Implications of wetland degradation for the potential denitrifying activity and bacterial populations with *nirS* genes as found in a succession in Qinghai-Tibet plateau, China

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Abstract: Alpine wetland in the Zoige Plateau has suffered from serious degradation during the last 30 years due to global climate change and anthropogenic impact. Denitrification is a key nitrogen removal process which can be performed by different microorganisms, including bacteria harboring *nirS*-genes. In this study, a degradation succession was used to study the effect on potential denitrification activity (PDA) and on bacterial communities harboring *nirS* genes. Based on the determination of the PDA, the abundance, structural diversity, and phylogenetic identity of the soil bacteria with *nirS* genes were further assessed by qPCR, terminal restriction fragment length polymorphism (T-RFLP), and DNA-sequencing, respectively. The results showed that soil PDA ranged from 8.78 to 52.77 ng N_2O-N g^{-1} dry soil h^{-1}, being lowest in sandy soil and highest in swamp soil. The abundance of *nirS* genes (copies g^{-1} soil) were also the lowest in the sandy soil while highest in the swamp soil. The average Shannon-Wiener diversity index of the *nirS* denitrifying bacterial structural ranged from 2.20 in the meadow soil to 3.07 in the swamp soil. Redundancy analysis (RDA) showed that the *nirS* denitrifying bacterial community correlated with soil water content and available phosphorus, with water content as the major factor in shaping the *nirS* denitrifying bacterial community. The results of this study suggest that the wetland degradation would decrease soil PDA, and abundance and structural diversity of the denitrifying bacteria with *nirS* genes. These findings can contribute to support a theoretical foundation for predicting the potential influences of wetland degradation on soil denitrifying bacteria in alpine wetlands.

Keywords: Wetland degradation; Potential denitrifying activity; *NirS* denitrifying bacterial community; T-RFLP; qPCR

1. Introduction

Qinghai-Tibet Plateau is a special geographical and ecological area that plays an important role in environmental change. Studying on the dynamics of nitrogen cycle in this area is critical to evaluate the response and feedback of biogeochemistry cycles to global climate change [1]. Zoige wetland on the eastern edge of the Qinghai-Tibetan Plateau is the highest and largest highland peat marsh in China [2]. Most alpine wetlands in the Zoige Plateau have suffered serious degradation, carbon and nitrogen loss and decline of biodiversity due to global climate change [3] and anthropogenic impact [4]. A lowered water table has resulted in loss of humus and peat horizons and transformation of these into meadow soil. Besides, long term overgrazing by cattle in the meadows has decreased vegetation coverage and disturbed their water regulating function. Moreover, draining the mining areas has influenced the water retention ability of peat swamps, and changed the wetland soil moisture conditions [2]. In a typical degradation succession in Zoige wetland, native swamp soil turns first into transition meadow soil and then into degraded sandy soil [5]. Although it is known that wetland degradation affects plant community composition and reduces diversity and above- and below-ground biomass [6], little is known about how the soil microbial community composition and diversity respond to wetland degradation.

Denitrification is a microbiological process which can be mediated by bacteria, archaea or fungi, and more than 50 genera of bacteria have been identified as denitrifying bacteria [7]. It is a key nitrogen removal process in wetlands and frequently limited by available nitrogen rather than carbon [8]. Given that the high phylogenetic diversity among denitrifying bacteria, detection methods based on assessment of genes such as 16S rRNA are not directly useful and therefore requires the use of functional genes which encode for enzymes directly involved in the denitrification process. There are two structurally different but functionally equivalent nitrite reductases: copper (*nirK*) and cytochrome

cd1-containing (*nirS*) enzymes, which are responsible for conversion of nitrite (NO_2^-) to nitric oxide (NO)[9], are frequently-used as the molecular marker for the cultivation independent studies on denitrifying bacteria as only denitrifiers possess this enzyme[10]. These functional genes have been used as molecular markers for the cultivation independent studies on denitrifying bacterial diversity in various environments. Recent studies showed that *nirS* and *nirK*-type denitrifying bacterial communities responded differently to environmental parameters such as nitrate and oxygen concentrations in rice fields, and probably the *nirK* denitrifying bacteria were active at the beginning of anaerobic incubation, while the *nirS* denitrifying bacteria were more active in the fully developed anaerobic conditions [11]. Moreover, *nirS* denitrifying bacteria were found to be far more abundant, more active, and more diverse than *nirK* denitrifying bacteria [12]. The presence of bacterial populations harbouring *nirS* genes may correlate with environmental factors [13]. To date, little is known about how the abundance and community composition of the denitrifying bacteria respond to Plateau wetland degradation. Due to the expected bigger role of *nirS* denitrifying bacteria and better sequence database coverage of *nirS*, studying the response of denitrifying bacterial community, with a focus on *nirS* denitrifying bacteria, to wetland degradation in Qinghai-Tibet Plateau is necessary to understand how nitrogen cycle responds to the changing climate in this area.

To this end, in combination with the measurement of denitrification potential activities, the abundance, structural diversity, and phylogenetic identity of the *nirS* denitrifying bacteria in three different soils (native swamp, degraded meadow, and sandy soils) were assessed by qPCR, T-RFLP, and DNA-sequencing, respectively. The objectives of this study were (1) to investigate the variation in *nirS* denitrifying bacterial community in the three different soils associated with wetland degradation succession, and (2) to understand the relationships between the *nirS* denitrifying bacterial community and soil physicochemical properties. We hypothesized that wetland degradation had a significantly

negative effect on soil potential denitrifying activity, and on the abundance, phylogenetic diversity and community composition of the *nirS* denitrifying bacteria.

2. Materials and Method

Soil sampling

The study site was on the Zoige Plateau, located at the northeastern part of the Qinghai-Tibetan Plateau (101°30′E-103°30′E, 32°20′N -34°00′N) (Table 1). The average altitude at Zoige Plateau is 3500 m and the mean annual temperature is 1.1°C, with the lowest monthly temperature of -10.3 °C in January and the highest of 10.9 °C in July. Annual precipitation is 560-860 mm. Swamp soil (SW1, SW2, and SW3) was covered with water all year round, and the vegetation was dominated by hydrophytes and sedge hydro-mesophytes (*Carex muliensis*, *Carex lasiocarpa* and *Carex meyeriana*). Meadow soil (MD1, MD2, and MD3) surface was in a humid state and the vegetation was dominated by mesophytes and hydro-mesophytes (*Kobresia tibetica*). Sandy soil (SD1, SD2, and SD3) surface was continuously dry and had little or only a few *Psammophytes* as vegetation cover [14]. Homogeneous silt and clay were the dominant soil parent materials of marsh soils [2]. The soil parent materials of meadow soil were accumulated residues of triassic slate, sandstones and siltstone [15]. Sandy soil, the top layer (0-30 cm) of which was sand, was composed of aeolian parent material [16].

The soils were sampled using an auger with 20 mm diameter × 100 cm length and only the surface (5-20 cm) soil samples were analyzed in this study. Each of the replicate samples represented a composite sample of 15 subsamples. Surface vegetation and roots were removed before collecting the samples. Samples (approximately 1.5 kg) were divided into two portions: one was air dried before removing roots and stones, and then sieved through a 2-mm mesh screen for physicochemical analyses. The other portion was stored at -80 °C for DNA extraction.

Soil physicochemical parameters and potential denitrifying activity analysis

Soil water content (WC) was determined after oven-drying at 105 °C for 48 h [17]. Soil total nitrogen (TN) and available nitrogen (AN) were determined using the alkaline hydrolysis diffusion method and semi-micro Kjeldahl method, respectively [18]. Soil organic carbon (SOC) and pH were determined by potassium dichromate oxidation-external heating method and potentiometry method, respectively [18]. Total phosphorus (TP) and available phosphorus (AP) were assessed by hydrofluoric acid-perchloric acid digestion-Mo-Sb colorimetry and sodium bicarbonate extraction-Mo-Sb colorimetry methods, respectively [18]. Total potassium (TK) and available potassium (AK) were determined by hydrofluoric acid-perchloric acid digestion-flame photometry and ammonium acetate extraction-flame photometry, respectively [18]. Potential denitrifying activity (PDA) was estimated using the C_2H_2 inhibition method [19].

Soil total DNA extraction

Total DNA was extracted from 0.5 g homogenized soil using a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). DNA concentration and quality was measured with a Nano-200 spectrophotometer (AoSheng, Hangzhou, China). DNA samples were stored at –20 °C for further analysis. For clone library construction, the template DNA was a mixture of an equal amount of DNA from the three replicates. The triplicate DNA samples were independently used for terminal restriction fragment length polymorphism (T-RFLP) and real-time PCR assays analysis.

Quantitative PCR

The *nirS* gene was quantified by qPCR with three technical replicates per sample. Standard curves to assess *nirS* gene abundance consisted of a 10-fold serial dilution (10^1-10^7) of a plasmid (299 ng μL^{-1}) containing the *nirS* gene fragments from the tested soil as described by Henry et al. [20]. The *nirS* genes were amplified with primer pair nirSCd3aF (5′-AACGYSAAGGARACSGG-3′) and

nirSR3cd (5′-GASTTCGGRTGSGTCTTSAYGAA-3′) [21]. For qPCR, 12.5 μL ABI Power SybrGreen qPCR Master Mix (Applied Biosystems, USA), 0.5 μM of each primer, 10 μM of soil DNA and 9.5 μL of sterile water were used to make up a final volume of 25 μL. The reaction was carried out on a ABI7500 sequence detection system (Bio-Rad) using the program of 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min and extension at 72 °C for 30 s. Absolute *nirS* copy number was calculated by interpolating from the standard curve. Gene abundances were standardized by the mass of DNA that was extracted per gram of dry soil and $\log_{10}$ transformed before analysis.

PCR amplification and T-RFLP of the *nirS* gene

The primer pair nirSCd3aF and nirSR3cd [21] was used to amplify the *nirS* gene in a C1000™ thermal cycler (Bio-Rad, Hercules, CA, USA). Amplification was done in a volume of 50 μL containing 10 μM diluted soil DNA, 5 μL of 10× PCR buffer, 200 μM of dNTP mix, 0.5 μM of each primer, 18.5 μL of ddH_2O and 2.5 U of Taq DNA polymerase (TAKARA, Otsu, Shiga, Japan) with the following cycling conditions: 5 min of denaturation at 95 °C, a 21 cycles touchdown program (94 °C for 30 s, annealing at 60 °C for 45 s, and 72 °C for 60 s) where the annealing temperature was lowered 1 °C after every three cycles, 22 cycles with 94°C for 30 s, 53 °C for 45 s, and 72 °C s for 60 s, and a 7 min extension at 72 °C.The PCR products were analyzed by electrophoresis on 1.0 % agarose gels and visualized by UV Transilluminator Model M-26 (UVP, USA) after ethidium bromide staining.

For T-RFLP, the forward primers were fluorescently labeled with 6-Fam (6-carboxyfluoresein). The *nirS* gene PCR products were digested with *HhaI* endonuclease (NEB, Ipswich, Massachusetts, USA) at 37 °C for 6 h, followed by further purifying by Tiangen DNA purification kit (Tiangen, China) to clean up the T-RFLP mix. T-RFLP profiles were generated by capillary electrophoresis using an ABI Prism 3100 Genetic Analyzer at Sangong Corporation (Shanghai, China). Sizes and

relative abundances of terminal restriction fragments (T-RFs) were quantified using PeakScan version 1.0 software (Applied Biosystems, Inc.). Only fragments with a signal greater than 1% of the sum of all peak heights were included in the analysis. The peak heights of T-RFs that differed in size by ≤2 bp in an individual profile were summed and considered as one fragment. To assign the peaks of samples to clone sequences, clones corresponding to different *in silico* T-RFs were also subject to T-RFLP analysis with the same procedure mentioned above.

Cloning and sequencing

The *nirS* gene PCR products were excised from agarose gels and purified using universal DNA Purification Kit (Tiangen Co, Beijing, China) following the manufacturer's instructions. PCR products were cloned into pGEM-T vector (Tiangen) and transformed into *Escherichia coli* strain DH-5α, followed by blue–white screening. Inserts were amplified with M13F and M13R primers and the size of the inserts were estimated by agarose gel electrophoresis. A total of 182 positive clones (approximately 60 clones from each site) were randomly selected for restriction screening by digesting with *HhaI* endonuclease, and 15 representative clones were sequenced. The *nirS* gene sequences were deposited in GenBank under the accession numbers KT783435-KT783449.

Statistical analysis

The means and standard deviations of the soil properties and *nirS* gene diversities were calculated and tested for normality and homogeneity of variances. Data was analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test when the ANOVA results were significant. Otherwise, Tamhane's T3 test was adopted using SPSS 21.0 software (IBM SPSS Statistics for Windows, Armonk, NY, USA). Two-sided $P< 0.05$ was considered statistically significant. To assess the correlation between soil physicochemical properties and *nirS* gene abundances, Pearson correlation coefficients were calculated using the Simple Correlation Method in

SPSS 21.0. Redundancy analysis (RDA), which was performed using CANOCO version 5, was used to assess the relationship between *nirS* denitrifying bacterial community and environmental variables [22]. The significance of the RDA results was tested with the Monte Carlo permutation test.

The clone sequences were aligned using ClustalX 1.83 [23], and a neighbor-joining tree was constructed using MEGA 5.0 [24]. Bootstrap analysis was used to estimate the reliability of the phylogenetic reconstruction (1000 replicates). The *nirF* (accession no. D50473) and *nirN* (accession no. D84475) gene sequences from *Pseudomonas aeruginosa* were used as out-groups for phylogenetic analysis due to that their proteins have recognizable sequence relatedness to *nirS* [25].

3. Results

Soil physicochemical properties and potential denitrifying activity

The soil water content (WC) was highest in swamp (79.4%) and lowest in the sandy soil (6.9%) (Table 2). Soil pH in meadow was higher than in swamp and sandy soil. Soil organic carbon (SOC) content was significantly higher in the swamp soil than in meadow and sandy soils. Soil TN and AN ranged from 0.79 to 5.71 g kg^{-1} and 41.22 to 1079.43 mg kg^{-1}, respectively. Swamp soil had the highest concentrations of soil total nitrogen (TN) and available nitrogen (AN). The lowest concentrations of TN and AN were in the sandy soil. Total phosphorus (TP) and available phosphorus (AP) varied from 0.44 to 1.43 g kg^{-1} and 3.45 to 26.01 mg kg^{-1}, respectively, with the highest values observed in swamp soil and the lowest in sandy soil. Total potassium (TK) and available potassium (AK) ranged from 6.79 to 23.13 g kg^{-1} and 33.42 to 443.45 mg kg^{-1}, respectively. The highest concentrations were in meadow soil and the lowest in sandy soil. Soil potential denitrifying activities ranged from 8.78 to 52.77 ng N_2O-N g^{-1} dry soil h^{-1}. Activities were highest in swamp soil and lowest in sandy soil. The potential denitrifying activity correlated positively with WC, SOC, TN, AN, TP and AP, and negatively with TK ($p<0.01$) (Table 3).

nirS gene abundance and diversity

The soil *nirS* gene copy numbers ranged from 2.77 $\log_{10}$ per gram dry soil in sandy soil to 5.57 $\log_{10}$ per gram dry soil in swamp soil (Fig.1), showing that wetland degradation significantly decreased the *nirS* denitrifying bacterial abundance. The *nirS* abundance correlated positively with soil potential denitrifying activity ($p<0.01$) (Table 3).

To assess the diversity of *nirS* carrying microbial populations, the mean values of richness, Shannon-Wiener index (H) and evenness were calculated based on the T-RFLP data (Table 4). The soil *nirS* denitrifying bacterial community α-diversity varied significantly among the swamp soils, meadow soils and sandy soils ($P<0.05$). The average Shannon-Wiener index ranged from 2.20 in the meadow soil to 3.07 in the swamp soil (Table 4).

nirS denitrifying bacterial community structure

The *nirS* T-RFLP profiles from triplicate samples of each soil were similar, indicating that the results were reproducible and representative for the *nirS* denitrifying bacterial communities in the Zoige Plateau wetland soils (Fig.2). Redundancy analysis (RDA) showed that the *nirS* denitrifying bacterial communities in the swamp soils, meadow soils and sandy soils formed separate clusters (Fig.3), indicating that *nirS* denitrifying bacterial communities from these three different soils related to wetland degradation succession were significantly different ($P<0.01$).

The relative abundances of *nirS* gene were also different in the swamp soils, meadow soils and sandy soils (Fig. 2). The relative abundance of the dominant 75-, 80-, and 115 bp T-RFs (>5%) were slightly different among the soils, indicating that the degradation of Zoige wetland had little impact on the core *nirS* denitrifying bacterial community. On the contrary, all the tested three soils had their specific minor *nirS* T-RFs (<5 %). For example, the 50-, 185-, 190- and 230 bp T-RFs were detected only in the swamp soil, the 260- and 280 bp T-RFs only in the meadow soil, and the 20-, 25-, 35- and

255 bp T-RFs in the sandy soil only.

Phylogenetic analysis

In the phylogenetic analysis, the *nirS* gene sequences were grouped into four clusters based on their relationship with *nirS* gene sequences from various habitats and some well characterized denitrifying bacteria (Fig. 4). The *nirS* sequences with T-RFs 76, 77, 116, 121, 129, and 227 bp in cluster 1 were related to *nirS* gene sequences retrieved from various environments such as estuary sediment soil (GI: KM891947) and wetland sediment (GI: KC468857). The sequences in cluster 2 with T-RFs 60, 113, and 115 bp were also related to the clones retrieved from sludge and sediment, and then grouped with the identified *nirS* gene sequence from gammaproteobacterial *Pseudomonas fluorescens* (GI: AF114792) and *Pseudomonas aeruginosa* (GI: X16452). The sequences in cluster 3 with T-RFs 260 bp were related to those from betaproteobacterial *Burkholderiaceae*, *Ralstonia* and *Thaueraterpenica*. The sequences in cluster 4 with T-RFs 255 and 280 bp were grouped into one clade with those from alphaproteobacterial *Paracoccus* sp.

The swamp soils, meadow soils and sandy soils harbored different *nirS* denitrifying bacterial communities that carried genes related to *nirS* genes retrieved from various habitats. In the swamp soil, the most abundant *nirS* genes which accounted for 91.2% of the *nirS* denitrifying bacterial communities were related to *nirS* genes retrieved from freshwater sediment habitats. In the meadow soil, the *nirS* that accounted for 72% of the total *nirS* detected were related to *nirS* genes isolated from wetland and farmland soil. For the sandy soil, the *nirS* genes in cluster 2 were related to *nirS* genes from sediment and sludge (Fig. 4).

Relationships between *nirS* denitrifying bacterial community and environmental parameters

In the redundancy analysis (RDA), the *nirS* denitrifying bacterial communities from the swamp soils, meadow soils and sandy soils were divided into three separate groups (Fig. 3). The RDA results

further confirmed that the *nirS* denitrifying bacterial communities in the soils were significantly different ($P<0.05$). Among the measured abiotic factors, water content appeared to be the major factor in determining the *nirS* denitrifying bacterial community composition ($P<0.05$). Soil *nirS* denitrifying bacterial community variance was also significantly linked to available phosphorus ($P<0.05$).

4. Discussion

Soil properties in response to wetland degradation

Microbiologically mediated denitrification is the key process of nitrogen removal from wetlands. In this study, the physicochemical properties of soils associated with the Zoige wetland degradation succession were analyzed. In earlier studies, the hydrological conditions affected the community structure, plant productivity, the import of organic matter, and the accumulation of soil organic carbon (SOC) and total nitrogen (TN) [26]. Decrease of the soil water content induced by wetland degradation increased organic matter decomposition rates and net N mineralization rates [27], leading to the loss of soil carbon, nitrogen, and phosphorus [28]. In the process of wetland restoration, concentrations of total and available nitrogen and phosphorus significantly decreased with a decrease in water content [29]. In our study, water and organic carbon contents and the concentrations of nitrogen and phosphorus all decreased with the succession of wetland degradation, indicating that water content is the major factor contributing to the decreases in carbon, nitrogen and phosphorus content that accompany Zoige wetland degradation. In an anaerobic environment, decomposition of animal and plant residues is slow, which possibly explains why SOC was highest in the flooded swamp soil and lowest in the sandy soil. An increase in net N mineralization leads to the loss of nitrogen [27, 28], and in accordance to that the nitrogen content was highest in the swamp soil. Higher SOC content may provide a larger sorption capacity for phosphates [30], plausibly explaining why the phosphorus content was higher in the swamp soil than in the meadow and sandy soils.

Wetland degradation decreased soil potential denitrifying activity (PDA)

High denitrifying capacity depended on the quantity and quality of the organic carbon needed to support bacterial growth [31], and such dependence could be described as a proportional relationship between the denitrification rate and organic carbon supply [32]. Decreased water content led to an increased decomposition rate of soil organic matter, which then decreased the denitrification activity and changed the dominant species of denitrifying bacterial community [33]. In accordance with the previous studies, the Pearson correlation analysis in this study showed that the PDA correlated significantly with SOC. In our study, the decrease in PDA is probably due to the variation in the response of soil parameters to wetland degradation [34]. Key factors that could have varied include the patchiness of particulate organic material in the wetlands [35], vegetation density [36], and the nature and extent of the aerobic/anaerobic interface in the sediments [37].

The *nirS* gene abundance decreased in response to wetland degradation

The composition of the denitrifying bacterial communities were affected by water regimes and hydrological factors such as water content were dominant in regulating bacterial communities in wetland soils [13]. The effect of water regime on the proportion of denitrification genes in the bacterial community can be explained by the different preferences of the denitrifying bacteria to oxygen conditions in the environment. Most denitrifying bacteria are facultative anaerobes, preferring oxygen as an electron acceptor. If conditions change from aerobic to anaerobic, they begin to use nitrogen oxide as an alternative electron acceptor [38]. Previous studies on different environments have shown that the *nirS* denitrifying bacteria prefer environments with constantly lower oxygen concentrations [25, 38]. Similarly, in the current study, the soil water content appeared to be the most important factor in affecting the *nirS* gene abundance. The swamp soil is generally anaerobic since soil is flooded with water and oxygen availability is restricted, while in the meadow and sandy soils, water

level fluctuation presumably creates more aerobic conditions.

The *nirS* denitrifying bacterial community responded to wetland degradation

Our main hypothesis that the denitrifying bacterial community structure and diversity would be negatively impacted by wetland degradation was not completely supported by the results. The three soils with different degradation succession in Zoige wetland hosted distinct denitrifying bacterial communities. As judged by the terminal restriction fragments (TRFs), the core of the communities in the three tested soils were similar yet more minor TRFs were detected in the swamp and sandy soils and each soil had its′ specific TRFs. This suggested that the minor TRFs contributed to the community structural differences. Similar result has been observed also in one previous study [39] indicating that the core denitrifying bacteria may not be sensitive to wetland degradation whereas some minor denitrifying bacteria with low relative abundance are critical when community responses are evaluated. Although the diversity index of the denitrifying bacterial communities in the swamp soil (native soil) were higher than those in meadow (transitional soil) and sandy (degraded soil), the diversity index of the denitrifying bacterial communities in the meadow soil was lower than that in sandy soil, indicating a positive effect of sandy soil on denitrifying bacterial diversity. Adsorbed small particles on a sand grain may provide binding sites for adsorption of organic matter and microbial cells [40], resulting in a larger number of microhabitats available for colonization. This in turn increases the potential of multiple species to coexist in close proximity without directly competing for resources and potentially increases bacterial diversity [40]. Thus, the increased denitrifying bacterial community diversity may be explained by the increased sand content with degradation.

In three farmland soils from Finland (62°55′N, 29°30′E), Germany (48°40′N, 11°04′E), and Sweden (58°20′N 13°30′E), the majority of the *nirS* gene clones were similar to the *nirS* from *Ralstonia* and *Paracoccus* bacteria, both members of the *Proteobacteria* [41]. The *nirS* genes in an

agricultural soil from Changsha, China, were also affiliated with *Paracoccus* [42]. In our study, the swamp soils, meadow soils and sandy soils in the Zoige Plateau harbored diverse *nirS* species. Meadow soil hosted *nirS* denitrifying bacteria closely related to *Thaueraterpenica* and *Paracoccus,* and the sandy soil clones were closely related to *nirS* from *Paracoccus* and *Pseudomonas*. The majority of swamp soil clones were related to uncultured strains, indicating a dearth of knowledge on the denitrifying bacterial community in the Zoige Plateau wetland.

Relationship between *nirS* denitrifying bacterial community structure and soil properties

Understanding the environmental variables that affect microbial community structure is a key goal in microbial ecology. Soil moisture was found to be an important abiotic factor in affecting the *nirS* denitrifying bacterial abundance and community composition [43]. Oxygen was also found to have a decisive effect on the structure of denitrifying bacterial communities in the continental margin sediments of the oxygen-deficient zone at the Pacific Coast of Mexico [44]. In addition, the relative abundances of *nirS* terminal restriction fragments responded variously to the O_2, NO_3^-, NO_2^- and depth gradients in the water column of the oxygen minimum zone in the Eastern South Pacific [45]. In our study, soil physicochemical parameters were important in shaping *nirS* denitrifying bacterial communities, and soil water content and available phosphorus were the most important factors in shaping *nirS* denitrifying bacterial communities in Plateau wetland.

Phosphorus was one of the soil parameters which correlated positively with the *nirS* gene abundances in this study. It was reported that the availability of phosphorus greatly affected soil microbial community [46]. A similar relationship between the abundance of *nirK* denitrifying bacteria and available phosphorus has been also observed in a spruce forest soil, where the available phosphorus content was closely connected with the nutrient availability [47]. In contrast, a negative relationship has been observed for the abundance of *nir* denitrifying bacteria at scales compatible with

339land management [46]. Given that phosphorus is a vital constituent component for microorganisms
340[46], it is not surprising to find a positive correlation across this and other studies [47, 48]. There may
341be a complex relationship between nitrogen-cycling and phosphorus-cycling microorganisms in
342wetland soil. It should be noted that the T-RFLP in this study could only reveal the most dominant
343*nirS* denitrifying bacterial community. To fully reveal the variation of the denitrifying bacterial
344community in the soils associated with Zoige wetland degradation, approaches based on high
345throughput targeted amplicon sequencing should be used.

346 In summary, the soil properties, potential denitrifying activity, and *nirS* denitrifying bacterial
347abundance, structural diversity and phylogenetic identity were different in the three tested soils
348associated with the Zoige wetland degradation succession. Although wetland degradation decreased
349the *nirS* gene phylogenetic diversity, the degraded soil (sandy) harbored relative higher *nirS* diversity
350index than the transition soil (meadow). Among the analyzed soil properties, soil water content was
351the most important factor in shaping *nirS* denitrifying bacterial community composition. The results of
352this study can contribute to support a theoretical foundation for predicting the potential influences of
353wetland degradation on soil denitrifying bacteria in alpine wetlands.

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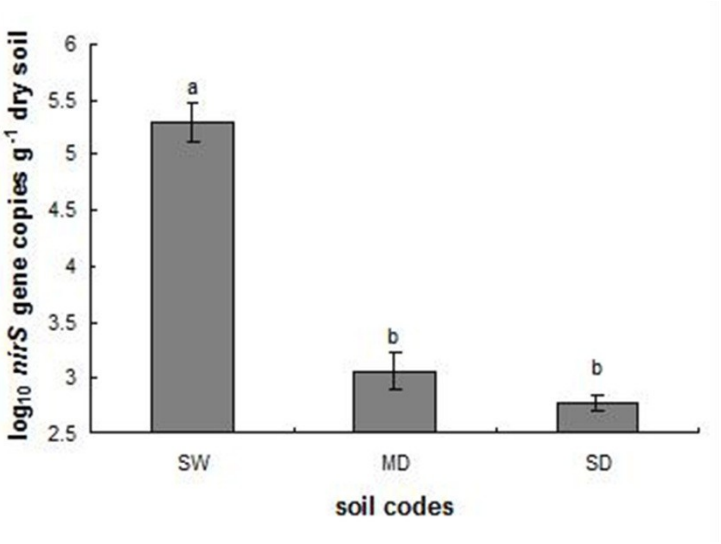
Figure legends

Fig. 1. Abundances of *nirS* genes in swamp soil (SW), meadow soil (MD) and sandy soil (SD). Different letters above the bars indicate significant differences based on protected LSD test ($P<0.05$).

Fig. 2. Average relative abundances of *nirS* terminal restriction fragments (T-RFs) in swamp soil (SW), meadow soil (MD) and sandy soil (SD). The relative abundance of T-RFs is given as a percentage of the total peak height. Fragment sizes (bp) of the experimental T-RFs are indicated on the right.

Fig. 3. Ordination diagram from redundancy analysis (RDA) on the basis of relative abundance of *nirS* T-RFs in swamp soil (SW), meadow soil (MD) and sandy soil (SD). AP, available phosphorus; WC, water content; TN, total nitrogen.

Fig. 4. Neighbor-joining phylogenetic tree of *nirS* gene sequences derived from swamp soil (SW), meadow soil (MD) and sandy soil (SD) in the Zoige Plateau. *nirN* gene (D84475) and *nirF* gene (D50473) from *Pseudomonas aeruginosa* were used as outgroups. Bootstrap values greater than 50% of 1000 resamplings are shown. The scale indicates the number of nucleotide substitutions per site. Numbers before the sample code indicate the sequenced *nirS* gene clones. Numbers in parentheses indicate the Genbank accession numbers.



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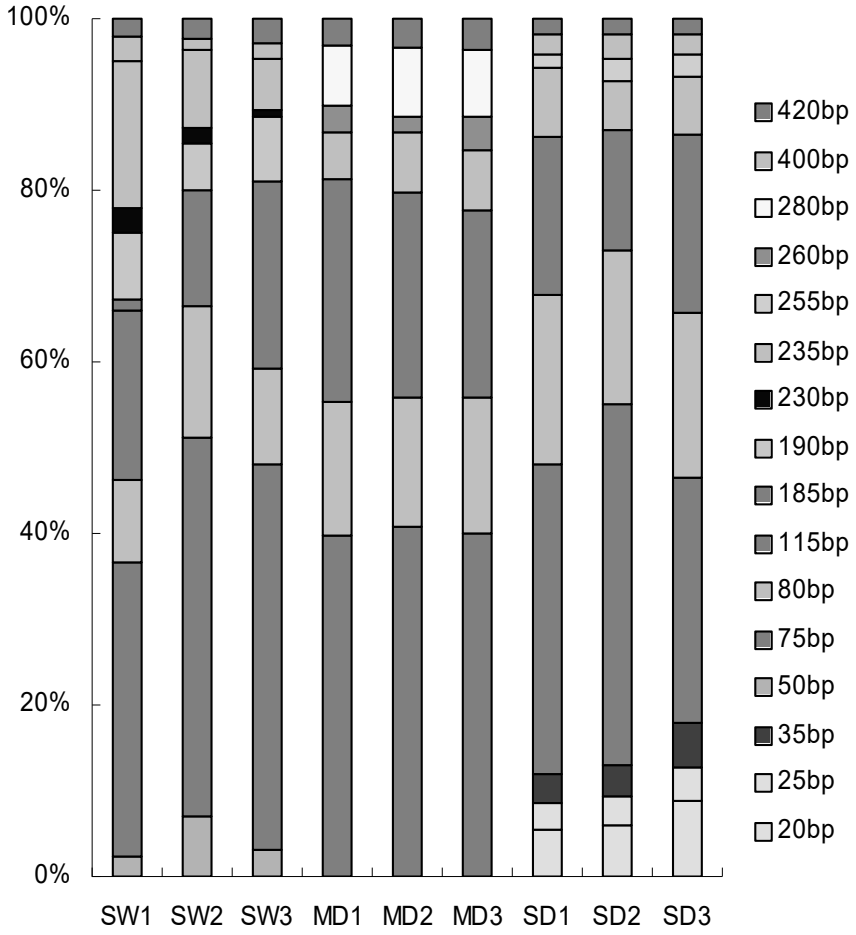

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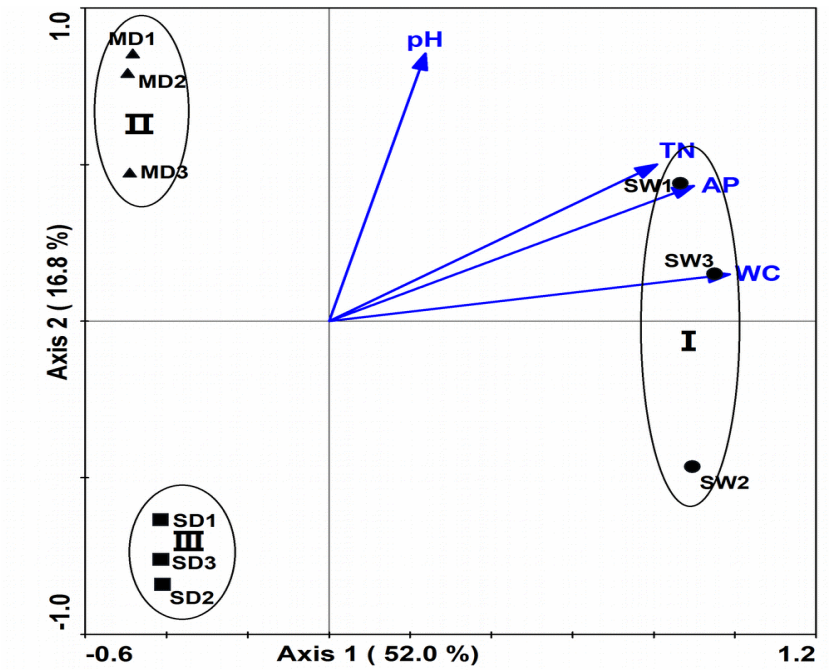

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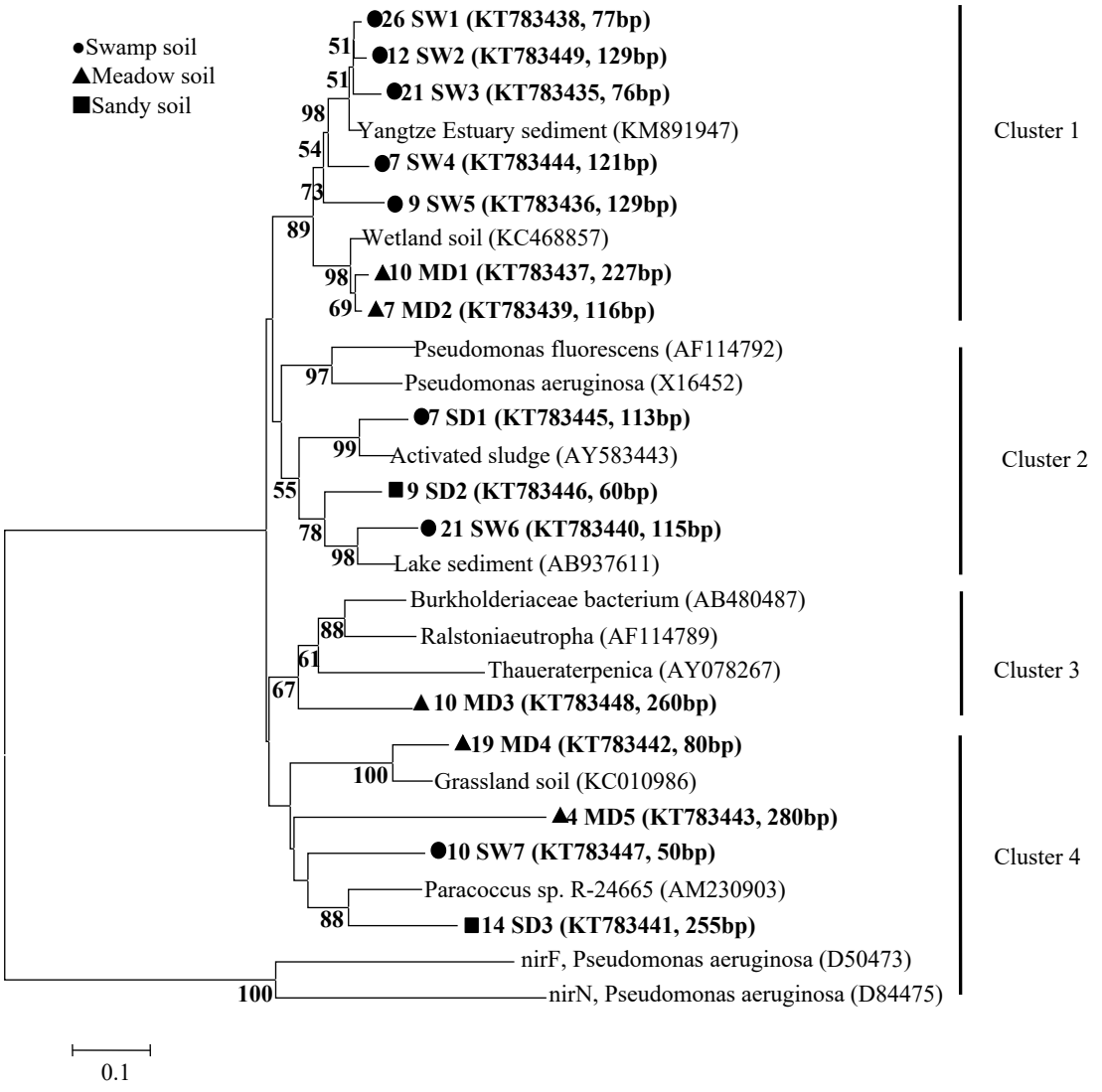

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Table captions

Table 1. Sampling sites in Zoige Plateau wetland.

Table 2. Physicochemical parameters of soil samples from Zoige Plateau wetland.

Table 3. Pearson correlation between soil physicochemical properties, potential denitrifying activity (PDA) and *nirS* gene abundance.

Table 4. *nirS* gene diversity indices of soil samples from Zoige Plateau wetland.

527 Table 1. Sampling sites in Zoige Plateau wetland

Sample code*	Sampling site	Longitude	Latitude	Altitude (m)	Soil temperature (°C)
SW1	Axi	102°49′ 02.8″N	33°54′57.0″E	3427	5
SW2	Heihe	102°48′ 48.2″N	33°54′58.2″E	3432	6
SW3	Fenqu	102°48′ 59.4″N	33°55′21.2″E	3432	5
MD1	Axi	102°52′ 08.5″N	33°47′37.4″E	3465	4
MD2	Huahu	102°56′ 94.9″N	33°38′99.6″E	3481	4
MD3	Ruoergai	103°13′ 81.7″N	33°11′99.8″E	3585	4
SD1	Tangke	102°27′ 07.6″N	33°17′55.4″E	3445	5
SD2	Tangke	102°43′40.5″N	33°31′32.8″E	3522	5
SD3	Tangke	102°40′35.5″N	33°25′16.9″E	3528	4

528* SW: swamp soil; MD: meadow soil; SD: sandy soil.

529 Table 2. Physicochemical parameters of soil samples from Zoige Plateau wetland

WC (%)	pH	SOC ($g \cdot kg^{-1}$)	TN ($g \cdot kg^{-1}$)	TP ($g \cdot kg^{-1}$)	TK ($g \cdot kg^{-1}$)	AN ($mg \cdot kg^{-1}$)	AP ($mg \cdot kg^{-1}$)	AK ($mg \cdot kg^{-1}$)	PDA N g
74.96±	7.53±	279.14±	5.15±	1.43±	7.44±	993.00±	26.01±	99.83±	33.5
0.33 a	0.02a	2.77a	0.44a	0.13a	0.45a	14.65a	1.36a	0.79a	2.14
76.53±	7.66±	283.09±	5.71±	1.41±	6.79±	1079.43±	22.63±	96.67±	52.7
2.34a	0.01a	4.30 a	0.03a	0.00a	0.10a	4.04a	0.16a	0.83a	3.53
79.40±	7.56±	281.25±	5.29±	1.32±	7.30±	1025.88±	24.72±	98.33±	48.6
1.36a	0.01a	5.67a	0.15a	0.01a	0.09a	7.00a	1.40 a	2.83a	2.37
16.12±	7.84±	66.34±	3.22±	0.98±	21.34±	246.33±	12.40±	365.42±	17.2
0.82c	0.01b	1.88 c	0.36c	0.11c	1.57c	6.11c	0.14c	4.37c	4.14
14.59±	7.75±	55.69±	2.99±	0.44±	23.13±	195.32±	11.87±	103.14±	19.3
0.12 c	0.01c	1.54b	0.34b	0.05c	0.44c	5.05c	0.29c	4.25c	2.42
16.56±	7.98±	72.83±	3.67±	0.95±	22.25±	277.35±	11.17±	443.45±	20.1
0.71c	0.01c	1.47b	0.41c	0.33c	0.23c	7.65c	0.23b	5.28c	1.17
7.23±	6.74±	8.55±	0.86±	0.84±	17.73±	65.44±	4.52±	51.24±	14.5
0.51d	0.02c	0.24d	0.15d	0.01d	0.44d	3.03d	0.76d	0.44d	0.24
7.01±	6.71±	8.24±	0.81±	0.76±	16.66±	61.23±	3.45±	33.42±	12.1
0.31d	0.01d	0.17c	0.01c	0.11d	0.19d	2.14d	0.12d	1.13d	0.78
6.89±	6.71±	8.02±	0.79±	0.72±	16.89±	41.22±	3.78±	41.27±	8.78
0.51d	0.01d	0.06c	0.03d	0.03d	0.14d	1.12d	0.15c	2.55d	1.03

530 Mean ± standard error (n=3). Values within the same column followed by the same letter do not differ

531 at $P < 0.05$. WC: water content; OC: organic carbon; TN: total nitrogen; TP: total phosphorus; TK:

532 total potassium; AN: available nitrogen; AP: available phosphorus; AK: available potassium. PDA:

533 Potential denitrifying activity. For sample codes, see Table1.

Table 3. Pearson correlation between soil physicochemical properties, potential denitrifying activity (PDA) and *nirS* gene abundance

	WC	pH	SOC	TN	TP	TK	AN	AP	AK	PDA
PDA	0.946**	0.466	0.945**	0.897**	0.811**	-0.804**	0.956**	0.895**	-0.086	1
Abundance	0.943**	0.292	0.931**	0.816**	0.841**	-0.897**	0.946**	0.842**	-0.238	0.963**

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. **: $P< 0.01$.

539Table 4. *nirS* gene diversity indices of soil samples from Zoige Plateau wetland

Soil codes	Shannon-Wiener index (H)	Richness	Evenness
SW1	2.86±0.06b	3.30±0.10c	0.58±0.02b
SW2	2.74±0.04bc	4.25±0.07a	0.37±0.15c
SW3	3.07±0.03a	3.89±0.10b	0.59±0.12b
MD1	2.22±0.04e	2.17±0.03f	0.72±0.01ab
MD2	2.31±0.02e	1.80±0.02g	0.81±0.00a
MD3	2.20±0.06e	1.97±0.03g	0.76±0.14ab
SD1	2.67±0.02cd	2.83±0.03c	0.69±0.00ab
SD2	2.57±0.02d	2.77±0.05cd	0.68±0.01ab
SD3	2.59±0.05cd	2.61±0.07e	0.74±0.009ab

540Mean ± standard error (n=3). Values within the same column followed by the same letter do not differ

541at $P< 0.05$. For sample codes, see Table1.