Applied Biochemistry and Biotechnology (2020) 192:22–41
https://doi.org/10.1007/s12010-020-03250-9

Characterization of Denitrifying Community for Application in Reducing Nitrogen: a Comparison of *nirK* and *nirS* Gene Diversity and Abundance

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Received: 15 August 2019 / Accepted: 8 January 2020 /
Published online: 25 March 2020

Abstract

Studies have shown that the addition of biochar to agricultural soils has the potential to mitigate climate change by decreasing nitrous oxide (N_2O) emissions resulting from denitrification. Rice paddy field soils have been known to have strong denitrifying activity, but the response of microbes to biochar for weakening denitrification in rice paddy field soils is not well known. In this work, compared with the chemical fertilizer alone, the chemical fertilizer + 20 t hm^{-2} biochar fertilizer slightly decreased denitrifying the nitrite reductase activity (S-NiR) and N_2O emission without statistic difference, whereas the chemical fertilizer + 40 t hm^{-2} biochar significantly boosted them. The abundance of *nir*-denitrifiers contributed to S-NiR and N_2O emission, especially *nirS*-denitrifiers, rather than the variation of community structure. Pearson correlation analysis showed that NO_2^--N was a key factor for controlling the abundance of *nir*-denitrifiers, S-NiR and N_2O emission. The biochar addition fertilization treatments strongly shaped the community structure of *nirK*-denitrifiers, while the community structure of *nirS*-denitrifiers remained relatively stable. In addition, *Paracoccus* and *Sinorhizobium* were revealed to be as the predominant lineage of *nirS*- and *nirK*-containing denitrifiers, respectively. Distance-based redundancy analysis (db-RDA) showed that changes in the *nir*-denitrifier community structure were significantly related to soil organic carbon, NO_3^--N, and total phosphorus. Our findings suggest that, although the *nirS*- and *nirK*-denitrifiers are both controlling nitrite reductase, their responses to biochar addition fertilization treatments showed significant discrepancies of diversity, abundance, and contribution to N_2O and *S-NiR* in a paddy soil.

Keywords *Nir*-denitrifiers · Reducing nitrogen application · Biochar · Nitrite reductase

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12010-020-03250-9) contains supplementary material, which is available to authorized users.

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Introduction

It is well known that nitrous oxide (N_2O) is a potent greenhouse gas and strongly catalyzes stratospheric ozone depletion [1]. Furthermore, recent reports have revealed that atmospheric N_2O concentration has been constantly rising and reached 328 ppb in 2016 (121% of preindustrial levels), mainly due to anthropogenic intervention in the nitrogen cycle [2]. Given that agriculturally managed soils emit 4.3–5.8 Tg N_2O yr^{-1}, developing effective mitigation strategies is a key challenge for the agricultural sector [3].

Rice is one of the most widely consumed staple foods in the world and is cultivated on nearly 155 million ha [4]. Paddy fields are an unavoidable component of many agricultural ecosystems. Irrigated rice cultivation often includes water regimes that alternate between flooded and drained conditions throughout the crop cycle, producing optimal conditions for denitrification [5]. Notably, the use of mid-season drainage sharply increases the flux of N_2O [6]. In addition, increasing N_2O levels in the atmosphere are significantly correlated with high nitrogen (N) and organic fertilizer application rates in agricultural ecosystems [7], especially paddy fields [8]. Therefore, the reduced application of chemical fertilizers in paddy fields would be a route to significantly decreasing N_2O emissions.

To achieve high yields of rice as well as decreasing N_2O, sustainable alternatives to these inorganic fertilizers are being sought [9, 10]. Biochar is generated by thermal decomposition of organic biomass under low oxygen conditions and has attracted a lot of attentions from agronomists and soil scientists because of its soil-enhancing properties and resistance to biodegradation [12, 13]. Furthermore, its potential function of inhibiting greenhouse gas emissions has been verified in a series of field and incubation experiments [14–16]. There are some evidences that, when biochar is added, soil pH tends to high and then inhibit the emission of N_2O [17, 18] due to certain physico-chemical properties [19]. In addition, factors such as the biochar source, the soil type, and the chemical form of the N fertilizer were found to affect the function of biochar decreasing N_2O [20]. However, the main mechanism leading to decreased N_2O emissions is still poorly understood [14, 20, 21].

Changes in soil properties can only explain the key mechanism of decreasing N_2O with difficulty. Undoubtedly, denitrification is mediated by soil microorganisms called denitrifiers, which reduced nitrate (NO_3^-) under oxygen-depleted conditions to produce gases such as N_2O and dinitrogen (N_2). They are, therefore, widely identified as the initiators of nitrogen loss from soil to atmosphere [22]. Denitrification results from a series of biological enzymes catalyzing the reactions of the different reduction steps [23]. Among these, the key reaction is the reduction of NO_2^- to NO, catalyzed by two structurally different, but functionally equivalent nitrite reductases, namely, cytochrome cd_1-containing reductase (*nirS*) and copper-containing reductase (*nirK*) [24]. This step causes dissolved N to become NO for the first time, and NO is then easily reduced to N_2O [25]. In other words, the activity of *nirS* and *nirK* genes determines the initial amount of N_2O, so these genes are widely used as molecular markers to investigate the micro-mechanism of N_2O loss. *NirS* and *nirK* are thought to be mutually exclusive among denitrifying species and to represent two ecologically distinct denitrifying groups [26], with diversity and abundance responding differently to various ecology systems [27–29]. Research has been carried out to explore the correlation between the N_2O flux and the diversity and abundance of denitrifiers, using denitrification enzyme activity (DEA) or potential denitrification activity (PDA) as indicators [30–32]. However, the DEA or PDA represented the whole of denitrification, rather than the known contribution of nitrite reductase. In this work, therefore, we have added nitrite reductase activity (S-NiR) as a

measure of the activity of *nirS* and *nirK* genes. Based on this more accurate indicator, the determination of the diversity and abundance of gene function has enabled us to identify the micro-mechanism of N_2O emission.

The purplish paddy soil in the southwest China was collected in this study [33]. We chose pot experiments as the main test method, in order to amplify the effect of a gradient reducing nitrogen application with biochar on *nirS*- and *nirK* denitrifier communities. Based on the discrepancy between *nirS* and *nirK* denitrifiers responding to different fertilizer treatments, the relationship between the N_2O flux, nitrite reductase, and denitrifiers can be identified. We hypothesize that (1) the medium amount of biochar application decreases the N_2O flux; (2) there is a significant species discrepancy between *nirS*- and *nirK*-denitrifier communities; (3) and the *nirS*-denitrifiers are mainly responsible for the N_2O flux.

Materials and Methods

Pot Experiments and Sampling

In the Southwest University greenhouse, a cultivation experiment with rice was established on March 10, 2017 with samples from the top 20 cm of soil at the National Monitoring Station of Soil Fertility and Fertilizer Efficiency on Purple Soils (30° 26′ N, 106° 26′ E, and 266.3 m above sea level) located in Beibei, Chongqing, China. The soil was a *Cabhaplic Stagnic Anthrosol* classified as a *Regosol* [34], derived from gray-brown purple sand shale parent materials in the Mesozoic Jurassic Shaxi Temple Group. It contained 13.95 $g{\cdot}kg^{-1}$ organic carbon (SOC), 121.52 $mg{\cdot}kg^{-1}$ available nitrogen (N), 264.25 $mg{\cdot}kg^{-1}$ available phosphorus (Olsen-P), and 206.32 $mg{\cdot}kg^{-1}$ available K and was at pH 7.86 prior to the start of the pot experiment.

The experiment included five fertilization treatments: (1) control (CK, no fertilizer); (2) chemical fertilizer (F, 1.20 g N pot^{-1} + 0.72 g P_2O_5 pot^{-1} + 0.96 g K_2O pot^{-1}); (3) chemical fertilizer +10 $t{\cdot}hm^{-2}$ biochar (LFB, 1.09 g N pot^{-1} + 0.69 g P_2O_5 pot^{-1} + 0.68 g K_2O pot^{-1} + 26.67 g biochar pot^{-1}); (4) chemical fertilizer +20 $t{\cdot}hm^{-2}$ biochar (MFB, 0.97 g N pot^{-1} + 0.67 g P_2O_5 pot^{-1} + 0.40 g K_2O pot^{-1} + 53.34 g biochar pot^{-1}); and (5) chemical fertilizer +40 $t{\cdot}hm^{-2}$ biochar (HFB, 0.74 g N pot^{-1} + 0.62 g P_2O_5 pot^{-1} + 0.16 g K_2O pot^{-1} + 106.68 g biochar pot^{-1}). Each treatment had six replicates and the 30 PVC pots (460 cm^2 × 23 cm) were arranged in randomized blocks. Based on the traditional local agricultural management, the amounts of N, P_2O_5, and K_2O were 0.2 $g{\cdot}kg^{-1}$, 0.12 $g{\cdot}kg^{-1}$, and 0.12 $g{\cdot}kg^{-1}$, respectively. The N, P, and K fertilizers used were urea (N 46%), calcium superphosphate (P_2O_5 12%), and potassium chloride (K_2O 60%). Except for urea, the chemical fertilizers and biochar were applied as basal fertilizers. The proportions of the urea used as basal and supplementary fertilizer were 60 and 40%, respectively, applied on May 6 and June 1. The biochar used in the experiment was provided by Sichuan Jiusheng Agriculture Co., Ltd. It was manufactured using rape straw as the raw material to char under anaerobic pyrolysis conditions of 500 °C for 2 h.

Gas Collection and Analysis

Gas samples were collected by in situ static chamber [35]. The chambers were made of stainless steel, with a cross-sectional area of 0.36 m^2 (60 × 60 cm) and a total height of 50 cm,

including both layers (see Hou et al. [36] for detailed description). There was a bottom layer (50 cm diameter × 3 cm high) under each pot, and the static chambers were covered by thermal-protective coating to avoid high temperature. The first gas sample was collected immediately after the rice was transplanted and the collecting interval was 7–10 days. The operating requirements and interval time for gas collection was carried out in accordance with the method of Yang et al. [11]. Gas samples were stored in a 50-mL syringe, and N_2O concentrations were analyzed using a gas chromatograph (Agilent GC-7890A, Agilent Science and Technology Ltd., USA).

Nitrite Reductase Measurement, Soil Sampling, and Analysis

The activity of nitrite reductase (S-NiR) was measured using a Nitrite Reductase Kit for soil (Comin Biotechnology Co. Ltd., Suzhou, China) according to the manufacturer's instructions.

Three soil samples without visible stones or plant residues were collected randomly from each pot after harvest and thoroughly mixed. The soil was air-dried and passed through different sieve types according to experimental requirements. The basic physical and chemical characteristics were analyzed by standard methods [37–39].

DNA Extraction and Cloning

The genomic DNA of soils was extracted using a Fast DNA SPIN Kit for soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. For amplification of *nirS*, the primer pair cd3aF [40] and R3cd [41] was used, while another primer pair 517F and 1055R was used for *nirK*-targeting polymerase chain reaction (PCR) [42]. The PCR was performed in a C1000™ thermal cycler (Bio-Rad, USA).

PCR-generated *nirS* and *nirK* fragments from soil DNA were excised from agarose gels and purified using a universal DNA Purification Kit (Tiangen Co. Ltd., Beijing, China) followed by molecular cloning with a pGM-T vector (Tiangen) into the *E. coli* strain DH5α. The 30 positive clones of *nirS* and *nirK* gene were randomly selected in each sample, respectively. In other words, each gene has 150 alternative clones from five samples. Screening for the correct size inserts was conducted via PCR with M13 forward and reverse primers (Tiangen Co. Ltd., Beijing, China). The correct size clones were sequenced, and BLAST was used to confirm the identity and validity of sequences. Typical *nirS* and *nirK* sequences, namely, operational taxonomic units (OTUs), were defined using 5% differences in the nucleotide sequences in the MOTHUR Program [43]. The typical *nirS* and *nirK* gene sequences were deposited in the GenBank database under the accession numbers *nirK*: MK521815-MK521827; *nirS*: MH316008 - MH316010, MH316012 - MH316013, MH316016 - MH316017, MH316019 - MH316020, MH316023, MH316025 - MH316029, MH316031 - MH316031, MH316037, MH316042 - MH316055.

PCR Amplification and Terminal Restriction Fragment Length Polymorphism

The *nir*S and *nirK* genes were amplified using the same primer pairs as the cloning. However, the forward primers were fluorescently labeled with carboxyfluorescein (FAM). PCR amplifications and thermocycling conditions were both same as for the cloning.

For each treatment, three individual PCR products were thoroughly mixed to avoid PCR artifacts and were gel-purified using Tiangen gel extraction kit (Tiangen Co. Ltd., Beijing,

China) to get exact DNA fragments. For T-RFLP, the *nirS* and *nirK* PCR products were digested with *HhaI* and *TaqI* (New England BioLabs Co. Ltd., UK) endonuclease at 37 and 65 °C for 10 min and then inactivated at 60 and 80 °C for 20 min, respectively. T-RFLP profiles were generated by the Sangni Corporation (Shanghai, China) using an ABI Prism 3100 Genetic Analyzer. In order to assign the peaks of T-RFs to clone sequences, clones corresponding to different in silico T-RFs were also subject to T-RFLP analysis following the same procedure described above except that monoclones were used as the template for PCR.

Quantification of Gene Abundance

The abundances of *nirS*- and *nirK*-denitrifiers were estimated via quantitative PCR (qPCR). The primer pairs nirScd3aF/R3cd [41] and nirK876/1040R [44] were used for *nirS* and *nirK* gene amplifications, respectively. Three duplications were performed per sample. Thermal cycling, fluorescence data collection, and data analysis were conducted with the iCycler iQ5 thermocycler (Bio-Rad, USA) detection system according to the manufacturer's instructions.

The standard curve was developed by methods described in the literature [44]. In order to test the potential inhibition of soil substance spiking in DNA, the soil DNA extracts were serially diluted and subject to qPCR analysis. Minimum inhibition was observed when the dilution was 20-fold. The efficiency of the reactions was 90% and 103% for *nirS* and *nirK*, respectively. The R^2 values for the standard curves were 0.998 and 0.997, respectively.

Statistical Analysis

The physico-chemical properties and PCR values were analyzed using the IBM SPSS statistical software package version 23 (IBM Corporation, New York, USA) with one-way ANOVA and Pearson's least significant difference ($p < 0.05$).

Size and relative abundances of T-RFs were quantified using Peak Scanner version 1.0 (Applied Biosystems, Inc., USA). The raw data was modified for noise and peak alignment and was presented in a data matrix [45]. Peaks with a fluorescence signal above 2% of the sum of all peak height signals in each sample were included in further analysis.

Based on the T-RFs, α-diversity (Shannon index, Margalef index and Pielou index) was calculated by the method of Atlas and Bartha [46]. The T-RFs composition of *nir*-denitrifiers was presented using Heatmap (HemI 1.0 version). Distance-based redundancy analysis (db-RDA) was chosen to explore the relationship between the community structure of *nirK*- and *nirS*-denitrifiers and soil environmental factors using Canoco 5 for Windows (Microcomputer Power, Ithaca, NY, USA) [47]. The clone sequences were input into MEGA 5.0. (version 5.0, www.megasoftware.net) [48], followed by alignment to construct the phylogenetic trees using neighbor-joining algorithm. The reliability of branching within the tree was tested with bootstrapping (1000 replicates), and the consensus tree was presented.

Results

Basic Soil Parameters

The application of chemical and biochar fertilizers impacted on soil physico-chemical properties (Table 1). The impacts of fertilization treatments were significant on soil NO_3^--N and

NH_4^+-N. Chemical fertilizer alone significantly increased soil NO_3^--N and NH_4^+-N, by approximately 51.23 and 3.02 units, respectively (Table 1). High and middle biochar addition fertilizations produced more significant impacts on the soil pH, soil organic carbon (SOC), total nitrogen (TN), and NO_2^--N (Table 1). Chemical as well as biochar addition fertilization treatments all significantly increased contents of total potassium (TK) by 3.88 g kg^{-1} and 2.98–4.94 g kg^{-1}, respectively. However, as the increasing biochar content in biochar addition fertilization treatments, the ability of increasing on TK was gradually weak.

N_2O Emissions and Nitrite Reductase Activity

Chemical and biochar addition fertilization treatments significantly altered N_2O flux, especially HFB treatment (Fig. 1). Meanwhile, the effects of fertilization treatments on N_2O flux were similar to *S-NiR*. Pearson correlation analysis indicates that the N_2O flux was positive correlation with S-NiR ($R^2 = 0.925$, $p < 0.01$). The N_2O flux ($R^2 = 0.960$, $p < 0.01$) and S-NiR ($R^2 = 0.925$, $p < 0.01$) were both significantly correlated with NO_2^--N, reaching an extremely significant level. In addition, pH, TN, and NH_4^+-N also significantly influenced N_2O flux and S-NiR (Supplementary Table S1).

Abundance of *Nir*-Denitrifiers

The copy numbers of *nirK*-denitrifiers was one order of magnitude higher than that of *nirS*-denitrifiers. Chemical fertilization significantly increased the copy numbers of *nirS*- and *nirK*-denitrifiers by 14.2 and 8.8%, respectively. Biochar addition fertilization treatments significantly increased the copy numbers of *nirS*-denitrifiers but significantly decreased that of *nirK*-denitrifiers. Notably, as the content of biochar decrease in mixed fertilization treatments, the impact of biochar addition fertilization treatments on the abundance of *nirK*-denitrifiers was more significant (Fig. 2).

The abundance of *nirS*-denitrifiers was significantly related to NO_2^--N (Table 2). The abundance of *nirK*-denitrifiers, however, was not correlated with any of the soil properties measured. In addition, the abundance of *nirS*-denitrifiers was related to soil S-NiR and N_2O (Table 2).

Community Structure of *Nir*-Denitrifiers

Based on the relative abundances of T-RFs of *nirS*- and *nirK*-denitrifiers, the characteristic indexes of α-diversity were calculated (Table 3). Obviously, the values of Margalef index *nirK*-denitrifiers were more than that of *nirS*-denitrifiers, further supported by Heatmap (Fig. 3). Chemical as well as biochar addition fertilization treatments all changed the α-diversity of *nir*-denitrifiers without statistic difference. The most impact on the α-diversity of *nirS- and nirK*-denitrifiers was from LFB and F treatments, respectively.

The T-RFLP data suggested a different community structure of *nirS*- and *nirK*-denitrifier communities between the CK, F, LFB, MFB, and HFB samples (Fig. 3), respectively. This pattern was further supported by db-RDA ordination plots, with a long straight distance between each sample for both *nirS*- and *nirK*-denitrifiers, especially the CK and F fertilizer (Fig. 5).

For the *nirS*-denitrifiers (Fig. 3a), sequences with T-RFs of 19, 21, and 25 bp disappeared in chemical fertilization, with T-RFs of 98 bp only being present. The addition of biochar

Table 1 Physico-chemical properties in soil following addition of the gradient inorganic fertilizer with biochar regimes

Treatment	pH	SOC g·kg^{-1}	TN g·kg^{-1}	TP g·kg^{-1}	TK g·kg^{-1}	NH_4^+-N mg·kg^{-1}	NO_3^-N mg·kg^{-1}	NO_2^-N mg·kg^{-1}
CK	7.40 ± 0.00c	18.81 ± 0.07b	1.40 ± 0.00c	1.35 ± 0.01d	47.45 ± 0.30c	12.31 ± 0.17d	15.07 ± 0.41f	0.001 ± 0.00d
F	7.31 ± 0.00d	17.14 ± 0.03c	1.58 ± 0.00b	1.44 ± 0.00a	51.33 ± 0.37b	15.33 ± 0.19a	66.30 ± 0.36a	0.282 ± 0.01b
LFB	7.30 ± 0.02d	18.64 ± 0.03b	1.41 ± 0.01c	1.39 ± 0.00c	52.39 ± 0.35a	12.14 ± 0.10d	25.60 ± 0.38d	0.121 ± 0.00c
MFB	7.46 ± 0.02b	20.38 ± 0.22a	1.58 ± 0.00b	1.41 ± 0.00bc	51.43 ± 0.32ab	13.24 ± 0.15c	39.43 ± 0.39c	0.304 ± 0.02b
HFB	7.55 ± 0.02a	20.62 ± 0.15a	1.62 ± 0.00a	1.42 ± 0.01b	50.93 ± 0.09b	14.44 ± 0.31b	41.52 ± 0.91b	0.853 ± 0.03a

CK no fertilizer control, *F* chemical fertilizer, *LFB* chemical fertilizer + 10 t hm^{-2} biochar, *MFB* chemical fertilizer + 20 t hm^{-2} biochar, *HFB* chemical fertilizer + 40 t hm^{-2} biochar. Error bars indicate standard error (from $n = 3$ replicates) for each treatment. Different letters within the same column indicate significant differences ($p < 0.05$)

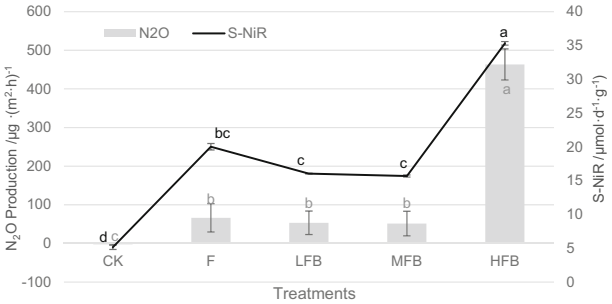

Fig. 1 Soil N_2O flux and S-NiR under chemical as well as biochar addition fertilization treatments. CK no fertilizer control, F chemical fertilizer, LFB chemical fertilizer + 10 t hm^{-2} biochar, MFB chemical fertilizer + 20 t hm^{-2} biochar, HFB chemical fertilizer + 40 t hm^{-2} biochar. Error bars indicate standard error (from $n = 3$ replicates) for each treatment. Different letters above the bar indicate significant differences among fertilizers ($p < 0.05$); letters in black stand for the S-NiR; letters in gray stand for the N_2O flux

increased the T-RFs of *nirS*-denitrifiers. Among biochar addition fertilization treatments, the ability to increase the T-RFs of *nirS*-denitrifiers was the most significant in the HFB treatment. Furthermore, the LFB and MFB treatments both increased the relative abundance of a T-RF of 16 bp, while decreasing that of 21 and 23 bp. The HFB treatment increased the relative abundance of T-RFs of 25 and 26 bp. In addition, Pearson correlation analysis indicated that N_2O and NO_2^--N had a significant positive relationship to T-RFs of 36 bp and 153 bp, and the latter also had a significant positive relationship to S-NiR (Supplementary Table S2).

Chemical and biochar addition fertilization treatments all significantly changed the community structure of *nirK*-denitrifiers (Fig. 3b). The T-RF of 16 bp only presented in the biochar fertilization treatments and showed high relative abundance. Compared with the T-RFs composition of *nirK*-denitrifiers in CK, the F treatment significantly increased the sequences of 105, 147,161, 192, and 215 bp, whereas biochar addition significantly decreased them. In addition, Pearson correlation analysis indicated that N_2O, S-NiR, and NO_2^--N had a significantly positive relationship to the T-RFs of 101 bp and 154 bp (Supplementary Table S3).

Fertilization treatments completely changed the T-RFs' composition of *nirK*-denitrifiers, whereas they changed the relative abundance of the T-RFs of *nirS*-denitrifiers. Cluster analysis

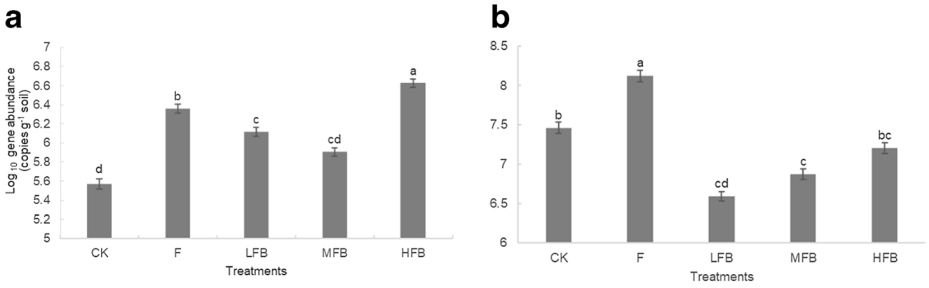

Fig. 2 Abundances of *nirS*- (**a**) and *nirK*-type (**b**) nitrate reductase genes (denitrifier abundance) under chemical as well as biochar addition fertilization treatments. CK no fertilizer control, F chemical fertilizer, LFB chemical fertilizer + 10 t hm^{-2} biochar, MFB chemical fertilizer + 20 t hm^{-2} biochar, HFB chemical fertilizer + 40 t hm^{-2} biochar. Error bars indicate standard error (from $n = 3$ replicates) for each treatment. Different letters above the bar indicate significant differences among fertilizers ($p < 0.05$)

Table 2 Correlation of *nirS*- and *nirK*-denitrifiers abundance with soil physico-chemical properties, N_2O flux and S-NiR

Abundance	Item	pH	SOC	TN	TP	TK	NH_4^+-N	NO_3^--N	NO_2^--N	N_2O	S-NiR
nirS	R^2	0.472	0.240	0.698	0.581	0.362	0.763	0.502	0.918*	0.928*	0.967**
	p	0.422	0.422	0.190	0.304	0.550	0.133	0.389	0.028	0.023	0.007
nirK	R^2	−0.357	−0.357	0.374	0.719	0.044	0.627	0.819	−0.087	−0.242	−0.009
	p	0.556	0.556	0.535	0.171	0.945	0.257	0.09	0.89	0.695	0.988

* Correlation is significant at the 0.05 level. ** Correlation is significant at the 0.01 level

indicated that the response of *nirK*-denitrifiers to biochar addition fertilization treatments was more discrepancy than that of *nirS*-denitrifiers.

Phylogenetic Analysis of *Nir*-Gene Sequences

Based on similar cultured and uncultured *nirS*-denitrifiers from the NCBI database, the 33 *nirS* OTUs from five fertilizer treatments divided into three clusters (Fig. 4a). Most sequences were identified as the Rhizobiales of the Alpha-proteobacteria and the Oxalobacteraceae and Burkholderiaceae families of the Beta-proteobacteria. The T-RFs of unknown sequences were not related to any culture bacteria. The sequence T-RFs sensitive to fertilization were scattered over the whole phylogenetic tree. In detail, sequences with T-RF 36 and 153 bp were identified as *Paracoccus* sp., and the latter was found in only one sequence from the HFB treatment; sequences with T-RF 158 and 98 bp were both identified as *Bradyrhizobium* sp.; sequences with T-RF 26 bp were identified as *Herbaspirillum* sp., and it was not clustered with T-RF 28 bp related to *Thauera* sp.; sequences with T-RF 101 bp were related to *Ralstonia* sp. and *Azoarcus* sp.

The 13 *nirK* OTUs were divided into two clusters in the phylogenetic tree (Fig. 4b). Most of the sequence T-RFs were grouped into Alpha-proteobacteria (T-RFs 36, 154, and 433 bp), which were closely identified to known *Bradyrhizobium* (Rhizobiales, Alpha-proteobacteria). The T-RF of 101 bp was also related to Rhizobiales, but it was identified to *Sinorhizobium*. The T-RF of 215 bp was identified to *Pannonibacter* (Rhodobacterales, Alpha-proteobacteria).

Table 3 α-diversity of the T-RFs of *nir*-denitrifier in soil following chemical as well as biochar addition fertilization treatments

Treatment	*nirS*			*nirK*		
	Shannon index	Margalef index	Pielou index	Shannon index	Margalef index	Pielou index
CK	1.69 ± 0.16a	1.43 ± 0.57a	0.68 ± 0.04a	1.51 ± 0.19b	1.25 ± 0.26a	0.61 ± 0.02a
F	1.53 ± 0.26a	1.00 ± 0.37a	0.64 ± 0.02a	2.25 ± 0.02a	2.61 ± 0.40a	0.66 ± 0.03a
LFB	1.19 ± 0.24a	0.86 ± 0.22a	0.50 ± 0.05b	1.97 ± 0.21ab	2.12 ± 0.60a	0.63 ± 0.02a
MFB	1.65 ± 0.21a	1.22 ± 0.08a	0.61 ± 0.07ab	1.44 ± 0.32b	1.41 ± 0.54a	0.57 ± 0.01ab
HFB	1.75 ± 0.16a	1.28 ± 0.30a	0.65 ± 0.01a	1.38 ± 0.28b	1.60 ± 0.42a	0.47 ± 0.08b

CK no fertilizer control, *F* chemical fertilizer, *LFM* chemical fertilizer + 10 t hm^{-2} biochar, *MFM* chemical fertilizer + 20 t hm^{-2} biochar, *HFM* chemical fertilizer + 40 t hm^{-2} biochar. Error bars indicate standard error (from $n = 3$ replicates) for each treatment. Different letters within the same column indicate significant differences ($p < 0.05$)

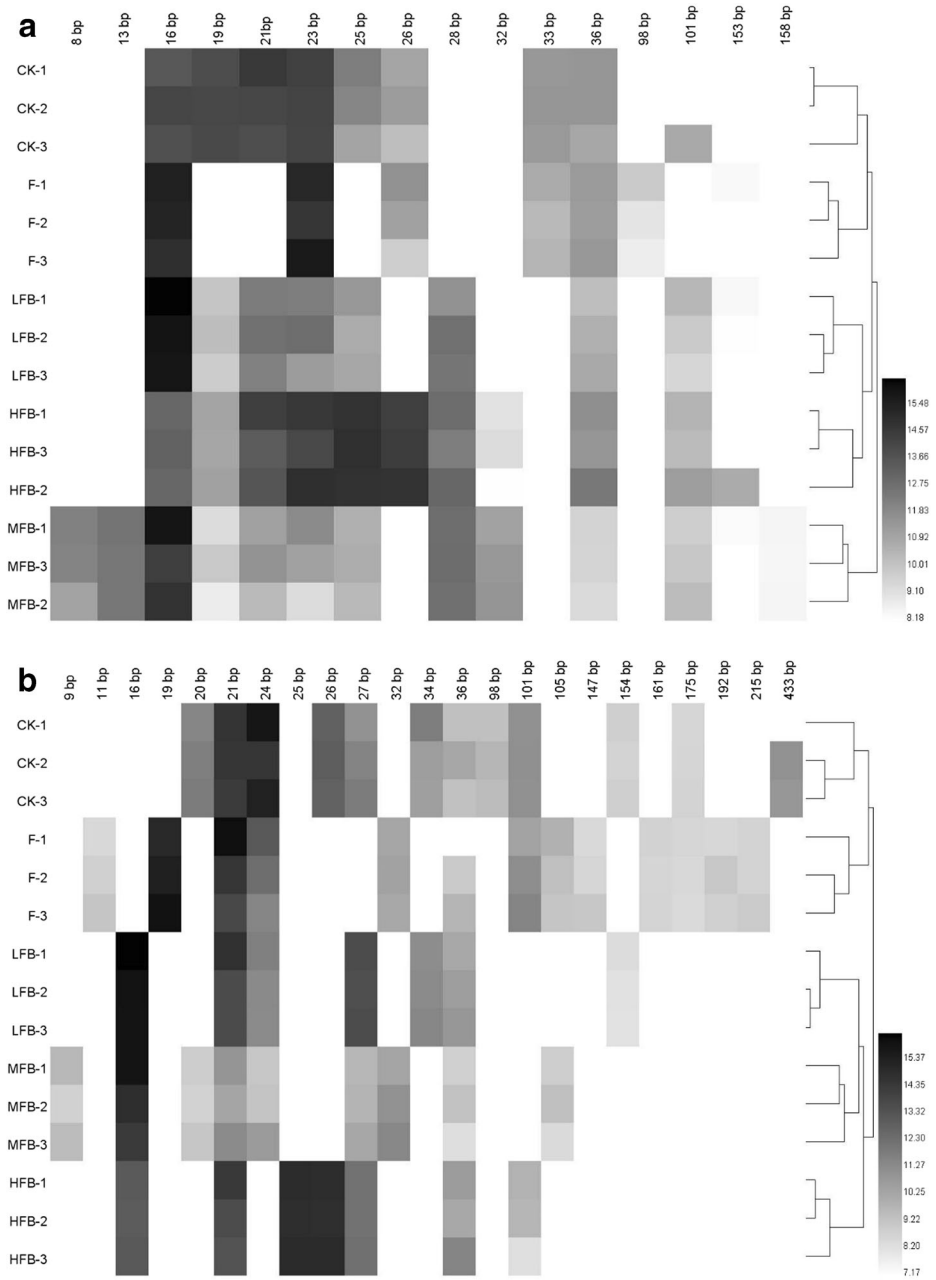

Fig. 3 Relative abundances of *nirS*- (**a**) and *nirK*- (**b**) terminal restriction fragments (T-RFs) in soils under chemical as well as biochar addition fertilization treatments. CK no fertilizer control, F chemical fertilizer, LFM chemical fertilizer + 10 t hm^{-2} biochar, MFM chemical fertilizer + 20 t hm^{-2} biochar, HFM chemical fertilizer + 40 t hm^{-2} biochar

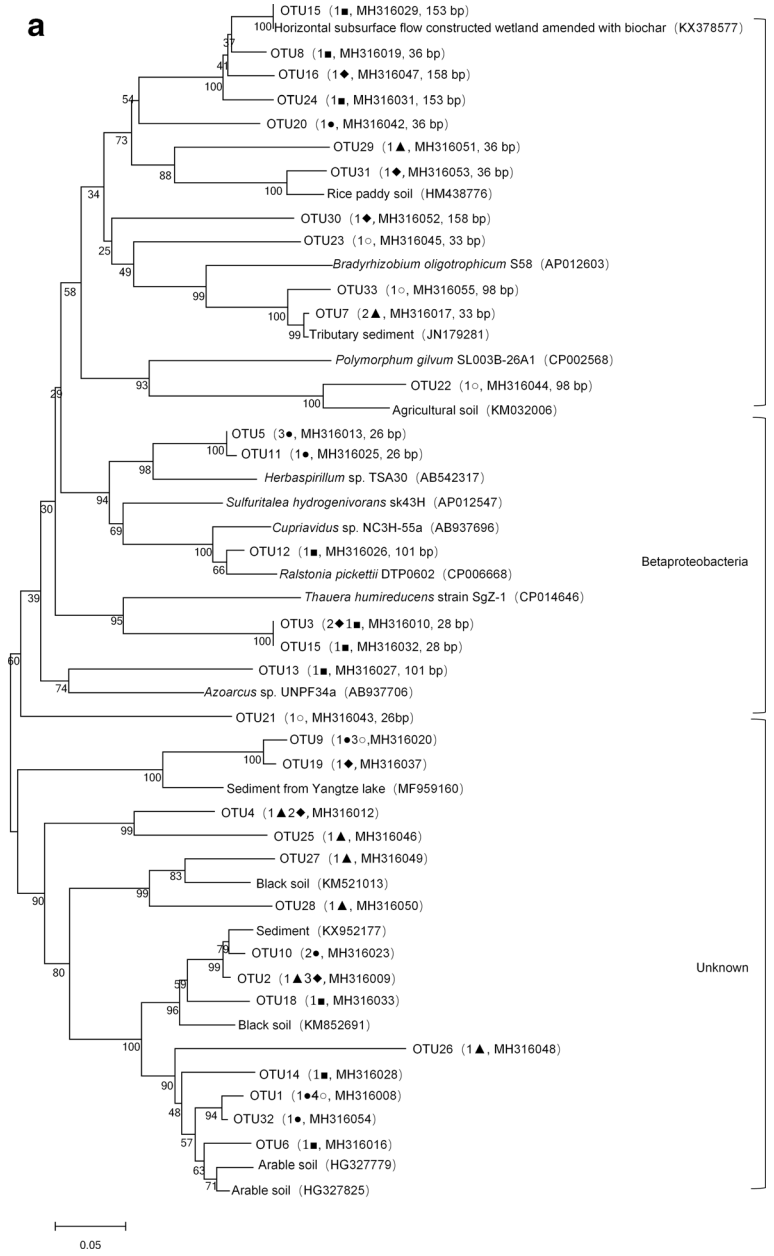

Fig. 4 Phylogenetic trees of nucleic acid of PCR-amplified *nirS* (**a**) and *nirK* (**b**) genes. Trees were constructed by neighbor-joining algorithm based on 13 (*nirK*) and 33 (*nirS*) nucleic acid OTUs. Clusters in *nirS* and *nirK* trees were both defined based on taxonomic grouping of characterized denitrifying taxa and BLAST results. The symbols in brackets stand for: ●, no fertilizer control; ○, chemical fertilizer; ▲, chemical fertilizer + 10 t hm^{-2} biochar; ◆, chemical fertilizer + 20 t hm^{-2} biochar; ■, chemical fertilizer + 40 t hm^{-2} biochar. The numbers in front of the symbols denote the amount of sequences in the fertilizer. The string in brackets is the accession number in the NCBI

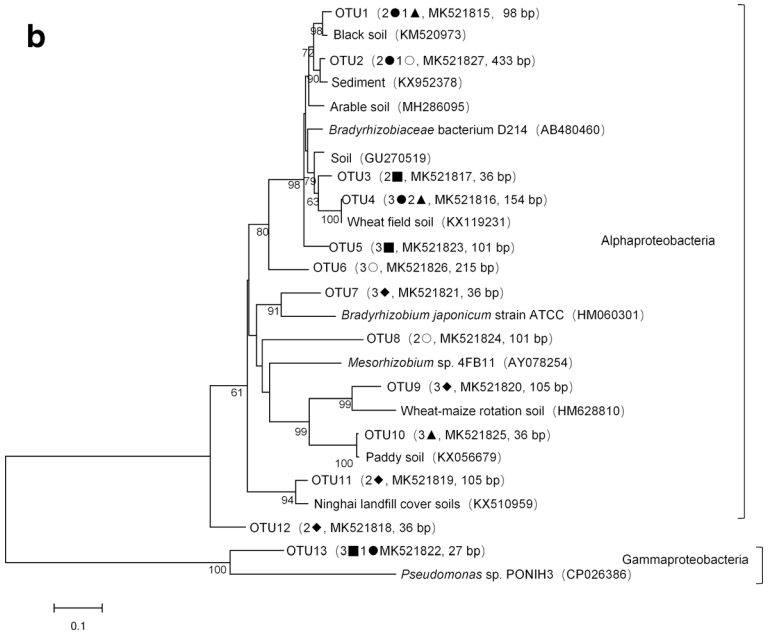

Fig. 4 continued

Although sequences with T-RF 105 bp cluster independent sub-clusters, it is closely related to *Bradyrhizobium* sp.

Relationship between *Nir*-Denitrifiers Communities, Soil Properties, N_2O, and S-NiR

The associations between environmental factors (soil physico-chemical properties, S-NiR, and N_2O) and the community structure of *nir*-denitrifiers were calculated by db-RDA (Fig. 5). The changes of *nirS*-and *nirK*-denitrifier communities across the treatments were significantly

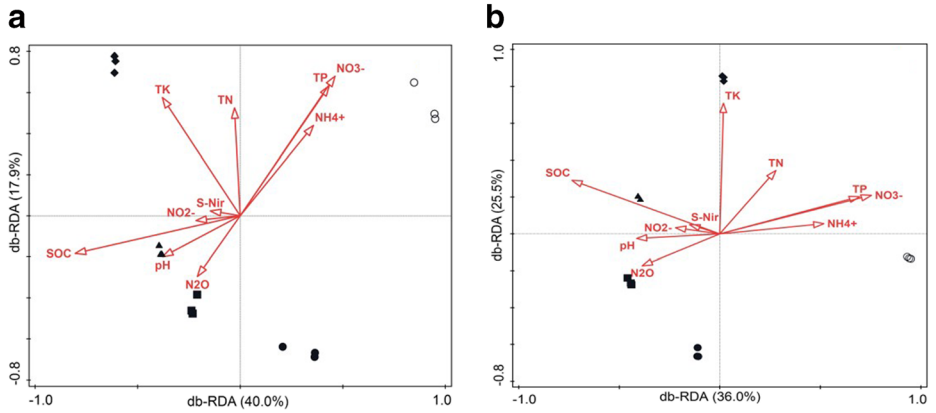

Fig. 5 Distance-based redundancy analysis (db-RDA) of correlation between community structures of *nirS*- (**a**) and *nirK*-denitrifiers (**b**) and soil environmental variables, N_2O flux and S-NiR. The symbols on the plots stand for: ●, no fertilizer control; ○, chemical fertilizer; ▲, chemical fertilizer + 10 t hm^{-2} biochar; ◆, chemical fertilizer + 20 t hm^{-2} biochar; ■, chemical fertilizer + 40 t hm^{-2} biochar

Table 4 Correlation of *nirS*- and *nirK*-denitrifiers community structures with soil physico-chemical properties, N_2O flux and S-NiR

Structure	Item	pH	SOC	TN	TP	TK	NH_4^+-N	NO_3^--N	NO_2^--N	N_2O	S-NiR
nirS	F	2.4	6.4	1.8	3.2	2.8	2.4	3.5	1.7	1.8	1.5
	p	0.056	0.002	0.126	0.026	0.026	0.04	0.01	0.14	0.142	0.172
nirK	F	3.4	5.0	3.8	5.0	0.044	4.5	5.4	3.8	4.2	3.6
	p	0.008	0.002	0.002	0.002	0.945	0.002	0.002	0.004	0.016	0.004

related to soil SOC, NO_3^--N, and TP, respectively (Table 4). For the *nirS* group, soil SOC had a higher correlation with its community structure compared to other physico-chemical properties. However, the *nirK* community was influenced by multiple physico-chemical properties. In addition, no matter *nirS*- or *nirK*-denitrifiers, the sample collected from HFB treatment had a high correlation with N_2O.

Discussion

Effects of Biochar and Fertilizer Addition on Soil Physico-Chemical Properties

In this work, whether the amendments were chemical fertilization or biochar, the variation of pH was relatively narrow (range from 7.3–7.5) and was less than in previous studies [49, 50]. In the literature, pH changes following biochar application have resulted from the inherent differences in biochar sources and the varying buffering capacities of the soils [49]. However, in paddy soil, the water also acts as a considerable buffer [51], and a previous pot experiment adding biochar to paddy soil showed a pH range from 5.75 to 5.88, which was similar to our results [52]. The higher soil NO_3^--N and NH_4^+-N values after chemical fertilizer application alone compared with biochar addition treatments (Table 1) confirmed the role of chemical fertilizer in contributing to dissolved organic N storage in soil. Krapfl et al. previously reported that, on increasing biochar addition content, SOC rose but TN was reduced [49], which was not completely in accordance with our results. In another previous study, Silber et al. reported that the K content of biochar is high and easily released, so adding it to soil significantly increased the content of total and available K [53]. However, high biochar content did not lead to high TK in our work. We hypothesize that high-content biochar addition has more effect of boosting rice to absorb K from soil.

Effects of Fertilizer Addition on N_2O Emissions and S-NiR

Previous studies concerning the effect of biochar addition on N_2O emissions showed variation according to conditions, with N_2O emissions suppressed [54], promoted [55], or unaffected [56]. In this study, whether chemical or biochar addition fertilization treatments, N_2O emissions were boosted, especially in the HFB treatment. Saarnio et al. reported that biochar increased soil moisture increasing N_2O emissions, which was difficult to explain our result, because of flooded conditions in this work [55]. Under flooded conditions in previous studies, biochar addition had a positive effect on soil nitrate, which is the initial substrate of denitrification and easily reduced to nitrite [11, 57]. In our work, nitrite showed a significant correlation with S-NiR, which was a key

stage in controlling denitrification [57]. In other words, nitrite has an important role indirectly influencing N_2O. As for NH_4^+-N, it is worth remembering that it provides the substrate for nitrification, generating N_2O as a byproduct [58]. Some previous studies also found an important role of NH_4^+-N in denitrification as well as pH and TN [59, 60]. As for the relationship between S-NiR and N_2O, previous studies mostly regarded the abundance and community structure of *nir*-denitrifiers as markers to explain the relationship, either closely relating [58, 61] or having no correlation [62]. Until now, there has been no general conclusion as to whether abundance, community, or both plays the key role in generating N_2O [59, 60]. The S-NiR was, therefore, aimed directly at showing the contribution of *nir*-denitrifiers to N_2O.

Effects of Fertilizer Addition on the Abundance of *Nir*-Containing Denitrifiers

Recently, more and more studies have reported that the size of *nirS* was always greater than that of *nirK* in paddy soils [27], which is opposite to our results. There was no doubt that inherent defects would impact the copy number of *nirS* and *nirK*, such as primer bias and multiple gene copies in a single cell. Yoshida et al. and Wang et al. [29, 63] also supported that the *nirK*-denitrifiers were more abundant than *nirS*-denitrifiers. It does not, however, necessarily indicate that *nirK*-denitrifiers contributed more to N_2O than *nirS*-ones. From the Table 2, we found the abundance of *nirS*-denitrifiers and not *nirK*-denitrifiers had a significant correlation with S-NiR and N_2O. Many previous studies have also arrived at this conclusion. Espenberg et al. found that N_2O emission was more primarily related to *nirS*-denitrifiers than *nirK*-denitrifiers [61], a view also supported by Dong et al. [58]. In addition, Tao et al. indicated that the contribution to S-NiR was not in the abundance of *nirK* ($R^2 = 0.0261$, $p = 0.6153$) but rather in that of *nirS* ($R^2 = 0.528$, $p = 0.0074$) [60].

From Table 2, it is notable that soil properties have little significant effect on the abundance of the *nirK*-denitrifiers. Although many previous studies have indicated that *nirK*-denitrifiers are more sensitive to environmental changes than *nirS*-denitrifiers [64, 65], we guess that, over time, waterlogging and eutrophication would decrease the sensitivity of *nirK*-denitrifiers. With long-term irrigation regimes, soil properties were not only uncorrelated with the abundance of *nirK*-denitrifiers but also their diversity and community structure [66]. Lee et al. also reported that the *nirS*-denitrifier community, rather than the *nirK*-one, should be the optimal indicator of microbial nitrite reduction processes in an estuarine system [67].

From previous studies, compared with controls, organic fertilizers have boosted the abundance of *nir*-denitrifiers [58, 60]. However, in our experiments, biochar addition inhibited the abundance of *nirK*-denitrifiers and increased *nirS*-denitrifiers. We found that measured soil properties cannot influence the abundance of *nirK*-denitrifiers (see Table 2). In the literature, dissolved oxygen was not a routine soil property but an important factor determining denitrifier abundance [57]. Li et al. indicated that a soil micro-environment with low dissolved oxygen was more suitable for *nirK*-denitrifiers, whereas there was no spatially heterogeneous variation for the *nirS*-denitrifier [68]. Huanhuan et al. also found that *nirK*-denitrifiers prefer deeper soils [69]. In our study, biochar was an abundant pore material, which would have the effect of increasing dissolved oxygen [70]. This may possibly explain the decrease in abundance of *nirK*-denitrifiers. To summarize, biochar influenced the abundance of *nirS*-denitrifiers through physico-chemical properties, whereas it possibly influenced that of *nirK*-denitrifiers through pore structure.

Effects of Biochar and Fertilizer Addition on *Nir*-Denitrifier Community Structure

From the abundance of *nir*-denitrifier results, we have found that the abundance of *nirS*-denitrifiers was more sensitive to environmental change than that of *nirK*-denitrifiers. However, the community structure seems to show the opposite result. Both the Shannon index (Table 3) and the Heatmap result (Fig. 3) showed that the diversity of *nirK*-denitrifiers was more sensitive to the variation of fertilizers than that of *nirS*-denitrifiers. In relation to the discrepancy of environmental sensitivity between *nir*-denitrifiers' abundance and community structure, Yin et al. have reported that the abundance of *nirS*-denitrifiers seems to be more sensitive than that of *nirK*-denitrifiers, while the community structure appears to be the opposite [30]. Certainly, the opposite result also has been reported. Yang et al. showed that the response of *nirS*-denitrifiers community to the gradient volume of N fertilization was more sensitive compared with that of *nirK*-denitifiers [59]. However, Luo et al. reported that N fertilizer had a clear effect on the community structure of *nirK*-denitrifiers but not the *nirS*-containing community [71]. Until now, few papers have examined the relationship between the *nir*-denitrifier community and gradient volumes of biochar addition. Consequently, we were unable to find any papers supporting our result that the LFB treatment obviously influenced the community structure of *nirK*-denitrifiers. We hypothesize that this phenomenon was correlated with the volume of dissolved oxygen carried from biochar into the soil.

Based on Figs. 3 and 4, we found that, with both *nirS*- and *nirK*-denitrifier communities, the dominant species were not playing a key role in controlling N_2O emission and S-NiR activity, but minority species were. In previous studies, the denitrifiers belonging to Rhodobacterales and Rhizobiales were commonly present [28–30]. In our work, the *nirS*-denitrifiers *Paracoccus* (sequence T-RF 153 bp) and the *nirK*-denitrifiers *Sinorhizobium* (sequence T-RF 101 bp) seem to the key genera relate to N_2O emission.

Previous papers stated that certain *Herbaspirillum* (Burkholderiales) strains and *Azospirillum* (Rhodospirillales) had the ability to emit N_2O [72, 73]. Yamane et al. reported that, in pure culture, *Paracoccus* have a significant ability of emitting N_2O [73]. From the above works, we have known that the NO_2^--N had a significant correlation with N_2O flux and *nirS*-denitrifiers, and the amount of NO_2^--N and N_2O emission both were significantly increased in HFB treatment. Previous studies have reported that *Paracoccus denitrificans* [74] and *Paracoccus pantotrophus* [39] can perform aerobic denitrification. Furthermore *P. denitrificans* can rapidly reduce NO_2^--N and was able to grow aerobically [75]. This may explain why *Paracoccus* was related to N_2O emission in the HFB treatment.

Notably, we found that the trend of abundance of *nirK*-denitrifiers was similar to the abundance of T-RF 101 bp, namely, *Sinorhizobium* (Figs. 2b and 3b). A previous study has reported that *Sinorhizobium* with the complete set of denitrification genes does not grow under anaerobic conditions with nitrate or nitrite as terminal electron acceptors [76], whereas under micro-oxic conditions it would use nitrate or nitrite as respiratory substrates [77]. In this work, combining the abundance of *nirK*-harboring *Sinorhizobium* (Figs. 1 and 3b), we guessed that the volume of oxygen in the HFB regime satisfied the demands of *Sinorhizobium*, and boosted it to use nitrate or nitrite as terminal electron acceptors, generating N_2O emission.

As for phylogenetic analysis, species types of *nirS*-denitrifiers were more diverse than *nirK*-ones in paddy soil. Yoshida et al. also explored the difference of *nirS*- and *nirK*-denitrifiers in paddy soil and got the same result [29]. Furthermore, we have previously researched another soil, the calcareous purple paddy soil, and found that *nirK* gene was hardly amplified in it [78].

Correlation between *Nir*-Denitrifier Community Structure and Properties

There is no doubt that environmental factors have a considerable effect on shaping *nir*-denitrifiers community structure [79, 80]. Yin et al. indicated that the community structure of two types of *nir*-denitrifiers responds differently to denitrifying enzyme activity, and the *nirK*-denitrifiers community was the true regulators [30]. The opposite result is also recorded, in which the N_2O emission was primarily related to the community structure of *nirS*-denitrifiers [61]. However, both the community structure of *nirK*- and *nirS*- denitrifiers were not related to S-NiR and N_2O in our work. Our findings were in line with the results of Dandie et al. who observed that the differences in denitrifier community composition were not related to denitrification rates and N_2O emissions [62].

As pointed out previously, soil NO_3^--N and SOC can serve as metabolic substrates, directly or indirectly influencing the denitrifying bacterial communities [59, 61]. It has also been shown that changes of soil phosphorus content were related to a structural shift in the *nir*-containing denitrifiers community [81]. In addition, there has been indication that the community structure of P-dissolving bacteria was related to soil nitrogen [82]. Therefore, the cycles of phosphorus and nitrogen are not absolutely independent. However, the mechanism of phosphorus intervening in the activity of denitrifiers is not well known. In our work, we showed that phosphorus plays a key role in shaping *nir*-denitrifiers community, but the exact evidence cannot be presented. In the future, this will be a large area of the research needed to understand denitrification.

Conclusions

Compared with chemical fertilizer, although the LFB and MFB treatments not significantly decreased denitrifying S-NiR and N_2O emission, the HFB treatment significantly increased the S-NiR and emit N_2O in paddy soil. The abundance of *nir*-denitrifiers was responsible for S-NiR and N_2O emission, rather than the variation of community structure. The NO_2^--N was a key factor of controlling the abundance of *nir*-denitrifiers, S-NiR and N_2O emission. Although the abundance of *nirK*-denitrifiers was more abundant than that of *nirS*-denitrifiers, S-NiR and N_2O emission showed no significant correlation to the abundance of the former but to the later one. Analysis of T-RFLP and phylogenetic tree data showed that the biochar additions greatly affected the richness, diversity, and structure of *nirS*- and *nirK*-denitrifier communities, especially *nirK*-denitrifers. In addition, the *nirS*-denitrifier *Paracoccus* and *nirK*- denitrifier *Sinorhizobium* may be a key species of emitting N_2O. Community structure was primarily influenced by SOC, NO_3^--N, and TP. Our findings suggest that, although *nirS*- and *nirK*-denitrifiers both mediate nitrite reductase to influence N_2O flux, their responds to gradient volume biochar addition were significant discrepancy.

Funding information The present study was financially supported by the National Key Research and Development Plan of China (Grant No. 2017YFD0800101).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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