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The roles of environmental variation and spatial distance in explaining diversity and biogeography of soil denitrifying communities in remote Tibetan wetlands

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Abstract

The relative importance of local environments and dispersal limitation in shaping denitrifier community structure remains elusive. Here, we collected soils from 36 riverine, lacustrine and palustrine wetland sites on the remote Tibetan Plateau and characterized the soil denitrifier communities using high-throughput amplicon sequencing of the *nirS* and *nirK* genes. Results showed that the richness of *nirS*-type denitrifiers in riverine wetlands was significantly higher than that in lacustrine wetlands but not significantly different from that in palustrine wetlands. There was no clear distinction in *nir* community composition among the three kinds of wetlands. Irrespective of wetland types, the soil denitrification rate was positively related to the abundance, but not the α-diversity, of denitrifying communities. Soil moisture, carbon availability and soil temperature were the main determinants of diversity (OTU number) and abundance of *nirS*-type denitrifier community, while water TOC, soil NO_3^- and soil moisture were important in controlling *nirK*-type denitrifier diversity and abundance. The *nirS* community composition was influenced by water electrical conductivity, soil temperature and water depth, while the *nirK* community composition was affected by soil electrical conductivity. Spatial distance explained more variation in the *nirS* community composition than in the *nirK* community composition. Our findings highlight the importance of both environmental filtering and spatial distance in explaining diversity and biogeography of soil *nir* communities in remote and relatively undisturbed wetlands.

Keywords: Denitrification, Marshes, Nitrogen cycles, Salt lakes, Species richness

Introduction

At the beginning of the 21st century, global anthropogenic and natural sources contributed approximately 413 Tg of reactive nitrogen (N) to terrestrial and marine ecosystems each year (Fowler *et al.* 2013). Excess N is directly linked to numerous environmental issues at different scales, including climate change, N deposition, river and lake acidification, water quality deterioration, forest productivity decline, and biodiversity loss (Driscoll *et al.* 2001; Magill *et al.* 2004; Compton *et al.* 2011; Liu *et al.* 2015). Denitrification is known to be the most important N removal process, converting nitrate (NO_3^-) to nitrous oxide (N_2O) and dinitrogen (N_2), which are then released into the atmosphere (Bettez and Groffman, 2012; Yao *et al.* 2016; Xiong *et al.* 2017). It has been estimated that global terrestrial, freshwater, and marine ecosystems can remove approximately 313 Tg N annually through the denitrification process (Seitzinger *et al.* 2006).

Dentrification is considered to be a facultative anaerobic process and is mediated by a range of microbes (Wallenstein *et al.* 2006). Before 1970, denitrification was generally considered to be a prokaryotic process and was thoroughly studied in several bacteria (Downey *et al.* 1954; Youatt 1954; Renner and Becker 1970; Anderson and Domsch 1973). However, recent studies have shown that fungal denitrification can occur under moderately reducing conditions and significantly contributes to greenhouse gas emissions (Seo and DeLaune 2010). The application of molecular tools has enhanced our understanding of denitrifying microorganisms and is beginning to address the importance of these organisms in

regulating the rate of denitrification (Wallenstein *et al.* 2006). However, the rate of denitrification is controlled by the sequential reduction processes, including the reduction of NO_3^- to N_2 through nitrite (NO_2^-), nitric oxide (NO) and N_2O (Sirivedhin and Gray 2006). Among of them, the process of NO_2^- to NO, known as the rate-limiting step of denitrification, is catalysed by copper- and cytochrome cd_1-containing nitrite reductases (encoded by *nirK* and *nirS* genes, respectively) (Zumft 1997; Braker *et al.* 1998). Therefore, the *nirK* and *nirS* genes, possessing equivalent function and physiology but different structure, have been widely used as molecular markers to determine the diversity, abundance and species composition of denitrifying microbial communities (Glockner *et al.* 1993; Throbäck *et al.* 2004; Brankatschk *et al.* 2011; Ligi *et al.* 2014; Gao *et al.* 2016; Liu *et al.* 2018).

Understanding which environmental variables regulate the spatial and temporal variations of soil microbial communities in different ecosystems is an important ecological topic (Xiong *et al.* 2012). Over the last several decades, many studies have indicated that the community structures of *nir*-harboring bacteria in wetland soils are strongly affected by a number of environmental and biotic factors, such as pH, temperature, oxygen concentration, carbon availability and vascular plants (Wallenstein *et al.* 2006; Jiang *et al.* 2017). Recently, increasing evidence has shown that geographical distance or dispersal limitation play a key, or even dominant, role in shaping the community structure of *nir* denitrifiers (Xiong *et al.* 2012; Shi *et al.* 2015). However, the relative contribution of environmental variation and geographical distance to *nir* community dissimilarity in aquatic habitats remains elusive, especially in high-altitude and relatively undisturbed areas.

The Tibetan Plateau is known as the "Third Pole" of the world. The altitude of this plateau is, on average, more than 4000 m above sea level, and it covers a total area of 2.57 million km^2 (Liu *et al.* 2017), of which 4.9% is possessed by wetlands, including rivers, lakes and marshes. However, with the rapid development of Tibetan economy and the intensification of human activities, some wetlands have been damaged to varying degrees, such as the decreasing in wetland area and landscape diversity, posing a threat to fragile plateau wetland ecosystems (Xing *et al.* 2009; Zhang *et al.* 2011; Li *et al.* 2015). As described above, denitrification, as a main nitrogen removal process, exerts the effects on the N_2O fluxes from wetlands in such special region. Nevertheless, the variation of *nir* community composition, depending on the stabilization of wetland ecosystems, can alter the N_2O fluxes, contributing to the region climate change (Kato *et al.*, 2011; Liu *et al.* 2017).

In this study, we investigated the denitrification rates and *nirK* and *nirS* denitrifier communities of soils collected from 36 riverine, lacustrine and palustrine wetland sites on the Tibetan Plateau. We tested the following three hypotheses. First, abundance, diversity, and community composition of soil *nir* denitrifiers would vary with the type of wetland. Second, denitrifier abundance and diversity would be positively related to soil denitrification rate. Third, we expected that both environmental variables and spatial distance would play an important role in shaping *nir* community structure.

Materials and methods

Study sites

The Tibetan Plateau contains diverse wetlands, including rivers, lakes, marshes and artificial wetlands (e.g., rice fields, fish ponds and reservoirs). The total area of rivers, lakes and marshes is 46,728, 41,832 and 13,261 km^2, respectively (Liu *et al.* 2017). Since the Tibetan Plateau is the source of a large number of major Asian rivers (e.g., the Yangtze, Yellow and Brahmaputra Rivers), these plateau wetlands are very important in providing water resources, improving water quality and regulating hydrological cycles (Liu *et al.* 2017; Yang *et al.* 2019).

Most parts of the Tibetan Plateau are characterized by cold and dry winters and cool-humid summers, with a mean annual temperature below 0°C. Annual precipitation in this plateau is generally less than 400 mm, over 80% of which falls during the summer monsoon season. In warm permafrost areas, the surface soils (0–50 cm depth) start to freeze at the end of October and begin to thaw in the beginning of May (Wu and Zhang, 2010). Biogeochemical processes and the associated soil microorganisms are particularly active in plant growth season from early May to late September (Chen *et al.* 2012).

Field sampling

In July 2014, when the soils had thawed, we sampled surface (0–10 cm) soils from 20 lacustrine (lake margin), 9 riverine, and 7 palustrine wetland sites on the Tibetan Plateau (Fig. S1 and Table S1). These wetlands were selected nonrandomly, mainly based on site accessibility, and were isolated from each other. For each wetland site, we randomly established a 1 × 1 m plot and sampled five soil cores (3 cm diameter and 10 cm deep) within the plot. Soil cores from the same wetland site were mixed thoroughly to create a composite soil sample, and finally we end up with 20 composite lacustrine samples, 9 composite riverine samples, and 7 composite palustrine samples for a total of 36 samples. 10-15 g of fresh soils from each wetland site were collected in a centrifuge tube and immediately stored in liquid N_2 until ready to be used for microbiological analysis. Other fresh soils from each wetland site were sealed in plastic bags and stored at 5°C in a portable refrigerator for measuring soil properties and denitrification rate.

Measurements of denitrifier community structure

Genomic DNA was extracted from each soil sample (approximately 0.25 g) using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. The DNA was purified using the UltraClean 15 DNA purification Kit (MoBio Laboratories, Carlsbad, CA, USA) and quantified with a NanoDrop 2000 Fluorospectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

Denitrification genes, i.e., *nirK* and *nirS*, were amplified by PCR using the commonly used primer sets F1aCu (5'-ATCATGGTSCTGCCGCG-3')/R3Cu (5'-GCCTCGATCAGRTTGTGGTT-3') and cd3aF (5'-GTSAACGTSAAGGARACSGG-3')/R3cd (5'-GASTTCGGRTGSGTCTTGA-3'), respectively (Hallin and Lindgren 1999; Throbäck *et al.* 2004), by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA), with an eight-base sequence barcode unique to each sample at the 5' end of forward and reverse primers, respectively. PCRs were performed in 25 µL volumes containing 2.5 µL of 10× buffer, 2 µL of 2.5 mM dNTPs, 0.5 µL of each primer (10 mM), 0.2 µL of rTaq polymerase and 1 µL of template DNA (10–100 ng/µL). The PCR operation conditions consisted of an initial 95°C for 3 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final extension at 72°C for 10 min.

Triplicate PCR amplicons were pooled for each sample before being purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union, CA, USA) and paired-end sequenced (2×300 bp) on an Illumina HiSeq 2000 sequencer (Illumina, San Diego, CA, USA) according to the standard protocols by Shanghai Majorbio Bio-pharm Technology Co.,Ltd. (Shanghai, China). The raw sequencing data were deposited into the NCBI Sequence Read Archive (SRA) database (accession number: SRP123686).

The raw *nirS* and nirK gene sequencing reads were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were

discarded; (ii) exact barcode matching, 2 nucleotide mismatch in primer matching, and reads containing ambiguous characters were removed; and (iii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. Reads that could not be assembled were discarded.

Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE (version 7.1, http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME.*et al.* One representative sequence from each OTUs was processed by the Ribosomal Database Project (RDP) functional gene (fungene) pipeline (Release7.3 http://fungene.cme.msu.edu/) for taxonomic assignment using confidence threshold of 0.7 (Fish *et al.*, 2013). The α-diversity indices (Chao1, Shannon and Simpson) were further generated using MOTHUR after normalization to 5482 (*nirK*) and 7184 sequences (*nirS*) per sample.

Quantification of denitrifier abundance

To quantify the abundance (i.e., copy number) of *nirK*, *nirS* and 16S rRNA genes, real-time quantitative polymerase chain reactions (qPCR) were performed in triplicate using a Roche LightCycler480 real-time PCR System (version 1.5.0; Roche Diagnostics, Mannheim, Germany) and the SYBR green fluorescent dye quantitative PCR method. Primer sets of nirK876/nirK1040, Cd3aF/R3cd and 357F/518R were utilized for the *nirK*, *nirS* and 16S rRNA genes, respectively (Muyzer *et al.* 1993; Henry *et al.* 2004; Throbäck *et al.* 2004). The 25 µL qPCR mixture contained 10 µL of SYBR Green qPCR Master Mix (2×), 1 µL of the primers (10 µM) and 2 µL of the DNA template. The primers and qPCR protocol can be found in our previous papers (e.g.,

Jiang *et al.* 2017). Standard curves were constructed with serial dilutions of a known amount of plasmid DNA containing a fragment of the *nirS* and *nirK* genes. Amplification efficiency was 77.6% and 88.7% for nirS and nirK genes, respectively, and r^2 values exceeded 0.99.

Determination of soil potential denitrification rate

We used the acetylene blocking technique to determine the potential denitrification rate of wetlands soils (Tiedje *et al.* 1989). The denitrification potential was determined in the presence of added carbon and NO_3^- and, therefore, provided an upper-bound estimate of *in situ* denitrification. Acetylene can inhibit the reduction of N_2O to N_2, causing the former to be the major end product of denitrification. When acetylene is not added, the N_2O is free to transform to N_2, allowing for the determination of the net N_2O emission rate (Liu *et al.* 2016a).

For the denitrification potential assays, 50 g of fresh soil from each wetland site was weighed into a 250-mL serum bottle with 30 mL of incubation solution (final concentrations: 0.1 g/L KNO_3, 0.18 g/L glucose and 1 g/L chloramphenicol). All of the serum bottles were then sealed and purged with N_2 gas for 2 min to induce anoxic conditions. Approximately 10% of the bottle headspace was replaced with acetylene to block the conversion of N_2O to N_2 during denitrification. These bottles were then incubated in the dark for 4 h at room temperature. The 4 h incubation time was determined after a trial, during which 5 random soil samples were incubated for 1, 2, 4, and 6 h to confirm that the N_2O accumulation was linear. At both the beginning

and end of the incubation, 10 mL samples of the headspace gas were collected from each bottle (after shaking vigorously), and their composition was determined by a gas chromatograph (Agilent 7890, CA, USA). Soil denitrification rates were calculated from the difference between the final and initial N_2O concentrations and were expressed as mg N kg^{-1} d^{-1}.

Determination of environmental factors

The climate factors, including mean annual temperature (MAT) and mean annual precipitation (MAP), were extracted from a climate dataset at a spatial resolution of 1 km in ArcGIS 10.0 (ESRI Inc., Redlands, CA, USA) in 2014.

Water depth of each sampling plot was measured if surface water was present. Several water quality parameters, including pH, temperature, and conductivity, were determined using a multiparameter water quality analyzer (*HI991301, Hanna Instruments, Padova, Italy*). Water samples were filtered through 47-mm diameter Whatman GF/C glass-fiber filters (pore size 1.2 µm). The total carbon (TC) and total organic carbon (TOC) concentrations of filtered water samples were determined by an elemental analyzer (Vario TOC cube, Elementar, Germany). The total N (TN) and total phosphorus (TP) concentrations of water were measured using the hydrochloric acid photometry and molybdenum blue spectrophotometric methods, respectively (Yao *et al.* 2016).

For the physicochemical properties of soils, we measured the temperature of 10 cm soil layer on-site using a temperature probe. Soil pH and conductivity were

determined in the laboratory at a 1:5 (v/v) soil to water ratio using a pH/Conductivity meter. Soil moisture was calculated gravimetrically after drying 50 g of wet soil at 105°C for 24 h. The soil total carbon (STC) content was analyzed by an elemental analyzer (Vario TOC cube, Elementar, Germany) using air-dried and sieved (100-mesh) soil samples. Soil total phosphorus (STP) was determined via colorimetry, according to the molybdenum blue method. Soil ammonia (NH_4^+) and NO_3^- concentrations were measured using an automatic nutrient analyzer (EasyChem plus, Systea, Italy), as described in detail in our previous work (Jiang *et al.* 2017).

Statistical analyses

Before statistical analyses, we checked the data for normal distributions using the Shapiro–Wilk test in PASW 18.0. If necessary, the data were either natural log (ln) or square root-transformed to improve normality. A one-way ANOVA with Tukey's post hoc test was used to determine the significance of the difference ($P < 0.05$) in denitrifier abundance and diversity among the three types of wetlands. Nonmetric multidimensional scaling (NMDS) was used to visualize the similarity in community composition among wetland sites in software PAST 3.16. Pearson correlation analyses were also performed to describe the relationships between diversity and abundance of *nir* community and environmental factors.

Redundancy analysis (RDA) was performed to examine the relationships between the species composition of *nir* communities and environmental factors using CANOCO 4.5 software (Microcomputer, Ithaca, NY, USA). The longest gradient length of detrended correspondence analysis (DCA) was used to evaluate whether the RDA

was the appropriate ordination form. Variation partitioning analysis was performed to examine the relative importance of environmental factors and spatial distance in explaining the *nir* community composition using the varpart function in the vegan package. Spatial scales were calculated based on the geographic coordinates of the wetlands by principal coordinates of neighbor matrices (PCNM) analysis. Forward selection method was used to get the spatial distance and environmental variables that correlated significantly with variation in composition of the *nirK* and *nirS* communities. All statistical analyses were performed in R version 3.3.1 (R Core Team, 2015) unless otherwise indicated.

Results

Denitrifier abundance and diversity

The abundance of the *nirS* gene varied between 4.79×10^5 and 2.30×10^7 copies g^{-1} fresh soil, and the *nirK* gene abundance ranged from 9.97×10^2 to 2.10×10^5 copies g^{-1} fresh soil (Table S2). The average abundance of the *nirS* gene in riverine, lacustrine and palustrine wetlands was 6.62×10^6, 4.74×10^6 and 2.31×10^6 copies g^{-1} fresh soil, respectively (Fig. 1). For the *nirK* gene, the mean abundance in riverine, lacustrine and palustrine wetlands was 4.84×10^4, 2.25×10^4 and 2.32×10^4 copies g^{-1} soil, respectively. No significant differences in *nirS* and *nirK* gene abundances were found among the three wetland types (Fig. 1). The relative abundance of *nirS* and *nirK* genes to 16S rRNA genes ranged from 0.54 to 23.84% and from 0.01 to 0.23%,

respectively. Wetland types also had no significant effect on the relative abundance of denitrification genes (Fig. 2).

There are 2480 and 3313 OTUs observed for *nirS* and *nirK* genes, respectively. The mean OTU number of the *nirS* gene in riverine wetlands was significantly higher than that in lacustrine wetlands, but not significantly different from that in palustrine wetlands (Fig. 1). Similarly, the riverine and lacustrine wetlands had the highest and lowest Chao1 richness, respectively. For the *nirK* gene, the Chao1 richness of riverine wetlands ranged from 88 to 502 with an average value of 290.73 (Table S2). The average values of OTUs number, Chao1 and Shannon indices in riverine wetlands were 267.86, 338.86 and 3.84, which were all slightly but not significantly higher than those in lacustrine and palustrine wetlands (Fig. 1).

When all wetland types were combined, both *nirS* and *nirK* gene abundances were positively correlated with the soil denitrification rates (Table S3). When we analyzed each wetland type separately, a significant relationship between denitrifier abundance and soil denitrification rates was only observed for the lacustrine wetlands (Table S3). There was no significant correlation between denitrifier diversity and soil denitrification rates (Table S3).

Denitrifier community composition

In riverine wetlands, the *nirS* denitrifiers were mainly composed of Bacteria_unclassified (35.79%), environmental_samples_noranked (34.50%) and *Proteobacteria*_unclassified (16.10%) (Fig. S2). In lacustrine wetlands, the *nirS*

denitrifiers consisted of Bacteria_unclassified (34.69%), Proteobacteria_unclassified (30.09%), unclassified genus of Betaproteobacteria (14.32%) and environmental_samples_noranked (13.82%). For the palustrine wetlands, the *nirS* denitrifiers were composed of Bacteria_unclassified (38.53%), environmental_samples_noranked (29.80%) and *Proteobacteria*_unclassified (16.09%) (Fig. S2). The NMDS ordination indicated that there was no distinct difference in the *nirS* denitrifier community structure among the three kinds of wetlands (Fig. S3).

Similarly, the *nirK* denitrifier consisted of Bacteria_unclassified (47.68%) and Unclassified Proteobacteria bacteria (20.56%) in soils of riverine wetlands. In lacustrine wetlands, the *nirK* denitrifier was also mainly composed of Bacteria_unclassified (47.94%) and Unclassified Proteobacteria bacteria (18.09%). However, in palustrine wetlands, the *nirK* denitrifier consisted of Bacteria_unclassified (57.63%) and Proteobacteria_unclassified (10%). Compared with riverine wetlands, the relative abundance of the genus Bradyrhizobium decreased, while the genus Afipia increased in lacustrine and palustrine wetlands (Fig. S4). The NMDS result showed that wetland type was not a key factor influencing the *nirK* denitrifier community structure (Fig. S3).

Environmental factors regulating denitrifier abundance and diversity

The abundance of the *nirS* gene was positively correlated with soil temperature, soil moisture and STC (Table 1). In addition, the OTU number of the *nirS* gene

showed negative relationships with soil moisture and STC. No significant relationship was found between water quality variables and the abundance and diversity of *nirS*-type denitrifiers (Table 1). For the *nirK* gene, the abundance was only significantly related to soil moisture, while the OTU number and Chao1 index of the *nirK* gene were negatively correlated with both water TOC and soil NO_3^- (Table 2).

Environmental factors regulating denitrifier community composition

For *nirS*-type denitrifiers, water TOC, TC and soil pH had positive correlations with *Proteobacteria*_unclassified, *Gammaproteobacteria*_unclassified, *Rhodobacter* and *Thauera* (Fig. 3). STC and soil NH_4^+ had no significant correlation with the *nirS* community composition at the genus level. For *nirK*-type denitrifiers, climate and water temperature had positive correlation with *Bosea*, *Achromobacter*, *Sinorhizobium*, *Rhizobiaceae*_unclassified_*rhizobiaceae*, *Ensifer* and unclassified genus of *Alcaligenaceae* (Fig. 4). Moreover, soil pH had negative relationships with both *Mesorhizobium* and *Betaproteobacteria*_unclassified, while positive correlations were found for soil NH_4^+.

The RDA results indicated that water conductivity, soil temperature and water depth had strong effects on the community composition of *nirS*-type denitrifiers (Fig. S5). For the *nirK* denitrifier communities, soil conductivity was the only significant environmental factor determining the composition of soil *nirK* denitrifier communities in Tibetan wetlands (Fig. S6).

The role of environmental and spatial factors in structuring denitrifier communities

Variation partitioning showed that environmental variables and spatial scales together explained 19.7% of the *nirS* community variation (Fig. 5a). Environmental variables, spatial scales, and their interaction accounted for 8.0%, 10.3% and 1.4% of the variation, respectively. For *nirK* communities, environmental and spatial variables could explain 17.2% of the variation (Fig. 5b). The environmental factors alone accounted for 12.5% of the variation. Spatial variables played a less important role in *nirK* community composition than in *nirS* community composition, accounting for 3.2% of the variation. These results also indicated that a large proportion of the variation was not explained by either measured environmental variables, spatial components of the PCNM, or their interactions (Fig. 5).

Discussion

Abundance, diversity and community composition of denitrifier in wetland soils

Denitrifying microorganisms played an important role in the N biogeochemical processes in different kinds of ecosystems (Deslippe *et al.* 2014; Shrewsbury *et al.* 2016; Regan *et al.* 2017; Yao *et al.* 2018). In this study, the mean abundance of the *nirS* gene (4.70×10^6 copies g^{-1} soil) was approximately two orders of magnitude higher than that of the *nirK* gene (2.81×10^4 copies g^{-1} soil) in soils of Tibetan wetlands. This is consistent with some studies showing that *nirS* denitrifying bacteria are far more abundant and diverse than *nirK* denitrifying bacteria in wetlands (e.g., Ligi *et al.* 2014). Both *nirS* and *nirK* gene abundances in Tibetan wetlands were

considerably lower than values detected in some previous studies. For example, Jiang *et al.* (2017) reported that *nirS* and *nirK* gene abundances were 1.07×10^{9} and 3.70×10^{7} copies g^{-1} sediment in Yangtze lakes, respectively. Wang *et al.* (2014) found that *nirS* and *nirK* gene abundances in sandy intertidal sediments along the Laizhou Bay in China were 2.54×10^{6} and 8.65×10^{5} copies g^{-1} sediment, respectively. Our results may imply that the denitrifier abundance in soils of Tibetan wetlands was at a lower level when compared with that of other wetlands in China.

In our study, *nirK*- and *nirS*-type denitrifiers showed no significant difference in soils of riverine, lacustrine and palustrine wetlands at both absolute and relative abundance levels (Fig. 1 and Fig. 2). This indicated that the wetland type was not an important factor in determining the denitrifier abundance in Tibetan wetlands. However, we found that riverine wetlands had relatively higher OTU number and Chao1 richness of *nirS*-type denitrifier communities when compared to lacustrine wetlands (Fig. 1). This may be because half of the studied lacustrine wetlands are saline marshes and have an average water conductivity of more than 13000 μS cm^{-1}. In most previous studies, salinity or conductivity was considered to be a main factor altering bacterial diversity, with high salinity decreasing the bacterial diversity (Bernhard *et al.* 2005; Ruhl *et al.* 2018). In line with our results, *nirS*-harboring bacterial diversity was found to be higher at the lower salinity than at the higher salinity sites (Yoshie *et al.* 2004; Zheng *et al.* 2015). Uygur (2006) reported that high salinity can inhibit the growth of bacteria by restricting the cell membrane and enzyme activity. Furthermore, the bacteria with low salt-tolerance have to assign more power against hyperosmosis due to high salinity, resulting insufficient power to

reproduce. Another possible reason is that microorganisms without salt-tolerance capacity may die because of phasmolysis and loss of cell viability (Chen *et al.* 2019). Although molecular methods are the best available approach to investigate *nir* communities, it should be noted that the commonly used *nirK* and *nirS* primer sets have low coverage over the entire gene pool. Bonilla-Rosso *et al.* (2016) did not find any existing primers that can detect clades of archaeal *nirS* and *nirK*. Ma *et al.* (2019) also reported that the primer set F1aCu/R3Cu covered only 60.7% of clade I *nirK* and the primer set cd3aF/R3cd could cover 77.7% of *nirS* clade I.

Our results showed that over half of the denitrifiers in the soils of the Tibetan wetlands are still not identified at both the genus and phylum levels (Fig. S2 and Fig. S4). For example, at the phylum level, we found that bacteria_unclassified and environmental_samples_nonrank accounted for 35.6% and 21.1% of the total *nirS* sequences, respectively. This is consistent with a study showing that the main phyla of *nirS*-type denitrifiers in sediments included Proteobacteria (66.28±3.52%), Bacteria_unclassified (26.33±2.75%) and environmental_samples (7.39±1.72%) in a shallow eutrophic reservoir in China (Zhou *et al.* 2016). However, our results are significantly different from those obtained in studies which investigated the well-studied wetland type following. For example, most denitrifiers in a micropolluted constructed wetland were identified, with *Rhodanobacter*, *Rubrivivax* and *Pseudomonas* being the dominant genera (Wu *et al.* 2017). Hence, our findings imply that more studies are still needed to separate and identify the main denitrifying bacteria using metagenomic or culture-dependent methods in remote and less accessible areas, such as the Tibetan Plateau.

Effects of environmental and spatial factors in structuring denitrifier communities

In this study, soil moisture had a positive relationship with abundances of both the *nirS* and *nirK* genes (Tables 1 and 2). It has been reported that *nirK* gene abundance increases rapidly in response to wet conditions in forest soils (Szukics *et al.* 2010). Moreover, Di *et al.* (2014) found that soil moisture status significantly affected the growth of *nir* communities in grassland soils. These results are not surprising because denitrifiers mainly reside in soil environments with higher moisture and lower oxygen contents (Jiang *et al.* 2017). Some studies reported that soil pH was an important factor influencing the diversity and biogeography of soil microbial communities at different spatial scales (Fierer and Jackson 2006). For example, Xiong *et al.* (2012) found that pH was the best predictor of bacterial community structure, phylotype richness and phylogenetic diversity in alkaline lake sediments across the Tibetan Plateau. However, in this study, pH was only found to have a significant correlation with some genera of denitrifiers such as *Mesorhizobium* and *Rhodobacter* (Figs 3 and 4). This may be because most soil samples in Tibetan wetlands are neutral or slightly alkaline and had a pH value between 7.5 and 8.5 (Table S1). Our results also indicate that although denitrifier abundance and diversity are less influenced by pH, there are some differences in the relationship between the composition of specific genus and pH.

Soil salinity can be assessed by the electrical conductivity (Van Horn *et al*. 2014). Previous studies have revealed that salinity was a major driver in determining microbial community structure in sediments of Tibetan lakes (e.g., Liu *et al.* 2016b;

Yang *et al.* 2016). In this study, we also found that conductivity was the most important factor influencing the community composition of soil denitrifiers in Tibetan wetlands (Figs. S5 and S6). However, consistent with some previous reports (e.g., Ikenaga *et al*. 2010), we failed to observe a significant relationship between conductivity and denitrifier abundance and diversity (Tables 1 and 2). These findings, together with previous reports, imply that denitrifier abundance and diversity may not be constrained by high salinity when denitrifying communities do not experience resource limitations or other environmental stresses. The water conductivity varied considerably across the 36 sampled wetlands on the Tibetan Plateau, ranging from 96 to 187400 μS cm^{-1}, while soil conductivity ranged between 61 and 2848 μS cm^{-1}. There are a number of mechanisms by which salinity or conductivity can affect the species composition of denitrifying communities in wetland soils (Yan *et al*. 2015). One obvious consideration is ionic strength, which has direct physiological effects (e.g., by inducing osmotic stress) or initiate a cascade of indirect effects (Franklin *et al*. 2017). Soluble salt generally increases the osmotic potential of soil water, drawing water out of cells which may reduce cell activity and eventually kill some microbes through plasmolysis (Yan *et al*. 2015). Besides ionic strength, higher salinity brings with it specific ions that may greatly affect soil denitrifying communities (Franklin *et al*. 2017). At a high salt concentration, elevated ions (e.g., Na^+, Cl^-, HCO_3^- and SO_4^{2-}) are toxic to metabolic activities of many microorganisms and can result in nutritional imbalances and ultimate loss of low-salt-tolerant species (Franklin *et al*. 2017). Third, studies have found that salinization can enhance ionic displacement and cause release of numerous chemical species (e.g., organic carbon and inorganic nitrogen) from wetland soils (Herbert *et al*. 2015). The increase in nutrient

availability may result in considerable changes in soil microbial community structure. The findings in this and previous studies together emphasize the key role of salinity in regulating the microbial communities in aquatic ecosystems on the Tibetan Plateau.

Environmental and spatial variables influenced the functional groups of bacterial communities involved in soil nitrogen cycling, such as denitrifiers (Regan *et al.* 2017). In this work, spatial variables could explain 1.7% and 2.1% of the variation in *nirS* and *nirK* community composition in wetland soils from the Tibetan Plateau, respectively. Xiong *et al.* (2012) reported that geographic distance contributed more to bacterial community variation (12.2%) than any other environmental variable in lake sediments across the Tibetan Plateau, although the environmental variables explained more variance when combined. These results may suggest that the community structure of soil microorganisms in wetlands across the Tibetan Plateau were significantly influenced by both local environmental factors and spatial distance. It should be noted that a large proportion of the variation in community structure of both *nirK* and *nirS* denitrifiers could not be explained by the environmental and spatial variables. The unexplained variation may be partly produced by unmeasured environmental factors in this study, such as oxygen availability and redox potential, which have been proven to be significant in shaping *nir* community structure in aquatic ecosystems (Knapp *et al.* 2009; Wittorf *et al.* 2016).

Conclusions

The present study represents an attempt to establish relationships between *nir* community structure and environmental and spatial factors in wetlands soils across the Tibetan Plateau. No clear distinction in *nir* community composition was found among the wetland types. The denitrifier abundance and diversity were mainly determined by soil properties such as moisture, carbon availability and temperature, while conductivity was the most important factors of variations of *nir* community composition. Our results highlight that environmental selection and spatial processes or dispersal limitation are important in explaining soil *nir* communities, especially *nirS*-type denitrifying communities, in relatively undisturbed plateau wetlands. Therefore, to predict the importance of environmental variables in structuring *nir* communities in plateau wetlands, water or soil conductivity should be a key concern.

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Conflict of Interest

The authors declare no conflict of interest.

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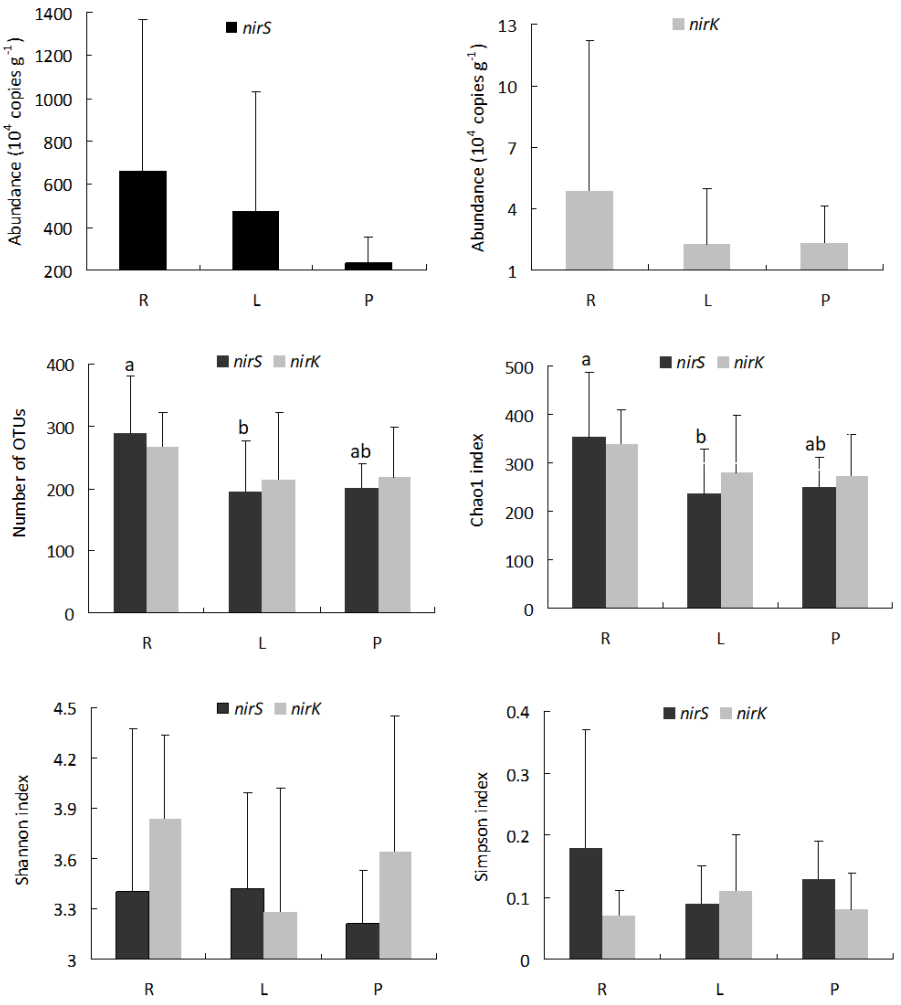

Figure 1 Abundance and diversity (mean±SD) of denitrifying communities in soils collected from riverine (R), lacustrine (L) and palustrine (P) wetland sites. Different lowercase letters over the bars indicate statistically significant differences among wetland types at $P< 0.05$.

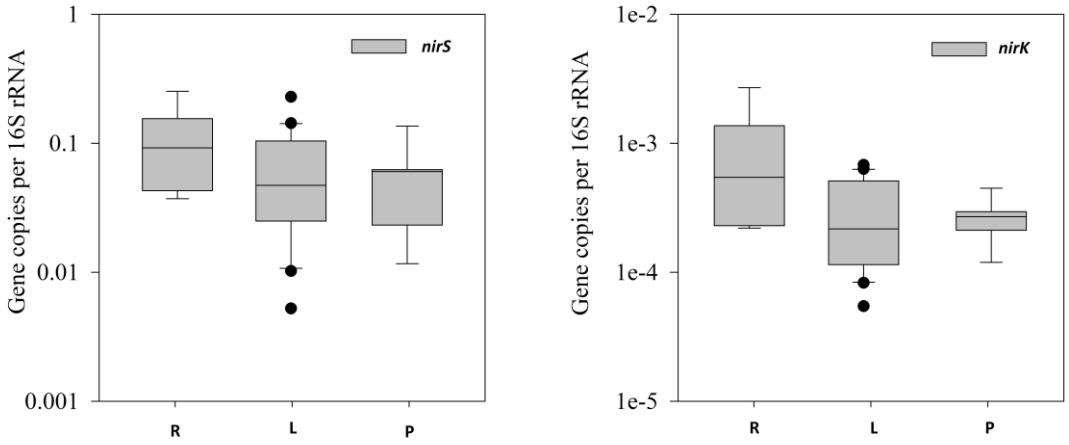

Figure 2 Relative abundances of *nirS* and *nirK* genes to the 16S rRNA gene in soils collected from riverine (R), lacustrine (L) and palustrine (P) wetland sites.

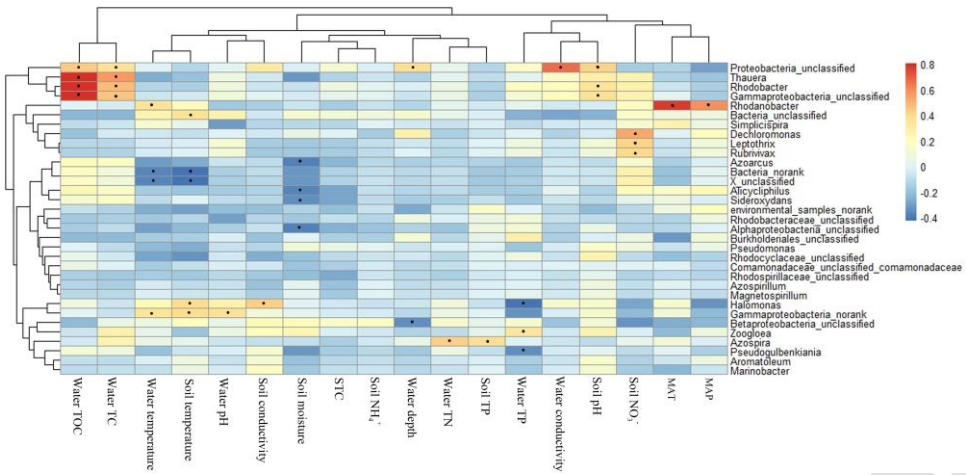

Figure 3 Heat maps showing the Pearson's correlation between the compositions of *nirS* denitrifier communities (genus level) and environmental factors.

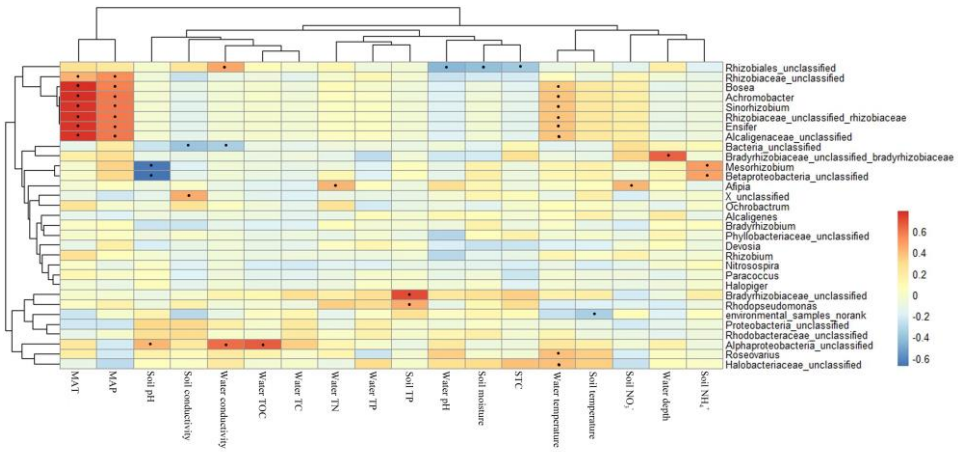

Figure 4 Heat maps showing the Pearson's correlation between the compositions of *nirK* denitrifier communities (genus level) and environmental factors.

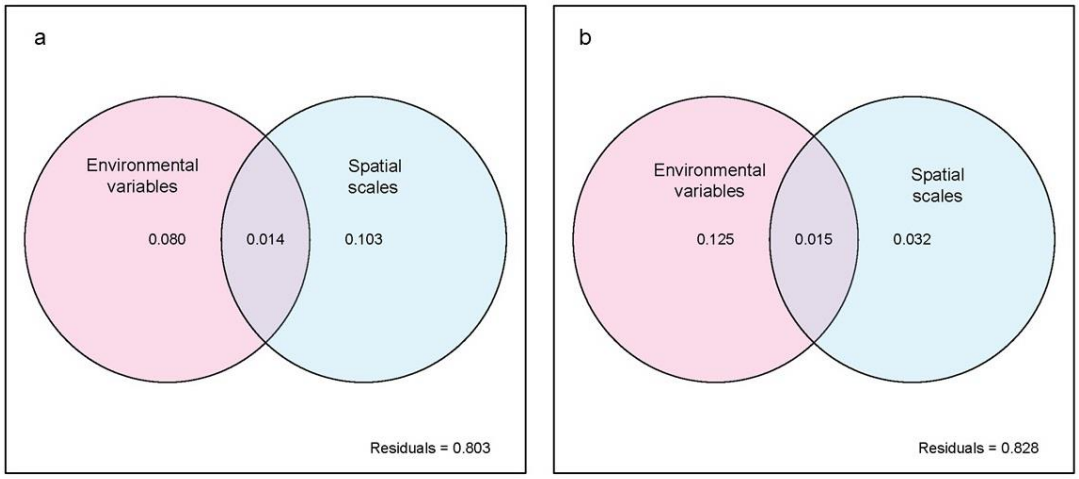

Figure 5 Variance partition analyses showing the relative importance of environmental and spatial factors in shaping *nirS* (a) and *nirK* (b) denitrifying communities.

Table 1 Pearson correlation coefficients between environmental factors and the abundance and diversity of *nirS*-type denitrifiers in Tibetan wetlands.

	Abundance	OTU number	Chao1	Shannon	Simpson
MAT (℃)	0.06	0.13	0.08	0.08	-0.01
MAP (mm)	0.08	0.16	0.16	0.18	-0.08
Water pH	0.15	-0.23	-0.24	0.03	-0.17
Water depth (m)	0.19	-0.06	-0.02	0.18	-0.20
Water temperature (℃)	0.34	0.05	0.05	0.18	-0.22
Water conductivity (μS cm^{-1})	-0.30	-0.16	-0.20	-0.17	0.05
Water TC (mg L^{-1})	-0.09	-0.07	-0.11	0.07	-0.16
Water TOC (mg L^{-1})	-0.18	-0.09	-0.15	0.13	-0.29
Water TN (mg L^{-1})	-0.15	-0.25	-0.27	-0.08	-0.13
Water TP (mg L^{-1})	-0.01	-0.24	-0.24	-0.06	-0.06
Soil pH	-0.25	-0.08	-0.10	-0.05	-0.01
Soil temperature (℃)	0.38*	0.12	0.12	0.26	-0.26
Soil conductivity (μS cm^{-1})	-0.09	-0.06	-0.04	-0.01	-0.13
Soil moisture (%)	0.43*	-0.40*	-0.33	-0.20	-0.15
STC (%)	0.45*	-0.43*	-0.32	-0.12	-0.25
Soil NH_4^+ (mg kg^{-1})	0.22	-0.14	-0.10	-0.16	0.04
Soil NO_3^- (mg kg^{-1})	0.22	-0.25	-0.21	0.15	-0.23
Soil TP (mg g^{-1})	-0.05	0.11	0.10	0.17	-0.09

* p <0.05; ** p<0.01

Table 2 Pearson correlation coefficients between environmental factors and the abundance and diversity of *nirK*-type denitrifiers in Tibetan wetlands.

	Abundance	OTU number	Chao1	Shannon	Simpson
MAT (℃)	-0.14	-0.15	-0.21	-0.01	0.02
MAP (mm)	0.06	-0.09	-0.10	0.05	0.04
Water pH	0.17	-0.16	-0.19	-0.26	0.19
Water depth (m)	0.02	-0.14	-0.13	-0.15	0.30
Water temperature (℃)	0.14	0.04	-0.01	0.03	-0.08
Water conductivity (μS cm^{-1})	-0.33	-0.27	-0.30	-0.04	-0.07
Water TC (mg L^{-1})	0.01	-0.34	-0.33	-0.18	0.10
Water TOC (mg L^{-1})	-0.02	-0.35*	-0.43*	-0.12	0.04
Water TN (mg L^{-1})	-0.33	-0.17	-0.24	-0.05	-0.07
Water TP (mg L^{-1})	0.03	-0.02	0.04	0.01	-0.10
Soil pH	-0.12	-0.19	-0.19	-0.19	0.08
Soil temperature (℃)	0.20	-0.01	0.01	-0.06	-0.03
Soil conductivity (μS cm^{-1})	-0.24	-0.11	-0.05	-0.29	0.21
Soil moisture (%)	0.38*	0.04	0.02	0.04	-0.17
STC (%)	0.34	0.16	0.14	0.05	0.06
Soil NH_4^+ (mg kg^{-1})	0.21	0.13	0.05	0.20	-0.16
Soil NO_3^- (mg kg^{-1})	0.07	-0.42*	-0.42*	-0.35	0.34
Soil TP (mg g^{-1})	0.09	-0.04	0.01	0.00	-0.10

* p <0.05; ** p<0.01