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Pyrosequencing of *nirS* gene revealed spatial variation of denitrifying bacterial assemblages in response to wetland desertification at Tibet plateau

GU Yun-fu†* https://orcid.org/0000-0001-8238-9360, e-mail: guyf@sicau.edu.cn

LIU Ting† https://orcid.org/0000-0002-4841-4099, e-mail: xixidayongshi@163.com

BAI Yan† https://orcid.org/0000-0002-4138-3912, e-mail: 18408211921@163.com

XIANG Quan-ju https://orcid.org/0000-0003-0155-7305, e-mail: xiangquanju@163.com

ZHANG Xiao-ping https://orcid.org/0000-0001-7199-4063, e-mail: zhangxiaopingphd@126.com

CHEN Qiang https://orcid.org/0000-0002-1831-4425, e-mail: cqiang@sicau.edu.cn

**Corresponding author; †Equal contributors*

Department of Microbiology, College of Resource Science and Technology, Sichuan Agricultural University, Chengdu 611130, China

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Abstract: Amplicon sequencing of functional genes is a powerful technique to explore the diversity and abundance of microbes involved in biogeochemical processes. One such key process, denitrification, is of particular importance because it can transform nitrate (NO_3^-) to N_2 gas that is released to the atmosphere. In nitrogen limited alpine wetlands, assessing bacterial denitrification under the stress of wetland desertification is fundamental to understand nutrients, especially nitrogen cycling in alpine wetlands, and thus imperative for the maintenance of healthy alpine wetland ecosystems. We applied amplicon sequencing of the *nirS* gene to analyze the response of denitrifying bacterial community to alpine wetland desertification in Zoige, China. Raw reads were processed for quality, translated with frameshift correction, and a total of 95,316 *nirS* gene sequences were used for rarefaction analysis, and 1011 OTUs were detected and used in downstream analysis. Compared to the pristine swamp soil, edaphic parameters including water content, organic carbon, total nitrogen, total phosphorous, available nitrogen, available phosphorous and potential denitrification rate were significantly decreased in the moderately degraded meadow soil and in severely degraded sandy soil. Diversity of the soil *nirS*-type denitrifying bacteria communities increased along the Zoige wetland desertification, and *Proteobacteria* and *Chloroflexi* were the dominant denitrifying bacterial species. Genus *Cupriavidus* (formerly *Wautersia*), *Azoarcus*, *Azospira*, *Thiothrix*, and *Rhizobiales* were significantly (P<0.05) depleted along the wetland desertification succession. Soil available phosphorous was the key determinant of the composition of the *nirS* gene containing denitrifying bacterial communities. The proportion of depleted taxa increased along the desertification of the Zoige wetland, suggesting that wetland desertification created specific physicochemical conditions that

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decreased the microhabitats for bacterial denitrifiers and the denitrification related genetic diversity.
Keywords: Wetland desertification; Amplicon sequencing; *nirS* bacteria; Differential abundance analysis

Introduction

Denitrification is an important microbial controlling pathway that removes excess nitrogen from wetland ecosystem. This anaerobic transformation reduces nitrate to nitrogen gas from nitrite, nitric oxide, and nitrous oxide that is generated in the denitrification process (Ward 2013). Wetlands and other water environments are 'key points' of nitrogen removal, in which the N loading from terrestrial environment were reduced (Veraart et al. 2017). Based on a meta-analysis, nitrogen rather than other nutrients was the key determinants in mediating the denitrification process in diverse aquatic environments, including wetlands, sediments, wastewater, oceans, and estuaries (Piña-Ochoa and Álvarez-Conbelas 2006). Although the abundance, diversity, and distribution of bacteria and archaea in alpine wetlands ecosystems has been reported (Yun et al. 2012), the abundance, diversity, and distribution of denitrifiers in alpine wetland ecosystems have received little attention.

Being widely distributed in unrelated phylogenetic groups, the commonly used 16S rRNA gene is not suitable for composition analysis of denitrifying bacterial communities (Veraart et al. 2017). Fortunately, several functional genes such as *nirS*, *nirK*, *cnorB*, *qnorB*, and *nosZ*, involved in the denitrification pathway, were successfully used as marker genes to study the denitrifying bacterial communities in natural environments (Wang et al. 2017; Yu et al. 2018). Among the proteins encoded by these genes involved in the denitrification process, two NiR enzymes with distinct structures but similar functions have been described. One is a cytochrome cd1 heme type reductase (NirS) encoded by *nirS*, and the other is a copper-oxidoreductase (*NirK*) encoded by *nirK*. The two enzymes are mutually exclusive as no denitrifier has been found to carry both types in their genome (Jones and Hallin 2010). Variations of the denitrifying bacterial community composition are often reported by using these functional genes as marker genes (Kim et al. 2011). Denitrifiers containing *nirS* and *nirK* gene were observed to be environment-specific in their distribution (Gao et al. 2017), and the prevalence of *nirS* and *nirK* denitrifying bacteria is closely related to the dissolved oxygen condition, with the former lower redox conditions (Gao et al. 2017). Furthermore, functional gene *nirS* is the more widely distributed gene in environment and is present in over 70% of known denitrifiers thus it is more suitable for revealing the diversity and composition of denitrifying bacteria community (Lisa et al. 2017).

As an ecologically vulnerable area, sensitive to climate change, Qinghai–Tibet Plateau (QTP) is an appropriate habitat to study the dynamics of nitrogen biogeochemical cycling in response to climate change (Kato et al. 2006). Zoige wetland in the QTP is a typical cold wetland, distinct from the boreal wetlands due to its location in low latitudes and a high altitude of 3400–3600 m. In the past 40 years, this world's largest plateau peatland has experienced severe desertification because of climate change and intensive anthropogenic disturbance, including drainage, over grazing, and peat harvesting (Wu et al. 2016). Zoige wetland desertification succession involves three stages: pristine swamp wetland is firstly changed into wet meadow and then into desertified land (Huo et al. 2013). Previously, we investigated the Zoige alpine wetland *nirS*-type denitrifying bacterial community composition using terminal restriction fragment length polymorphism (T-RFLP), clone libraries and Sanger sequencing (Gu et al. 2017). However, as has been the case in many other environmental studies of denitrifying bacteria based on clone libraries (Abell et al. 2013), these methods fell short of covering the highly diverse and complex denitrifier communities present in wetlands and sediments. Our previous study showed that the dominant denitrifying bacterial species in the soils at different stages of the Zoige plateau wetland desertification were similar; differences were attributed to a few rare species, which suggested that these rare species may be critical for mediating the denitrification process in the plateau wetland desertification (Gu et al. 2017).

Recently, amplicon sequencing has been applied in studying the *nirS* denitrifying bacterial communities in various environments. For

example, Lee and Francis (2017) investigated the distribution and dynamics of denitrifying communities in San Francisco Bay, and Wang et al. (2017) analyzed the diversity and community composition of denitrifiers in tannery wastewater treatment plants. Thus, to deeply understand the response of the diversity and composition of *nirS* functional gene community to the Zoige plateau wetland desertification, amplicon sequencing of the *nirS* gene was performed to further explore the diversity and composition of the *nirS*-type denitrifying bacterial community. Our aim was to (1) deeply understand the variation of the *nirS* gene community composition in soils related to the Zoige wetland desertification, (2) analyze the specific taxa significantly affected by wetland desertification, and (3) reveal the relationships between specific taxa and soil properties. We hypothesized that (i) the *nirS*-type denitrifying bacterial communities in the pristine soil would be more diverse than those found in the degraded soil due to the deterioration of soil nutritional conditions, and (ii) certain *nirS* denitrifying bacterial taxa would be enriched or depleted by the wetland desertification.

1 Materials and Methods

1.1 Soil sampling

The Zoige alpine wetland is located at the northern edge of Sichuan province, China, between 32°20'-34°00'N and 101°30'-103°30'E (Appendix 1), at the convergence of the East Asian Monsoon and Indian Summer Monsoon systems in Eastern Asia. Zoige wetland is well known as the "Yellow River reservoir" because it supplies up to 35% of the Yellow River's water in the dry season. The average altitude of this area is approximately 3500 m, and the mean annual precipitation is approximately 654 mm (Cui and Graf 2009). More than half of the precipitation (ca. 52%) is delivered from June to August due to the influence of warm and wet air from southwesterly and southeasterly monsoons. The mean relative humidity is approximately 69%, and the frost period lasts five to six months (Cui and Graf 2009). The variation in the soil water content that ranges from 30% to 90% is closely related to the natural slope, the soil depth and wetland types (e.g., the swamp, the meadow and the sandy area), and is also influenced by the vegetation coverage and the peat layer thickness.

The three stages of the Zoige wetland desertification are characterized by three different soils (Gu et al. 2017). The pristine swamp soil was covered with water all year round, and the dominant plant species were hydrophytes and sedge hydro-mesophytes like *Carex muliensis*, *Carex lasiocarpa* and *Carex meyeriana*. Mesophytes and hydro-mesophytes (*Kobresia tibetica*) characterized the meadow soil that was in a humid state. Only some *Psammophytes* grew in the desertificated sandy soil. The parent materials of swamp soil, meadow soil, and sandy soil were homogeneous silt clay and triassic slate residues, sandstones and siltstone, and aeolian material, respectively (Huo et al. 2013).

Briefly, three soil cores below the litter layer at 0-20 cm were collected using a steel corer and mixed into one homogenized composite sample (about 1.5 kg). Roots were removed prior to homogenizing the soil. The corer was sterilized using 70% ethanol between samplings. Soil samples were stored in Minigrip bags on ice and transported to the laboratory. The homogenized soils were divided into two parts: one for soil physicochemical properties analyses, and the other for total DNA isolation. The latter were stored at -80°C.

1.2 Soil edaphic properties and potential denitrification capacity

Soil edaphic properties were analyzed as previously described (Gu et al. 2017). Briefly, soil pH was determined in a soil-to-water ratio of 1:5, and soil organic carbon was analyzed by dichromate oxidization method. Soil gravimetric water was measured using an oven-drying method. Soil total nitrogen and available nitrogen were analyzed by Kjeldahl digestion and alkaline hydrolysis diffusion method, respectively. Soil total phosphorus and available phosphorus, total potassium and available potassium were assessed with molybdenum blue method and flame photometry method, respectively. Potential denitrification capacity was estimated following the method described by Dambreville et al. (2006).

1.3 Soil microbial DNA extraction

Soil total DNA was extracted from 0.5g homogenized soil with the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union, CA, USA) was used to purify the DNA. The purified DNA was quantified using a Qubit 2.0 spectrophotometer (Invitrogen, Carlsbad, CA, USA). The extracted DNA was stored at -25°C.

1.4 Preparation of *nirS* gene amplicon library and sequencing

An approximately 450 bp *nirS* fragment was amplified using the primers cd3aF (5′-GTSAACGTSAAGG-ARACSGG-3′) with a unique 8-mer barcode and R3cd (5′-GASTTCGGRTGSGTCTTGA-3′) (Smith et al. 2007). Primers were produced by Shangon Biotech (Shanghai, PR China). PCR amplification was done in triplicate with an ABI GeneAmp® 9700 PCR System in a total volume of 50 μl. PCR reaction mixture consisted of 5 μl of 10 × Ex Taq Buffer, 2 μl of dNTP Mix (2.5 mM each), 0.5 μl of each primer (10 μM), 5 μl of template DNA (20 ng/μl), 31.5 μl of double distilled water (ddH_2O) and 2.5 U of Taq DNA polymerase (TAKARA, Otsu, Shiga, Japan). The *nirS* gene PCR procedure was 4 min at 94°C, followed by thirty-two cycles of 20 s at 94°C, 20 s at 55°C, and 30 s at 72°C, and a final extension at 72°C for 10 min.

The triplicate PCR products were pooled, purified with PCR Clean-up Purification Kit (MP Biomedicals, California, USA), and quantified using Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). Amplicon sequencing targeting the *nirS* gene was done using 454 pyrosequencing on a GS-FLX platform (454 Life Sciences/Roche, Branford, CT, USA) at Macrogen (Seoul, South Korea). The amplicons of *nirS* gene were sequenced and processed with the shotgun protocol. All 454 sequence data from this study were deposited to NCBI Sequence Read Archive (SRA) under the accession no. SUB3738482.

1.5 Sequencing data analyses

QIIME quality filter was applied to select reads with phred-quality score higher than 20 and length over 300 bps for further analyses, and UCHIME with *de novo* strategy was used to detect and remove the chimeric sequences (Caporaso et al. 2012). FrameBot tool was applied to adjust the frame shift errors (Wang et al. 2013). The *nirS* gene reads were subsequently translated into deduced amino acid sequences (DACS).

To determine the DACS clustering threshold, we established a linear regression model between 16S rRNA genes and *nirS* DACS. We firstly downloaded all *nirS* DACS derived from isolates from FunGenes database, and then matched them with corresponding 16S rRNA genes downloaded from GenBank database using an in-house Python script. We filtered those sequence pairs with ambiguous matching, and with invalid sequence length (*nirS* DACS: 120-143 residues; 16S rRNA genes: 1450-1650 bps) during matching. Sixty-seven sequence pairs were retained for regression analysis after filtering. ARB software package version 5.5 was used to calculate sequence dissimilarity matrix (D) for both DACS and 16S rRNA gene sequences (Ludwig et al. 2004). The similarity matrices were calculated with equation: $S=1-D$, and used to establish linear regression model in R. Based on the regression model, we calculated the *nirS* DACS clustering cutoffs at each classification level. Using UCLUST pipeline, the *nirS* DACS were clustered into OTUs at 95% sequence similarity. The representative sequences were picked using an in-house Python script with an algorithm similar with QIIME pipeline.

To accurately assign *nirS* OTUs, we firstly combined representative sequences with reference *nirS* DACS downloaded from FunGenes database. Then we clustered the combined sequence set at 83% sequence similarity (Family level) using UCLUST algorithm. The sequences were clustered and assigned into OTUS at the family level with an in-house Python script. For a particular cluster, we proceeded to next parsing when no representative sequence was detected. When no reference sequence was detected, OTUs were assigned using an artificial number. For representative sequences that could be clustered with more than one reference, one of the references was randomly selected to assign these OTUs. The OTU abundance table was generated based on OTU clustering and assignment results with Python script. A depth of

1515 tags per sample (totally 95,316 sequences for all three soils) was applied in rarefaction analysis and resampling, and 1011 OTUs were detected and used in downstream analysis (Appendix 2). Alpha diversity indices were calculated using QIIME pipeline based on resampled OTU abundance table.

1.6 Statistical analyses

Soil properties were compared using random permutation test, and further used to perform principal component analysis (PCA) to assess differences among soils. After calculating the relative abundances of taxa at family level, principal coordinates analysis (PCoA) was used to assess community composition in the soils on the basis of Bray-Curtis dissimilarities with package vegan (Oksanen et al. 2018) in R (R Development Core Team 2010). Distance based redundancy analysis (dbRDA) was performed to determine factors driving community variation. Effect significance of the dbRDA model factors was calculated using "permutest" function with a maximum of 999 permutations in R package vegan. The goodness of fit for each edaphic factor was tested using "envfit" function with 999 permutations in R package vegan. The relationships between soil edaphic properties and *nirS* gene community composition were evaluated using Mantel test.

The R package DESeq2 was applied to analyze differential abundances at family level (Love et al. 2014). Differential abundance analysis was performed using a Wald test (Love et al. 2014). False discovery rate (FDR) control based on the Benjamini-Hochberg procedure was used to correct for multiple testing (Love et al. 2014). Taxa with significant abundance change were assumed as taxa with FDR-adjusted $P < 0.1$ and absolute differential abundance >1.0. Differentially abundant taxa were picked out to construct a phylogenetic tree. To minimize the phylogenetic tree, we randomly selected one representative sequence from the sequence pool of each taxon using a Python script. The sequences were aligned against *nirS* reference sequences using BLAST (e value: 1e-5). The reference sequences with best alignment were combined with query sequences, and neighbor-joining tree was generated and monitored with 1000 replicates for bootstrap testing using MEGA7 (Kumar et al. 2016) and visualized using iTOL with a web interface.

2 Results

2.1 Edaphic properties and potential denitrification rates (PDR)

Soil water content (WC), organic carbon content (SOC), and concentration of total nitrogen (TN), total phosphorous (TP), and available nitrogen (AN) and available phosphorus (AP) decreased as the swamp soil desertificated to sandy soil ($P<0.05$) (Table 1). Soil pH and total and available potassium (TK, AK) were highest in the meadow soil. Soil pH was lowest in the sandy soil. Soil TK concentration was lowest in the swamp soil, and AK was lowest in the sandy soil. The highest potential denitrifying rate (PDR) was determined

Table 1 Physicochemical parameters of soil samples from Zoige plateau wetland

Soil codes	WC (%)	pH	SOC	TN	TP	TK	AN	AP	AK
			g.kg^{-1}				mg.kg^{-1}		
SW1	74.96±0.33a	7.53±0.02a	279.14±2.77a	5.15±0.44a	1.43±0.13a	7.44±0.45a	993.00±14.65a	26.01±1.36a	99.83±0.79a
SW2	76.53±2.34a	7.66±0.01a	283.09±4.30a	5.71±0.03a	1.41±0.00a	6.79± 0.10a	1079.43±4.04a	22.63±0.16a	96.67±0.83a
SW3	79.40±1.36a	7.56±0.01a	281.25±5.67a	5.29±0.15a	1.32±0.01a	7.30±0.09a	1025.88±7.00a	24.72±1.40a	98.33±2.83a
MD1	16.12±0.82c	7.84±0.01b	66.34±1.88c	3.22±0.36c	0.98±0.11c	21.34±1.57c	246.33±6.11c	12.40±0.14c	365.42±4.37c
MD2	14.59±0.12c	7.75±0.01c	55.69±1.54b	2.99±0.34b	0.44±0.05c	23.13±0.44c	195.32±5.05c	11.87±0.29c	103.14±4.25c
MD3	16.56±0.71c	7.98±0.01c	72.83±1.47b	3.67±0.41c	0.95±0.33c	22.25±0.23c	277.35±7.65c	11.17±0.23b	443.45±5.28c
SD1	7.23±0.51d	6.74±0.02c	8.55±0.24d	0.86±0.15d	0.84±0.01d	17.73±0.44d	65.44±3.03d	4.52±0.76d	51.24±0.44d
SD2	7.01±0.31d	6.71±0.01d	8.24±0.17c	0.81±0.01c	0.76±0.11d	16.66±0.19d	61.23±2.14d	3.45±0.12d	33.42±1.13d
SD3	6.89±0.51d	6.71±0.01d	8.02±0.06c	0.79±0.03d	0.72±0.03d	16.89±0.14d	41.22±1.12d	3.78±0.15c	41.27±2.55d

Notes: Mean ± standard error (n=3). Values within the same column followed by the same letter do not differ at $P < 0.05$. WC: water content; SOC: soil organic carbon; TN: total nitrogen; TP: total phosphorus; TK: total potassium; AN: available nitrogen; AP: available phosphorus; AK: available potassium. AK: available potassium; SW: swamp; MD: meadow soil; SD: sandy soil.

Table 2 Potential denitrifying rate (PDR) of the three soils associated with Zoige wetland desertification.

	SW1	SW2	SW3	MD1	MD2	MD3	SD1	SD2	SD3
PDR (ng N_2O-N g^{-1} ds h^{-1})	33.52±2.14a	52.77±3.53a	48.63±2.37a	17.25±4.14c	19.39±2.42c	20.17±1.17b	14.52±0.24d	12.13±0.78d	8.78±1.03c

For sample codes, see Table1.

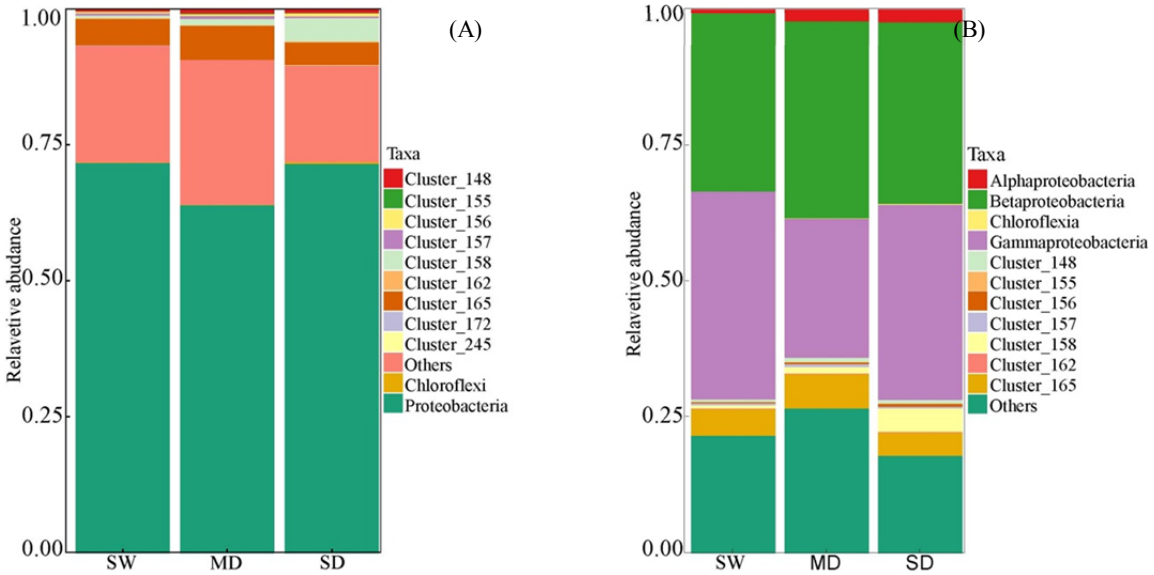

Figure 1 Relative abundances of the most abundant phyla (A) and classes (B) in the soils associated with the Zoige wetland desertification. 'Others' refer to taxa less abundant than the most abundant taxa. SW: swamp soil; MD: meadow soil; SD: sandy soil.

in pristine swamp soil while lowest in the sandy soil (Table 2).

2.2 Diversity and composition of nirS-type denitrifying bacterial community

Amplicon sequencing of the *nirS* gene resulted in 95,316 sequences with an average sequence length of 367±3 bp. The Good's coverage for all the samples were >90%, suggesting that the numbers of sequences were sufficient for diversity analysis (Appendix 3). Significant differences were observed in OTU numbers (P< 0.01) and Shannon- wiener index (P < 0.01) (Appendix 4). Both the Shannon-wiener index and OTU numbers were lowest in pristine swamp soil and highest in sandy soil. In the principal component analysis, the first axis explained 73.12% of the variation and separated the communities in pristine swamp soils from those in the degraded meadow and sandy soils (Appendix 5). The other axis explained 22.32% of the variation and separated the sandy soil communities from meadow soil communities.

Proteobacteria was the most abundant phylum (Figure 1A). The phyla with relative abundance over 0.01 included Cluster158, Cluster165, and *Chloroflexi*. *Betaproteobacteria* accounted 0.32 to 0.36 of the relative abundance of *Proteobacteria*, followed by *Gammaproteobacteria* (0.27 to 0.38) and *Alphaproteobacteria* (0.01 to 0.03) (Figure 1B).

2.3 Differential abundance analysis

Taxa that were significantly affected by wetland desertification were identified by differential abundance analysis. Enriched taxa (e-taxa) and depleted taxa (d-taxa) were defined as taxa with more than two times higher or lower relative abundance (P<0.05) when comparing samples. Altogether seventeen differentially abundant taxa were detected at family level (Figure 2).

Compared with the pristine swamp soil, three e-taxa (*Nevskiales*, Cluster 215, and Cluster 225) and ten d-taxa (Cluster 212, Cluster 214, Cluster 216, Cluster 217, Cluster 225, Cluster 246, Cluster 247, Cluster 248, *Rhizobiales* and one unclassified taxon) were determined in the sandy soil (Figure 2). Cluster 214 and Cluster 217 were determined as d-taxa also in the meadow soil (Figure 2). Compared

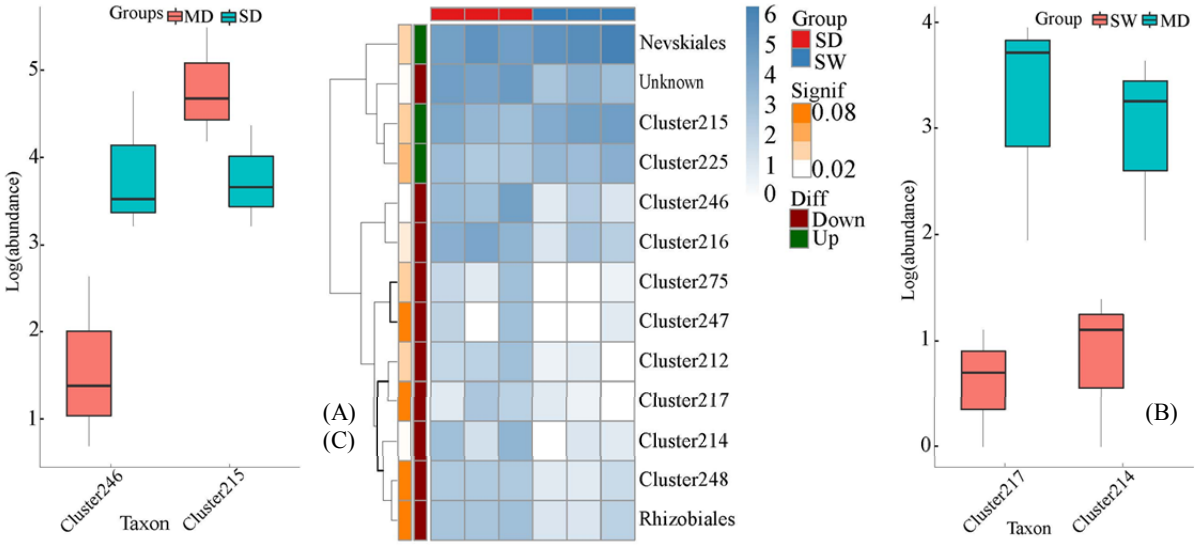

Figure 2 Differential abundance analyses of the *nirS* taxa that were significantly enriched (blue) and depleted (red) in three soils associated with Zoige wetland desertification. (A) Swamp soil (SW) vs. Meadow soil (MD), (B) Swamp soil (SW) vs. Sandy soil (SD), (C) Meadow soil (SW) vs. Sandy soil (SD)

with the meadow soil, Cluster 215 and Cluster 246 were determined as d-taxa and e-taxa, respectively, in the sandy soil (Figure 2).

Based on the Mantel test, the differentially abundant taxa correlated with multiple edaphic factors (P<0.05) (Figure 3). Cluster 215 correlated positively with pH, SOC, WC, TN, TP, AN, AP, and AK (P<0.05). Cluster 212 correlated negatively with pH, WC, SOC, TN, TP, AN, AK, and AP (P<0.05) (Figure 3).

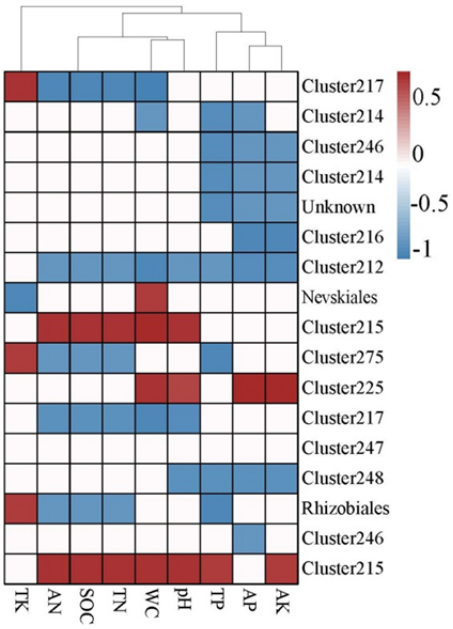

Figure 3 Correlations between the differential abundant taxa and soil physicochemical parameters. WC, soil gravimetric water content; SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus; TK, total potassium; AN, available nitrogen; AP, available phosphorus (Olsen phosphorus); and AK, available potassium.

2.4 Phylogenetic analysis of differentially abundant taxa

In the *nirS* gene phylogenetic tree, the representative sequences of differentially abundant taxa grouped into *Alphaproteobacteria* and *Betaproteobacteria* (Figure 4). The *Alphaproteobacteria* related differentially abundant taxa grouped into a clade consisting of species from cultured *Thiothrix lacustri* and unknown denitrifying bacteria. Cluster 216 and Cluster 217 were related to *Thiothrix lacustri* with 77% and 80% identity, respectively. Cluster 247 was related to bacterium CYCU-0207 with 77% identity.

The *Betaproteobacteria* related taxa Cluster 212, Cluster 214, Cluster 215, Cluster 225, Cluster 246, Cluster 248, and Cluster 275 grouped into three major clusters. Cluster 212 and Cluster 275 were related to *Cupriavidus* sp. with 74% and 75% identity, respectively. Cluster 215 and Cluster 225 were both related to *Azospira* sp. with 77 and 79% identity, respectively. Cluster

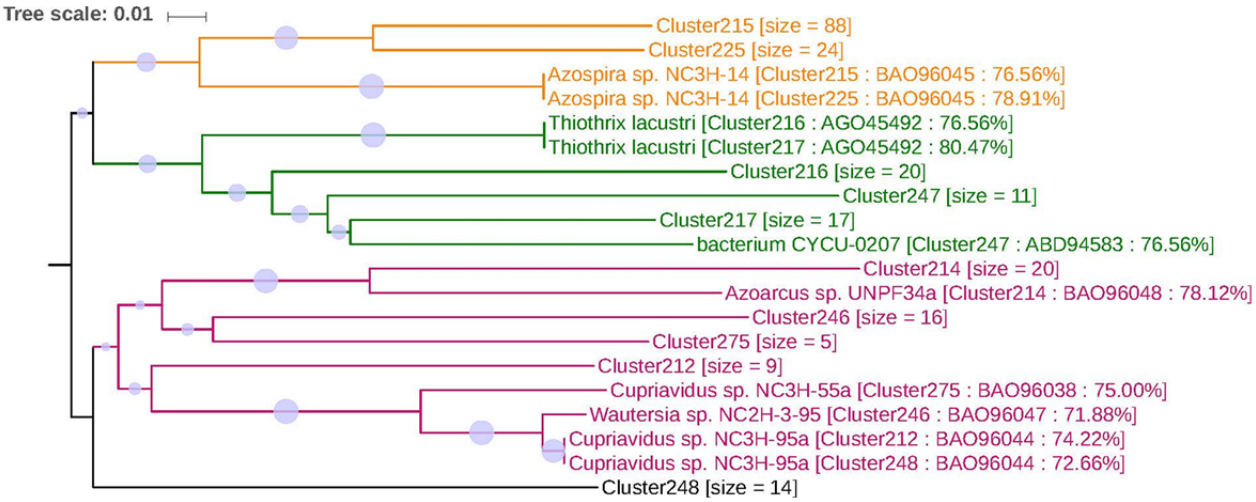

Figure 4 Phylogeny of the differentially abundant taxa. Size indicates sequence abundance. Cluster215: BAO96045: 76.56% indicates that *nirS* sequence BAO96045 is the closest matching sequence for Cluster215 with 76.56% identity. Numbers next to branches indicate the bootstrap values

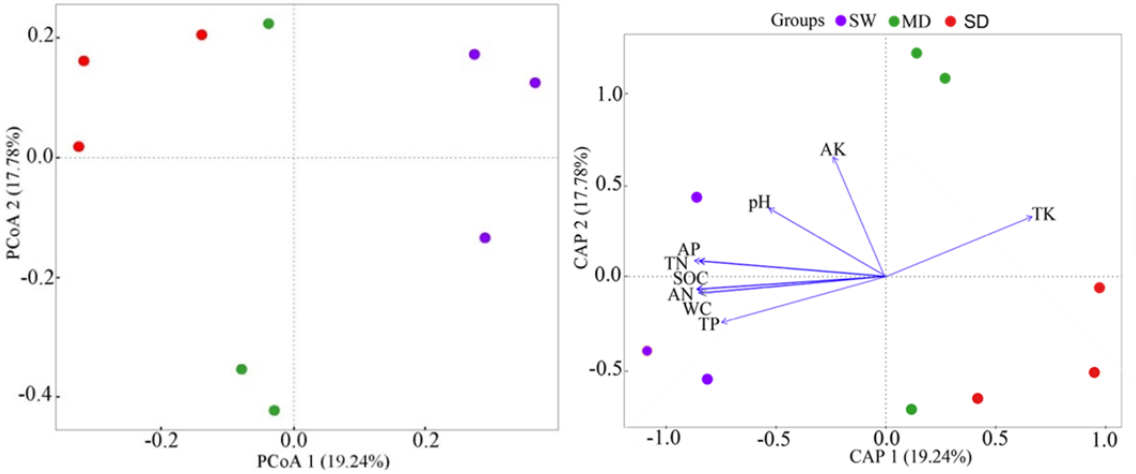

Figure 5 *nirS*-type denitrifying bacterial communities and their relation with edaphic factors across the Zoige plateau wetland soils. (A) Principal coordinates analysis (PCoA) of *nirS*-type denitrifying bacterial communities. (B) Quantifying the effects of edaphic factors on *nirS*-type denitrifying bacterial community composition by distance-based redundancy analysis. WC, soil gravimetric water content; SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus; TK, total potassium; AN, available nitrogen; AP, available phosphorus (Olsen phosphorus); and AK, available potassium. SW: swamp soil; MD: meadow soil; SD: sandy soil.

214 and Cluster 246 were related to *Azoarcus* and *Wautersia* with 78% and 71% identity, respectively.

2.5 Community composition and variation

Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarities separated the *nirS*-type denitrifying bacterial community in the pristine soil from those in the desertificated soils (Figure 5), indicating that the wetland desertification in Zoige resulted into different *nirS*-type denitrifying bacterial communities. The influence of edaphic parameters on *nirS* gene community composition was quantified by the distance-based redundancy analysis (dbRDA). Of all constrained nine edaphic factors, available phosphorus (AP) (r^2=0.9505, P=0.001) was the most potential determinant (Figure 5). Soil water content (WC) was also an important factor affecting *nirS*-type denitrifying bacterial community composition. Both the Mantel and partial Mantel tests further confirmed that that soil AP and WC were the most influential abiotic variables in shaping the communities of *nirS* gene

containing denitrifying bacteria during the desertification process of Zoige plateau wetland (Figure 6).

3 Discussion and Conclusion

Microorganisms are essential for keeping the stability of ecosystems and biogeochemical cycling of nutrients, especially nitrogen cycling. Denitrification which is mediated by bacteria, archaea and fungi is fundamentally important in nitrogen removal from wetlands (Veraart et al. 2017). Zoige plateau wetland in China, the largest alpine peat wetland in the world, has been desertificated rapidly in the past 30 years. In our previous study, variation of the denitrifier communities caused by Zoige plateau wetland desertification was evaluated by using terminal restriction fragment length polymorphism (T-RFLP) and clone libraries (Gu et al. 2017). In this study, the diversity of *nirS*-type denitrifiers was remarkably high when using high-throughput amplicon sequencing targeting the functional *nirS* gene. Previously, using 16S rRNA amplicon sequencing, considerable number of *Proteobacteria* which might be involved in carbon cycling were identified, yet only a few OTUs belonging to denitrifying *Proteobacteria* were detected among Zoige wetland soil bacteria (Gu et al. 2018). *Proteobacteria* is highly diverse and members of this phylum are active in biogeochemical cycling. Revealing the roles of these microorganisms in a natural community is not feasible on the basis of 16S rRNA gene sequencing. The results of our present study results suggested that amplicon sequencing the functional *nirS* gene makes detailed study of denitrifiers achievable.

Similar to a previous study (Wang et al. 2017), in our study the *nirS* gene sequences were mostly affiliated with *Proteobacteria*. However, unlike in Wang et al. (2017) and Yang et al. (2018), *Alphaproteobacteria* and *Gammaproteobacteria* accounted for 0.32 to 0.36 and 0.27 to 0.38 of the total sequences in *Proteobacteria*, respectively. Diversity of the functional genes related to denitrification in *Gammaproteobacteria* has been previously reported (Katsuyama et al. 2013). Although many of the *nirS* sequences were

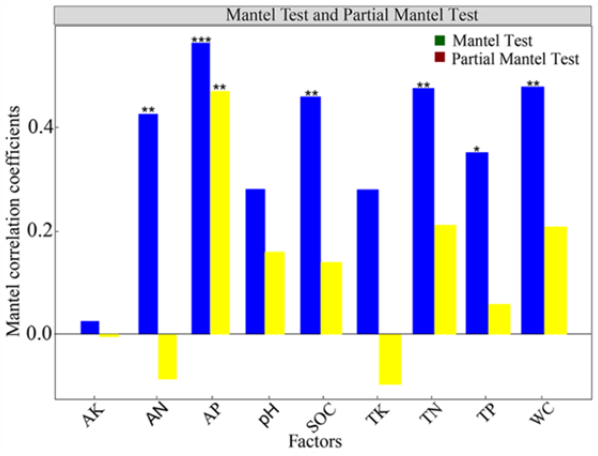

Figure 6 Mantel and partial Mantel tests between the relative abundances of *nirS* gene and edaphic factors in the Zoige wetland soils. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. WC, soil gravimetric water content; SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus; TK, total potassium; AN, available nitrogen; AP, available phosphorus (Olsen phosphorus); AK, available potassium.

affiliated with *Gammaproteobacteria*, none of these sequences were identified at the genus level, which may indicate a dearth of knowledge on the *nirS*-denitrifying bacteria diversity in the alpine wetland. Based on the diversity and composition of the *nirS* denitrifying bacteria communities in the soils from different stages of wetland desertification, we could provide an insight into the composition of *nirS* gene communities in this ecosystem. The unidentified *Gammaproteobacteria nirS* gene sequences may represent ecologically important strains, yet determining the roles of the strains requires further study. Thus, using culture dependent methods to study these novel *nirS* denitrifying bacteria will increase our understanding of their physiological functions and ecological importance.

Soil edaphic properties parameters play critical roles in affecting the abundance, diversity, and composition of soil microbial communities. Therefore, exploring the relations between soil properties and functional microbial communities is one of the key goals in microbial ecology (Liang et al. 2011). Previous studies showed that water level and content was the key driving force leading to the desertification of Zoige wetland, playing an important role in the decrease in soil fungal community abundance and prokaryotic diversity (Wu et al. 2016). With the decline in the water

table, some wetland plant species that are essential in swamp soil formation are being replaced by meadow vegetation (Zhong et al. 2017). Different vegetation types altered the soil bacterial communities due to their different root exudates, which are nutrients for soil bacteria (Bremer et al. 2007). In this study, SOC, WC, TN, TP, AN, and AP contents all decreased during the process of the wetland desertification. Only soil potassium increased in the meadow soil, possibly partly due to the decrease in soil water content which may decrease the leaching of soil potassium, and partly due to the growth of the plant roots that provide more sorption sites for soil potassium (Chmolowska et al. 2016). The decreasing contents resulting from wetland desertification make them valuable indices for evaluating desertification. Similar to our previous study (Gu et al. 2017), soil AP content was also a key factor in affecting the community of *nirS* gene containing denitrifying bacteria in the three different soils. A similar relationship between the abundance of *nir* denitrifiers and AP content has been found in a spruce forest soil, where the AP content was closely related to the nutrient availability in soil (Báttard et al. 2010). Given that phosphorus is needed to synthesize phosphorus-rich RNA that is essential for protein synthesis, it is not surprising that a similar positive correlation relationship was found in an earlier (Yin et al. 2015) and our study. Soil fungi play critical roles in transforming soil phosphorus, and their symbiotic relations with plants may inhibit the growth of denitrifiers via decreasing the availability of soil nutrients essential for denitrifying microorganisms, thereby altering the denitrifier community composition (Bender et al. 2014). Accordingly, there may be a complex relationship between nitrogen cycling and phosphorus cycling microorganisms in this alpine wetland.

Contrary to our main hypothesis that more diverse *nirS*-denitrifying bacterial communities would be observed in the pristine wetland, both the diversity and species richness of denitrifying bacteria increased during the Zoige alpine wetland desertification. Seemingly many of the denitrifying bacteria were adapted to the deteriorated soil conditions. However, many taxa were depleted in the degraded soils, which may indicate that the taxa that are specialized in the pristine swamp conditions did not tolerate the wetland desertification. These inconsistent results suggest that the decreasing supply of soil nutrients during the wetland desertification may have changed the original niche space, leading to conditions under which the e-taxa could thrive at the expense of the d-taxa.

As shown by the differential abundance analysis, the *nirS*-type denitrifying bacterial community structure in the pristine soil differed from those in the degraded meadow and sandy soils. This distinction may have resulted from the direct or indirect effects of wetland desertification. In line with our hypothesis, many taxa were depleted in the degraded soil when compared with the pristine swamp soil. Out of the depleted taxa, *Azoarcus* is a physiologically versatile group encompassing bacteria with diverse functions and widely found in aquatic heterotrophic denitrifying communities (Hong et al. 2010). Hwang et al. (2006) reported that at high pH environments *Azoarcus*-like populations were of low diversity yet predominant. Moreover, high NaCl concentrations (1%-3%) would stimulate the growth of the genera *Azoarcus* and *Methylophaga* (Osaka et al. 2008). In this study, pH decreased as the pristine swamp soil desertificated into the sandy soil. In Zoige plateau wetland swamp soil has been found more saline than sandy soil (Wang et al. 2017). Therefore, the *Azoarcus*-like populations could thrive in the high pH and saline swamp soil. The results indicated that frequently detected bacterial genera like *Azoarcus* might be critical in mediating the biogeochemical cycling of nitrogen in the Zoige alpine wetland and correlate with the denitrification process in this environment. Out of the enriched OTUs, the genus *Azospira* belongs to *Betaproteobacteria*, and many *Azospira* strains have denitrification or nitrogen fixation ability, suggesting that these bacteria play vital roles in nitrogen cycling and organic compounds decomposition (Arroyo et al. 2015).

Rhizobial nitrate reduction and denitrification have been widely detected in terrestrial and aquatic environments, dominating the denitrification process in wastewater treatment plants and meadow soil (Rungkitwatananukul et al. 2016). Rhizobia can convert NO_3^- to N_2 under both reducing and oxidizing conditions. Members of the genus *Thiothrix* are filamentous and colorless

sulfur-oxidizing bacteria that are metabolically versatile and capable of autotrophic, mixotrophic and heterotrophic growth. *Thiothrix* spp. can perform nitrate-mediated sulfide oxidation, and adapt to micro-aerobic conditions (Cytryn et al. 2005). In the pristine swamp soil, *Thiothrix* spp. possibly play important roles in the biogeochemical cycling of nitrogen and sulfur, as they are able to use NO_3^- or NO_2^- as electron acceptors to oxidize inorganic and organic sulfur compounds.

In summary, this study showed that the diversity of the soil *nirS* gene community increased along the Zoige wetland desertification. The composition of *nirS* gene community also varied significantly in the three soils associated with the desertification process. Among the identified *nirS* denitrifier species, *Proteobacteria* and *Chloroflexi* were the dominant phyla in the soils. *Azoarcus*, *Azospira*, *Wautersia*, *Cupriavidus*, *Rhizobiales*, and *Thiothrix* were significantly affected by the wetland desertification. Changes in the *nirS*-type denitrifying bacterial communities were related to the changes in available phosphorus content. The unclassified and novel taxa observed in this study require further studying. The results of this study contribute to comprehending the roles of *nirS* denitrifying bacteria in predicting wetland desertification in Qinghai-Tibet plateau.

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