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# Interactions between biochar and nitrogen impact soil carbon mineralization and the microbial community

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ABSTRACT

Adding biochar (BC) to soils is proposed to enhance carbon sequestration in agricultural soils. However, there is limited knowledge regarding the effects of the interactions between BC and nitrogen (N) on soil organic carbon (SOC) mineralization at an interannual scale. We conducted a three-year field experiment to systematically reveal the impacts of BC combined with N fertilizer on a silty clay soil, including its physicochemical properties, soil respiration characteristics and microbial community. BC applied once at 0, 20 and 40 t $ha^{-1}$ (B0, B1 and B2, respectively) was combined with three N fertilization levels (0, 120 and 240 kg N $ha^{-1}$; N0, N1 and N2, respectively). Only winter wheat (*Triticum aestivum* L.) was cultivated, and it was cultivated in a winter wheat-summer fallow crop system. BC application persistently increased the SOC content by 36.3–91.6% over three years. Several soil parameters were also improved by BC combined with N after one year, such as bulk density, $NO_3$- and available P content. Compared with its values with B0 treatments with N1 or N2, the total soil $CO_2$ emitted decreased by 6.7–8.9% in response to BC combined with N1 but increased by 5.9–7.5% in response to BC combined with N2. B1N1 not only significantly increased microbial biomass but also decreased $qCO_2$. In addition, B1N1 in particular increased the relative abundances of members of the microbial population associated with increased microbial C use efficiency, such as *Rhizopus* and *Helotiaceae*. However, B2N1 and B2N2 strongly disturbed the soil microbial ecosystem; moreover, the lowest alpha diversity of the bacterial community was observed in B2N2 soils. In summary, adding BC at a high rate to N-fertilized soil strongly disturbs the soil microbial ecosystem and reduces the C-sequestering potential of soil, while B1N1 treatment is recommended to enhance soil C sequestration and improve soil fertility under dryland farming.

## 1. Introduction

The benefits of using biochar (BC) include not only enhancing carbon sequestration (Woolf et al., 2010; Smith, 2016) but also improving soil fertility (Liu et al., 2016). However, soil biological properties are related to the profound influences of BC on soil carbon stabilization, including affecting respiration, microbial communities and enzymatic activities (Major et al., 2010a; Lehmann et al., 2011). These processes significantly affect soil organic carbon (SOC) dynamics (Fang et al., 2018). Before BC is widely used as a soil amendment, it is important to thoroughly understand its impacts on SOC mineralization and the underlying mechanisms.

Studies conducted in both the laboratory and the field have shown that BC can induce significant changes in soil microbial activity and community composition that in turn affect the mineralization of SOC (Foster et al., 2016; Chen et al., 2017; Li et al., 2017a). Whether BC acts as a sink or source of C is a heated debate (Zimmerman et al., 2011). The primary mineralization of native soil organic matter has been mostly attributed to the labile C contained in BC, which serves as a substrate to stimulate microorganism activities and the growth of some taxon-specific microbial communities (Mitchell et al., 2015; Zheng et al., 2016). However, the labile pool of BC (approximately 3% of total C) can degrade rapidly within several months after incorporation into soil, while the recalcitrant pool can persist for several millennia (Pei et al., 2017). Therefore, positive responses of soil $CO_2$ fluxes to BC amendment are generally found in short-term incubation studies (Liu

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et al., 2016).

In contrast, BC can stabilize native SOC through physical, chemical and biological interactions between BC, native SOC and soil biota in the long term (Fang et al., 2014; Singh and Cowie, 2014). For instance, BC can increase the stability of macroaggregates and protect organic matter, including BC, from mineralization (Pronk et al., 2012; Herath et al., 2013). In addition, BC can promote microbial community shifts towards increasing microbial C use efficiency in field sites (Ameloot et al., 2014) and reduce microorganisms that are capable of decomposing recalcitrant C (Zheng et al., 2016). Therefore, BC amendment may result in a negative priming effect under field conditions in a few years or longer (Prayogo et al., 2014). However, the interannual effects of BC on soil C mineralization, microbial community structure and function have rarely been systematically evaluated in natural dryland farming systems.

Furthermore, the application of biochar to N-fertilized soils has received increasing attention due to its potential to sequester carbon and improve soil fertility (Alburquerque et al., 2013; Sarma et al., 2018). Notably, BC or N might importantly impact SOC dynamics by changing the diversity and activity of microbial communities (Fierer et al., 2012; Gul et al., 2015). Several studies suggested that BC can offset the negative effects of N on bacterial diversity and the positive effects of N on microbial biomass C, which depends on the BC and N dose (Li et al., 2018a). Our previous 108-day incubation experiment indicated that apple branch BC application at a 1% rate (w/w) decreases the soil respiration rate in N-fertilized alkaline soil, which reverses when the BC application rate is greater than 2% (Li et al., 2017a). Sui et al. (2016) reported that urea N with 14.8 t $ha^{-1}$ BC significantly enhances $CO_2$ emissions over a short term in paddy soils. In contrast, Senbayram et al. (2019) using a $\delta^{13}C$ stable isotope approach, found that four types of BC (corn cob, cotton stalk, olive mill and pistachio shell) at 4 t $ha^{-1}$ coapplication with N fertilizer cause negative priming in an alkaline clay soil and decrease $CO_2$ emissions up to 12% compared to those of the N-added control treatment. Additionally, the role of BC in regard to extracellular enzymes, in which the primary means of soil microorganisms degrade insoluble macromolecules into smaller, soluble molecules (Burns et al., 2013), is also dependent on the N level (Foster et al., 2016; Li et al., 2017a). Overall, to date, there is no comprehensive understanding of how the interaction of BC and N fertilizer affects SOC mineralization in natural fields.

Based on existing knowledge, we hypothesized that soil microbial metabolic characteristics, microbial communities and C cycling-associated enzyme activities are affected by interactions of BC and N, which closely depend on the addition rate of BC and N fertilizer. We conducted a BC and N interaction field experiment for three years in the Loess Plateau of northern China (1) to explore the microbial mechanisms underlying the impacts of BC on soil respiration at different N levels and (2) to understand the effects of BC and N interaction on soil physiochemical properties, C mineralization characteristics and the soil microbial community.

## 2. Materials and methods

### 2.1. Site, soil and BC descriptions

The long-term BC experimental plot was set up in Yangling, Shaanxi, China (34°18′15″ N, 108°02′30″ E; 530 m elevation), from October 2015 to July 2018. The experimental site is located in the southern region of the Loess Plateau. The climate of the area is characterized as semihumid. During the experimental period, the average temperature was 14 °C, and the annual precipitation was 599 mm. The mean monthly air temperature and precipitation data during the experiment were collected from a nearby weather station, and they are presented in Fig. S1 (Yangling weather station, Yangling Meteorological Bureau, 2018).

The soil (0-20-cm layer) physical and chemical properties before the experiment are shown in Table S1. The soil type is Lou soil (Eum-Orthic Anthrosol), which is considered silty clay according to the USDA system. BC was derived from apple branches (*Malus pumila* Mill.) through slow pyrolysis using a dry distillation method. The furnace temperature was increased from nearly 20 °C to 450 °C at a rate of 30 °C $min^{-1}$, and the temperature was maintained at 450 °C for 8 h. The physicochemical properties of the BC are shown in Table S2. The methods for determining the nutrient contents and other detailed characteristics of the BC are described by Li et al. (2017a).

### 2.2. Experimental design and soil management

The two experimental factors were BC and N fertilizer levels. BC was applied at 0, 20 and 40 t $ha^{-1}$ (termed B0, B1 and B2, respectively); N fertilizer was applied in the form of urea at 0, 120 and 240 kg N $ha^{-1}$ (termed N0, N1 and N2, respectively). BC was manually added once to the plow layer (0–20 cm) of the soil using a shovel and was mixed before sowing occurred in October 2015. Basal application of urea to the plow layer was performed once a year before sowing (i.e., in mid-October of 2015, 2016 and 2017). All treatments were arranged in a randomized complete block design with three replicates, and each plot had an area of 16 $m^2$ (i.e., 4 m × 4 m). The plots were separated by pathways (0.5 m wide) to avoid cross contamination and treatment effects. Only winter wheat (*Triticum aestivum* L. cv., Xiaoyan No. 22) was cultivated, with a 25-cm row spacing at a seeding rate of 150 kg $ha^{-1}$. No tillage occurred during the growth stage, and weeds were regularly removed by hand. Wheat was harvested manually at maturity on 28 May 2016, 2 June 2017 and 29 May 2018 by cutting the aboveground biomass and removing it from the plots. Approximately one month after harvesting, the 0-20-cm soil layer was plowed using a manual shovel. During the study, natural rainfall was the only water supply for each plot.

### 2.3. Soil sampling

To determine the soil properties, soil (200–300 g fresh weight) was collected as a composite sample from five locations in each plot using a soil auger with a 4-cm inner diameter after the wheat harvest each year. Roots and other debris were removed, and all samples were sieved through a 2-mm screen. A portion of the samples was placed in a 10-mL centrifuge tube and stored at -80 ℃ for soil DNA extraction. A portion of the samples was transported to the laboratory to determine the soil microbial biomass, enzyme activities, and soil inorganic N contents. The remaining soil samples were dried at room temperature to measure the soil physicochemical properties.

### 2.4. Analysis of soil physicochemical properties

To determine the soil bulk density (BD), undisturbed soil cores (100 $cm^3$) were collected using a cutting ring from a depth of 0–20 cm in each plot after harvest in 2017 and 2018. Soil pH was measured at a soil-to-water ratio of 1:2.5 (w/v) with a pH meter. For the analysis of SOC, soils were placed in tiny tin cups and pretreated with 1 M HCl to remove carbonates. Soils were oven-dried at 80 °C and then directly combusted with a C/N elemental analyzer (vario MACRO cube, Elementar, Germany) to determine the total C and N. Soils were suspended in water (1:2, soil: water) for 1 h and filtered through 0.45-μm membranes to determine the contents of dissolved organic C (DOC) and dissolved organic N (DON) (Mukherjee et al., 2016). DOC was determined using an automated total organic C analyzer (Shimadzu, TOC-Vwp, Japan), and DON was measured by the Kjeldahl method (Bremner and Mulvaney, 1982). $NO_3^-$ and $NH_4^+$ were extracted by 2 M KCl solution and analyzed using flow injection analysis (TRAACS 2000, Bran and Luebbe, Norderstedt, Germany). The total P content was determined using the molybdenum blue method after digestion with $H_2SO_4HClO_4$ at 300 °C for 2 h. Available P was extracted with 0.5 M Na

bicarbonate and quantified by the molybdenum blue method (Jin et al., 2016).

### 2.5. Soil respiration and temperature sensitivity, microbial biomass and metabolic quotient

Soil respiration rates were determined using an automated soil $CO_2$ flux system equipped with a portable chamber 20 cm in diameter (Li-8100, Lincoln, NE, USA). All visible living organisms were manually removed from the chamber pedestal before measurement. The soil temperature at a 5-cm depth was measured using a Li-Cor thermocouple probe (Delta-T Devices, Cambridge, England). During the experimental period, soil respiration was measured at least once per month from June 2016 to July 2018. The investigation was conducted from 9:30 am to 11:00 am on each measurement day (Wang et al., 2018). The temperature sensitivity of soil respiration ($Q_{10}$) was derived by fitting $R_s$ (μmol $m^{-2}$ $s^{-1}$) at different temperatures to an exponential function (Fang and Moncrieff, 2001):

$$R_s = \beta_0 e^{\beta 1 T} \quad (1)$$

$$Q_{10} = e^{10\beta 1} \quad (2)$$

where $\beta_0$ and $\beta_1$ are fitted parameters and T is the measured soil temperature (°C) at a soil depth of 5 cm.

The soil microbial biomass C ($C_{mic}$) content was determined via the fumigation-extraction method (Vance et al., 1987), and the conversion factor was 0.45 (Wu et al., 1990), as described in Li et al. (2017a). The metabolic quotient ($qCO_2$) was quantified following Zhou et al. (2017). Briefly, $qCO_2$ was calculated as $R_s$ per unit microbial biomass and was expressed as mg $CO_2$-C $g^{-1}$ $C_{mic}$ $h^{-1}$. For the calculation of $qCO_2$, area-related $CO_2$ data (per $m^2$) were converted to $CO_2$ emitted per g soil in the 0–20 cm soil depth by applying treatment-specific bulk densities (Table S3). In general, $qCO_2$ was determined in periods without vegetation and extreme influences such as fertilization events (Bamminger et al., 2018). $R_s$ data for $qCO_2$ were calculated by summing the $CO_2$-C emission of two consecutive gas sampling events after harvest (from 2 June to 7 July in 2017, from 28 May to 7 July in 2018) and divided by the total time between the gas sampling events.

### 2.6. Soil DNA extraction and sequencing

The DNA of 0.5 g of soil was extracted using MoBio Power Soil™ DNA Isolation Kits (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The integrity of the DNA extracts was confirmed by 1% agarose gel electrophoresis. The V4-V5 region of the bacterial 16S ribosomal RNA gene was amplified using primers 515 F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 907R (3′−CCGTCAATT CCTTTGAGTTT-5′) (Caporaso et al., 2011). The fungal ITS1 region gene was amplified using primers ITS5-1737 F (5′-GGAAGTAAAAGTCGTAA CAAGG-3′) and ITS2-2043R (3′-GCTGCGTTCTTCATCGATGC-5′) (Caporaso et al., 2011). PCRs were performed in a volume of 30 μL containing 2 μL of sterile ultra-pure water, 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 3 μL of 6 μM primers, and 10 μL of template DNA (approximately 10 ng). Thermal cycling consisted of initial denaturation at 98 ℃ for 1 min, followed by 30 cycles of denaturation at 98 ℃ for 10 s, annealing at 50 ℃ for 30 s, and elongation at 72 ℃ for 30 s, and a final step at 72 ℃ for 5 min. Triplicate PCR amplicons were mixed with the same volume of 1 × loading buffer (containing SYB green) and subjected to electrophoresis on a 2% agarose gel for detection. Then, mixed PCR products were purified with a Qiagen Gel Extraction Kit (Qiagen Co., Ltd, Germany). Sequencing was performed using the Illumina HiSeq 2500 platform at Novogene Bioinformatics Technology Co., Ltd., Beijing, China. Raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Numbers: PRJNA522864 and PRJNA522870).

### 2.7. Processing of sequencing data

The raw sequence data were analyzed using QIIME (Version 1.7.0) (Caporaso et al., 2010). Chimeric sequences were eliminated using the Uchime algorithm (Edgar et al., 2011). High-quality sequences were clustered by UPARSE software (Edgar, 2013) and assigned to operational taxonomic units (OTUs) at similarities of 97%. Taxonomy was assigned using the Ribosomal Database Project classifier. Community diversity indicators, including the observed OTUs, Shannon index, Chao1 estimator and ACE, were calculated using QIIME.

### 2.8. Enzymatic activities

We performed hydrolytic enzyme assays for three enzymes targeting labile to intermediately reactive C (breakdown of carbohydrates and polysaccharides): β-glucosidase (BG; EC: 3.2.1.21; cellulose degrading), β-xylosidase (BX; EC: 3.2.1.37; hemicellulose degrading) and β-D-cellubiosidase (CBH; EC: 3.2.1.91; cellulose degrading). Enzyme activities were measured using a fluorometric measurement protocol according to Bell et al. (2013) with minor modifications. Briefly, a well-homogenized soil slurry was prepared by adding 100 mL of sterile distilled water to 3.00 g of fresh soil and shaking at 200 rpm for 1 h at 25 °C. Then, 150 μL of sample slurry was pipetted into wells of a 96-well microplate, and 50 μL of substrate (200 μM) was quickly added using an 8-channel pipette. The microplates were incubated at 25 °C in the dark for 3 h and then oscillated for 1 min using a miniature oscillator. The fluorescence of the supernatants was quantified using a microplate fluorometer (Synergy, BioTek, USA) with 365-nm excitation and 460-nm emission filters. Standard curves were prepared by incubating soil suspensions in the presence of increasing concentrations of 4-methylumbelliferone (MUB) standard for each soil sample. Eight replicate wells were tested for each sample. Potential enzymatic activities were calculated as nmol product released $g^{-1}$ dry soil $h^{-1}$.

### 2.9. Statistical analysis

The significance of differences in the soil physiochemical properties, $CO_2$ emissions, $C_{mic}$, $qCO_2$ and $Q_{10}$, alpha diversity of bacteria and fungi, and enzymatic activities was tested by two-way ANOVA. The repeatedly measured data (soil physiochemical properties, $C_{mic}$, $qCO_2$ and enzyme activity) were also analyzed via repeated-measures ANOVA. Pearson correlation analysis was performed to explore the relationship of soil parameters with $CO_2$ emissions, $C_{mic}$, $qCO_2$ and $Q_{10}$, and enzymatic activities. Two-way ANOVA, repeated-measures ANOVA and Pearson correlation analysis were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Nonmetric multidimensional scaling (NMDS) ordination plots were used to identify differences in bacterial and fungal community composition, and the significance of the differences was determined using analysis of similarities (ANOSIM). Linear discriminant analysis (LDA) effect size (LEfSe) (http://huttenhower.sph.harvard.edu/lefse/) was used to identify features that differed significantly among samples. A significance alpha of 0.05 and an effect size threshold of 4 were used for all of the biomarkers evaluated. Canonical correspondence analysis (CCA) was performed to identify the soil physiochemical factors related to the microbial community. NMDS, ANOSIM and CCA were conducted using the vegan package in R-3.4.3.

## 3. Results

### 3.1. Soil physiochemical properties

Compared with the B0 treatments, the BC treatment significantly increased SOC by 36.3–91.6% at each N level throughout the study (Table S3). BC and N significantly influenced SOC, DOC, $NO_3^-$, total P and available P, and these variables showed an increasing trend with BC application (Table 1). Additionally, BD decreased significantly in

**Table 1**
Results of two-way ANOVA and repeated-measures ANOVA (F-values and significance) for soil physiochemical properties.

| Parameter | Year | BC | N | Year | BC × N | BC × Year | N × Year | BC × N × Year |
|---|---|---|---|---|---|---|---|---|
| Bulk density | 2017-2018 | **19.4↓*** ** | **14.4*** ** | 3.5 | 0.2 | 0.7 | 1.1 | 0.6 |
| | 2017 | **17.5↓*** ** | **14.9*** ** | | 0.4 | | | |
| | 2018 | **6.8↓** * | **4.2*** | | 0.4 | | | |
| pH | 2016-2018 | **4.6*** | **31.0↓*** ** | **86.8*** ** | **3.36*** | **10.3*** ** | **10.5*** ** | **6.3*** ** |
| | 2016 | **30.2*** ** | **4.5*** | | *0.7†* | | | |
| | 2017 | 0.7 | **11.5↓*** ** | | *2.1†* | | | |
| | 2018 | 0.5 | **63.1↓*** ** | | **26.2*** ** | | | |
| SOC | 2016-2018 | **190.5↑*** ** | **5.1*** | **4.2*** | **4.4*** | **2.8*** | 0.8 | 0.4 |
| | 2016 | **108.2↑*** ** | **4.1*** | | 0.8 | | | |
| | 2017 | **123.6↑*** ** | **5.6*** | | 6.2** | | | |
| | 2018 | **24.4 ↑*** ** | 0.4 | | 0.8 | | | |
| Total N | 2016-2018 | **12.4↑*** ** | **9.7*** ** | **96.1*** ** | 2.2 | **5.0** ** | **3.2*** | **4.3*** ** |
| | 2016 | 1.1 | **38.1↑*** ** | | **23.0*** ** | | | |
| | 2017 | **25.3↑*** ** | **4.0*** | | 1.1 | | | |
| | 2018 | 2.1 | 0.6 | | 0.9 | | | |
| DOC | 2016-2018 | **9.8↑*** ** | **46.9↑*** ** | **16.1*** ** | 1.3 | 0.3 | **4.9** ** | 0.7 |
| | 2016 | **4.2↑*** | **16.3*** ** | | 0.7 | | | |
| | 2017 | 1.4 | **24.4*** ** | | 1.4 | | | |
| | 2018 | *2.8†* | **7.0** ** | | 0.5 | | | |
| DON | 2016-2018 | **73.9↓*** ** | **11.0*** ** | **222.8*** ** | **24.0*** ** | **109.1*** ** | **103.4*** ** | **21.0*** ** |
| | 2016 | **5.7*** | **748.2↑*** ** | | **165.5*** ** | | | |
| | 2017 | **136.6↓*** ** | **34.5*** ** | | **18.2*** ** | | | |
| | 2018 | 1.9 | **5.7↑*** | | 1.2 | | | |
| $NO_3^-$ | 2016-2018 | **55.8↑*** ** | **1412.1↑*** ** | **274.3*** ** | **27.4*** ** | **15.2*** ** | **162.7*** ** | **17.44*** ** |
| | 2016 | **531.1*** ** | **1025.0↑*** ** | | **191.5*** ** | | | |
| | 2017 | **14.5↑*** ** | **149.1↑*** ** | | **8.1*** ** | | | |
| | 2018 | **33.9↑*** ** | **933.3↑*** ** | | **37.9*** ** | | | |
| $NH_4^+$ | 2016-2018 | **8.1** ** | **13.6*** ** | **160.6*** ** | **11.9*** ** | **5.1** ** | **3.6*** | **3.0*** |
| | 2016 | **9.1↓** ** | **6.0↓** ** | | 1.1 | | | |
| | 2017 | 2.8 | *3.4†* | | **5.8** ** | | | |
| | 2018 | **12.8↑*** ** | **14.6↑*** ** | | 1.0 | | | |
| Total P | 2016-2018 | **13.0↑*** ** | **8.4** ** | **48.9*** ** | **3.8*** | **9.9*** ** | **29.8*** ** | **7.1*** ** |
| | 2016 | **11.6↑*** ** | 2.3 | | 0.3 | | | |
| | 2017 | 0.1 | **46.9*** ** | | **15.0*** ** | | | |
| | 2018 | **26.9↑*** ** | 2.3 | | 0.1 | | | |
| Available P | 2016-2018 | **107.9↑*** ** | 1.7 | **127.8*** ** | **21.0*** ** | **5.6*** ** | **3.6*** | **16.0*** ** |
| | 2016 | **43.5↑*** ** | **5.7↑*** | | **4.5*** | | | |
| | 2017 | **39.3↑*** ** | *2.9†* | | **26.7*** ** | | | |
| | 2018 | **27.7↑*** ** | 0.5 | | *2.8†* | | | |

Note: Significant effects and trends are in bold/italics and are indicated by $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^\dagger 0.05 < P < 0.1$. Arrows show the direction of significant BC or N effects but not the cases of their interaction (BC × N). DOC and DON are water-dissolved soil organic carbon and nitrogen, respectively.

response to BC application (Table 1). N addition significantly decreased the soil pH, and the soil pH of B0N2 was lower than that of B0N0 by 0.16 pH units in 2018 (Table 1 and S3). However, the pH values of B1N2 and B2N2 were significantly higher than that of B0N2 in 2018 (Table S3).

### 3.2. Soil respiration, metabolic quotient and temperature sensitivity

Soil respiration showed similar seasonal and annual variations among all treatments (Fig. 1A), increasing gradually from February to July and decreasing after September. The highest soil respiration rate was recorded in July 2017. N levels and the interactions of BC and N significantly affected the total $CO_2$ emitted (Table 2 and Fig. 1B). Compared with the B0N0 treatment, B0N1 and B0N2 treatments significantly increased the total $CO_2$ emitted by 12.8% and 12.6% ($P < 0.05$), respectively. However, the effects of BC on the total $CO_2$ emission varied with N level (Fig. 1B). Compared with that of B0N1, the total $CO_2$ emitted decreased in response to B1N1 and B2N1 by 6.7% and 8.9% ($P < 0.05$), respectively. In contrast, the total $CO_2$ emissions of B1N2 and B2N2 soils were significantly higher than that of B0N2 soils by 7.5% and 5.9% ($P < 0.05$), respectively. The total $CO_2$ emitted was positively correlated with DOC and the $NO_3^-$ content but was negatively correlated with BD and pH (Table 3).

BC, N and their interactions significantly influenced $C_{mic}$ and $qCO_2$ (Table 2). During 2017–2018, B1 treatments generally increased $C_{mic}$, but B2 treatments other than B2N2 decreased $C_{mic}$ (Table S4). $C_{mic}$ increased with N fertilization and was positively correlated with the soil $NO_3^-$ and $NH_4^+$ contents (Table 3). $qCO_2$ generally decreased with N and was significantly correlated with some soil parameters (Tables 2 and 3). Compared with B0 treatments with N1 or N2, B1 treatments combined with N fertilization decreased $qCO_2$ by 1.1–26.1%, but the B2N1 treatment showed a positive effect on $qCO_2$ (Table S4). The $Q_{10}$ value was 1.66–1.98 across all treatments, and it was significantly affected by the interactions between BC and N (Table 2). BC application generally resulted in an increase in $Q_{10}$ with N1, in contrast to the response observed with N2 (Table S4).

### 3.3. Microbial community diversity

The minimum sequencing depth for 29,318 sequences of bacteria and 46,792 sequences of fungi at the same survey effort level was randomly selected from each sample. The coverages of bacteria and fungi exceeded 97.0% and 99.5%, respectively. A total of 1,773,470 bacterial quality sequences with an average of 65,684 sequences per sample (approximately 373-bp read length) were clustered into 94,767 OTUs. A total of 1,839,541 fungal quality sequences with an average of 68,131 per sample (approximately 252-bp read length) were clustered into 29,393 OTUs. Overall, the numbers of observed OTUs and the alpha diversity indices of either bacteria or fungi were hardly influenced by BC and the interaction between BC and N (Table 4). However, N addition significantly decreased the number of bacterial OTUs ($P < 0.05$); in addition, the number of bacterial OTUs of B2N2 was

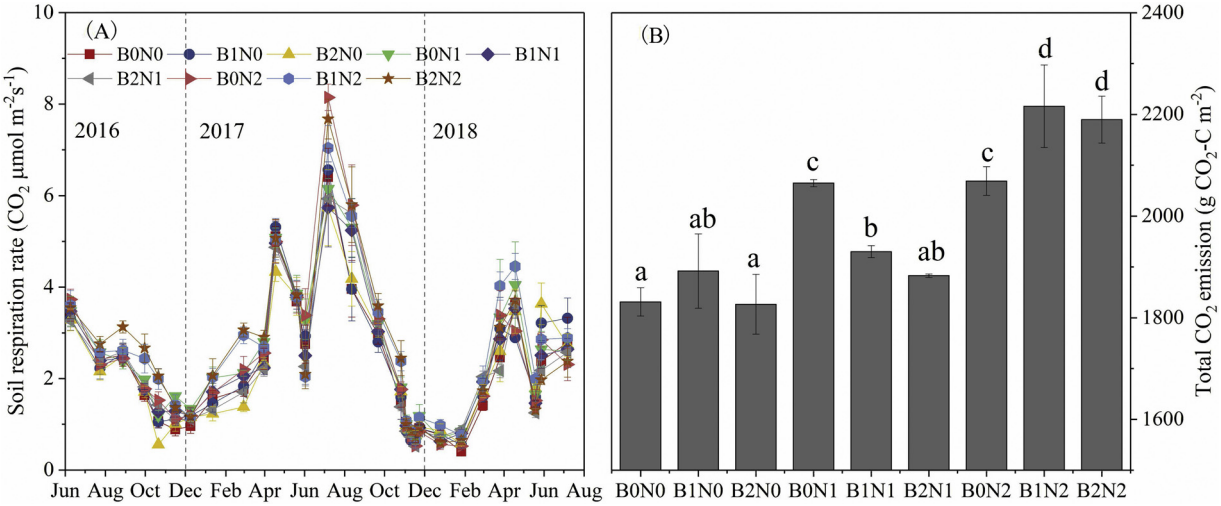


**Fig. 1.** Dynamics of soil respiration and the total $CO_2$ emitted during the three years (mean ± SE). B0, B1 and B2 refer to without biochar and biochar application in the plow layer soil at 20 t $ha^{-1}$ and 40 t $ha^{-1}$, respectively. N0, N1 and N2 refer to nitrogen fertilization at 0 kg N $ha^{-1}$, 120 kg N $ha^{-1}$ and 240 kg N $ha^{-1}$, respectively. Different letters on bars indicate significant differences ($P < 0.05$).

significantly lower than that of B0N2 by 6.5% ($P < 0.05$, Table 4).

### 3.4. Microbial community composition

The dominant bacterial phyla across all soil samples were *Proteobacteria* (29.5% average relative abundance), *Acidobacteria* (17.8%) and *Actinobacteria* (16.3%) (Fig. 2A). The dominant fungal phyla across all soil samples were *Ascomycota* (59.3%), *Zygomycota* (20.5%) and *Basidiomycota* (17.3%) (Fig. 2B). BC, N and their interactions significantly affected the relative abundances of some dominant bacteria and fungi at the phylum and genus levels (Table S5), which were related to soil parameters such as BD, DON, $NO_3^-$ and $NH_4^+$ (Table S6). For example, compared with the B0N2 treatment, B1N2 and B2N2 treatments exerted negative effects on the relative abundance of *Acidobacteria* (Fig. 2A). BC combined with N fertilizer increased the relative abundance of *Skermanella* (Fig. 2B). BC application alone decreased the relative abundance of *Ascomycota,* but BC combined with N fertilizer increased it (Fig. 2C). B1N1 treatment particularly increased the relative abundances of *Rhizopus* and *Ochrocladosporium* (Fig. 2D). Additionally, the levels of some bacterial and fungal genera with low abundance (< 0.01%) were significantly different among treatments (Figs. S2 and S3).

LEfSe analyses were performed to determine the statistical significance of the differential abundances of taxa that were chosen as specific microbial biomarkers (Fig. 3, LDA values are shown in Fig. S4). The relative abundances of *Actinobacteria*, *Rhodospirillales* and *Xylarialesas* were primarily changed in B2N2. The biomarkers of B1N2 soils were *Nitrosomonadaceae* and *Mortierellales*, whose abundances were significantly positively correlated with the soil $NO_3^-$ content (Table S6). The biomarker of B1N1 soil was *Helotiaceae*, whose abundance was closely related to SOC (Table S6).

An NMDS plot based on the Bray-Curtis distance dissimilarity (number of OTUs) clearly identified a large change in the bacterial structure of B2N2 soil (Fig. 4A), and this finding was supported by an ANOSIM test (Table S7). In addition, the bacterial communities of the BC soils were slightly different from those of the B0 soils with N1 and N2, as indicated by the BC treatments being separated from the B0 treatments along the NMDS2 axis (Fig. 4A). However, the fungal community was barely changed by BC at each N level (Fig. 4B and

**Table 3**
Pearson correlations of soil $CO_2$ emissions and the metabolic quotient with soil parameters.

| Parameter | $CO_2$ emission | $C_{mic}$ | $qCO_2$ | $Q_{10}$ |
|---|---|---|---|---|
| Bulk density | **−0.34*** | −0.01 | **−0.30*** | −0.01 |
| pH | **−0.39**** | −0.08 | **0.58**** | 0.05 |
| SOC | −0.07 | 0.06 | 0.11 | 0.18 |
| Total N | 0.14 | −0.10 | **−0.55**** | 0.06 |
| DOC | **0.44**** | 0.03 | **−0.39**** | −0.22 |
| DON | −0.06 | 0.19 | **0.29*** | −0.13 |
| $NO_3^-$ | **0.81**** | **0.33*** | −0.07 | −0.25 |
| $NH_4^+$ | −0.06 | **0.30*** | **0.62**** | 0.01 |
| Total P | −0.25 | −0.24 | **−0.43**** | 0.04 |
| Available P | −0.02 | 0.00 | **0.58**** | −0.10 |

Note: DOC and DON are water-dissolved organic carbon and nitrogen, respectively. *$P < 0.05$, **$P < 0.01$.

**Table 2**
Results of two-way ANOVA and repeated-measures ANOVA (F-values and significance) for $CO_2$ emissions, $C_{mic}$, $qCO_2$ and $Q_{10}$.

| Parameter | Year | BC | N | Year | BC × N | BC × Year | N × Year | BC × N × Year |
|---|---|---|---|---|---|---|---|---|
| Total $CO_2$ emission | 2016-2018 | 2.3 | **97.3↑***** | | **10.4***** | | | |
| $C_{mic}$ | 2017-2018 | **22.1***** | **10.9↑***** | **16.9***** | **4.5*** | 2.4 | 1.3 | 1.2 |
| | 2017 | **15.9***** | **8.1↑**** | | 2.0 | | | |
| | 2018 | **12.4***** | **5.9↑**** | | **6.1**** | | | |
| $qCO_2$ | 2017-2018 | **13.4***** | **12.3↓***** | **204.2***** | **4.7**** | **9.2**** | ***3.5*** | 1.0 |
| | 2017 | **12.3***** | **5.9↓*** | | 0.7 | | | |
| | 2018 | **6.7 ↑**** | **21.7↓***** | | **15.4***** | | | |
| $Q_{10}$ | 2017-2018 | 0.6 | 1.1 | | **3.7*** | | | |

Note: Significant effects and trends are in bold/italics and are indicated by *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. Arrows show the direction of significant BC or N effects but not the cases of their interaction (BC × N). $C_{mic}$, $qCO_2$ and $Q_{10}$ are the soil microbial biomass C, metabolic quotient and temperature sensitivity of soil respiration, respectively.

**Table 4**
Community diversity indices of bacteria and fungi (at 97% sequence similarity) based on the 16S and ITS rRNA genes (mean ± SE).

| Variables | | N0 | | | N1 | | | N2 | | | Two-way ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | B0 | B1 | B2 | B0 | B1 | B2 | B0 | B1 | B2 | BC | N | BC × N |
| Observed OTUs | Bacteria | 2829 ± 107$^{b}$ | 2921 ± 17$^{b}$ | 2867 ± 78$^{b}$ | 2724 ± 203$^{ab}$ | 2837 ± 37$^{b}$ | 2771 ± 93$^{ab}$ | 2803 ± 40$^{b}$ | 2819 ± 106$^{b}$ | 2592 ± 102$^{a}$ | 2.6 | **3.6**↓* | 1.5 |
| | Fungi | 932 ± 20$^{a}$ | 883 ± 95$^{a}$ | 974 ± 94$^{a}$ | 859 ± 53$^{a}$ | 903 ± 97$^{a}$ | 947 ± 145$^{a}$ | 948 ± 94$^{a}$ | 893 ± 150$^{a}$ | 971 ± 131$^{a}$ | 1.1 | 0.3 | 0.2 |
| Shannon index | Bacteria | 9.60 ± 0.05$^{b}$ | 9.64 ± 0.03$^{b}$ | 9.63 ± 0.07$^{b}$ | 9.51 ± 0.21$^{ab}$ | 9.64 ± 0.01$^{b}$ | 9.59 ± 0.03$^{b}$ | 9.56 ± 0.02$^{ab}$ | 9.58 ± 0.08$^{b}$ | 9.41 ± 0.08$^{a}$ | 1.8 | *3.2*† | 1.7 |
| | Fungi | 5.93 ± 0.82$^{ab}$ | 5.07 ± 0.72$^{ab}$ | 5.81 ± 0.78$^{ab}$ | 5.08 ± 0.19$^{ab}$ | 4.53 ± 0.94$^{a}$ | 6.14 ± 0.62$^{b}$ | 5.80 ± 0.39$^{ab}$ | 5.63 ± 1.12$^{ab}$ | 6.00 ± 0.90$^{ab}$ | *3.1*† | 1.2 | 0.8 |
| Chao1 estimator | Bacteria | 3626 ± 510$^{a}$ | 3896 ± 56$^{a}$ | 3674 ± 237$^{a}$ | 3591 ± 311$^{a}$ | 3569 ± 7$^{a}$ | 3476 ± 423$^{a}$ | 3594 ± 162$^{a}$ | 3675 ± 217$^{a}$ | 3411 ± 137$^{a}$ | 1.0 | 1.1 | 0.3 |
| | Fungi | 1110 ± 24$^{a}$ | 1157 ± 108$^{a}$ | 1116 ± 21$^{a}$ | 1109 ± 150$^{a}$ | 1100 ± 36$^{a}$ | 1102 ± 171$^{a}$ | 1080 ± 117$^{a}$ | 1021 ± 144$^{a}$ | 1101 ± 130$^{a}$ | 0.0 | 0.6 | 0.3 |
| ACE | Bacteria | 3727 ± 438$^{ab}$ | 3974 ± 73$^{b}$ | 3805 ± 232$^{ab}$ | 3672 ± 259$^{ab}$ | 3662 ± 65$^{ab}$ | 3564 ± 303$^{ab}$ | 3701 ± 103$^{ab}$ | 3792 ± 220$^{ab}$ | 3393 ± 184$^{a}$ | 1.7 | 1.9 | 0.7 |
| | Fungi | 1142 ± 21$^{a}$ | 1167 ± 105$^{a}$ | 1163 ± 50$^{a}$ | 1138 ± 114$^{a}$ | 1153 ± 46$^{a}$ | 1129 ± 148$^{a}$ | 1105 ± 122$^{a}$ | 1055 ± 170$^{a}$ | 1145 ± 144$^{a}$ | 0.1 | 0.6 | 0.2 |

Note: N0, N1 and N2 refer to nitrogen fertilization at 0 kg N ha$^{-1}$, 120 kg N ha$^{-1}$ and 240 kg N ha$^{-1}$, respectively. B0, B1 and B2 refer to no biochar application and biochar application in the plow layer soil at 20 t ha$^{-1}$ and 40 t ha$^{-1}$, respectively. Different letters in a row indicate significant differences among treatments ($P < 0.05$). The results of two-way ANOVA are indicated by F-values and significance. Significant effects are in bold/italics and indicated by *$P < 0.05$, †$0.05 < P < 0.1$. Arrows show the direction of significant BC or N effects but not the cases of their interaction (BC × N).

Table S7). A CCA indicated that BD, $NO_3^-$, and other soil parameters were closely correlated with the microbial community structure (Fig. 5).

### 3.5. Soil enzyme activities

Three carbohydrate-mineralizing enzyme activities were significantly affected by the interactions of BC and N and by sampling year (Table 5). Generally, BC combined with N fertilizer decreased the potential activities of BG and BX in 2017; however, the trend for BX reversed in 2018 (Table 5). The potential activity of CBH increased with the BC addition rate with N0, but it decreased with BC application with N1 and N2 throughout the investigation (Table S8). These enzyme activities were closely related to some soil parameters such as pH, total N and $NH_4^+$ (Table S9).

## 4. Discussion

### 4.1. Effects of BC combined with N fertilizer on soil properties

The application of BC alone or combined with N fertilization significantly increased SOC throughout the three years of the field study (Table 1 and S3), which confirmed the role of BC in contributing to C storage as reported by previous studies (Smith, 2016; Bamminger et al., 2018). The reason for this behavior is that more than 97% of the carbon contained in BC is recalcitrant and thus dominated by aromatic compounds that are resistant to biological degradation (Wang et al., 2016). Additionally, several soil properties such as BD, pH, $NO_3^-$ and the available P content continued to improve after three years of BC amendment (Table 1). These soil environment factors were significantly correlated with soil respiration and the microbial population and communities (Table 3 and Fig. 5). Previous studies suggested that BC adsorbs $NO_3^-$ for a long period, thus resulting in less available N for plant uptake (Haider et al., 2016). However, BC ages biotically and abiotically after long-term culture with wheat (Hale et al., 2011), and the adsorption capacity of BC may decrease over time, mainly due to pore-filling processes (Ren et al., 2016). In our work, regardless of the N level, BC weakly decreased the $NO_3^-$ content after one year of BC application but had a positive effect on the $NO_3^-$ content in the last two years (Tables 1 and S3). The improved N availability was attributed to the fact that "aged" BC accelerates soil N mineralization and reduces leaching (Yoo et al., 2014). In addition, the aging process can enhance the interactions of BC with native organic matter and clay minerals and thus increased the amount of nutrients retained in the soil (Major et al., 2010b).

BC amendment is an optimal nutrient-management strategy with multiple benefits to agriculture, including slowing the dangerous rates of anthropogenic acidification (Lehmann, 2007; Zheng et al., 2016). In this study, high N fertilization (N2) decreased the soil pH by approximately 0.17 units in three years (Tables 1 and S3); however, BC treatments restricted the pH decrease (Table S3). Two possible mechanisms contribute to the increase in soil pH: the high alkaline pH of BC (Table S2) and the negatively charged groups (e.g., phenolic and hydroxyl) on BC surfaces binding $H^+$ ions from the soil solutions, thereby reducing the $H^+$ concentration in these solutions (Gul et al., 2015; Li et al., 2017a). Hence, BC showed the potential to slow the soil acidification process resulting from N fertilization in alkaline dryland soils.

### 4.2. Effects of BC combined with N fertilizer on soil respiration

Compared with the B0N0 treatments, N fertilizer significantly enhanced total $CO_2$ emissions by 12.8% (B0N1) and 12.6% (B0N2) (Fig. 1). The stimulating effects of N fertilization on soil respiration were most directly attributed to N stimulating the size of the soil microbial population (Table 2). The positive correlation between $C_{mic}$ and

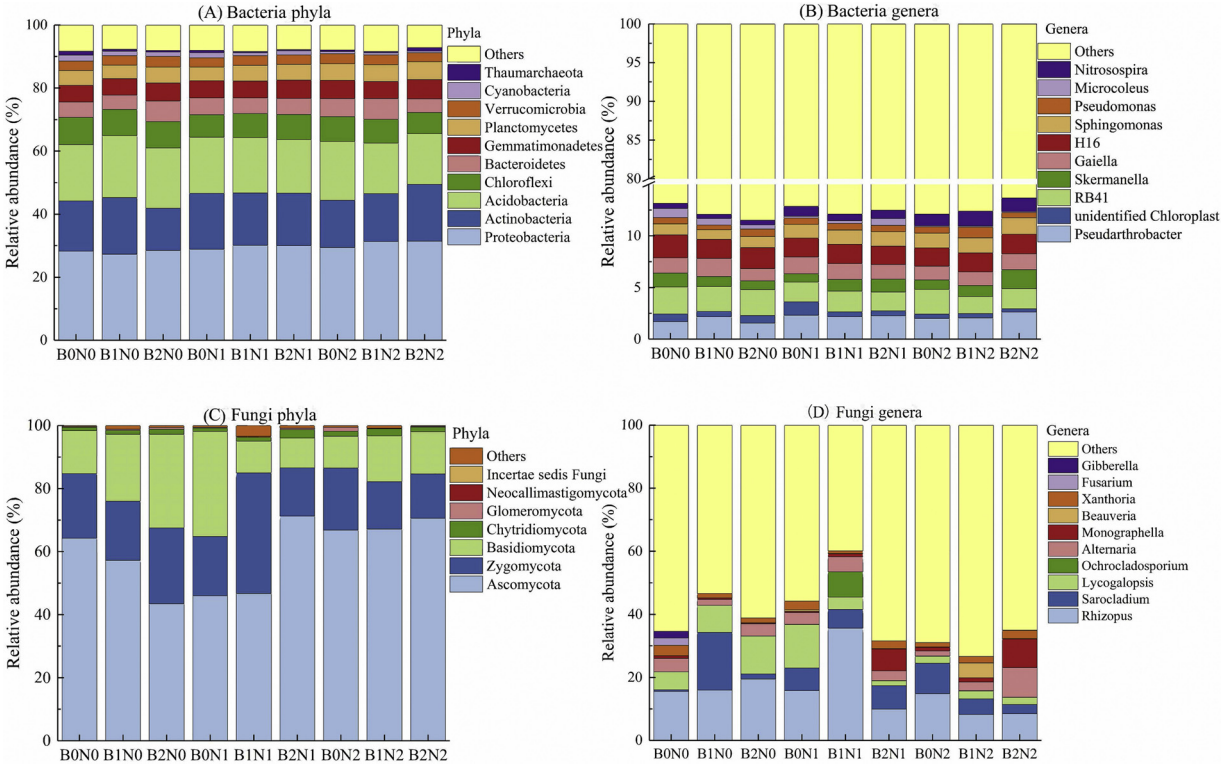


**Fig. 2.** Changes in the microbial communities at the phylum and genus levels. A and B: bacteria; C and D: fungi. B0, B1 and B2 refer to without biochar and biochar application in the plow layer soil at 20 t ha$^{-1}$ and 40 t ha$^{-1}$, respectively. N0, N1 and N2 refer to nitrogen fertilization at 0 kg N ha$^{-1}$, 120 kg N ha$^{-1}$ and 240 kg N ha$^{-1}$, respectively.

the contents of mineral N ($NO_3^-$ and $NH_4^+$) supported this fact (Table 3). However, BC combined with N1 significantly decreased the total $CO_2$ emitted, which was opposite to the trend with N2 (Fig. 1). This result clearly illustrates that the interaction of BC and N fertilizer affected the total $CO_2$ emitted (Table 2). Several studies have demonstrated that the labile C in BC is completely exhausted by microorganisms within one year after BC is incorporated into soils (Wang et al., 2016). However, BC has a high porosity and large surface area (Li et al., 2017a), which decreases the soil BD and may improve aeration and water retention (Castellini et al., 2015). Additionally, BC increases soil nutrient contents ($NO_3^-$, $NH_4^+$ and available P, etc.) and provides suitable habitats for microbial growth and protection from predators (Warnock et al., 2010; Lehmann et al., 2011). Hence, $C_{mic}$ and changes in microorganism activities in BC treatments are related to changes in the soil environment (Table 3).

B1N1 not only had a significant positive effect on $C_{mic}$ but also decreased $qCO_2$ (Table S4), which suggested increased microbial C use efficiency and enhanced SOC retention (Bardgett and Saggar, 1994;

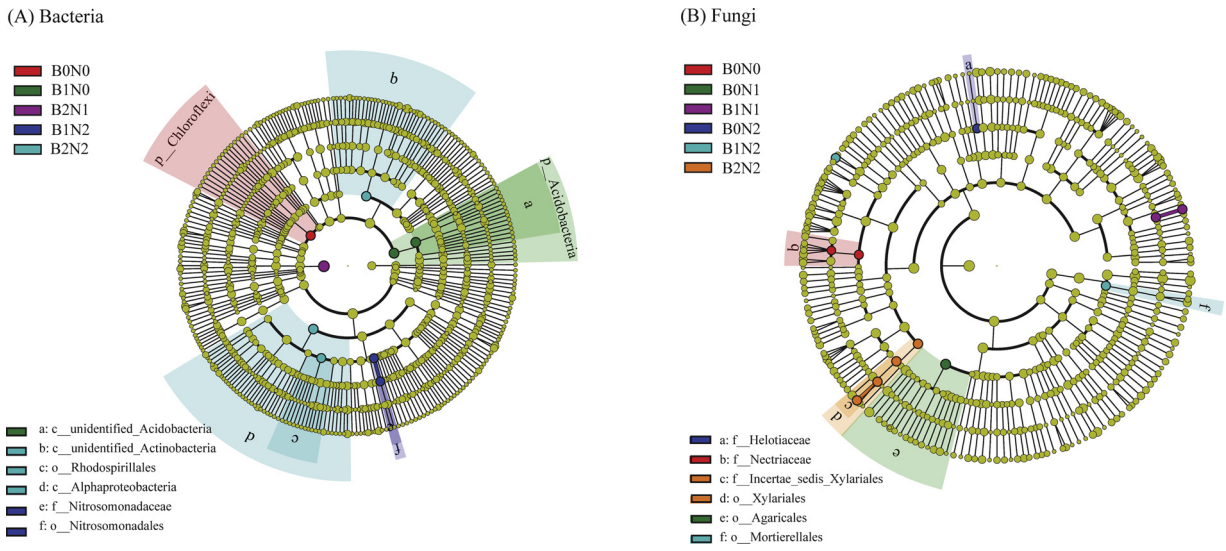


**Fig. 3.** A linear discriminant analysis effect size (LEfSe) method identifies significant differences in the abundance of bacterial (A) and fungal (B) taxa in all treatments. The taxa with significantly different abundances among treatments are represented by colored dots, and from the center outward, they represent the kingdom, phylum, class, order, family, and genus levels. The colored shadings represent trends of the significantly different taxa. Each colored dot has an effect size LDA score, as shown in Fig. S4. Only taxa meeting an LDA significance threshold of > 4 are shown.

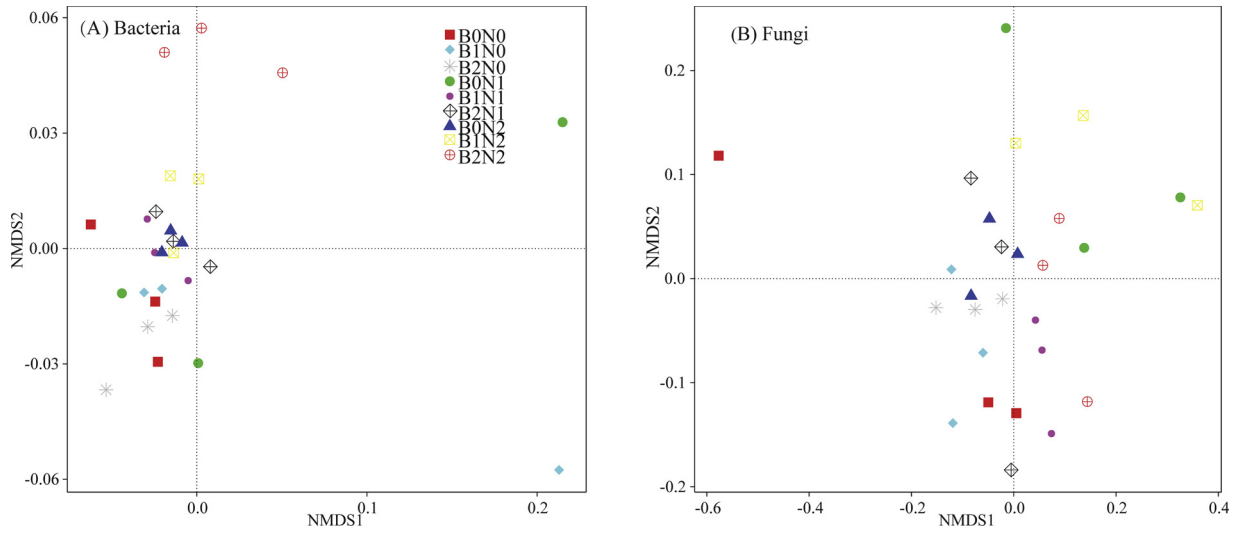


**Fig. 4.** Nonmetric multidimensional scaling (NMDS) plot of all samples' microbial communities (OTU abundance). A: bacteria; B: fungi. B0, B1 and B2 refer to without biochar and biochar application in the plow layer soil at 20 t ha$^{-1}$ and 40 t ha$^{-1}$, respectively. N0, N1 and N2 refer to nitrogen fertilization at 0 kg N ha$^{-1}$, 120 kg N ha$^{-1}$ and 240 kg N ha$^{-1}$, respectively.

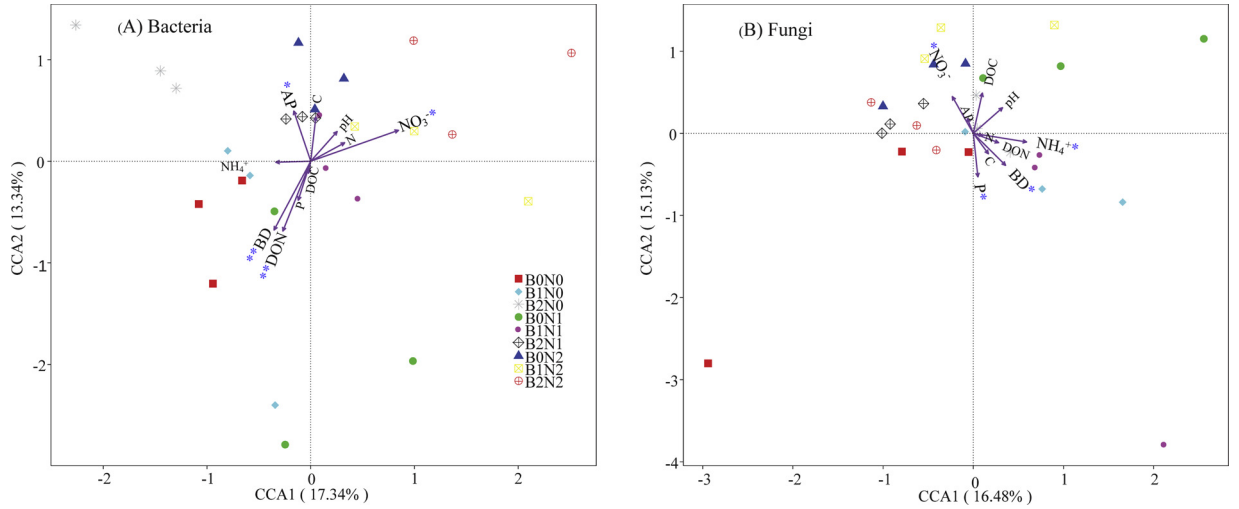


**Fig. 5.** Canonical correspondence analysis (CCA) of microbial community changes with environmental variables. A: bacteria; B: fungi. B0, B1 and B2 refer to without biochar and biochar application in the plow layer soil at 20 t ha$^{-1}$ and 40 t ha$^{-1}$, respectively. N0, N1 and N2 refer to nitrogen fertilization at 0 kg N ha$^{-1}$, 120 kg N ha$^{-1}$ and 240 kg N ha$^{-1}$, respectively. BD and AP are soil bulk density and available P, respectively. DOC and DON are soil water dissolved organic carbon and nitrogen, respectively. *$P < 0.05$, **$P < 0.01$.

**Table 5**
Results of two-way ANOVA and repeated-measures ANOVA (F-values and significance) for soil enzyme activities.

| Variable | Year | BC | N | Year | BC × N | BC × Year | N × Year | BC × N × Year |
|---|---|---|---|---|---|---|---|---|
| β-Glucosidase | 2017-2018 | **4.6↓*** | **4.9*** | **19.4***** | **8.0***** | **11.7***** | **3.7*** | **5.7**** |
| | 2017 | **15.1↓***** | **14.3↓***** | | **7.3***** | | | |
| | 2018 | **4.4*** | 0.5 | | **6.9***** | | | |
| β-Xylosidase | 2017-2018 | **18.8↓***** | **33.2↑***** | **327.9***** | **21.4***** | **56.6***** | **11.5***** | **9.3***** |
| | 2017 | **40.4↓***** | **22.4↑***** | | **16.4***** | | | |
| | 2018 | **27.4↑***** | **16.3↑***** | | **4.7**** | | | |
| β-D-Cellubiosidase | 2017-2018 | 1.2 | **20.4↑***** | **195.7***** | **93.2***** | **12.1***** | 2.1 | **15.9***** |
| | 2017 | **7.4**** | **11.3***** | | **61.8***** | | | |
| | 2018 | **6.8**** | **8.1↑**** | | **25.8***** | | | |

Note: Significant effects and trends are in bold and are indicated by *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Arrows show the direction of significant BC or N effects, but not the cases of their interaction (BC × N).

Zhou et al., 2017). However, the decreased $C_{mic}$ and increased $qCO_2$ of B2N1 were probably due to the higher number of BC particles in this treatment than in B1N1 retaining more nutrients (Gul et al., 2015; Li and Shangguan, 2018) and thus forming a nutrient-limiting microenvironment for microorganisms, limiting their population size. In addition, a high $qCO_2$ usually indicates soil degradation under intensive land use and could be interpreted as having a positive priming effect on the decomposition of the labile soil SOC pool (Zhou et al.,

2017). Hence, the increased DOC content found in B2N1 throughout the investigation contributed to the priming effect (Table S3), but the decreased soil microbial population limited soil $CO_2$ emissions. However, the increased soil nutrients (e.g., DOC and $NO_3^-$) in the treatments where BC was combined with N2 could remove nutrient limitations and increase $C_{mic}$ and microbial activities (Tables 3 and S3), thus enhancing soil respiration. In addition, the decreased $qCO_2$ of B2N2 in 2018 may imply a shift of SOC towards a form that is more recalcitrant against microbial degradation (Zheng et al., 2016). BC may improve soil aggregation and consequently organic mineral-microbial interactions, thus limiting soil respiration in the long run (Smith et al., 2010; Zhou et al., 2017), but confirmation of this hypothesis requires further research.

A small variation in the temperature sensitivity of soil respiration (often expressed as $Q_{10}$) can cause a large bias in predicting soil $CO_2$ released into the atmosphere, especially under ever-changing climate conditions in the future (Wang et al., 2018). In our case, $Q_{10}$ was not changed by the main effects of BC and N, but it was significantly affected by the interaction between BC and N (Table 2). Bamminger et al. (2018) also found that BC applied alone had no effect on the $Q_{10}$ in a long-term soil warming experiment. Hence, the temperature sensitivity of BC-C mineralization may be similar to that of SOC (Fang et al., 2014). Alternatively, BC was not degraded in large enough amounts to influence $Q_{10}$ (Bamminger et al., 2018). However, soil nutrients are affected by the combination of BC and N, which could alter the stability of the substrate and influence $Q_{10}$ (Conant et al., 2011; Wang et al., 2018). In addition, stable SOC is more sensitive than labile SOC to increasing soil temperature (Conant et al., 2011). Therefore, the decreased nutrient content and increased stable carbon content for B2N1 compared to B1N1 resulted in a low microbial C use efficiency, which consequently contributed to the higher $Q_{10}$ in B2N1.

### 4.3. Effects of BC combined with N fertilizer on the microbial community

Microbial diversity plays a crucial role in influencing ecosystem stability, productivity and resilience towards stress (Zheng et al., 2016). Increased microbial diversity is usually detected in soils amended with BC for a long period of time (Grossman et al., 2010; Zheng et al., 2016). Our results indicated that bacterial and fungal alpha diversity hardly changed after two years of addition of BC alone or combined with N fertilizer, except in response to B2N2. Similarly, long-term BC amendment field studies in a black soil of northeastern China also showed that fungal diversity is not influenced by BC (Yao et al., 2017). In addition, in our work, N fertilizer generally decreased the number of observed bacterial OTUs, and B2N2 treatment resulted in the lowest bacterial alpha diversity index (Table 4). Additionally, the bacterial communities changed with BC application at each N level, especially at N2 (Fig. 4A). However, no distinct group separation among the fungal communities of different treatments was observed (Fig. 4B), which is inconsistent with the results reported by Yao et al. (2017). Therefore, BC interacting with a high level of N would strongly influence bacterial diversity, resulting in community destabilization for the bacterial but not the fungal community.

Many studies have demonstrated that BC has an impact on bacterial and/or fungal community compositions on short- or long-term scales (Nielsen et al., 2014; Zheng et al., 2016; Awasthi et al., 2017). However, the changes in community composition caused by the interaction of BC and N remain unclear. In this study, the bacterial community composition was influenced by BC combined with N at the phylum and genus levels (Figs. 2A and B; S2). The application of BC alone or combined with N fertilizer significantly decreased the relative abundance of *Chloroflexi*, which was the biomarker of B0N0 (Fig. 3A). *Chloroflexi* species are usually predicted to degrade plant compounds with pathways commonly identified for the degradation of cellulose, starch and long-chain sugars. In addition, *Chloroflexi* species likely compete for labile carbon with other organisms (Hug et al., 2013). Therefore, the lower relative abundance of *Chloroflexi* in BC-amended soils than in other soils may limit the organic material degradation rate.

With regard to fungi, the relative abundances of phyla and genera also showed different distributions among the samples (Figs. 2C and D; S3), which is in line with the results from Zheng et al. (2016). Mineralizable C has been reported to significantly decrease the relative abundance of *Basidiomycota,* and most fungal OTUs from *Basidiomycota* were assigned as nonsaprotrophs (Dai et al., 2018). Hence, the decreased relative abundance of *Basidiomycota* in BC treatments at each N level may be due to BC increasing the SOC and DOC (Table 1). As a microbial C source, the DOC probably promotes saprotroph growth and enhances their competitive capacity, leading to an overall decrease in diversity and a decline in fungal pathogens (Dai et al., 2018). Furthermore, the *Nectriaceae* were identified as a fungal biomarker of B0N0 (Fig. 3). Most species of the ascomycete family *Nectriaceae* are weak to virulent or soil-borne saprobes and facultative or obligate plant pathogens, and several species have also been reported as important opportunistic pathogens of humans (Lombard et al., 2015). Therefore, the finding of rare *Basidiomycota* and *Nectriaceae* in BC treatments suggested that BC amendment may be beneficial for suppressing the occurrence of some crop and human diseases over the long term.

In particular, B1N1 treatment increased the relative abundances of *Rhizopus* and *Ochrocladosporium* (Fig. 2 D). *Rhizopus* is probably the best-known genus in the class of *Zygomycetes* fungi, which normally live on dead and decaying plant material. These fungi exhibit a complex metabolism and produce a variety of enzymes that enable them to utilize a wide range of nutrients (Lennartsson et al., 2014). *Ochrocladosporium* is a hyphomycete that has morphological and ontogenic characteristics similar to those of *Cladosporium* (Ren et al., 2012). The common saprobic members of *Cladosporium* usually occur on senescing or dead leaves and stems of herbaceous vegetation (Crous et al., 2007) and have the capacity to degrade a wide range of organic matter (Anastasi et al., 2005). Additionally, the biomarker of B1N1 treatments was the *Helotiaceae*, which were positively correlated with the SOC content (Table S6). The *Helotiaceae* include many dark septate endophytes and are a diverse group of filamentous ascomycetes (Detheridge et al., 2016) that may be involved in mutualistic interactions with higher plants and play an important role in plant nutrition (van der Heijden et al., 2015). Therefore, B1N1 treatments may enhance soil organic matter turnover through accelerating litter decomposition, which requires further research to verify.

B1N2 soils had the highest relative abundance of *Nitrosomonadales* and *Mortierellales*. As a marker gene for nitrification sequences, amoA usually forms clusters with *Nitrosomonadales*, including *Nitrosomonas* and *Nitrosospira*, which oxidize ammonia to nitrite (Segawa et al., 2014). This finding suggested that the increased $NO_3^-$ content in B1N2 soils may be due to the treatment's promotion of soil nitrification. *Mortierellales*, which include fast-growing saprobic fungi that mainly utilize simple soluble substrates, are associated with high cellulose content in the soil (Li et al., 2017b). These fast-growing saprobic fungi may utilize many simple soluble substrates, thus leading to increased $CO_2$ emissions. However, B2N2 soils had a higher relative abundance of *Actinobacteria* and *Rhodospirillales*. *Actinobacteria*, as gram-positive bacteria, play a vital role in organic matter turnover, including the decomposition of cellulose and chitin (Ali et al., 2019). *Rhodospirillales* enrichment has been attributed to the increased C/N ratio due to BC application (Abujabhah et al., 2018), which may enhance microbial nitrogen fixation (Berkelmann et al., 2018). The enriched populations of *Actinobacteria* and *Rhodospirillales* in B2N2 soils may symmetrically accelerate microbial organic matter decomposition, which partly drives the higher soil $CO_2$ emissions. Additionally, B2N2 had the lowest relative abundance of *Xylariales* (Fig. 3B), whose secondary metabolites are usually signaling compounds that play important roles in the interaction between fungi and bacteria in soil (Pazoutova et al., 2013). This result indicated that high amounts of BC combined with high levels of N addition may decrease the abundance of signaling compounds and

thus strongly disturb the microbial community.

The combination of BC with N altered the soil properties (e.g., BD, $NO_3^-$, $NH_4^+$, TP, available P, DOC and DON) (Fig. 5), which were closely associated with the soil microbial community structure in this study. Likewise, several studies suggested that the impacts of long-term biochar amendment on the soil microbial community occurred indirectly as a result of the alteration of soil physiochemical properties (Lucheta et al., 2016; Gul et al., 2015). The high content of $NO_3^-$ and low BD were the main factors for the dissimilarity of the bacterial community of B2N2 soil in this three-year field study (Fig. 5A). Our previous two-year field study indicated that long-term N fertilization at 240 kg N $ha^{-1}$ in conjunction with BC at 40 t $ha^{-1}$ leads to large amounts of residual $NO_3^-$ without increased plant productivity but dramatically reduces the availability of $NO_3^-$ (Li et al., 2019). On the other hand, a large amount of BC input into silty clay soil forms pores that are too large (Castellini et al., 2015) and increases the connectivity of the pores between particles (Barnes et al., 2014), which is not conducive to maintaining moisture (Li et al., 2018b). Therefore, the fixation of $NO_3^-$ and the moisture shortage might constitute the dominant mechanism for the distinctive community structure of B2N2 soils. Furthermore, soil pH has been shown to decrease with N fertilization and is usually a good indicator of microbial community changes (Feng et al., 2014). BC has the potential to slow the soil acidification rate caused by a high amounts of chemical N fertilizer; thus, the effect of BC and N interaction on soil pH may directly influence the microbial community and thus needs constant attention over the long term.

### 4.4. Effects of BC combined with N fertilizer on enzyme activities

BG, BX and CBH are often associated with organic carbon catalysis (Luo et al., 2017). In general, the activity of carbohydrate-mineralizing enzymes decreases after two years of BC addition, as suggested by Lehmann et al. (2011). BC reduces extracellular enzyme activity through the functional groups present in BC, which tend to bind substrates and extracellular enzymes, thus interfering with the rate of substrate diffusion to the catalytic active site of enzymes (Li et al., 2017a). Another possible explanation for this change is the colocation of C and microorganisms on BC surfaces, which may improve efficiency and reduce the need for enzyme production (Lehmann et al., 2011).

However, the interaction effects of BC and N on the potential activities of BG and BX changed after three years (Table 5). For example, B2N1 treatment significantly increased the activities of BG in 2018, which means that B2N1 treatment might accelerate cellulose degradation and the subsequent release of monomer glucose to microorganisms (Luo et al., 2017). Additionally, the application of BC combined with N2 increased BX activities in 2018, which indicated that BC may accelerate the reduction of cellulose from xylan under long-term high-N fertilization (Foster et al., 2016). These results contradicted previous findings from either short-term laboratory incubation or long-term field studies (Chen et al., 2017; Zheng et al., 2016). Because BX and BG are known to cycle C substrates, increasing activities may increase substrate for microorganism metabolism, thus enhancing soil respiration. In addition, shifts in BX and BG extracellular enzymatic activities may be a proxy for changing metabolic pathways in soil (Bell et al., 2013). Therefore, the application of BC combined with N fertilizer at an appropriate level should maintain the stability of the soil microbial ecosystem.

## 5. Conclusions

The application of BC combined with N fertilizer persistently increased SOC and improved several soil parameters over three years. However, BC combined with N1 decreased soil $CO_2$ emission, whereas the opposite response was observed when BC was combined with N2. This result was associated with changes in microbial biomass, $qCO_2$ and the microbial community due to BC and N. B1N1 treatment not only increased microbial biomass but also decreased $qCO_2$. B1N1 treatment enriched some species that contributed to increased microbial C use efficiency and decreased SOC mineralization. Nevertheless, adding BC at a high rate to N-fertilized soil (e.g., B2N2) strongly disturbed the soil microbial ecosystem and decreased bacterial community diversities, thus decreasing the C sequestration potential of BC. Ultimately, B1N1 treatment is recommended to enhance soil C sequestration and improve soil fertility in the long term.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.still.2019.104437.

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