Biochar Impacts Soil Microbial Community Composition and Nitrogen Cycling in an Acidic Soil Planted with Rape

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Supporting Information

ABSTRACT: Biochar has been suggested to improve acidic soils and to mitigate greenhouse gas emissions. However, little has been done on the role of biochar in ameliorating acidified soils induced by overuse of nitrogen fertilizers. In this study, we designed a pot trial with an acidic soil (pH 4.48) in a greenhouse to study the interconnections between microbial community, soil chemical property changes, and N₂O emissions after biochar application. The results showed that biochar increased plant growth, soil pH, total carbon, total nitrogen, C/N ratio, and soil cation exchange capacity. The results of high-throughput sequencing showed that biochar application increased α -diversity significantly and changed the relative abundances of some microbes that are related with carbon and nitrogen cycling at the family level. Biochar amendment stimulated both nitrification and denitrification processes, while reducing N₂O emissions overall. Results of redundancy analysis indicated biochar could shift the soil microbial community by changing soil chemical properties, which modulate N-cycling processes and soil N₂O emissions. The significantly increased *nosZ* transcription suggests that biochar decreased soil N₂O emissions by enhancing its further reduction to N₂.

INTRODUCTION

Long-term overuse of nitrogen fertilizers and acid deposition have been shown to accelerate the soil acidifying processes in China,^{1,2} and increase N₂O emissions.³ Acidification can affect both the soil biota and biogeochemical processes,⁴ thus decreasing agricultural production. Furthermore, a lower soil pH leads to a higher N₂O: N₂ ratio of soil emitted gases^{5,6} thus posing environmental risks, such as climate change. Although several technologies (e.g., liming and amendments from plant materials) have been developed to remediate acidified soils, they all have drawbacks (e.g., unavailable in many areas, high costs, short lasting of ameliorating effects).⁷ A cost-effective soil amendment is required to solve this issue, and biochar amendment has been proposed as one of such solutions.

Biochar has been reported to modify soil quality characteristics, thereby increasing crop yields.⁸ Because it is usually alkaline, biochar can increase the pH of acidic soils.^{9,10} Furthermore, biochar application has also been promoted as a means of contributing to the mitigation of climate change by reducing soil N₂O emissions.^{11,12} However, not all studies have shown that biochar could suppress soil N₂O emissions. Some has shown no effect¹³ and some that biochar increased soil N₂O emissions.¹⁴

In addition to the uncertain effect of biochar on N₂O emission, the factors and mechanisms of the influence of biochar on N₂O remain unclear due to the complex N₂O formation pathways. N₂O can be produced from several closely related biological processes in soil, including nitrification, nitrifier denitrification, denitrification, codenitrification, dissimilatory nitrate reduction to ammonia, nitrate assimilation, and chemodenitrification, among which nitrification and denitrification are predominant.¹⁵ Since N₂O can be the final or intermediate product of denitrification (including nitrifier denitrification), there are two possible ways to decrease denitrification N₂O emissions: (i) a decrease in the total N denitrified; and (ii) an increase in the further reduction of N₂O to N₂. Although the ways to decrease nitrification N₂O have not been well characterized, it has been shown that the decreases of soil N₂O emissions were often accompanied by lower nitrification rates.¹⁶ Several hypotheses have been proposed for N₂O decreases by biochar, including: (i) biochar reduces levels of denitrification via improving soil aeration;¹⁷ (ii)

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biochar increases the adsorption of NO_3^- , thus decreasing substrate availability for denitrification;¹⁸ (iii) biochar increases soil pH therefore driving denitrification thorough to N_2 ;¹⁸ (iv) biochar sorbs N_2O ;¹⁹ and (v) biochar contains ethylene which could inhibit nitrification and thus the formation of NO_3^- and N_2O .²⁰ More studies are needed to clarify the effect of biochar, especially when it is used as a soil amendment in agricultural production.

Nitrification and denitrification are both performed by soil microbial communities, however, few of the hypotheses stated above have been well supported by microbial studies conducted by advanced methods. Although the majority of biochar-C is thought to be largely unavailable to microbes,²¹ some studies based on traditional microbial methods have shown that biochar addition to soil could increase soil microbial biomass and soil respiration.^{22,23} However, only a few studies have investigated the changes of soil microbial community composition²⁴ and N_2O -related functional gene abundances and transcripts^{25–27} after biochar application. More work is needed to find out the potential effect of biochar on soil microbial community composition and soil microbes, especially those underpinning N_2O -related N-transformation processes, by using advanced methods. Recently, barcoded Illumina paired-end sequencing (amplicon sequencing) method has provided a low cost-effective way to study the soil microbial community.²⁸

To study the effects of biochar application on the soil microbial community and to explore the microbial mechanisms of biochar-induced changes in N_2O emissions in agricultural production, we designed a pot trial in a greenhouse using rape, which is a widely planted vegetable in China with an appropriate growth season for the study. We applied Illumina sequencing technology to analyze shifts in the soil microbial community and chose *nirK* and *nirS*, as well as *nosZ* as denitrifying bacteria gene markers,^{29,30} and gene *amoA* (including archaeal *amoA* and bacterial *amoA*) as nitrification functional markers.³¹ The objectives of this study were as follows: (i) to study the effects of biochar on soil properties and N_2O emissions from soil with and without rape; (ii) to study the shift in microbial community composition after biochar addition; (iii) to reveal the responses of nitrifier and denitrifier populations to biochar addition; and (iv) to determine the mechanisms of increasing or reducing N_2O emissions by biochar amendment.

■ EXPERIMENTAL SECTION

Soil Characteristics. The soil (belonging to Acrisol according to the World Reference Base for Soil Resources) was collected from the plough layer (0–20 cm in depth) of a greenhouse (16 years old) in a vegetable production center (31°50' N, 118°28' E) located in He County, Anhui Province, China. This area belongs to a subtropical humid monsoon climate region, with a mean annual rainfall of 1067 mm, a mean annual temperature of 15.8 °C, featuring four distinct seasons. Muskmelon was planted in spring and rape was planted in autumn in the greenhouse with potassium sulfate as base fertilizer and fertigation during the growth season. Upon arrival at the lab, the soil was passed through a 2 mm sieve and air-dried before mixed with biochar. The water content of the air-dried soil was $4.39\% \pm 0.05\%$ (standard deviation). The basic chemical properties and element composition of the soil are shown in Supporting Information (SI) Tables S1 and S2.

Biochar Preparation and Characteristics. Biochar used in this experiment was made from rice straw, sourced from Xiamen, Fujian, China. Air-dried rice stalks were charred at 500 °C for 4 h in a closed container under oxygen-limited conditions using nitrogen as the medium gas in a muffle furnace (Isotemp, Fisher Scientific, U.S.A.). Biochar was milled to pass a 2 mm sieve before analysis and application to the soils. The basic properties and element composition of the biochar are shown in SI Tables S1 and S2.

Rape Preparation and Pot Trial. The type of the rape was Jingguan (*Brassica campestris* L., cv. Jingguan), a hybrid cultivated by the National Engineering Research Center for vegetables. The rape could grow easily, and was strongly resistant to disease and heat, with a growing period of 40–45 d.

In this pot trial, four treatments with four replicates were carried out: addition of 5% (w/w) rice straw biochar with rape (BP), addition of 5% (w/w) rice straw biochar without rape (B), untreated soil with rape (CP), and untreated soil without rape (C). The main procedures are described as follows. We mixed 21.9 kg air-dried soil (equals to 20.9 kg oven dry basis) and 1.1 kg biochar, weighed 2 kg soil or mixture of soil and biochar into each pot, and activated the soil microbes by incubating the soil for a week at a water content of about 50% WHC (water holding capacity). After a week, uniform rape seedlings (the preparation of the seedlings is shown in the SI) were transplanted into the pots (17.5 cm diameter, 20 cm height). After being transplanted into the pots, the rape plants were grown in a greenhouse at a temperature of 28–36 °C and a relative humidity of 90%. The water content was maintained at about 50% WHC and no fertilizer was added during the incubation.

Nitrous oxide (N_2O) fluxes were measured by using the closed chamber method (see SI) every week for 6 weeks. Gas samples (60 mL each time) were taken 0 and 2 h after chamber closure. The N_2O concentrations were analyzed with the robotized incubation system.³² Six weeks later, a soil sample from each pot was blended carefully, divided into parts and stored differently as required. One subsample was immediately frozen in liquid N_2 and stored at –80 °C until used for DNA and RNA extraction. The remainder was sieved with a 2 mm sieve, after which some was stored at 4 °C for determination of moisture, nitrate, ammonium, potential nitrification rate (PNR), and denitrification enzyme activity (DEA) within 3 days, and some was air-dried for analysis of soil pH, cation exchange capacity (CEC), total carbon (TC), and total nitrogen (TN).

Soil and Plant Chemical Analysis. Soil pH was determined with a soil to water ratio of 1:2.5 (w/v) using a pH-meter (accumet excel XL 60, Fisher Scientific, Singapore). CEC was determined by the BaCl_2 replacement method. Soil nitrate and ammonium were extracted with 2 M KCl at a soil to solution ratio of 1:10 (w/v) and determined by a Continuous Flow Analyzer (SAN++, Skalar, Breda, Holland). PNR was measured using a chlorate inhibition method.³³ DEA was determined by using the acetylene (C_2H_2) inhibition technique.³⁴ Total nitrogen and total carbon were determined by an Element Analyzer (Vario EL III, Elementar, Hanau, Germany). Moisture content was calculated by the weight of soils before and after being oven-dried for 48 h at 105 °C.

Rape biomass was weighed after washing with deionized water and dried 48 h at 80 °C. Inorganic nitrogen was determined using fresh rape. Rape nitrate and ammonium contents were determined by grinding extraction method (see

Table 1. Chemical Properties of Soils under Different Treatments^a

treatments	pH	TN (g kg ⁻¹)	TC (g kg ⁻¹)	C/N	NO ₃ ⁻ (mg kg ⁻¹)	NH ₄ ⁺ (mg kg ⁻¹)	CEC (cmol kg ⁻¹)
C	4.48 ± 0.02d	2.3 ± 0.1c	14.1 ± 0.2b	6.09 ± 0.03d	166.8 ± 2.3a	5.47 ± 0.41a	0.021 ± 0c
CP	4.56 ± 0.02c	2.2 ± 0.1d	14.7 ± 0.3b	6.65 ± 0.12c	51.76 ± 1.09c	5.48 ± 0.36a	0.023 ± 0b
B	6.03 ± 0.02b	3.1 ± 0.1a	41.3 ± 1.0a	13.53 ± 0.27b	152.0 ± 1.2b	4.32 ± 0.03b	0.027 ± 0a
BP	6.17 ± 0.01a	2.8 ± 0.1b	39.9 ± 0.6a	14.17 ± 0.13a	51.96 ± 3.01c	5.29 ± 0.30a	0.028 ± 0a

^aDifferent letters in a single column indicate significant difference between the treatments at $p < 0.05$. C: biochar untreated soil without rape, CP: biochar untreated soil with rape, B: biochar treated soil without rape, and BP: biochar treated soil with rape.

SI). Rape nitrite was determined by spectrometric method according to National food safety standards (see SI).

DNA Extraction and Illumina Sequencing. DNA was extracted from 0.5 g soil using the FastDNA SPIN kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instruction. The DNA was dissolved in 50 μ L of sterilized deionized and free of nucleases water and stored at -20°C before use for Illumina sequencing.

The V3 region of the 16S rRNA gene was used as the bacterial-specific fragment using the primers 338F (5'-ACT CCT ACG GGA GGC AGC AG-3') and 533R (5'-TTA CCG CGG CTG CTG GCA C-3').³⁵ Each pair of primers used to amplify a certain soil sample were barcoded with a unique error-correcting eight-base barcode³⁶ on both forward and reverse primers. All amplifications were performed in 4-fold 25 μ L reactions, while each reaction volume contained 1 μ L of DNA template (about 20 ng), 0.5 μ L of each appropriate primer (at a final concentration of 0.2 μ M), 0.25 μ L of bovine serum albumin (BSA, at a final concentration of 6 mM) (Takara, Japan), 12.5 μ L of 2 $\times$ DreamTaq Green PCR Master Mix (Thermo Scientific, U.S.A.). The PCR conditions, performed in a LifePro Thermal Cycler (Hangzhou Bioer Technology Co. Ltd., Hangzhou, China), consisted of an initial denaturation step at 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final extension step at 72°C for 5 min. 4-fold reactions of each sample were pooled and purified by agarose gel electrophoresis with a MinElute PCR Purification Kit (Qiagen, Germany). The concentrations of purified products were quantified using SpectraMax M5 (Molecular Devices) with PicoGreen.³⁷ The pooled samples were sent to BGI and sequenced on an Illumina HiSeq2000 platform (Illumina, San Diego, CA, U.S.A.).

RNA Extraction and Real-Time PCR. RNA was extracted based on the bead-beating method previously described by Griffiths et al.³⁸ with slight modification (see SI). Total RNA was purified using the AllPrep DNA/RNA Mini Kit (Qiagen, Germany) and digested with DNase I (Thermo Scientific, Canada). Reverse transcription of 16S rRNA was performed with random hexamer primers (0.5 μ g) using the Reverse Transcription System (Promega Corporation, Madison, U.S.A.) according to the manufacturer's instruction, while those of all the functional genes used gene-specific primers in the RT-PCR (see SI Table S3).

Quantification of archaeal and bacterial 16S rRNA, and archaeal and bacterial *amoA*, *nirK*, *nirS*, and *nosZ* transcripts were performed on a 7500 real-time PCR system (Applied Biosystems, Germany). For the bacterial 16S rRNA, the 20 μ L PCR mixture contained 10 μ L of Premix Ex Taq (TaKaRa, Japan), 0.2 μ M of each primer, 0.2 mg mL⁻¹ BSA, 0.4 μ M Taqman Probe, 0.4 μ L ROX Reference Dye and 1–10 ng cDNA. For the other genes, the 20- μ L reaction mixtures contained 10 μ L of SYBR Premix Ex TaqII (TaKaRa, Japan), 0.2 μ M of each primer, 0.25 μ L of 0.2 mg mL⁻¹ BSA, 0.4 μ L

ROX Reference Dye and 1–10 ng cDNA. Thermal cycling conditions and primers used for each reaction are described in SI Table S3. Plasmid pGEM-T Easy Vector (3015 bp, Promega, Madison, U.S.A.) was used in the cloning of gene fragments to establish standard curves for quantitative PCR (see SI).

High-Throughput Sequencing Data Preprocessing.

Data yielded from Illumina sequencing were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME 1.6.0) toolkit.³⁹ The process of analyses is described as follows: (i) removed any low quality or ambiguous reads; (ii) assigned taxonomy of the qualified sequence reads using RDP classifier at a 97% identity threshold; (iii) removed putative chimeras and singletons from OTU tables generated from high-throughput sequencing data; (iv) calculated α -diversity indices (Shannon-Weaver index (H), PD whole tree, observed species and chao1) to assess the internal (within-sample) complexity of individual microbial populations; and (v) computed β -diversity using the unweighted UniFrac metric.⁴⁰

Statistical Analysis. Univariate statistical and correlation analyzes were performed using SPSS 18.0 (SPSS Inc., Chicago, III, U.S.A.) and Sigmaplot 12.0 (Systat Software, Inc., San Jose, CA). The statistical significance of the differences between samples was determined by Duncan's multiple range test. P -values < 0.05 were considered significant. RDA were performed using R (2.14.0, <http://www.r-project.org/>) with the community ecology package "vegan (2.0–10)".⁴¹ Envfit function (999 permutations) was used to choose significant environmental factors. Adonis was used to test the microbial community composition significance between treatments.

RESULTS

Soil Chemical Properties. Changes in the chemical properties of soil in the four treatments are displayed in Table 1. The addition of biochar significantly increased soil pH from 4.48 ± 0.01 to 6.03 ± 0.01 without rape, and from 4.56 ± 0.02 to 6.17 ± 0.02 with rape. TC, TN, C/N ratio, and CEC were also significantly increased with biochar application. There was no difference in final NO₃⁻ concentration between CP and BP, which were much lower than those in soils without rape. Final NO₃⁻ and NH₄⁺ concentrations in B were lower in those with in C. Planting rape also increased soil pH, but the effect was not as strong as those treated with biochar. Planting rape had no effect on TC, however decreased TN, resulting in the increase of the C/N ratio.

Biochar addition increased rape shoot biomass from 2.31 ± 0.19 to 4.23 ± 0.21 g dry weight per pot (standard error). Biochar also decreased total N and inhibited NO₂⁻ accumulation in rape shoot, but had no effects on shoot total C, ammonium, and nitrate.

N₂O Emissions, Nitrification and Denitrification Activities. Biochar addition reduced N₂O emissions regardless of plantation (Figure 1). For the treatments without plant, biochar decreased N₂O emissions from the second week, while

Figure 1. N₂O emissions in greenhouse soil every week during the rape growth period ($n = 4$; error bars represent standard errors). C: biochar untreated soil without rape, CP: biochar untreated soil with rape, B: biochar treated soil without rape, and BP: biochar treated soil with rape.

for the treatments with plant, biochar addition only significantly decreased N₂O emission in the second and fourth week. Rape planting also had some effect on N₂O emission. For the untreated soils, rape planting increased N₂O emissions during the first 2 weeks and decreased from the third week.

We summed the total N₂O emission during the sampling period (over 2 h) determined each week (SI Figure S1). According to SI Figure S1, treatment C emitted the highest cumulative N₂O, followed by CP and B. The total N₂O accumulation in BP is negative, which shows that the soil with the biochar amendment and rape planting could be more likely a sink for N₂O. Generally, biochar decreased N₂O accumulation significantly irrespective of planting.

Potential nitrification rates and denitrification enzyme activity of soils treated with biochar, are much higher than those without biochar (Figure 2). Nitrification and denitrification showed similar responses to biochar addition and rape planting. BP had the highest nitrification and denitrification activities, followed by the treatment B.

Shift of Microbial Community Composition under Biochar Application. After demultiplexing and quality filtering, 723 854 high-quality sequences were obtained in total (more information see SI). To compare the microbial community diversity among all the soils, a sequencing depth of 19 772 sequences were randomly selected from each sample in the sequencing library. Rarefaction of observed species showed that even at a sequencing depth of 19 772, the diversity of soil bacteria continued to rapidly increase with increasing

sequencing depth (SI Figure S2), which illustrated the high diversity of soil bacteria. All diversity indices showed that bacterial community diversities in treatments with biochar amendment were higher than those in control soils (SI Table S4).

The most abundant phyla were Proteobacteria, Acidobacteria, Chloroflexi, Actinobacteria, Firmicutes, TM7, Gemmatimonadetes and Bacteroidetes, and these taxa accounted for more than 91% of the bacterial sequences in all soils of the four treatments (SI Figure S3). As shown in SI Figure S4, Acidobacteria was the most sensitive phylum to biochar, with relative abundances decreasing from 17.8% to 6.1%, 17.2% to 5.9% with and without rape, respectively. Chloroflexi was also significantly decreased by biochar, from 15.2% to 10.7% with rape planting, and from 14.7% to 10.3% without rape planting. On the other hand, Bacteroidetes, Gemmatimonadetes, and TM7 were noticeably increased after biochar application. Treatment B had the highest Firmicutes abundance (19.1%), while the abundances in C, CP, and BP were 12.5%, 13.3%, and 13.5%, respectively. Proteobacteria, Actinobacteria, and Cyanobacteria showed no response to biochar treatment or rape planting. Rape planting was observed to only have an effect on Firmicutes and Gemmatimonadetes with biochar amendment, and had no obvious effects on the other phyla relative abundances.

Families, those have a relative abundance higher than 1.5% in at least two treatments, were selected for analysis. As shown in Table 2, at the family level the relative abundances of Acidobacteriaceae, Koribacteraceae, Solibacteraceae, Bradyrhizobiaceae, Gaiellaceae, and Rhodospirillaceae were significantly lower in biochar treatments than those in the controls, while the relative abundances of Sphingomonadaceae, Flammeovirgaceae, Hyphomicrobiaceae, and Chitinophagaceae were significantly higher after biochar application. Without rape, biochar amendment significantly decreased the relative of Xanthomonadaceae, and increased the relative abundance of Bacillaceae. Although the relative abundances of Clostridiaceae were higher than 2%, there was no significant difference between treatments.

The RDA plots of the bacterial communities at the family level clearly indicated that the bacterial community composition of the samples was strongly changed by biochar application (Figure 3). Upon ADONIS analysis, the soil bacterial community composition was significantly changed by biochar. However, rape only had significant effect on the soil bacterial community composition with biochar amendment (BP and B), but not without biochar (CP and C). The first axis could

Figure 2. Potential nitrification rates and denitrification enzyme activities in the greenhouse soil under different treatments. Significant differences are indicated by different letters ($p < 0.05$). C: without biochar amendment; B: with biochar amendment; -P: without rape planting; +P: with rape planting.

Table 2. Relative Abundances of Abundant Families in Four Treatments^{a,b}

Families	C	CP	B	BP
Acidobacteriaceae	1.95 ± 0.13b	2.22 ± 0.30a	0.36 ± 0.03c	0.27 ± 0.03c
Koribacteraceae	6.31 ± 0.50a	5.47 ± 2.02a	2.61 ± 0.94b	1.73 ± 0.94b
Solibacteraceae	3.29 ± 0.60a	3.40 ± 1.11a	1.07 ± 0.13b	1.05 ± 0.13b
Gaiellaceae	2.07 ± 0.37a	1.90 ± 0.46a	1.15 ± 0.15b	0.94 ± 0.15b
Bradyrhizobiaceae	1.29 ± 0.24a	1.84 ± 0.75a	0.23 ± 0.07b	0.29 ± 0.07b
Rhodospirillaceae	2.08 ± 0.12a	1.89 ± 0.28a	1.20 ± 0.22b	1.37 ± 0.22b
Xanthomonadaceae	7.74 ± 1.57a	7.25 ± 1.47a	3.66 ± 0.90b	6.11 ± 0.90a
Flammeovirgaceae	0.01 ± 0.00c	0.01 ± 0.00c	2.49 ± 0.60b	3.09 ± 0.60a
Chitinophagaceae	0.41 ± 0.10b	0.77 ± 0.27b	4.76 ± 1.30a	4.83 ± 1.30a
Sphingomonadaceae	1.71 ± 0.21b	1.64 ± 0.20b	2.44 ± 0.29a	2.74 ± 0.29a
Hyphomicrobiaceae	1.54 ± 0.15c	1.82 ± 0.28b	1.83 ± 0.20b	2.05 ± 0.20a
Bacillaceae	4.99 ± 1.66b	5.27 ± 1.27b	8.44 ± 1.04a	4.93 ± 1.04b
Clostridiaceae	2.60 ± 0.46a	2.75 ± 0.28a	2.80 ± 0.48a	2.67 ± 0.48a

^aDifferent letters in a single column indicate significant difference between the treatments at $p < 0.05$. ^bC: biochar untreated soil without rape, CP: biochar untreated soil with rape, B: biochar treated soil without rape, and BP: biochar treated soil with rape.

Figure 3. Redundancy analysis (RDA) of soil samples based on bacterial community composition obtained using the family abundances matrix in each sample. C: biochar untreated soil without rape, CP: biochar untreated soil with rape, B: biochar treated soil without rape, BP: biochar treated soil with rape.

explain 57.90% of the total soil microbial community variation. TC, pH, TN, C/N ratio, CEC, and ammonium, moisture, and nitrate were used in the environmental factor matrix to calculate their contribution to the soil microbial community variation among the four treatments. After filtering using the envfit function (999 permutations) with R, pH, TC, TN, C/N ratio, CEC, and ammonium were chosen in the biplot of RDA. In total, these factors could explain 78.7% of the soil microbial community composition variation. TC, pH, TN, C/N ratio, and CEC explained 73.4% of the soil microbial community composition variation, but the greatest part of the explanation may be their coeffect due to their significant correlation ($p \leq 0.01$) with each other (SI Table S5).

Transcript Abundances of 16S rRNA and Functional Marker Genes. The gene copy numbers in cDNA produced from the reverse transcription of RNA were used to demonstrate the active expressions of total archaea, total bacteria, ammonia-oxidizing archaea, ammonia-oxidizing bacteria and denitrifiers (Figure 4). There were no significant differences among all the four treatments in the transcription of archaeal 16S rRNA and bacterial 16S rRNA. Similarly, bacterial *amoA*, *nirS*, and *nirK* gene transcripts showed no responses to biochar either. Among all the genes, only archaea *amoA* and *nosZ* gene transcripts were significantly stimulated by biochar addition. Transcriptions of archaeal *amoA* increased from 2.04×10^5 to 1.43×10^6 and 2.16×10^5 to 7.29×10^5 transcript

Figure 4. Transcript copy numbers of archaeal and bacterial 16S rRNA, archaeal and bacterial *amoA*, *nirK*, *nirS*, and *nosZ* genes in the greenhouse soil under different treatments. Significant differences are indicated by different letters ($p < 0.05$). C: without biochar amendment; B: with biochar amendment; -P: without rape planting; +P: with rape planting.

copies per g dry soil with and without rape planting, respectively. Transcriptions of *nosZ* increased from 3.86×10^5 to 1.43×10^6 and 2.16×10^5 to 7.29×10^5 transcript

10^6 to 8.59×10^6 and 3.70×10^6 to 6.38×10^6 transcript copies per g dry soil with and without rape planting, respectively. Planting rape slightly inhibited transcriptions of *nosZ* where biochar was added.

DISCUSSION

Nitrification, Denitrification and N_2O Emissions.

Nitrification is thought to be an important pathway of soil N_2O production. However, in this study, the increased nitrification rates by biochar did not result in higher N_2O emissions. As nitrification is an acidifying process,⁴² alkaline biochar may create much more favorable conditions for nitrifiers thus increasing nitrification rates due to its liming effect.^{43,44} The decreased N_2O emission and increased nitrification rates demonstrate that the relative contribution of nitrification to N_2O emissions is much less than that of denitrification (including nitrifier denitrification) in this soil. This result shows that biochar inhibition on nitrification is unlikely to be the mechanism of N_2O reduction in the soil.

Although the acetylene inhibition method has limitations, these limitations have been shown under longer incubation (168 h) rather than in short time incubation systems.⁴⁵ In contrast to the hypotheses that biochar may inhibit denitrification by increasing soil aeration or adsorption of NO_3^- , denitrification enzyme activity in this study was stimulated by biochar. Soil pH may be the main factor that induces the enhancement of denitrification, as pH was increased about 2 units by biochar. It is generally agreed that the rate of denitrification (production of $N_2O + N_2$) is optimal in the range of pH 7.0–8.0 while increasing the pH of acidic soil will accelerate denitrification.⁴⁶ Biochars may increase the activity of N_2O -reductase enzymes of denitrifiers through increasing soil pH, thereby enhancing N_2 formation from N_2O while stimulating denitrification.⁴⁷ Therefore, hypothesis (iii) is the mechanism resulting in decreasing N_2O emission here: biochar suppresses N_2O emissions by means of further reducing it to N_2 . Nevertheless, this conclusion should not be applied to all conditions since the effect of biochar on N_2O emissions may depend on soil properties and biochar characteristics.

Changes of Soil Chemical Properties and Microbial Community Caused by Biochar. Biochar addition changed soil chemical properties, including increasing soil pH, TN, TC, C/N ratio, and CEC, and shifted the bacterial community composition. As biochar has been considered unlikely to be used by soil microbes before initial-oils or condensates are decomposed,²¹ and it can not directly influence soil microbial community, the major contribution of chemical properties to the soil microbial community variation and significant correlation between the changed relative phylum abundances and soil chemical properties (SI Table S6) indicates that biochar may affect soil microbial community via improving soil chemical properties. In many previous studies, pH has always proven to be a prime variable in shaping the soil bacterial community, but mostly from biogeographical perspectives.^{48,49} In this study, soil pH, variation induced by biochar amendment, was also observed to be an important factor in shifting the soil microbial community, contributing 56.5% of the total variation. Högberg et al.⁵⁰ have reported that the C/N ratio was as good as pH in predicting the soil microbial community. However, in this study the C/N ratio was increased somewhat artificially by the addition of C in the biochar, which was unavailable to microbes in a short incubation period, unlike the natural system

investigated by Högberg et al.⁵⁰ Thus, in envfit test, C/N ratio was found to be significant in shaping the soil microbial community composition, which could be due to its high correlation with soil pH. Rape planting also showed some influence on the soil microbial community composition but only in treatments with biochar. This result demonstrates that plantation contributes less than chemical properties caused by biochar to the soil microbial community variation; though this should not be generalized as plant root systems vary greatly between species.

Neither total archaeal 16S *rRNA* nor bacterial 16S *rRNA* transcription was changed by biochar. Similar results for bacterial abundance at the DNA level were observed in an incubation experiment with biochar amendment under anoxic conditions.²⁶ These results demonstrate that biochar may not change microbial size, but shift the microbial community composition by increasing soil microbial α -diversity and modifying taxa relative abundances (SI Figures S1 and S4).

At the family level, the relative abundances of Flammeovirgaceae, Chitinophagaceae, Sphingomonadaceae, and Hyphomicrobiaceae were increased by biochar. Flammeovirgaceae and Chitinophagaceae belong to the same order Sphingobacteriales. They play an important role in organic matter decomposition and carbon cycling.⁵¹ *Sphingomonas* and *Novosphingobium* of Sphingomonadaceae have been reported to be able to degrade refractory pollutants⁵² and aromatic-compound,⁵³ respectively. Of Hyphomicrobiaceae, a species of *Devosia* has been shown to modulate and fix nitrogen symbiotically with plants,⁵⁴ and *Hyphomicrobium* is a genus capable of methyl-trophic denitrification.⁵⁵ These results showed that biochar amendment changed the relative abundances of microbes that underpin carbon and nitrogen cycling in this soil.

Here we also observed an increase in rape shoot biomass after biochar addition. This can be attributed to the shift in soil microbial community composition by biochar, which may drive nutrient (e.g., C, N, and P) cycling thus increasing nutrient availability to crops. Biochar addition has been shown to increase nutrient availability.⁵⁶ These results are of practical economic value when biochar is used for acidic soil amelioration.

Expression of N_2O -Related Functional Genes. The changes in nitrification and denitrification could be due to the fact that biochar amendments change the microbial diversity and taxa relative abundances as biochar did not change total expression of archaeal and bacterial. Biochar increased the activity of nitrifiers and denitrifiers. Archaea may contribute more than bacteria to the enhanced nitrification by biochar, as the expression of archaeal *amoA* abundances were considerably increased by biochar, but not bacterial *amoA* (Figure 4). In previous studies, at DNA level, biochar has been shown to increase both archaeal *amoA* and bacterial *amoA* compared to control at the last wet cycle of an incubation.²⁷ Correspondingly, Ducey et al.²⁵ observed a higher bacterial *amoA* abundance in the presence of biochar at the end of an incubation of 6 months. In this experiment, no fertilizer was added, and ammonium is likely derived from the mineralization of organic matter, to which AOA is more adapted,^{57,58} resulting in no effect on bacterial *amoA* transcription by biochar.

Although the denitrification process was stimulated by biochar, *nirS* and *nirK* transcripts did not change significantly. This may be because nitrite reductase encoded by *nirS* or *nirK* was not the limiting factor of denitrification in this study. There is also a possibility that some quantification of bacterial

functional genes could not represent all denitrifiers, as denitrification can be performed by bacteria, archaea, and fungi.^{59,60} The enhanced expression of *nosZ* supports the inference that biochar mitigates N₂O emissions by further reducing it to N₂. In their incubation experiment using water-saturated soil microcosms, Harter et al.²⁶ also observed that biochar increased the transcript copy numbers of *nosZ* genes, while having no significant effect on the abundances of *nirK* and *nirS* genes. Similar results of incubation experiments have been reported, at DNA level, *nosZ* gene abundance was significantly higher in the presence of biochar.^{25,27} Thus, our findings suggest that enhanced *nosZ* gene transcripts by biochar may be the main reason for N₂O emission reduction regardless of the experimental conditions. Although the soil was only sampled at the end of the incubation and the results of the soil functional genes are limited in explaining N₂O emissions during the entire incubation, they could explain the lower cumulative N₂O emission in B than in C. Because rape may have a more important effect on soil N₂O emission, biochar did not have a significant effect on the 6th week when the soil was collected, but also result in much lower cumulative N₂O emission with rape.

In summary, when used in acidified soil amelioration, biochar can increase crop yield through improving soil chemical conditions and changing the availability of nutrients. It can also impact soil microbial community (increasing α -diversity of soil microbes and changing relative abundances of taxa) via changing soil chemical properties, thus influencing soil nutrient (e.g., C, N) cycling and controlling greenhouse gas emissions. By contrast, biochar can also enhance soil N losses to the atmosphere by stimulating both nitrification and denitrification, thus decreasing the efficiency of N-fertilizer utilization. Therefore, the effect of biochar on the use efficiency of N-fertilizer should be considered when it is widely recommended for soil amendments.

■ ASSOCIATED CONTENT

■ Supporting Information

Basic properties of the soil and biochar used in biochar amendment pot trial study in greenhouse (Table S1), elemental composition of the soil and biochar used in biochar amendment pot trial study in greenhouse (Table S2), primers and PCR conditions used for the real-time PCR in biochar amendment pot trial study (Table S3), comparison of α -diversity indices in four treatments with or without biochar amendment (Table S4), correlations of soil properties (Table S5), correlations of abundant phyla with soil properties (Table S6), N₂O accumulation during determination period; the accumulation of N₂O is calculated by adding N₂O emission in 2 h determined each week; the letters were used to show the differences among treatments (Figure S1), rarefaction of observed species at a sequencing depth of 19 772 sequences per sample (Figure S2), relative abundances of bacterial community composition at phylum level detected in biochar treated and untreated soils (Figure S3), relative abundances of taxa at class level in the eight dominant phyla detected in biochar treated and untreated soils (I: Proteobacteria, II: Firmicutes, III: Acidobacteria, IV: Actinobacteria, V: Chloroflexi, VI: Gemmatimonadetes, VII: Bacteroidetes, VIII: TM7); the abundance is presented in terms of an average percentage of four replicates, classified by RDP Classifier at a confidence threshold of 97%; "Other" refers to the sum of unclassified sequences and all other taxa unnamed. C: biochar untreated soil without rape, CP: biochar untreated

soil with rape, B: biochar treated soil without rape, BP: biochar treated soil with rape; the letters were used to show the differences among treatments at phylum level (Figure S4), redundancy analysis (RDA) of bacterial community composition obtained using the family abundances matrix in each sample; and the symbols represent different taxa at the family level (Figure S5). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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