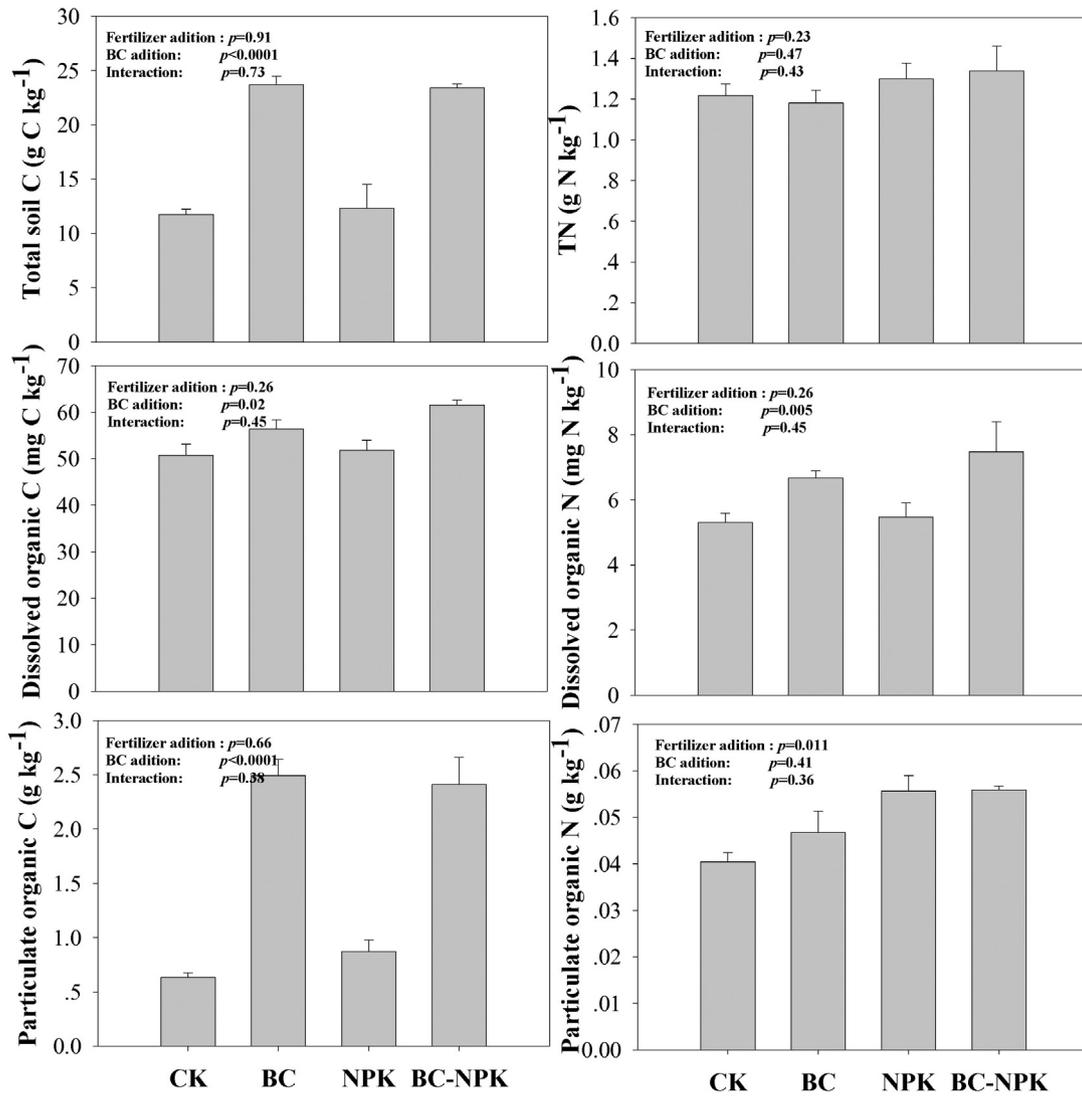[a] The contribution (%) means the percentage of each factor for explaining overall variance.

Fig. 1. Soil organic matter fractions with and without biochar addition and NPK fertilization. Error bars represent standard error of the means (*n* = 3); CK: control; BC: biochar addition; NPK: NPK fertilizer addition; BC-NPK: combined biochar and NPK fertilizer addition.

contribution of fertilizers only accounted for 19.6% of the variation (Table 1). The LAP activity increased in the following order: CK ≈ NPK < BC < BC-NPK (*p* < 0.05; Table 1 and Fig. 4).

3.4. Microbial metabolic functions

The BC amendment clearly affected the microbial metabolic functions (Table 1). The addition of BC (BC, BC-NPK) increased the total metabolic activity (indicated as AWCD) by 36.2–58.4% compared with the CK and NPK treatments, but it did not affect the metabolic diversity (indicated as Shannon-Wiener, H′) (Tables 1 and 2). Remarkably, BC addition increased the microbial utilization intensity of amino acids and amines (BC, BC-NPK) (*p* < 0.05; Tables 1 and 2).

4. Discussion

4.1. Effects of biochar and fertilizer addition on organic matter fractions

The higher soil total C content after BC application alone or combined with fertilization (BC-NPK) compared with CK and NPK application treatments (Table 1 and Fig. 1) confirmed the role of BC in contributing to C storage in soil (Lehmann et al., 2006; Demisie et al., 2014). Biochar is a very C-rich substrate, and it is also resistant to

decay due to its aromatic structure; e.g., only approximately 6% of the initially added BC was mineralized to CO_2 during the first 8.5 years of incubation (Kuzyakov et al., 2014). BC application, therefore, can increase C stock in paddy soil.

The addition of BC (BC, BC-NPK) increased the POC content (Table 1 and Fig. 1). Specifically, BC amendment may have directly contributed to the POC fraction in our study. A significant portion of the BC was associated with non-physically protected OM and OM occluded into aggregates after density fractionation (Llorente et al., 2010). Therefore, it is likely that a significant portion of BC is localized in the POM, thus resulting in higher content, especially for POC. Although we cannot conclusively prove this relationship because we did not extract BC from the POM in this study, the increased POC: PON ratio after BC addition indicated that a significant portion of BC might be localized in the POM fraction. POC is associated with nutrient cycling (Liebig et al., 2002), SOM sequestration (Carter and Gregorich, 2010), and the formation and stability of macro-aggregates in soil (Six et al., 2000). Our results clearly indicate that BC addition to soil may have a higher potential for improving soil quality. The higher DOC and DON concentrations in response to BC-NPK application compared with the CK and NPK only treatment (Fig. 1) may be attributed to both the indirect BC effect and the synergistic effects of BC and fertilization. Because the C originating from litter and humus constitutes a significant proportion of DOM (Kalbitz et al.,
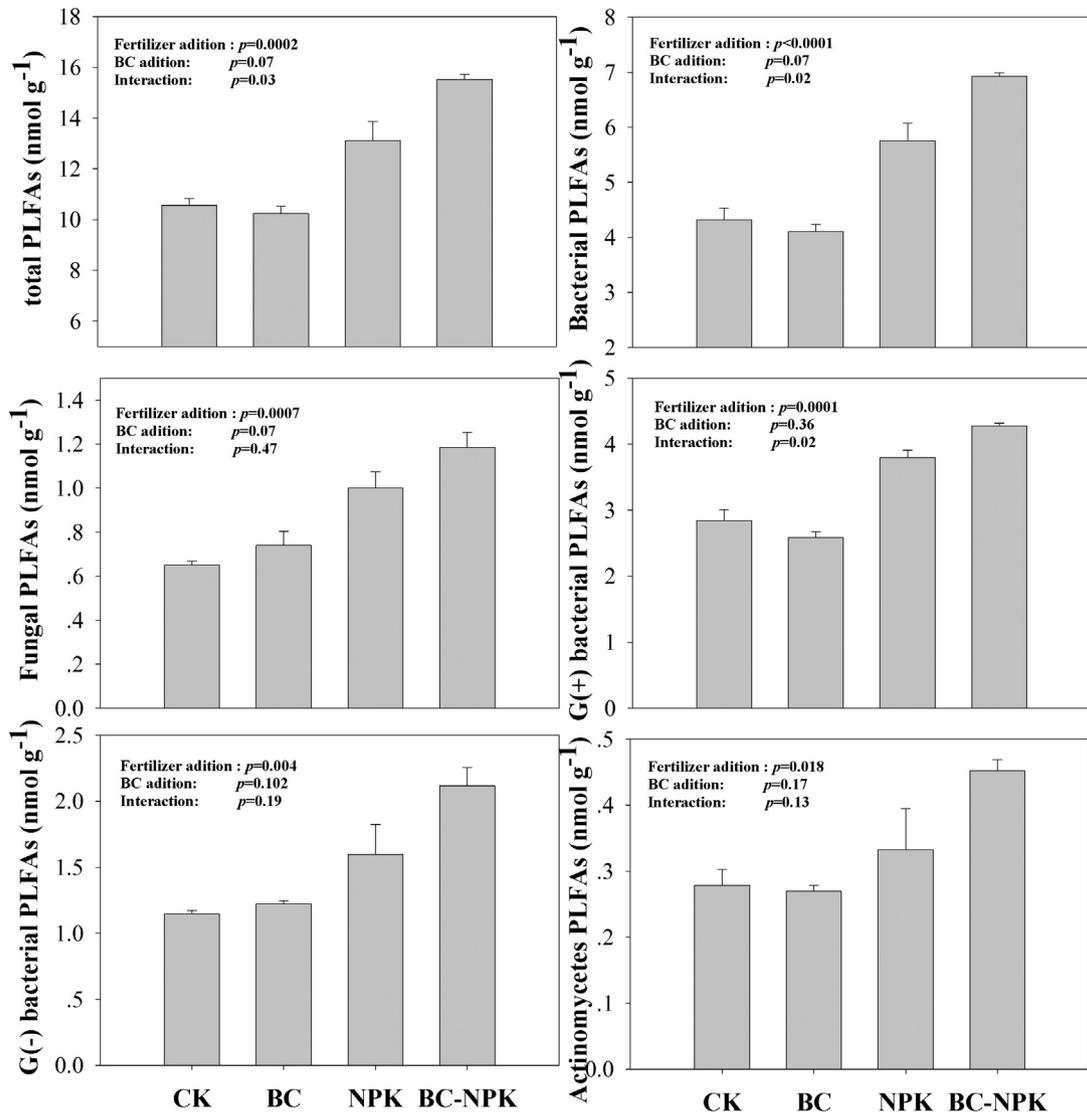
Fig. 2. Abundance of microbial biomarker groups with and without biochar addition and NPK fertilization. Error bars represent standard error of the means ($n = 3$); CK: control; BC: biochar addition; NPK: NPK fertilizer addition; BC-NPK: combined biochar and NPK fertilizer addition.
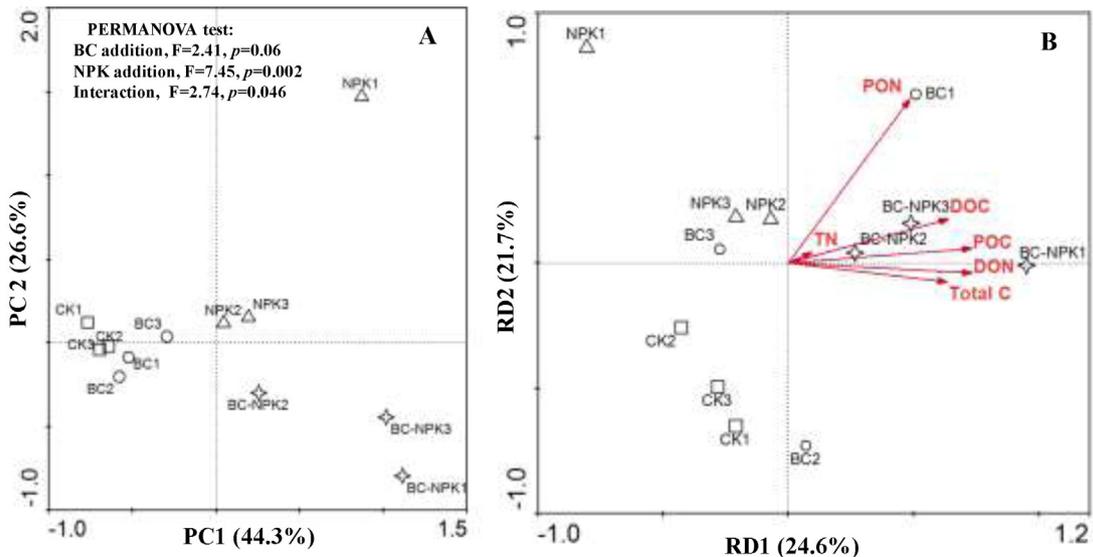

Fig. 3. Principal component analysis (PCA) of the soil microbial communities (A) and redundancy analysis (RDA) of the soil microbial communities constrained by soil SOM fractions (B).
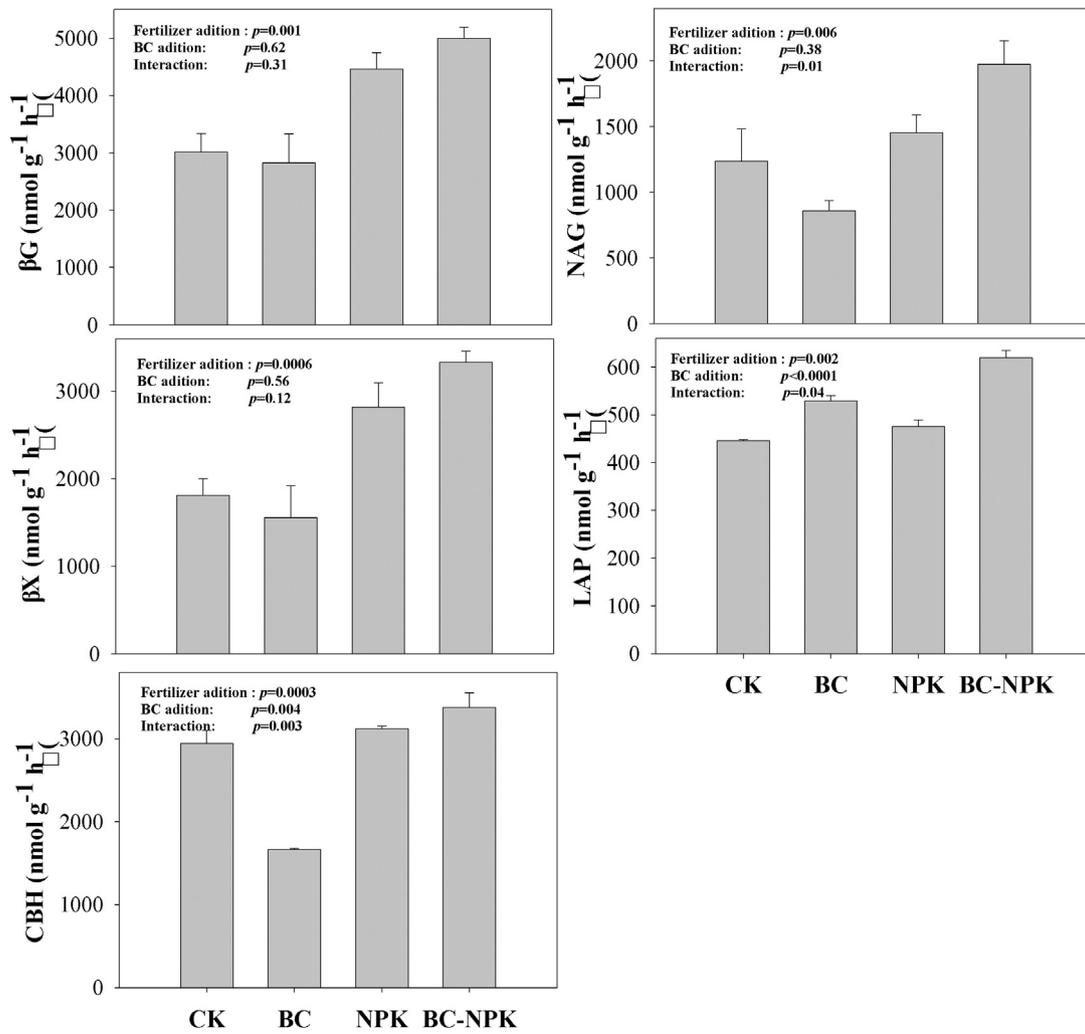
Fig. 4. Enzyme activities with and without biochar addition and NPK fertilization Error bars represent standard error of the means ($n = 3$); CK: control; BC: biochar addition; NPK: NPK fertilizer addition; BC-NPK: combined biochar and NPK fertilizer addition.

2000; Haynes, 2005), the increase in the DOC and DON may be partly attributed to the higher plant biomass or the larger roots biomass and exudation after BC and fertilizer amendment. Indeed, the aboveground biomass was significantly higher after BC-NPK amendment than after the other three treatments (7996 kg ha^{-1} vs. 5543, 5490, and 5623 kg ha^{-1}). According to our results, the combined application of BC and NPK (BC-NPK treatment) could be a promising sustainable agricultural management strategy than BC only addition. Specifically, BC and fertilization synergistically interact to accumulate and sequester C and enhance SOM cycling.

4.2. Effects of biochar and fertilizer addition on microbial community structure

Fertilizer was the main factor influencing microbial abundance and community structure (Table 1, Fig. 2 and Fig. 3A). This observation was supported by a meta-analysis that revealed a 15.1% increase in microbial biomass after mineral fertilizers application (Geisseler and Scow, 2014). Interestingly, compared with the CK, the addition of only BC did not change microbial abundance and community structure. However, BC altered the microbial abundances and community structure in

Table 2
Microbial metabolic activity (AWCD), Shannon–Wiener diversity (H′) and microbial utilization of C substrates with and without biochar addition and NPK fertilization.

Treatment	AWCD	Shannon–Wiener diversity (H′)	Intensity of substrates utilization					
			Carbohydrates	Carboxylic acids	Amino acids	Amines	Polymers	Miscellaneous
CK	0.77(0.04)	2.95(0.07)	6.60(1.59)	7.51(0.19)	4.24(0.40)	1.41(0.14)	3.39(0.36)	1.67(0.15)
BC	1.22(0.14)	3.05(0.03)	5.80(0.40)	7.96(0.78)	9.08(0.47)	2.72(0.11)	4.00(0.97)	1.70(0.46)
NPK	0.80(0.03)	2.90(0.04)	4.62(0.52)	6.64(1.11)	4.32(0.41)	1.87(0.14)	4.48(0.40)	1.84(0.06)
BC-NPK	1.09(0.18)	3.06(0.03)	4.77(0.25)	8.32(1.43)	7.45(1.40)	2.33(0.03)	3.63(0.59)	1.54(0.05)

Numbers in bracket represent standard error of the means ($n = 3$).
CK: control; BC: biochar addition; NPK: NPK fertilizer addition; BC-NPK: combined biochar and NPK fertilizer addition.

conjunction with NPK fertilization (BC-NPK) (Fig. 2, Fig. 3A, and Table 1). Previous studies reported an increase in bacterial abundance and altered microbial communities after BC addition under controlled laboratory conditions, which was attributed to the physic-chemical properties of BC (e.g., sorption, pH, chemical properties, habitats) (Anderson et al., 2011; Hale et al., 2015; Pietikäinen et al., 2000). However, such short-term positive effects of the physico-chemical properties of BC on microorganisms under controlled laboratory conditions were not as notable in our long-term field study. The altered microbial community under BC-NPK vs. BC can partly be attributed to N limitation for crops and microbial N immobilization caused by sole BC addition (Nelissen et al., 2012; Steiner et al., 2008). Inorganic fertilizer addition may reduce N limitation for crop and weaken plant-microbial competition for N, therefore improving crop growths and affecting microorganisms by direct mineral nutrients input and indirect crop effects under BC-NPK treatment. This agrees with previous studies that showed that higher plant biomass usually induced higher SOM fractions contents (e.g., particulate organic matter, rhizodeposition), which could act as major sources and energy for microorganism (Haynes, 2005; Tian et al., 2013). Higher abundances of microbial groups under BC-NPK conditions coincide with higher aboveground biomass in our field study. This response also corresponds to our finding that changes in PON were the main SOM fractions for influencing the microbial community (Fig. 3B). Previous reports showed that particulate organic matter was the primary source of mineral N (Zeller and Dambrine, 2011).

In addition to the main effects of fertilizer (by direct mineral nutrients input and indirect crop effect) on microorganisms, a synergic positive BC effect on microorganisms under BC-NPK treatment resulted in a 15.5% higher total microbial biomass and 17.0% higher bacterial biomass in BC-NPK vs. NPK treatment. This may be attributed to improved N fertilizer use efficiency by BC addition (Steiner et al., 2008), thus leading to better amplified fertilizer effects compared with only NPK addition. Similar to our study, Doan et al. (2014) observed a 59% greater bacterial abundance after combination of BC and inorganic N application compared with only mineral fertilizer addition in a 3-year mesocosm study. Thus, the hypothesized synergistic effects of BC and mineral fertilizers on microorganisms were confirmed by pronounced increase in microbial populations under BC-NPK treatment.

4.3. Effects of biochar and fertilizer addition on soil enzymes and metabolic microbial functions

In contrast to the increase in the peptidase activity (LAP) after only BC application, the activity of enzymes that decompose plant residues slightly (βG and βX) or significantly (CBH) decreased (Fig. 4). Furthermore, the decrease of βG and CBH activities by BC positively correlated with the BC application rate (Jin, 2010; Bailey et al., 2011). This decrease in the activity of C-cycling enzymes after BC application alone may be ascribed to the co-localization of C and microorganisms on the BC surfaces, which may improve the carbon use efficiency and reduce the need for enzyme production (Lehmann et al., 2011). It should be noted that the decreased enzymes activities (e.g. βG) could also be caused by BC sorption (Lammirato et al., 2011). However, a decrease in enzyme activity by mere sorption to biochar is less likely, as suggested by Lehmann et al. (2011). Nevertheless, it is important to consider reducing sorption-driven artificial phenomenon in order to most accurately report the activities of soil enzymes in the presence of BC in future studies. For example, Bailey et al. (2011) strongly recommend the use of fluorescence-based assays for measuring enzymes activities in BC-related studies.

Interestingly, the combined application of NPK and BC significantly increased the C-cycling enzyme activities compared with the application of BC alone, confirming our hypothesis. This finding may be attributed to increased microbial turnover induced by NPK fertilization, which over-compensates for the effect of BC-NPK treatment. Thus, sole application of BC may cause low soil C mineralization and nutrient

cycling; however, inorganic fertilizer amendment will compensate for this shortage.

Although the Shannon-Wiener index was not affected, BC application increased the metabolic activity, which affected the substrate utilization pattern. The altered substrate utilization pattern, as indicated by an increase in the utilization of amino acids and amines (Table 2), reflects that microbes mine the SOM for N to compensate for the high C: N ratio after BC amendment. Similarly, a 10% greater utilization of amino acids was observed after the addition of complex substrates (with high C:N) compared with the addition of simple compounds (with low C:N) (Orwin et al., 2006). Nevertheless, the accelerated utilization of N compounds caused by BC in our study (up to 50%) was much more pronounced than the 10% increase observed by Orwin et al. (2006). This was evidently because the complexity of the substrate directly correlates with the number of enzymes required for its degradation. Therefore, the utilization of the complex BC substrate required more N. Similar to our observations, the substrate use pattern of microorganisms changed after BC addition in a forest organic soil horizon (Pietikäinen et al., 2000), and these changes were attributed to the microbial community structure and enzyme activities. The latter is supported by our results because the altered substrate utilization pattern after BC amendment was mainly due to changes in the soil peptidase activity (LAP).

5. Conclusions

The addition of BC alone did not alter the structure of the soil microbial community, but it significantly increased the peptidase activity to accelerate the decomposition of soil proteins. Therefore, the BC management strategy may accelerate organic N turnover and consequently has important implications for N cycling in agricultural ecosystems. If BC application induces N limitation for crops and microbial N immobilization, NPK fertilization could compensate for these negative effects on the SOM and microbial turnover. Further studies are required to determine the trade-off in N uptake between microorganisms and crops after BC and NPK fertilization by using long-term field plots. Furthermore, future studies are necessary to investigate the impact of BC and fertilization on the dynamics of microbial community and functions and the interactions between SOM and the microorganisms.

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