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Consistent increase in abundance and diversity but variable change in community composition of bacteria in topsoil of rice paddy under short term biochar treatment across three sites from South China

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ABSTRACT

Biochar functionality related to soil microbial community changes has not yet been fully understood. In this study, we present a cross site field experiment on bacterial community changes of rice paddies among three sites (Jiangxi province, JX; Hunan province, HN; and Sichuan province, SC) from South China with biochar amended (BSA) at 0, 20 and 40 t ha^{-1} before rice plantation in 2010. Changes in bacterial abundance and diversity of topsoil (0-15 cm) sampled at rice harvest were assessed. Increases in soil pH, soil organic carbon, total N, soil microbial biomass, as well as bacterial gene copy numbers and diversity indices (phylogenetic diversity, Shannon, Chao1 and OTU richness) were consistently observed under BSA at 40 t ha^{-1}, though generally insignificant at 20 t ha^{-1} across the sites. Cluster analysis of both terminal restriction fragment length polymorphism (T-RFLP) profiles and pyrosequencing of the 16S gene indicated a strong impact of biochar on bacterial community composition, though the changes were variable across the sites. In particular, BSA at 20 and 40 t ha^{-1} greatly increased the relative abundance of Betaproteobacteria (by 54% and 80%) and Deltaproteobacteria (by 164% and 151%) in JX while decreased Betaproteobacteria (by 46% and 52%) and increased Chloroflexi (by 27% and 61%) in SC site, respectively. However, no significant changes were detected in HN site. In addition, some significant but variable changes were observed in the abundance of nitrifying, denitrifying and N-fixing bacteria groups with biochar addition among sites. This study suggested a potential role of biochar in enhancing bacterial abundance, community diversity and modifying the community compositions, particularly of the bacteria involved in N cycling. However, changes in soil microbial structure and functioning related to biochar treatment deserve further studies.

1. Introduction

Incorporating of biochar of crop residues via pyrolysis into croplands has been considered a promising option to enhance soil organic carbon (SOC) sequestration and sustain crop productivity (Lehmann, 2007; Sohi, 2012). In comparison to field burning of crop residues, this approach could allow better controlled and cleaner combustion, reduce CO_2 emission, improve the recycling of nutrients and offer renewable energy (Knoblauch et al., 2011; Clare et al., 2014). The role of biochar soil amendment (BSA) had been well addressed in reducing non-CO_2 greenhouse gas (GHG) emissions in a number of field experiments (Hammond et al., 2013; Liu X.-y. et al., 2012; Zhang et al., 2010, 2013). Being usually alkaline, high in negatively-charged surface area and rich in recalcitrant carbon with highly porous structure, biochar could generally enhance crop productivity (Jones et al., 2012; Major et al., 2010) by a single or a combination of liming, moistening, aggregating and possible nutrient enhancing effects (Liu et al., 2013, 2014). However, biochar's functionality related to soil biochemical process associated with microorganisms is still poorly understood (Lehmann et al., 2011).

Functioning and sustaining soil fertility is known to be governed largely by the activity of soil microorganisms (Anderson,

Abbreviations: BSA, biochar soil amendment; SOC, soil organic carbon; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; T-RFLP, terminal restriction fragment length polymorphism; qPCR, quantitative real-time PCR.

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2003). Changes in crop productivity and reduction of GHG emissions could be regulated by the changes in microbial communities. Constituting more than 90% of the total soil microbial biomass, bacteria together with fungi are the key regulators of soil organic matter dynamics and nutrient transformation (Six et al., 2006). Community diversity and activity of bacteria are considered more sensitive than other biota in response to soil condition changes such as pH, aeration and C and N status (Griffiths et al., 2001). Biochar amendment could alter both biotic and abiotic soil properties, which could potentially modify either microbial biomass or community composition or both in biochar amended soils (Lehmann et al., 2011). For example, increase in gram-negative bacterial biomass was observed in soils with addition of glucose and yeast derived biochar in a greenhouse experiment via phospholipid fatty acid analyses (Steinbeiss et al., 2009). With the help of nano-scaled electron microscopy, soil microorganisms were shown in close association with and/or colonizing on biochar particles in amended soil (Jin, 2010; Lehmann and Joseph, 2009). Moreover, improved crop productivity and soil quality with BSA could be related to an alteration in bacterial community composition and abundance (Lehmann et al., 2011; Graber et al., 2010; Kolton et al., 2011; Steiner et al., 2008). In particular, Terra preta, an anthropogenic dark earth rich in black carbon in the Amazon basin, has shown greater bacterial biomass and diversity than the adjacent soils which generally showed poor fertility (Kim et al., 2007). In a work by Graber et al. (2010), enhanced pepper plant development with BSA could be partly explained by the presence of some plant growth promoting and/or bio-controlling bacteria in the biochar-treated pots. Recently, there have been increasing observations of biochar induced systemic resistance (ISR) to pathogens of plants with plant growth-promoting rhizobacteria between plant roots in pot experiments (Elad et al., 2010; Meller Harel et al., 2012). However, robust changes in bacterial abundance and functional diversity under BSA have not yet been well addressed in field conditions.

Rice paddy soils were characterized by low carbon turnover with a dominance of anaerobic microbial communities under mostly anoxic conditions (Conrad and Klose, 1999; Lu et al., 2004). Covering approximately 20% of the world total irrigated croplands (Frolking et al., 2002), rice paddy soils of China are also important due to their high C storage and sequestration potential (Pan et al., 2003). BSA has been suggested to be effective in reducing N_2O emissions from rice paddies in field experiments across sites of China (Zhang et al., 2010; Liu X.-y. et al., 2012), but biochar's potential role to induce changes in microbial communities related to C and N cycling processes are still poorly understood. In particular, BSA even caused reduction in CH_4 emission from rice paddy soils (Feng et al., 2012; Liu et al., 2011), suggesting potential changes in functioning of taxa-specific soil bacterial/archaeal community in biochar-treated soils. Increased bacterial but declined fungal gene abundance with shifted community structure as a consequence of BSA amendment was also observed in a slight acid rice paddy from South China (Chen et al., 2013). So far, the consistence of these changes with the same biochar across soils has not yet been assessed.

In-depth understanding of the changes in soil bacterial community composition following BSA with powerful tools would be crucial for biochar application in agriculture. Next-generation sequencing technology, such as 454 pyrosequencing, is capable to massively deep-sequence microbial communities in environmental samples without the time-consuming cloning procedure (Sundberg et al., 2013), and has been widely used in microbial ecology studies in various environments including soils (Roesch et al., 2007) and sewage treatment systems (Zhang T. et al., 2012). In a study with biochar amendment in pot experiment, Anderson et al. (2011) assigned the T-RFLP peaks to a database obtained with 454 pyrosequencing. In their work, some specific groups of bacteria involved in N cycling processes were found positively influenced by biochar, including Bradyrhizobiaceae of the class Alphaproteobacteria, Hyphomicrobiaceae of the order Rhizobiales within the class Alphaproteobacteria and Streptosporangineae as a suborder of *Actinomycetales* within the phylum Actinobacteria. Using pyrosequencing of 16S rRNA fragments from root-associated bacterial communities also in a pot experiment, Kolton et al. (2011) reported an improved growth of sweet pepper with BSA due to an augment of a few genera-specific bacteria in the rhizophere (eg. *Flavobacterium* and *Chitinophaga* affiliated with phylum Bacteroidetes, possessing an arsenal of extracellular enzymes involved in organic matter mineralization). Therefore, these shifts in groups of bacterial community revealed by pyrosequencing technology could provide fundamental information of biochar's role in modifying soil microbial community abundance and composition, and thus mediating the biogeochemical cycling of C and N in amended soils.

The aim of the present study was to explore the potential impact of BSA on bacterial abundance, diversity and community composition in rice paddies and the consistency across sites and soil conditions. Here, a randomized block design with and without BSA was laid out across three sites to assess the effects of biochar, site and their interaction. Chemical and microbiological analyses were conducted on topsoil samples collected from rice paddies under BSA (0, 20 and 40 t ha^{-1}) after a whole rice growing season across three sites from South China. The impact of biochar on bacterial abundance and diversity was evaluated using quantitative real-time PCR (qPCR), T-RFLP and 454 pyrosequencing technologies.

2. Materials and methods

2.1. Sites and field experiment

A cross-site field experiment with BSA to rice paddy was conducted in the typical rice production areas of South China (Liu X.-y. et al., 2012). Included in this study, the three sites were respectively JX at the Experimental Farm of Jiangxi Institute of Red Soils (28°15′N, 116°20′E) in Jinxian County of Jiangxi Province, HN at Ganshan Township (28°08′N, 113°12′E) in Changsha Municipality of Henan Province, and SC at Xigao Township (31°03′N, 104°10′E) in Guanghan Municipality in Sichuan Province, of China. Site conditions and soil properties of the three rice paddies are listed in Table 1.

The biochar used in this study was produced in a biochar plant, with a business scale technology of continuous pyrolysis using a vertical kiln (Pan et al., 2011) at Sanli New Energy Company Henan, China. With this technology, wheat straw was continuously pyrolysed at 350–550 °C in a vertical kiln to produce tons of biochar in a day, which was aggregated and homogenized to a large biochar stock. Biochar material from the same stock was ground by machine to pass through a 2 mm sieve and used consistently across sites when the rice paddy field amendment was operated. The basic properties of biochar were measured of repeated samples of the stock before the field use and were widely reported in our previous studies (Zhang A. et al., 2012; Zhang et al., 2010, 2013; Liu X.-y. et al., 2012). On average, the biochar material contained on dry base organic carbon of 467 g kg^{-1}, total N of 5.9 g kg^{-1}, ash of 20.8% and had a surface area of 8.92 m^2 g^{-1} with a bulk density of 0.59 g cm^{-3} and pH (H_2O) of 10.42 as well as a cation exchange capacity of 21.7 cmol kg^{-1}.

The set-up and performance of the field experiment has been previously described in detail (Liu X.-y. et al., 2012). Briefly, biochar was amended at rates of 0 (C0, control), 20 (C1) and 40 (C2) t ha^{-1} before rice (*Oryza sativa* L.) transplantation in May 2010. The biochar material was spread to soil surface and tilled into a depth of approximately 0–12 cm by raking. Urea was applied for rice

Table 1
Site condition and basic soil properties of the topsoil from the studied rice paddies before biochar amendment.
Data of site condition from Liu X.-y. et al. (2012).

Site	MAT (°C)	MAP (mm)	Crop rotation	pH (H_2O)	SOC (g kg^{-1})	Total N (g kg^{-1})	BD (g cm^{-3})	Sand (%)	Silt (%)	Clay (%)
JX	17.7	1400	Double rice	4.89 ± 0.08	17.70 ± 0.33	1.59 ± 0.12	1.24 ± 0.03	42	38	20
HN	17.1	1500	Double rice	6.21 ± 0.04	18.76 ± 0.46	1.79 ± 0.02	0.91 ± 0.02	54	28	18
SC	16.5	807	Rice–wheat	5.99 ± 0.02	20.11 ± 0.55	1.81 ± 0.04	1.10 ± 0.02	52	32	16

MAT and MAP, mean annual temperature and precipitation; BD, bulk density.

season as N fertilizer at a total amount of 240 kg ha^{-1} in SC and HN sites, and 300 kg ha^{-1} in JX site. Rice production had been managed with local cultivars and conventional fertilization practices consistently across the treatments in a single site. Each treatment was conducted in an area of 4 m × 5 m with triplicates (n = 3), and laid out in a randomized block design. Details of N fertilization application and rice crop management were reported in Liu X.-y. et al. (2012).

2.2. Soil sampling and analysis

Topsoil samples at depth of 0–15 cm were collected from each plot after rice harvest in October 2010. Twelve soil cores (approximately 5 cm diameter) were taken from each plot with a stainless shave and thoroughly mixed to form one composite sample. Each composite sample was placed in a sterilized plastic bag, sealed and stored in an ice box before shipping to laboratory within two days. Visible plant fragments and gravels, if any, were manually removed prior to sieving through a 2 mm sieve. A portion was further air-dried at room temperature and passed through a sieve of 0.25 mm for chemical assays. A second portion was stored at 4 °C prior to microbial biomass C and N analysis. The remaining portion was stored at −70 °C prior to DNA extraction.

All analyses of soil properties were performed following the procedures described by Lu (2000). Soil moisture content was measured by oven-drying to constant weight at 105 °C for 24 h. Soil pH was determined with a soil to water ratio of 1:2.5 using a precision pH meter (Mettler Toledo Seveneasy, Switzerland). Soil organic carbon (SOC) content was determined by wet digestion with $K_2Cr_2O_7$ oxidation. Total nitrogen (TN) was analyzed with semi-Kjeldahl method. Topsoil bulk density was measured while sampling in field, with a steel cylinder of 100 cm^3 in volume. The chloroform fumigation–extraction protocol with extraction by K_2SO_4 solution described by Vance et al. (1987) was employed for soil microbial biomass C (MBC) and N (MBN) determination using a 15 g oven-dry equivalent field-moist soil sample. The MBC and MBN were calculated using a k_{EC} factor of 0.45 (Wu et al., 1990) and a k_{EN} factor of 2.22 (Brookes et al., 1985), respectively.

2.3. DNA extraction and real-time PCR assay of bacterial abundance

Two DNA extractions from 0.5 g soil of each sample were performed with the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc., CA) according to the manufacture's instruction, with the following minor modifications: the bead tubes were held in a MoBio Vortex Adapter tube holder and vortexed at maximum speed (3200 rpm) for 15 min to ensure the detachment and break down of the cells from biochar aggregates (Chen et al., 2013). The two DNA extracts, i.e., technical replicates of the soil samples, were then pooled prior to analysis. The concentration of double stranded DNA in each sample was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The DNA concentration of each sample was adjusted to 10 ng μl^{-1} and used as template for subsequent PCR amplification.

To estimate the bacterial population size, quantitative real-time PCR (qPCR) assay was performed using universal eubacterial 16S rRNA gene primers 338F and 518R described by Fierer et al. (2005). The bacterial 16S rRNA gene copy numbers were determined in triplicates following previously described protocols (Liu Y. et al., 2012). Based upon flourometric monitoring with SYBR Green I dye by an iCycler IQ5 Thermocycler (Bio-Rad Hercules, CA), high amplification efficiency of quantification of 99%, with a R^2 value of 0.992 and a slope of −3.337 was obtained in this study. A melting curve analysis was conducted following each assay to confirm specific amplification. A 2% agarose gel electrophoresis was further performed on the PCR amplification products and blanks to check whether an appropriate size was achieved. Standard curve was created using tenfold dilution series of plasmids containing the eubacterial 16S rRNA gene from soil samples. The number of copies for each sample was calculated from the concentration of extracted plasmid and expressed on the basis of dry soil.

2.4. T-RFLP analysis of bacterial community

For T-RFLP analysis, primers 8F (Amann et al., 1995) (labeled with 6-carboxyfluorescein (6-FAM) at the 5′ end) and 926R (Muyzer et al., 1995) were used to amplify bacterial 16S rRNA gene fragments. The reaction mixture contained 25 μl Go Taq® Green Master Mix (Promega, Madison, WI), 1 μl of 10 μM each primer, 22 μl of sterile ddH_2O and 1 μl of DNA template. Soil DNA from each sample was amplified using an Eppendorf Gradient Cycler (Eppendorf, Hamburg, Germany) with the following program: 5 min of incubation at 94 °C, followed by 35 cycles of 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min 30 s, and a final extension step at 72 °C for 10 min. For each sample, the PCR products of two reactions were pooled and run on 2% agarose gel to test their quality prior to purification with PCR Fragment Purification Kit (TaKaRa Biotech, Dalian, China). For T-RFLP analysis, approximately 200 ng purified amplicons were digested using restriction enzymes *Msp*I (TaKaRa Biotech, Dalian, China). Fragment analysis was achieved by capillary electrophoresis (3730 Genetic Analyzer, Applied Biosystems, CA), using a GeneScan ROX-labeled GS500 internal size standard. All T-RFLP GeneScan reads were repeated in duplicates. True terminal restriction fragments (T-RFs) were determined and aligned to the nearest integer with T-REX as described by Culman et al. (2009). The relative abundance of a true T-RF within a given T-RFLP pattern was generated as a ratio of the respective peak area. Besides, T-RFs having a proportion below 1% and size below 50 bp were excluded from subsequent analyses.

2.5. PCR amplification and pyrosequencing

PCR amplification for pyrosequencing was conducted using the primers 515F (*CGTATCGCCTCCCTCGCGCCATCAG*<u>NNNNNNN</u>GTGCCAGCMGCCGCGG) and 926R (*CTATGCGCCTTGCCAGCCCGCTCAG*<u>NNNNNNN</u>CCGTCAATTCMTTTRAGTTT), in which the italicized sequence is that of a 454 Life Sciences® primer and the underlined sequence is a barcode sequence tag. The PCR reaction mixture consisted of 1 × PCR buffer (Mg^{2+} Plus), 0.2 mM dNTP mixture, 1 U TaKaRa Taq HS polymerase (TaKaRa Biotech, Dalian, China), 0.5 μM each primer, 1 μl of 10-fold diluted DNA sample in a final volume of 50 μl reaction. PCR reaction was carried out in a thermal cycler

(Bio-Rad Laboratories, Hercules, CA) using the following program: 5 min of incubation at 94 °C, followed by 35 cycles of 94 °C for 1 min, 55 °C for 45 s, and 72 °C for 45 s, with a 7 min extension at 72 °C. The obtained PCR products were checked on 1.5% agarose gel to ascertain the specificity of bacterial 16S rRNA gene amplification with a size of approximately 420 bp. The appropriate fragments were cut and purified with Agarose Gel DNA Extraction Kit (TaKaRa Biotech, Dalian, China). The concentrations of the purified fragments were then measured via spectrometry (NanoDrop Technologies, Wilmington, DE). Finally, the amplicons from different samples were sent out for pyrosequencing on the Roche 454 FLX Titanium platform (Roche) at the University of Tongji, Shanghai, China.

The obtained sequences from the 27 samples were demultiplexed according to their unique 6 bp barcodes and processed using QIIME version 1.7.0 (Caporaso et al., 2010a) according to the guideline of standard operation procedure described at http://qiime.org/tutorials/tutorial.html. Primers and barcodes were removed before the raw reads were quality filtered. Sequences were removed if they had long homopolymeric regions (>6 nt), were smaller than 200 nt and had average quality scores lower than 25, or if they were identified as being chimeric. The high quality sequences were then clustered into operational taxonomic units (OTUs) at a 97% sequence identity cut-off by default, and consensus sequences were chosen for each OTU. The representative OTU sequences were aligned with the Python Nearest Alignment Space Termination (PyNAST) (Caporaso et al., 2010b) and taxonomic identity was assigned with the RDP classifier from genus to phylum at hierarchical levels (Wang et al., 2007). Diversity indices including the observed OTU richness, the Chao1 index (Chao and Bunge, 2002), the phylogenetic diversity (PD) index (Faith, 1992) and the Shannon index (Hill et al., 2003) were also calculated using QIIME software. Rarefaction curves were generated to reveal the alpha diversity based on these two metrics: the observed species metric is simply the count of unique OTUs found in the sample, and Shannon index. To correct for survey effort, we used a randomly selected subset of 3000 sequences per soil sample to compare relative differences between samples.

2.6. Data processing and statistical analysis

The presentation and treatment of all the measurement data were processed with Microsoft Excel 2003, and the results were expressed as the means ± S.D. of the three replicates. A similarity matrix (Bray–Curtis method) of T-RFLP profile was constructed based on the T-RFs observed across all soil samples and their relative abundances in individual samples using PRIMER (Clarke and Warwick, 1998). The hierarchical clustering was in turn constructed based on the similarity matrix and the unweighted pair-group methods with arithmetic means (UPGMA) were used to portrait the type of dendrogram. Cluster analysis of the bacterial community obtained with pyrosequencing was conducted using PRIMER software to elucidate microbial community structures based on a similarity matrix generated using the Bray–Curtis method with the relative abundance of phyla and proteobacterial classes. Statistical analysis to evaluate the effects of the biochar treatments on the measured biochemical parameters of the three sites was conducted by two-way ANOVA following the general linear model (GLM) procedure using the Tukey HSD pairwise multiple comparison test at alpha level of 5% with SPSS version 16.0 software.

2.7. 16S rRNA gene amplicon pyrosequencing data accession number

The pyrosequencing-generated raw reads of bacterial 16S rRNA gene in rice paddies in this study have been deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under project accession number SRP029423.

3. Results

3.1. Soil properties and bacterial gene abundance

Basic soil properties were different among the sites (Table 1). Soil from site JX was a clay loam and the other two were sandy loam in soil texture. SOC and total N content was higher in SC and HN soils than in JX, respectively. Original soil pH was in an order of HN > SC > JX. However, after BSA, there were significant changes in pH, SOC and total N contents of soil across sites (Table 2). There were consistent increases in SOC (by 50–55%) and total N (by 4–10%) but decreases in bulk density (by 3–10%) both under C1 and C2 treatments across sites. Under BSA, increase in soil pH was seen slight (by ~2%) under C1 treatment but significant (by over 5%) under C2 treatment across sites. In detail, BSA at 40 t ha^{-1} (C2) caused a significant increase over control (C0) in pH by 0.24, 0.28 and 0.72 in JX, HN and SC, respectively.

In line with the changes in soil properties, changes in microbial biomass carbon and nitrogen were observed under BSA treatments. Compared to C0, both C1 and C2 treatments exerted much higher contents of soil MBC and MBN in HN and SC soils, except in JX site. Bacterial 16S rRNA gene copy numbers ranged from 1.2×10^{10} to 2.6×10^{10} copies g^{-1} dry soil in the three sites, and were greatly increased under BSA at 40 t ha^{-1} by 45%, 37% and 60% in JX, HN and SC over control, respectively. As revealed by the GLM analysis, both BSA and site condition affected significantly soil physicochemical properties, MBC, MBN and bacterial gene copy number ($p < 0.01$) (Table 2). Pairwise multiple comparisons further showed that both C1 and C2 were significantly different to C0 for SOC, total N, bulk density, MBN and gene copy numbers, while significant differences between C1 and C2 were also observed for SOC and bacterial gene copy number. Besides, BSA and site condition had significantly ($p < 0.05$) interactive effects on both pH and MBC (Table 2). Effects of biochar on pH and MBC were enhanced particularly in SC site but weakened in JX site.

3.2. T-RFLP of bacterial community

In this study, the *Msp*I enzyme used for digestion of bacterial 16S rRNA gene fragments gave a total of 55 unique terminal restriction fragments (T-RFs). On average, 12 of the 55 T-RFs occupied 57.3% of the total fragment abundance of each profile. Most of the T-RFs exhibited insignificant changes under BSA in the relative abundance. While the numbers of T-RFs varied among sites (ranging from 24 to 33) (Supplementary Fig. 1), there was a slight increase under BSA in the number of T-RFs across the sites. For example, T-RFs of 128 bp and 281 bp in JX site, 263 bp and 508 bp in HN site, T-RFs of 159 bp, 179 bp, 427 bp, 437 bp and 444 bp in SC site appeared only in biochar-amended soils. Most of the above T-RFs showed a relative small proportion (less than 2.5%) to the total abundance. As indicated by the dendrogram based on the Bray–Curtis similarity index (Fig. 1), bacterial communities were related stronger within individual sites than between biochar treatments (the similarity between the group averages of JX and of the two soils of HN and SC was 43% only) (Fig. 1), indicating a major control by site/soil conditions on bacterial community composition of rice soils. The cluster analysis also showed similar bacterial community structure among replicates, confirming the reproducibility of our experimental setup. Moreover, the community structure under BSA showed a slight divergence from that under non-biochar amendment in a single site.

Table 2
Selected physicochemical properties, MBC, MBN and bacterial gene copy number of topsoil under biochar treatment in the three rice paddies.

Site	Treatment	pH (H_2O)	SOC ($g\,kg^{-1}$)	Total N ($g\,kg^{-1}$)	Bulk density ($g\,cm^{-3}$)	MBC ($mg\,kg^{-1}$)	MBN ($mg\,kg^{-1}$)	Gene copy number ($\times 10^{10}\,g^{-1}$ dry soil)
JX	C0	4.88 ± 0.05	16.48 ± 0.54	1.83 ± 0.02	1.19 ± 0.02	338.86 ± 18.82	20.27 ± 7.59	1.86 ± 0.09
	C1	4.99 ± 0.13	19.29 ± 0.66	1.89 ± 0.02	1.09 ± 0.07	336.775 ± 3.75	42.63 ± 5.37	2.08 ± 0.17
	C2	5.12 ± 0.01	25.43 ± 2.89	1.89 ± 0.04	1.11 ± 0.07	321.703 ± 6.85	32.52 ± 3.15	2.70 ± 0.33
HN	C0	6.01 ± 0.06	18.05 ± 1.20	1.65 ± 0.06	0.87 ± 0.04	397.86 ± 7.37	26.98 ± 6.61	2.60 ± 0.23
	C1	6.15 ± 0.02	23.03 ± 1.42	1.79 ± 0.09	0.81 ± 0.03	481.40 ± 37.69	41.17 ± 2.91	3.32 ± 0.24
	C2	6.29 ± 0.15	28.11 ± 2.14	1.73 ± 0.09	0.79 ± 0.03	483.25 ± 31.04	53.06 ± 11.01	3.56 ± 0.14
SC	C0	5.54 ± 0.23	20.14 ± 0.29	1.80 ± 0.06	1.09 ± 0.01	602.67 ± 52.21	50.47 ± 3.00	1.31 ± 0.06
	C1	5.89 ± 0.05	22.52 ± 2.77	1.87 ± 0.02	1.07 ± 0.04	750.76 ± 78.85	62.35 ± 7.15	1.69 ± 0.20
	C2	6.26 ± 0.17	30.22 ± 3.98	1.98 ± 0.06	1.06 ± 0.01	750.22 ± 46.07	60.41 ± 9.72	2.10 ± 1.22
Biochar[a]		***	***	**	**	**	***	***
Site[a]		***	**	***	***	***	***	***
B × S[a]		*	NS	NS	NS	*	NS	NS

NS, not significant ($p > 0.05$).
[a] Significance levels for two-way ANOVA with biochar amendment and site as main effects.
* $p < 0.05$ significant levels.
** $p < 0.01$ significant levels.
*** $p < 0.001$ significant levels.

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3.3. Tag-encoded amplicon pyrosequencing

To examine the robust changes in bacterial community compositions of the rice paddies under biochar amendment, a 454 pyrosequencing assay was performed. Via the QIIME pipeline processes, a total of 324,850 quality sequences (ranging from 3063 to 37,718 of a single soil sample without subsampling) were obtained, with an average of 12,031 sequences per sample and an average read length of 376 bp across the total 27 treatment plot samples. Following the RDP Classifier (Wang et al., 2007), the quality sequences with the same number (3000, subsampled) were assigned to taxonomical hierarchy. More than 99% of total obtained bacterial sequences were identified, of which 9–18% was returned as unclassified. When grouped at a 97% similarity level, there was a total of 27,838 operational taxonomic units (OTUs) in the complete data set.

As shown in Table 3, BSA had a significant ($p < 0.001$) effect on the bacterial alpha diversity. Tukey HSD pairwise multiple comparisons further showed that all the diversity indices both under C1 and C2 treatments were significantly ($p < 0.05$) higher over control, while Chao1 and OTU richness was both significantly higher under C2 treatment than C1 treatment. Biochar amendment, particularly at 40 $t\,ha^{-1}$, remarkably increased the bacterial diversity (PD and Shannon index) and richness (Chao1 and OTU richness), while the site effect was significant only on PD and Shannon index. Notably, the values of PD in JX and Shannon index in HN were significantly ($p < 0.05$) higher than in the other two sites as indicated by pairwise multiple comparisons. However, no biochar and site interaction effect was observed on all the four diversity indices. Soils under BSA exhibited a higher bacterial community diversity, which was further evidenced by the rarefaction at 0.03 distance of each sample using the same number of sequences from a single site (Fig. 2), where soils under BSA exhibited higher numbers of observed OTUs than the non-amended soils.

The relative abundance of bacteria under a single treatment was examined at the levels of phylum, class and genus based on the quality sequences. In total, 24 phyla, 63 classes, 173 families and 529 genera were represented by all the 27 samples. The most frequent phyla or proteobacterial classes for all samples were Acidobacteria, Chloroflexi, Betaproteobacteria and Deltaproteobacteria (Supplementary Fig. 2), indicating a predominance of these groups in the rice paddies. Though most of the phyla or

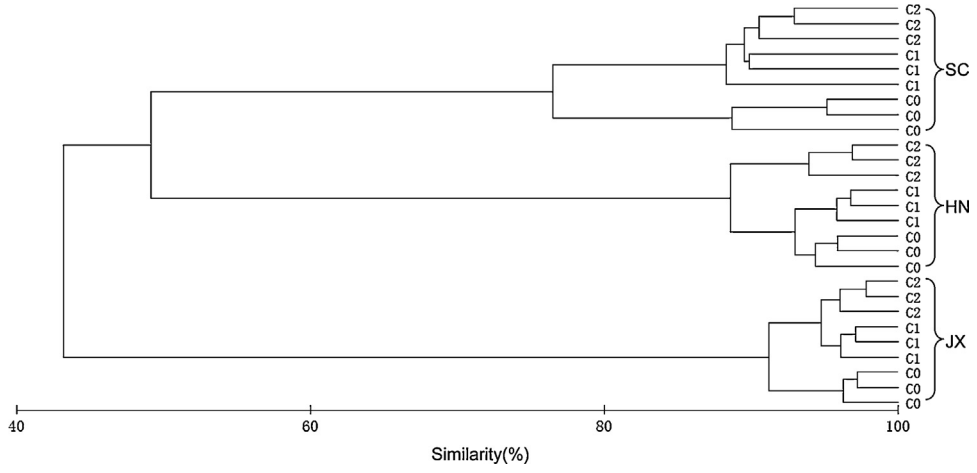

Fig. 1. Cluster analysis of T-RFLP profiles of amplified 16S rRNA gene fragments of bacterial community in biochar-amended (C1 and C2) and non-amended (C0) rice paddy in three sites (JX, HN and SC). Similarity matrix was calculated from the relative abundance of T-RFs in individual samples and the samples were clustered with a UPGMA algorithm using PRIMER software.

Table 3
Alpha diversity at depth of 3000 sequences of bacteria of topsoil from the rice paddies under biochar treatment.

Site	Treatment	Phylogenetic diversity (PD)	Shannon index	OTU richness	Chao1
JX	C0	162 ± 5	9.51 ± 0.08	1496 ± 67	5307 ± 705
	C1	185 ± 10	9.94 ± 0.16	1715 ± 94	7225 ± 989
	C2	187 ± 7	10.04 ± 0.14	1755 ± 80	7457 ± 808
HN	C0	147 ± 6	9.94 ± 0.04	1628 ± 62	5357 ± 829
	C1	155 ± 9	10.12 ± 0.09	1736 ± 82	6108 ± 761
	C2	166 ± 2	10.28 ± 0.01	1861 ± 18	7450 ± 317
SC	C0	136 ± 11	8.98 ± 0.74	1488 ± 142	5018 ± 694
	C1	153 ± 6	9.99 ± 0.11	1718 ± 31	6133 ± 183
	C2	166 ± 10	10.23 ± 0.20	1851 ± 54	7191 ± 282
Biochar[a]		***	***	***	***
Site[a]		***	*	NS	NS
B × S[a]		NS	NS	NS	NS

NS, not significant ($p > 0.05$).
[a] Significance levels for two-way ANOVA with biochar amendment and site as main effects.
* $p < 0.05$ significant levels.
*** $p < 0.001$ significant levels.

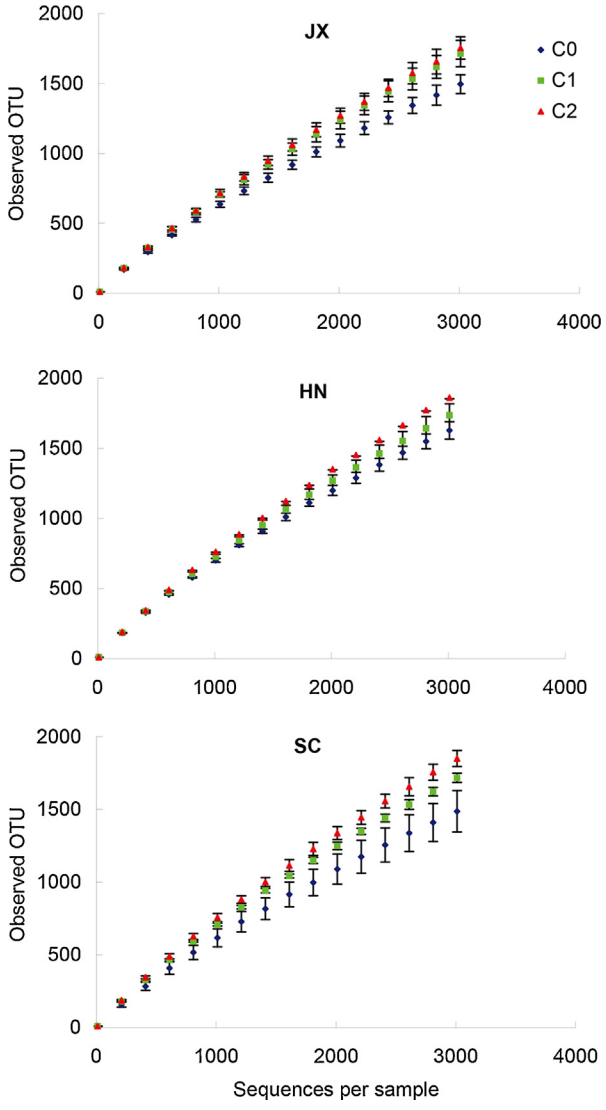

Fig. 2. Rarefaction analysis at 3% dissimilarity level under treatments of C0, C1 and C2 using sequences with the same number in a single site.

proteobacterial classes showed no significant changes with the BSA treatments in a single site, some significant changes in the community composition were still noted under BSA (Supplementary Fig. 2). Two-way ANOVA showed significant changes in the abundances of the altered bacterial phyla and proteobacterial classes across sites though variable with biochar amendment rates (Table 4). There was no significant effect of BSA on the groups of Chloroflexi, Betaproteobacteria, Deltaproteobacteria and Gemmatimonadetes. Clearly, site exerted the strongest influence on the bacteria groups followed by biochar/site interaction (Table 4). In detail, Alphaproteobacteria was significantly increased by 123% under C2 treatment compared to C0 in SC, while Nitrospirae and Actinobacteria were increased by 94% and 269% under C2 treatment over C0 respectively in JX and SC. Whereas, the mean relative abundance of Chloroflexi was decreased by 8% but increased by 64% under C2 treatment compared to C0 in JX and SC site, respectively. In contrast, the relative abundances of Betaproteobacteria were increased by 54% and 80% in JX but greatly decreased by 46% and 52% in SC under C1 and C2 over the controls, respectively. Varying greatly between the sites, the relative abundances of Deltaproteobacteria were increased by 164% and 151% under C1 and C2 over the control in JX, respectively. Furthermore, biochar amendment also significantly influenced the relative abundance of Spirochaetes ($p < 0.01$) and Bacteroidetes ($p < 0.05$) though there were no significant differences between C1 and C2.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2015.02.012.

The dendrogram resulting from the cluster analysis of pyrosequencing data showed that the samples clustered according to their sites at a 61.73% similarity between the group average of JX against the group averages of the other two soils of HN and SC sites (Fig. 3), which was much higher than that from the results of T-RFLP. This indicated a greater difference between the sites than between biochar treatments in bacterial composition. Besides, the SC site showed a higher variable community structure than the other two sites, which was indicated by the low similarity (73.16%) between the group average of C0 and the amended soils (C1 and C2). Nevertheless, the dendrogram also showed that the three replicates of C0 in JX site were grouped together and differed from the biochar-amended samples (C1 and C2), demonstrating an influence of biochar on bacterial community composition. However, such effect was not clearly seen in the other two sites.

Table 4
Frequency of the altered bacterial phyla and proteobacterial classes indicated in % of all classified sequences with biochar treatment in the studied soils.

Site	Treatment	Chloroflexi	Alphaproteobacteria	Betaproteobacteria	Deltaproteobacteria	Nitrospirae	Actinobacteria	Gemmatimonadetes	Bacteroidetes	Spirochaetes
JX	C0	19.81	2.96	3.02	2.24	0.71	3.18	1.10	0.57	0.04
	C1	15.51	4.00	4.67	5.93	1.54	1.98	0.72	1.32	0.36
	C2	18.18	3.67	5.43	5.63	1.38	2.53	0.84	1.34	0.34
HN	C0	14.54	4.66	10.68	19.16	3.49	1.32	0.58	3.22	0.37
	C1	14.78	4.98	10.71	17.70	3.23	1.74	0.66	2.80	0.41
	C2	14.90	4.89	12.56	16.78	2.92	1.47	0.59	4.20	0.50
SC	C0	10.12	2.58	32.01	8.27	1.93	1.33	1.49	1.69	0.09
	C1	12.83	2.72	17.17	9.07	2.56	1.30	2.41	2.79	0.16
	C2	16.34	5.74	15.38	9.06	1.52	4.92	1.54	1.50	0.06
Biochar[a]		NS	**	NS	NS	**	***	NS	*	**
Site[a]		***	**	***	***	***	***	***	***	***
B × S[a]		NS	*	**	**	**	***	*	**	**

NS, not significant ($p > 0.05$).
[a] Significance levels for two-way ANOVA with biochar amendment and site as main effects.
* $p < 0.05$ significant levels.
** $p < 0.01$ significant levels.
*** $p < 0.001$ significant levels.

The most frequent classified genera of the soil samples under BSA treatments were shown in Table 5. All the listed genera varied significantly among the three rice paddies, which was emphasized by the results of the ANOVA, whereas biochar had a less effect on them as only 5 of 19 genera showed significant changes under BSA. Significant differences, such as Gp1, Gp2, *Rhizomicrobium* and *Geobacter*, were found in relation to biochar treatments, sites and site/biochar interactions (Table 5). Gp1 (belonging to *Acidobacteria*), the most dominant genus in JX site followed by *Ktedonobacter* and Gp3, was significantly ($p < 0.05$) affected by BSA at C2, and showed a great reduction by 35% and 44% compared to their controls in JX and SC sites, respectively. The relative abundance of *Aminomonas* was also remarkably decreased by 75% over the control under BSA at C2 in JX. In contrast, the relative abundances of Gp2 and *Geobacter* were significantly increased by over 5-folds under BSA at C2 in JX. Nevertheless, the majority genera showed insignificant changes with BSA across the sites.

Some known functional bacterial genera (Uroz et al., 2010) are shown in Table 6. Again, site condition showed a significant effect on the functional bacterial genera. The soils in this study contained a majority of nitrifying bacteria, such as *Nitrosococcus*,*Nitrosospira* and a large number of *Nitrospira*. Biochar amendment significantly ($p < 0.05$) affected *Nitrosospira* abundance. Significant higher abundances of *Nitrosospira* were found under BSA at C2 compared to C0 across the three sites, but no significant differences between C1 and C0 were observed by pairwise multiple comparisons. Notably, striking increases by 7.5 and 6.5 folds in *Geobacter* were also observed under BSA under both C1 and C2 in JX site, respectively. Nitrogen-fixing bacteria *Bradyrhizobium* was also observed to increase under C2 by 228%, 137% and 24%, respectively, in JX, HN and SC site. Significant increases in *Rhizomicrobium* were also noted in both JX and HN sites.

4. Discussion

4.1. Effect of biochar amendment on soil bacterial abundance

Soil type or site conditions are well known as the determinant factors for changes in soil microbial abundance and community structure, with soil pH as principal parameter (Girvan et al., 2003; Berg and Kornelia, 2009). In this study, there were great variations (CV in range of 10–15%) of soil pH, bulk density and soil texture but slight variations (CV < 8%) of SOC and total nitrogen across sites (Table 1), which could result in a great difference in soil microbial response to biochar between the sites. As shown by the two-way ANOVA analysis, soil microbial properties (MBC, MBN and bacterial gene copy numbers) were strongly affected by site conditions despite of an insignificant interaction of site and biochar factors (Table 2). Nevertheless, consistent increases were observed in microbial biomass carbon (MBC) and nitrogen (MBN) under BSA at 40 t ha^{-1} in the rice paddies across sites, except for MBC in JX, in this study. Moreover, bacterial gene copy numbers were consistently increased under BSA by 25–60% across sites. This observed positive effect of biochar on soil microbial community growth in rice paddies is in agreement with reports of non-paddy soils (Ameloot et al., 2013; Kolb et al., 2009; Liang et al., 2010). Significant and consistent increases across sites in bacterial 16S rRNA gene copy numbers determined by qPCR further supported an enhanced growth of soil bacterial community since bacteria were the most abundant and diverse group of soil microorganisms, being sensitive to soil pH changes (Gans et al., 2005). Such increase in microbial biomass was also reported in a single soil under short term incubation of both swine manure and willow wood biochars (Ameloot et al., 2013). Similarly, increases in microbial biomass and activity with increasing biochar addition were well characterized

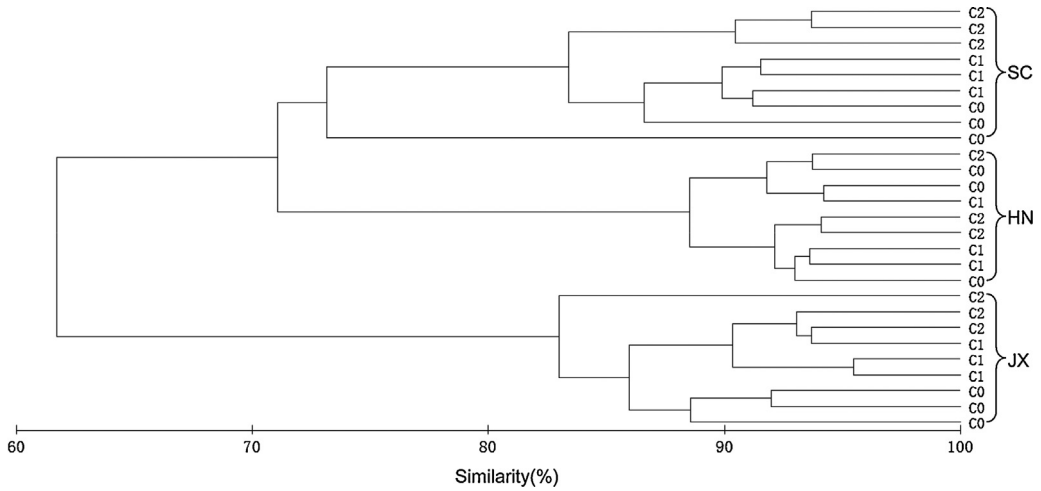

Fig. 3. Cluster analysis of bacterial communities based on abundance data of phyla and proteobacterial classes in the biochar amended and non-amended soils over the three sites.

by Kolb et al. (2009) across four distinct temperate soils (Mollisol, Alfisol, Entisol and Spodosol).

Increased microbial biomass and bacterial abundance could relate to the increased soil organic matter, increased soil pH and/or mixed benefits with improved soil microhabitats as discussed in our previous study (Chen et al., 2013). Here, the relative increase over the control in bacterial gene copy numbers in a single soil was positively correlated with the relative increase in pH ($r = 0.84$, $p = 0.04$) and in SOC ($r = 0.75$, $p = 0.08$) under BSA. This could indicate improved microhabitats with BSA as a neutral reaction favoring bacterial growth and porous biochar particles stimulated bacterial colonization (Kasozi et al., 2010; Pietikäinen et al., 2000; Thies and Rillig, 2009). In line, Lehmann et al. (2011) reported positive effects of biochar on abiotic and biotic soil properties.

Compared to gene copy numbers, the changes in MBC and MBN with boichar in this study were less significant and consistent. Significant decreases in MBC had been reported in coarse textured soil with *Eucalyptus* biochar (Dempster et al., 2012) and in a rice soil with bamboo biochar (Liu et al., 2011). When a large amount of biochar was added, there could occur potential sorption of lysed cells to biochar particles. As a result, enhanced microbial biomass carbon would not be easily measured using a fumigation–extraction procedure, raising an issue of underestimation of microbial biomass (Durenkamp et al., 2010). This could explain, in part, the divergent results of MBC with varying biochar additions. Liang et al. (2010) argued that the recovery of microbial biomass was 21–41% lower in biochar-rich Terra preta soils than in biochar-poor adjacent soils based on the recovery of ^{13}C-labeled microbial biomass added to the soils. This study provided evidence that BSA could enhance soil bacterial growth in rice paddy soils, however the mechanism behind is still not clear.

4.2. Effect of biochar amendment on soil bacterial diversity

As commonly hypothesized, microbial diversity is playing a key role in influencing ecosystem stability, productivity and resilience towards stress and disturbance through mediating the soil biogeochemical processes. So far, there are scant reports on potential biochar induced changes in soil microbial diversity using estimation and comparison of OTUs based on 16 S rRNA pyrosequencing technology. Despite the relatively lower resolution of T-RFLP fingerprinting profiles compared to the pyrosequencing, we found a few increases with BSA in the number of T-RFs despite of their relative small proportion to the total abundance. This indicated enhanced growth of a few novel bacterial groups with low relative abundance under biochar amendment. Moreover, ANOVA analysis of the pyrosequencing data revealed strongly significant and consistent differences in bacterial community diversity between biochar treated and non-treated plots across the three sites (Table 3). By using the same number of sequences for all samples, significant increases in Shannon index, PD diversity index, OTU richness and Chao1 were found under BSA over the control across the sites, though the initial diversity (such as PD and Shannon index) varied across sites. The PD in the control was lower by 10% in HN site and by 16% in SC site compared to that in JX site while Shannon diversity was similar in HN site but lower by 6% in SC site, compared to JX site. However, the diversity increases with BSA were significant (by 5-15%) under both C1 and C2 treatments across the sites. The results here confirmed our previous finding for a single paddy soil (Chen et al., 2013). Terra Preta, an old biochar-rich oxic soil, was characterised by approximately 25% greater species richness of bacteria than adjacent forest soil (Kim et al., 2007). Recent study on this soil further demonstrated a greater overall richness of bacterial community and a number of novel sequences (less than 98% similarity matched in databases) (Grossman et al., 2010), which could indicate a potential role of biochar in improving soil bacterial diversity. In contrast, Khodadad et al. (2011) observed an overall loss of microbial diversity but slight increases in some specific taxa in two forest topsoils treated with laboratory-generated acid oak biochar and alkaline grass biochar in a short term laboratory incubation study. Wheat biochar used in this study was shown relatively rich in nutrients (Joseph et al., 2013) and could promote bacterial development through diversified nutrient supply and microhabitats in waterlogged rice paddy (Zheng et al., 2008). In the present study, the greater bacterial diversity under BSA could be attributed firstly to increased soil pH, which had been accepted as a primary abiotic factor for bacterial growth (Fierer and Jackson, 2006). Indeed, in a study by Lauber et al. (2009), an overall phylogenetic diversity of the bacterial communities was observed to correlate with soil pH, with the greatest diversity found in the soils with near-neutral pH. In this study, consistent increases in soil pH were found across the three sites, indicating a liming effect of biochar (Liu et al., 2013). Again, the relationship between phylotype richness and soil pH observed by Fierer and Jackson (2006) could help to interpret the diversity changes in individual soils with BSA. The greater diversity

Table 5
Frequency of the most abundant classified bacterial genera indicated in % of all classified sequences of topsoil under biochar treatment in three sites.

Site	Treatment	Gp1	*Ktedonobacter*	Gp3	*Longilinea*	*Aminomonas*	Gp7	*Gemmatimonas*	*Leptolinea*	*Thermodesulfovibrio*	*Litorilinea*
JX	C0	26.26	8.16	3.53	2.78	1.10	1.21	1.10	0.72	0.43	0.51
	C1	21.31	5.86	3.09	2.01	0.48	1.26	0.72	0.82	0.87	0.39
	C2	17.07	6.83	3.59	2.67	0.28	1.09	0.84	0.77	0.91	0.33
HN	C0	0.54	0.02	1.30	3.17		0.96	0.58	0.79	2.43	0.18
	C1	0.57	0.00	1.41	3.27		1.12	0.66	0.79	2.04	0.21
	C2	0.74	0.01	1.19	3.42		1.27	0.59	0.92	1.93	0.31
SC	C0	3.07	0.12	1.17	4.13		1.64	1.49	0.43	0.49	0.18
	C1	3.98	0.16	1.68	5.38		1.84	2.41	0.58	1.28	0.36
	C2	1.71	0.13	1.19	5.50		1.89	1.54	0.72	0.58	0.54
Biochar[a]		*	NS	NS	NS	NA	NS	NS	NS	NS	NS
Site[a]		***	***	***	***	NA	**	***	*	***	NS
B × S[a]		*	NS	NS	NS	NA	NS	*	NS	**	NS

Site	Treatment	Gp6	*Telmatobacter*	*Conexibacter*	*Bellilinea*	Gp2	*Rhizomicrobium*	Gp18	*Geobacter*	Bryobacter
JX	C0	0.33	0.53	0.28	0.34	0.46	0.28	0.28	0.20	0.20
	C1	0.42	0.48	0.29	0.36	2.57	0.66	0.61	1.70	0.14
	C2	0.42	0.37	0.44	0.59	3.03	0.42	0.76	1.51	0.12
HN	C0	1.66		0.06	1.56	0.02	0.01	1.40	1.92	0.03
	C1	1.97		0.08	1.68	0.06	0.06	1.51	1.71	0.04
	C2	2.03		0.07	1.76	0.10	0.09	1.62	1.54	0.09
SC	C0	3.33	0.17	0.03	0.28	0.19	0.16	0.68	0.84	0.19
	C1	3.86	0.20	0.02	0.46	0.10	0.08	0.83	1.69	0.08
	C2	4.86	0.09	0.18	0.53	0.11	0.03	0.44	1.03	0.09
Biochar[a]		NS	NA	**	NS	***	*	NS	*	NS
Site[a]		***	NA	***	***	***	***	***	*	*
B × S[a]		NS	NA	NS	NS	***	**	NS	*	NS

NS, not significant ($p > 0.05$); NA, not applicable.
[a] Significance levels for two-way ANOVA with biochar amendment and site as main effects.
* $p < 0.05$ significant levels.
** $p < 0.01$ significant levels.
*** $p < 0.001$ significant levels.

with BSA over control could be attributed also to a physical shelter provided by the porous structure of the added biochar, through providing a wide range of niches or microhabitats thereby contributing to increases in both abundance and diversity of microorganisms. The observed increases in microbial biomass and bacterial diversity could be of ecological significance, as enhanced methane oxidation activity was observed with higher methane-oxidizing bacterial gene diversity in a rice paddy receiving long term organic amendment (Zheng et al., 2008). Nevertheless, additional surveys are needed to unravel the ecology and mechanisms of increased bacterial diversity under biochar amendment.

Table 6
Number of sequences known of functional bacterial genera indicated in ‰ of total of topsoil under biochar treatment in the studied rice paddies.

Site	Treatment	Nitrifying bacteria			Denitrification bacteria		N fixation bacteria		
		Nitrosococcus	*Nitrosospira*	*Nitrospira*	*Anaeromyxobacter*	*Geobacter*	*Rhizobacter*	*Rhizomicrobium*	*Bradyrhizobium*
JX	C0	0.67	0.33	2.56	2.22	2.00		2.78	0.78
	C1	2.44	1.56	6.44	4.89	17.00		6.56	1.44
	C2	2.33	1.00	4.22	6.33	15.11		4.22	2.56
HN	C0	0.11	0.89	7.56	6.89	19.22		0.11	0.89
	C1	0.33	1.33	9.56	8.11	17.11	0.11	0.56	0.89
	C2	0.44	1.56	8.22	7.44	15.44	0.44	0.89	2.11
SC	C0		1.56	14.00	7.89	8.44	0.11	1.56	0.45
	C1		3.78	12.33	5.22	16.89	0.33	0.78	0.44
	C2		3.11	9.33	7.78	10.33	0.22	0.33	0.56
Biochar[a]		NA	*	NS	NS	*	NA	*	**
Site[a]		NA	**	***	*	*	NA	***	**
B × S[a]		NA	NS	NS	NS	*	NA	**	NS

NS, not significant ($p > 0.05$); NA, not applicable.
[a] Significance levels for two-way ANOVA with biochar amendment and site as main effects.
* $p < 0.05$ significant level.
** $p < 0.01$ significant level.
*** $p < 0.001$ significant level.

4.3. Biochar effect on bacterial community composition

Traditional culture-independent molecular fingerprinting analyses (i.e., T-RFLP used in this study) of 16S rRNA gene fragments provided some clear divergences in bacterial community structure under biochar amendment. However, such fingerprinting method offers little specific phylogenetic information on bacterial community structure or described communities at very coarse levels of taxonomic resolution (Fierer and Jackson, 2006), because the T-RFs detected were quite limited and the abundance in low density could hardly be recognized via construction of clone libraries of 16S rRNA gene fragments as we did previously (Chen et al., 2013). Additionally, despite its importance in microbial ecology studies, T-RFLP has some drawbacks that have to be taken into account, e.g., a single T-RF might not represent a single phylotype or species. Although the pyrosequencing technique also has certain limitations (Petrosino et al., 2009), it could be better suited for assessing changes in the relative abundances of major taxa. In this study, pyrosequencing analysis revealed a much higher Shannon index of diversity of bacterial community than T-RFLP analysis, and provided a more in-depth comparison of soil bacterial community compositions, due to its capacity to identify greater number of bacteria than T-RFLP. For example, 24 phyla, 63 classes, 173 families and 529 genera bacteria were identified based upon the subsampled quality sequences in the studied rice paddy soils. Besides, a large portion of effective bacterial sequences could not be assigned to any taxa of different level, indicating the extent of novel sequences captured by this technology in the present study.

Previous studies had reported that BSA had a pronounced impact on the bacterial community structure (Lehmann et al., 2011; Khodadad et al., 2011; Chen et al., 2013). However, its impact on bacterial community structures could depend rather on abiotic attributes of soil structure and chemistry in a single site than on amendments across sites (Girvan et al., 2003). In this study, the three rice paddy sites were physically, chemically and geographically different from each other, thereby providing heterogeneous niches to sustain microbial diversity and exhibiting divergent bacterial community compositions among sites (Table 4). This was in coincidence with the results from cluster analyses of both T-RFLP and pyrosequencing of 16S gene as well as with the results of the two-way ANOVA (Tables 4 and 5) related to the site-effect. Of these differences, soil texture and pH were the primary determinants of the composition of bacterial community. *Proteobacteria* was the most abundant phylum of the major taxa in HN and SC sites, accounting for 39–49% and 34–59% of total effective bacterial sequences, respectively. This is consistent to the analytical results of bacterial communities in agricultural soils (Roesch et al., 2007), rice rhizosphere (Somenahally et al., 2011) and sewage treatment systems (Zhang T. et al., 2012), in which *Proteobacteria* was also the most dominant community. However, in JX soil, Acidobacteria rather than *Proteobacteria* was the predominant phylum detected in all the treatment plots, comprising 28–46% of each soil community. This was likely due to the low pH of the soil in JX, which was an acid sandy loam derived from quaternary red clay. Abundance of Acidobacteria was recently found prone to pH change, with the highest abundances found in very acidic environments (Fierer and Jackson, 2006; Fierer et al., 2007; Jones et al., 2009). Moreover, significant decreases in Gp1 (within Acidobacteria), which was predominant in JX and SC soils, further reflected an impact of pH on Acidobacterial communities. Relative to the other two soils, the predominance of Acidobacteria in JX soil was likely impacted by its low pH of 4.89. Although soil pH was considered the best predictor of bacterial community composition relative to the other soil characteristics (Lauber et al., 2009), large variability of bacterial community structure was not observed corresponding to soil pH increase under BSA in a range of 0.11–0.72 (Table 2). Similarly, Anderson et al. (2011) reported less statistically significant changes in relative bacterial abundance of major taxa from soils treated with biochar for nearly 12 weeks compared to the control. Therefore, bacterial community compositions under BSA could be likely influenced by the liming effect of biochar, and the extent depended on the soil type in the short-term experiment.

Although varying among sites, some significant changes in relative abundance of bacteria at phylum level demonstrated potential influences of biochar on soil bacterial communities. While a few specific taxa of bacteria had been altered by biochar addition in the paddy soils, the impact has been found to be site-specific (Table 4). In particular, as the most striking change with BSA, abundance of Actinobacteria was increased by 269% under C2 treatment (BSA at 40 t ha^{-1}) over control in SC site. The group of Actinobacteria, known to grow readily on carbon-rich refractory materials (O'Neill et al., 2009), could be potentially involved in the decomposition of organic materials or complex substrates (e.g., cellulose and chitin) in biochar-added soils. The increased Actinobacteria in this study was in agreement with previous studies by Khodadad et al. (2011), who reported increase in specific taxa such as Actinobacteria despite of an overall loss of bacterial diversity in forest soils added with pyrogenic chars, suggesting an active role of this group in soil metabolism of pyrogenic carbon substrates. Additionally, the relative abundance of Chloroflexi in SC soils was also notably increased with BSA. Bacteria included in genera Chloroflexi, such as the *Anaerolineae* lineage, were a group of bacteria consisting of anaerobic heterotrophs that could decompose carbohydrates and amino acids (Yamada and Sekiguchi, 2009), and was also concerned as a potential degrader of relatively recalcitrant carbon compounds such as phenol (Fang et al., 2006) and 4-methylbenzoate (Wu et al., 2001). Although Chloroflexi were ubiquitous and abundant members of bacterial communities in agricultural soils, an ecological understanding of this important phylum remained elusive due to the lack of cultured representatives. With biochar amendment, Betaproteobacteria was found hardly changed in HN site, significantly decreased in SC site but increased in JX soil indicating an inconsistent response of this group to BSA. Likewise, Kolton et al. (2011) reported that Proteobacteria was decreased from 71% to 47% under citrus wood biochar amendment compared to the non-amendment in a sandy soil poor in organic matter. Nevertheless, there were still a few specific taxa of bacteria that showed divergent responses to BSA, probably because the biochar-induced physicochemical changes are complex and dynamic in soils, particularly in the rice fields with shifting water and nutrient regime conditions.

In our previous works, a reduction in N_2O emission under BSA in rice paddies was proposed to relate with changes in microbially mediated processes of nitrification and denitrification (Liu X.-y. et al., 2012; Zhang et al., 2010). However, the potential impact of BSA on functional groups involved in the N cycle, i.e., resulting in a reduction of N_2O emission, has not yet been well characterized (Cayuela et al., 2014). Being of potential ecological significance, here we demonstrated some changes with BSA in the relative abundance of nitrifying, denitrifying and N-fixing bacteria in rice paddy soils though they varied among sites. It has been demonstrated that ammonia-oxidizing bacteria (AOB) (involved in the first step of nitrification) contributed greatly to nitrification in agricultural soils (Jia and Conrad, 2009). In the present study, soils receiving biochar addition showed generally higher nitrifying bacteria abundance, such as *Nitrosospira*, indicating a potential enhancement of the nitrification. These results were in agreement with the findings by Ball et al. (2010), who found significant increases in AOB and enhanced nitrification in dry montane forest soils influenced by charcoal. Of the detected denitrifying bacteria, *Geobacter* was increased by almost eight times under BSA at both

20 and 40 t ha^{-1} in JX site. It has been evidenced that *Geobacter* could retrieve electrons directly from a poised graphite electrode that served as electron donors for microbial respiration, and used these electrons to reduce nitrate to nitrite (Gregory et al., 2004). Hence, a great increase in the relative abundance of *Geobacter* could accelerate the processes involved in the nitrate reduction under BSA. Besides, significant increases in the relative abundance of *Bradyrhizobium* observed under BSA also indicated enhancement of the N fixation and N_2O reduction processes mediated by these microorganisms under BSA, because *Bradyrhizobium* were both N_2-fixing bacteria and denitrifiers, and could possess the denitrification enzyme NosZ, which is responsible for the reduction of N_2O to N_2 (Anderson et al., 2011). Our obtained molecular evidences could partially support the recent findings by Cayuela et al. (2013), who demonstrated the role of biochar in facilitating the transfer of electrons to soil denitrifying microorganisms, also called "electron shuttle", which could promote the reduction of N_2O to N_2. In fact, increased *nosZ* gene copy numbers were reported with addition of activated switchgrass biochar in an aridic subsoil (Ducey et al., 2013) and increased Bradyrhizobiaceae family by up to 11% was observed with Monterey pine biochar addition (Anderson et al., 2011). All these studies provided additional evidences that biochar addition could potentially mediate the process of soil N cycling by affecting the abundances of nitrifiers and denitrifiers. This study suggested a potential promotion of nitrification under BSA, with the increased nitrifying genera and with the enhanced abundance of denitrifying bacteria such as *Geobacter* and *Bradyrhizobium* for complete reduction of nitrate to nitrogen gas.

5. Conclusions

This study suggested a potential role of biochar in enhancing bacterial abundance, community diversity as well as modifying more or less the community compositions, particularly the bacteria involved in N cycling, in field conditions. Such changes, bacterial community composition in particular, were found to be site-specific as the properties differed among the studied soils. Besides, pyrosequencing technology is suggested to be a powerful tool for an in-depth comparison of soil bacterial community compositions with biochar amendment in agricultural soils. However, the link of these BSA induced changes to soil functioning and the inconsistent changes in community composition across soils remained unclear.

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