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1Implications of wetland degradation for the potential denitrifying activity and bacterial populations
2with *nirS* genes as found in a succession in Qinghai-Tibet plateau, China

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18

19**Abstract:** Alpine wetland in the Zoige Plateau has suffered from serious degradation during the last
2030 years due to global climate change and anthropogenic impact. Denitrification is a key nitrogen
21removal process which can be performed by different microorganisms, including bacteria harboring
22*nirS*-genes. In this study, a degradation succession was used to study the effect on potential
23denitrification activity (PDA) and on bacterial communities harboring *nirS* genes. Based on the
24determination of the PDA, the abundance, structural diversity, and phylogenetic identity of the soil
25bacteria with *nirS* genes were further assessed by qPCR, terminal restriction fragment length
26polymorphism (T-RFLP), and DNA-sequencing, respectively. The results showed that soil PDA
27ranged from 8.78 to 52.77 ng N₂O-N g⁻¹ dry soil h⁻¹, being lowest in sandy soil and highest in swamp
28soil. The abundance of *nirS* genes (copies g⁻¹ soil) were also the lowest in the sandy soil while highest
29in the swamp soil. The average Shannon-Wiener diversity index of the *nirS* denitrifying bacterial
30structural ranged from 2.20 in the meadow soil to 3.07 in the swamp soil. Redundancy analysis (RDA)
31showed that the *nirS* denitrifying bacterial community correlated with soil water content and available
32phosphorus, with water content as the major factor in shaping the *nirS* denitrifying bacterial
33community. The results of this study suggest that the wetland degradation would decrease soil PDA,
34and abundance and structural diversity of the denitrifying bacteria with *nirS* genes. These findings can
35contribute to support a theoretical foundation for predicting the potential influences of wetland
36degradation on soil denitrifying bacteria in alpine wetlands.

37**Keywords:** Wetland degradation; Potential denitrifying activity; *NirS* denitrifying bacterial
38community; T-RFLP; qPCR

39

401. Introduction

41 Qinghai-Tibet Plateau is a special geographical and ecological area that plays an important role in
42 environmental change. Studying on the dynamics of nitrogen cycle in this area is critical to evaluate
43 the response and feedback of biogeochemistry cycles to global climate change [1]. Zoige wetland on
44 the eastern edge of the Qinghai-Tibetan Plateau is the highest and largest highland peat marsh in China
45 [2]. Most alpine wetlands in the Zoige Plateau have suffered serious degradation, carbon and nitrogen
46 loss and decline of biodiversity due to global climate change [3] and anthropogenic impact [4]. A
47 lowered water table has resulted in loss of humus and peat horizons and transformation of these into
48 meadow soil. Besides, long term overgrazing by cattle in the meadows has decreased vegetation
49 coverage and disturbed their water regulating function. Moreover, draining the mining areas has
50 influenced the water retention ability of peat swamps, and changed the wetland soil moisture
51 conditions [2]. In a typical degradation succession in Zoige wetland, native swamp soil turns first into
52 transition meadow soil and then into degraded sandy soil [5]. Although it is known that wetland
53 degradation affects plant community composition and reduces diversity and above- and below-ground
54 biomass [6], little is known about how the soil microbial community composition and diversity
55 respond to wetland degradation.

56 Denitrification is a microbiological process which can be mediated by bacteria, archaea or fungi,
57 and more than 50 genera of bacteria have been identified as denitrifying bacteria [7]. It is a key
58 nitrogen removal process in wetlands and frequently limited by available nitrogen rather than carbon
59 [8]. Given that the high phylogenetic diversity among denitrifying bacteria, detection methods based
60 on assessment of genes such as 16S rRNA are not directly useful and therefore requires the use of
61 functional genes which encode for enzymes directly involved in the denitrification process. There are
62 two structurally different but functionally equivalent nitrite reductases: copper (*nirK*) and cytochrome

63cd1-containing (*nirS*) enzymes, which are responsible for conversion of nitrite (NO_2^-) to nitric oxide
64(NO)[9], are frequently-used as the molecular marker for the cultivation independent studies on
65denitrifying bacteria as only denitrifiers possess this enzyme[10]. These functional genes have been
66used as molecular markers for the cultivation independent studies on denitrifying bacterial diversity in
67various environments. Recent studies showed that *nirS* and *nirK*-type denitrifying bacterial
68communities responded differently to environmental parameters such as nitrate and oxygen
69concentrations in rice fields, and probably the *nirK* denitrifying bacteria were active at the beginning
70of anaerobic incubation, while the *nirS* denitrifying bacteria were more active in the fully developed
71anaerobic conditions [11]. Moreover, *nirS* denitrifying bacteria were found to be far more abundant,
72more active, and more diverse than *nirK* denitrifying bacteria [12]. The presence of bacterial
73populations harbouring *nirS* genes may correlate with environmental factors [13]. To date, little is
74known about how the abundance and community composition of the denitrifying bacteria respond to
75Plateau wetland degradation. Due to the expected bigger role of *nirS* denitrifying bacteria and better
76sequence database coverage of *nirS*, studying the response of denitrifying bacterial community, with a
77focus on *nirS* denitrifying bacteria, to wetland degradation in Qinghai-Tibet Plateau is necessary to
78understand how nitrogen cycle responds to the changing climate in this area.

79 To this end, in combination with the measurement of denitrification potential activities, the
80abundance, structural diversity, and phylogenetic identity of the *nirS* denitrifying bacteria in three
81different soils (native swamp, degraded meadow, and sandy soils) were assessed by qPCR, T-RFLP,
82and DNA-sequencing, respectively. The objectives of this study were (1) to investigate the variation in
83*nirS* denitrifying bacterial community in the three different soils associated with wetland degradation
84succession, and (2) to understand the relationships between the *nirS* denitrifying bacterial community
85and soil physicochemical properties. We hypothesized that wetland degradation had a significantly

86negative effect on soil potential denitrifying activity, and on the abundance, phylogenetic diversity and
87community composition of the *nirS* denitrifying bacteria.

88

892. Materials and Method

90Soil sampling

91 The study site was on the Zoige Plateau, located at the northeastern part of the Qinghai-Tibetan
92Plateau (101°30'E-103°30'E, 32°20'N -34°00'N) (Table 1). The average altitude at Zoige Plateau is
933500 m and the mean annual temperature is 1.1°C, with the lowest monthly temperature of -10.3 °C in
94January and the highest of 10.9 °C in July. Annual precipitation is 560-860 mm. Swamp soil (SW1,
95SW2, and SW3) was covered with water all year round, and the vegetation was dominated by
96hydrophytes and sedge hydro-mesophytes (*Carex muliensis*, *Carex lasiocarpa* and *Carex meyeriana*).
97Meadow soil (MD1, MD2, and MD3) surface was in a humid state and the vegetation was dominated
98by mesophytes and hydro-mesophytes (*Kobresia tibetica*). Sandy soil (SD1, SD2, and SD3) surface
99was continuously dry and had little or only a few *Psammophytes* as vegetation cover [14].
100Homogeneous silt and clay were the dominant soil parent materials of marsh soils [2]. The soil parent
101materials of meadow soil were accumulated residues of triassic slate, sandstones and siltstone [15].
102Sandy soil, the top layer (0-30 cm) of which was sand, was composed of aeolian parent material [16].
103 The soils were sampled using an auger with 20 mm diameter × 100 cm length and only the
104surface (5-20 cm) soil samples were analyzed in this study. Each of the replicate samples represented a
105composite sample of 15 subsamples. Surface vegetation and roots were removed before collecting the
106samples. Samples (approximately 1.5 kg) were divided into two portions: one was air dried before
107removing roots and stones, and then sieved through a 2-mm mesh screen for physicochemical
108analyses. The other portion was stored at -80 °C for DNA extraction.

109 Soil physicochemical parameters and potential denitrifying activity analysis

110 Soil water content (WC) was determined after oven-drying at 105 °C for 48 h [17]. Soil total
111 nitrogen (TN) and available nitrogen (AN) were determined using the alkaline hydrolysis diffusion
112 method and semi-micro Kjeldahl method, respectively [18]. Soil organic carbon (SOC) and pH were
113 determined by potassium dichromate oxidation-external heating method and potentiometry method,
114 respectively [18]. Total phosphorus (TP) and available phosphorus (AP) were assessed by
115 hydrofluoric acid-perchloric acid digestion-Mo-Sb colorimetry and sodium bicarbonate extraction-
116 Mo-Sb colorimetry methods, respectively [18]. Total potassium (TK) and available potassium (AK)
117 were determined by hydrofluoric acid-perchloric acid digestion-flame photometry and ammonium
118 acetate extraction-flame photometry, respectively [18]. Potential denitrifying activity (PDA) was
119 estimated using the C₂H₂ inhibition method [19].

120 Soil total DNA extraction

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

127 Quantitative PCR

128 The *nirS* gene was quantified by qPCR with three technical replicates per sample. Standard
129 curves to assess *nirS* gene abundance consisted of a 10-fold serial dilution (10¹-10⁷) of a plasmid (299
130 ng μL⁻¹) containing the *nirS* gene fragments from the tested soil as described by Henry et al. [20]. The
131 *nirS* genes were amplified with primer pair nirSCd3aF (5'-AACGYSAAGGARACSSGG-3') and

132nirSR3cd (5'-GASTTCGGRTGSGTCTTSAYGAA-3') [21]. For qPCR, 12.5 μ L ABI Power
133SybrGreen qPCR Master Mix (Applied Biosystems, USA), 0.5 μ M of each primer, 10 μ M of soil
134DNA and 9.5 μ L of sterile water were used to make up a final volume of 25 μ L. The reaction was
135carried out on a ABI7500 sequence detection system (Bio-Rad) using the program of 95 $^{\circ}$ C for 10 min
136followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 15 s, annealing at 60 $^{\circ}$ C for 1 min and extension at
13772 $^{\circ}$ C for 30 s. Absolute *nirS* copy number was calculated by interpolating from the standard curve.
138Gene abundances were standardized by the mass of DNA that was extracted per gram of dry soil and
139log₁₀ transformed before analysis.

140PCR amplification and T-RFLP of the *nirS* gene

141 The primer pair nirSCd3aF and nirSR3cd [21] was used to amplify the *nirS* gene in a C1000™
142thermal cycler (Bio-Rad, Hercules, CA, USA). Amplification was done in a volume of 50 μ L
143containing 10 μ M diluted soil DNA, 5 μ L of 10 \times PCR buffer, 200 μ M of dNTP mix, 0.5 μ M of each
144primer, 18.5 μ L of ddH₂O and 2.5 U of Taq DNA polymerase (TAKARA, Otsu, Shiga, Japan) with the
145following cycling conditions: 5 min of denaturation at 95 $^{\circ}$ C, a 21 cycles touchdown program (94 $^{\circ}$ C
146for 30 s, annealing at 60 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C for 60 s) where the annealing temperature was lowered
1471 $^{\circ}$ C after every three cycles, 22 cycles with 94 $^{\circ}$ C for 30 s, 53 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C s for 60 s, and a
1487 min extension at 72 $^{\circ}$ C. The PCR products were analyzed by electrophoresis on 1.0 % agarose gels
149and visualized by UV Transilluminator Model M-26 (UVP, USA) after ethidium bromide staining.

150 For T-RFLP, the forward primers were fluorescently labeled with 6-Fam (6-carboxyfluorescein).
151The *nirS* gene PCR products were digested with *HhaI* endonuclease (NEB, Ipswich, Massachusetts,
152USA) at 37 $^{\circ}$ C for 6 h, followed by further purifying by Tiangen DNA purification kit (Tiangen,
153China) to clean up the T-RFLP mix. T-RFLP profiles were generated by capillary electrophoresis
154using an ABI Prism 3100 Genetic Analyzer at Sangong Corporation (Shanghai, China). Sizes and

155relative abundances of terminal restriction fragments (T-RFs) were quantified using PeakScan version
1561.0 software (Applied Biosystems, Inc.). Only fragments with a signal greater than 1% of the sum of
157all peak heights were included in the analysis. The peak heights of T-RFs that differed in size by ≤ 2 bp
158in an individual profile were summed and considered as one fragment. To assign the peaks of samples
159to clone sequences, clones corresponding to different *in silico* T-RFs were also subject to T-RFLP
160analysis with the same procedure mentioned above.

161Cloning and sequencing

162 The *nirS* gene PCR products were excised from agarose gels and purified using universal DNA
163Purification Kit (Tiangen Co, Beijing, China) following the manufacturer's instructions. PCR products
164were cloned into pGEM-T vector (Tiangen) and transformed into *Escherichia coli* strain DH-5 α ,
165followed by blue–white screening. Inserts were amplified with M13F and M13R primers and the size
166of the inserts were estimated by agarose gel electrophoresis. A total of 182 positive clones
167(approximately 60 clones from each site) were randomly selected for restriction screening by digesting
168with *HhaI* endonuclease, and 15 representative clones were sequenced. The *nirS* gene sequences were
169deposited in GenBank under the accession numbers KT783435-KT783449.

170Statistical analysis

171 The means and standard deviations of the soil properties and *nirS* gene diversities were calculated
172and tested for normality and homogeneity of variances. Data was analyzed using one-way analysis of
173variance (ANOVA), followed by Tukey's multiple comparison test when the ANOVA results were
174significant. Otherwise, Tamhane's T3 test was adopted using SPSS 21.0 software (IBM SPSS
175Statistics for Windows, Armonk, NY, USA). Two-sided $P < 0.05$ was considered statistically
176significant. To assess the correlation between soil physicochemical properties and *nirS* gene
177abundances, Pearson correlation coefficients were calculated using the Simple Correlation Method in

178SPSS 21.0. Redundancy analysis (RDA), which was performed using CANOCO version 5, was used
179to assess the relationship between *nirS* denitrifying bacterial community and environmental variables
180[22]. The significance of the RDA results was tested with the Monte Carlo permutation test.

181 The clone sequences were aligned using ClustalX 1.83 [23], and a neighbor-joining tree was
182constructed using MEGA 5.0 [24]. Bootstrap analysis was used to estimate the reliability of the
183phylogenetic reconstruction (1000 replicates). The *nirF* (accession no. D50473) and *nirN* (accession
184no. D84475) gene sequences from *Pseudomonas aeruginosa* were used as out-groups for phylogenetic
185analysis due to that their proteins have recognizable sequence relatedness to *nirS* [25].

1863. Results

187Soil physicochemical properties and potential denitrifying activity

188 The soil water content (WC) was highest in swamp (79.4%) and lowest in the sandy soil (6.9%)
189(Table 2). Soil pH in meadow was higher than in swamp and sandy soil. Soil organic carbon (SOC)
190content was significantly higher in the swamp soil than in meadow and sandy soils. Soil TN and AN
191ranged from 0.79 to 5.71 g kg⁻¹ and 41.22 to 1079.43 mg kg⁻¹, respectively. Swamp soil had the
192highest concentrations of soil total nitrogen (TN) and available nitrogen (AN). The lowest
193concentrations of TN and AN were in the sandy soil. Total phosphorus (TP) and available phosphorus
194(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
195observed in swamp soil and the lowest in sandy soil. Total potassium (TK) and available potassium
196(AK) ranged from 6.79 to 23.13 g kg⁻¹ and 33.42 to 443.45 mg kg⁻¹, respectively. The highest
197concentrations were in meadow soil and the lowest in sandy soil. Soil potential denitrifying activities
198ranged from 8.78 to 52.77 ng N₂O-N g⁻¹ dry soil h⁻¹. Activities were highest in swamp soil and lowest
199in sandy soil. The potential denitrifying activity correlated positively with WC, SOC, TN, AN, TP and
200AP, and negatively with TK (p<0.01) (Table 3).

201 ***nirS* gene abundance and diversity**

202 The soil *nirS* gene copy numbers ranged from 2.77 log₁₀ per gram dry soil in sandy soil to 5.57
203 log₁₀ per gram dry soil in swamp soil (Fig.1), showing that wetland degradation significantly
204 decreased the *nirS* denitrifying bacterial abundance. The *nirS* abundance correlated positively with soil
205 potential denitrifying activity ($p < 0.01$) (Table 3).

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

211 ***nirS* denitrifying bacterial community structure**

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

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

224255 bp T-RFs in the sandy soil only.

225Phylogenetic analysis

226 In the phylogenetic analysis, the *nirS* gene sequences were grouped into four clusters based on
227their relationship with *nirS* gene sequences from various habitats and some well characterized
228denitrifying bacteria (Fig. 4). The *nirS* sequences with T-RFs 76, 77, 116, 121, 129, and 227 bp in
229cluster 1 were related to *nirS* gene sequences retrieved from various environments such as estuary
230sediment soil (GI: KM891947) and wetland sediment (GI: KC468857). The sequences in cluster 2
231with T-RFs 60, 113, and 115 bp were also related to the clones retrieved from sludge and sediment,
232and then grouped with the identified *nirS* gene sequence from gammaproteobacterial *Pseudomonas*
233*fluorescens* (GI: AF114792) and *Pseudomonas aeruginosa* (GI: X16452). The sequences in cluster 3
234with T-RFs 260 bp were related to those from betaproteobacterial *Burkholderiaceae*, *Ralstonia* and
235*Thaueraterpenica*. The sequences in cluster 4 with T-RFs 255 and 280 bp were grouped into one clade
236with those from alphaproteobacterial *Paracoccus* sp.

237 The swamp soils, meadow soils and sandy soils harbored different *nirS* denitrifying bacterial
238communities that carried genes related to *nirS* genes retrieved from various habitats. In the swamp
239soil, the most abundant *nirS* genes which accounted for 91.2% of the *nirS* denitrifying bacterial
240communities were related to *nirS* genes retrieved from freshwater sediment habitats. In the meadow
241soil, the *nirS* that accounted for 72% of the total *nirS* detected were related to *nirS* genes isolated from
242wetland and farmland soil. For the sandy soil, the *nirS* genes in cluster 2 were related to *nirS* genes
243from sediment and sludge (Fig. 4).

244Relationships between *nirS* denitrifying bacterial community and environmental parameters

245 In the redundancy analysis (RDA), the *nirS* denitrifying bacterial communities from the swamp
246soils, meadow soils and sandy soils were divided into three separate groups (Fig. 3). The RDA results

247further confirmed that the *nirS* denitrifying bacterial communities in the soils were significantly
248different ($P<0.05$). Among the measured abiotic factors, water content appeared to be the major factor
249in determining the *nirS* denitrifying bacterial community composition ($P<0.05$). Soil *nirS* denitrifying
250bacterial community variance was also significantly linked to available phosphorus ($P<0.05$).

2514. Discussion

252Soil properties in response to wetland degradation

253 Microbiologically mediated denitrification is the key process of nitrogen removal from wetlands.
254In this study, the physicochemical properties of soils associated with the Zoige wetland degradation
255succession were analyzed. In earlier studies, the hydrological conditions affected the community
256structure, plant productivity, the import of organic matter, and the accumulation of soil organic carbon
257(SOC) and total nitrogen (TN) [26]. Decrease of the soil water content induced by wetland degradation
258increased organic matter decomposition rates and net N mineralization rates [27], leading to the loss of
259soil carbon, nitrogen, and phosphorus [28]. In the process of wetland restoration, concentrations of
260total and available nitrogen and phosphorus significantly decreased with a decrease in water content
261[29]. In our study, water and organic carbon contents and the concentrations of nitrogen and
262phosphorus all decreased with the succession of wetland degradation, indicating that water content is
263the major factor contributing to the decreases in carbon, nitrogen and phosphorus content that
264accompany Zoige wetland degradation. In an anaerobic environment, decomposition of animal and
265plant residues is slow, which possibly explains why SOC was highest in the flooded swamp soil and
266lowest in the sandy soil. An increase in net N mineralization leads to the loss of nitrogen [27, 28], and
267in accordance to that the nitrogen content was highest in the swamp soil. Higher SOC content may
268provide a larger sorption capacity for phosphates [30], plausibly explaining why the phosphorus
269content was higher in the swamp soil than in the meadow and sandy soils.

270 **Wetland degradation decreased soil potential denitrifying activity (PDA)**

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

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

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

293level fluctuation presumably creates more aerobic conditions.

294**The *nirS* denitrifying bacterial community responded to wetland degradation**

295 Our main hypothesis that the denitrifying bacterial community structure and diversity would be
296negatively impacted by wetland degradation was not completely supported by the results. The three
297soils with different degradation succession in Zoige wetland hosted distinct denitrifying bacterial
298communities. As judged by the terminal restriction fragments (TRFs), the core of the communities in
299the three tested soils were similar yet more minor TRFs were detected in the swamp and sandy soils
300and each soil had its' specific TRFs. This suggested that the minor TRFs contributed to the community
301structural differences. Similar result has been observed also in one previous study [39] indicating that
302the core denitrifying bacteria may not be sensitive to wetland degradation whereas some minor
303denitrifying bacteria with low relative abundance are critical when community responses are
304evaluated. Although the diversity index of the denitrifying bacterial communities in the swamp soil
305(native soil) were higher than those in meadow (transitional soil) and sandy (degraded soil), the
306diversity index of the denitrifying bacterial communities in the meadow soil was lower than that in
307sandy soil, indicating a positive effect of sandy soil on denitrifying bacterial diversity. Adsorbed small
308particles on a sand grain may provide binding sites for adsorption of organic matter and microbial cells
309[40], resulting in a larger number of microhabitats available for colonization. This in turn increases the
310potential of multiple species to coexist in close proximity without directly competing for resources and
311potentially increases bacterial diversity [40]. Thus, the increased denitrifying bacterial community
312diversity may be explained by the increased sand content with degradation.

313 In three farmland soils from Finland (62°55'N, 29°30'E), Germany (48°40'N, 11°04'E), and
314Sweden (58°20'N 13°30'E), the majority of the *nirS* gene clones were similar to the *nirS* from
315*Ralstonia* and *Paracoccus* bacteria, both members of the *Proteobacteria* [41]. The *nirS* genes in an

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

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

323 Understanding the environmental variables that affect microbial community structure is a key
324 goal in microbial ecology. Soil moisture was found to be an important abiotic factor in affecting the
325 *nirS* denitrifying bacterial abundance and community composition [43]. Oxygen was also found to
326 have a decisive effect on the structure of denitrifying bacterial communities in the continental margin
327 sediments of the oxygen-deficient zone at the Pacific Coast of Mexico [44]. In addition, the relative
328 abundances of *nirS* terminal restriction fragments responded variously to the O₂, NO₃⁻, NO₂⁻ and depth
329 gradients in the water column of the oxygen minimum zone in the Eastern South Pacific [45]. In our
330 study, soil physicochemical parameters were important in shaping *nirS* denitrifying bacterial
331 communities, and soil water content and available phosphorus were the most important factors in
332 shaping *nirS* denitrifying bacterial communities in Plateau wetland.

333 Phosphorus was one of the soil parameters which correlated positively with the *nirS* gene
334 abundances in this study. It was reported that the availability of phosphorus greatly affected soil
335 microbial community [46]. A similar relationship between the abundance of *nirK* denitrifying bacteria
336 and available phosphorus has been also observed in a spruce forest soil, where the available
337 phosphorus content was closely connected with the nutrient availability [47]. In contrast, a negative
338 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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357

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482 **Figure legends**

483 Fig. 1. Abundances of *nirS* genes in swamp soil (SW), meadow soil (MD) and sandy soil (SD).

484 Different letters above the bars indicate significant differences based on protected LSD test ($P < 0.05$).

485

486 Fig. 2. Average relative abundances of *nirS* terminal restriction fragments (T-RFs) in swamp soil

487 (SW), meadow soil (MD) and sandy soil (SD). The relative abundance of T-RFs is given as a

488 percentage of the total peak height. Fragment sizes (bp) of the experimental T-RFs are indicated on the

489 right.

490

491 Fig. 3. Ordination diagram from redundancy analysis (RDA) on the basis of relative abundance of

492 *nirS* T-RFs in swamp soil (SW), meadow soil (MD) and sandy soil (SD). AP, available phosphorus;

493 WC, water content; TN, total nitrogen.

494

495 Fig. 4. Neighbor-joining phylogenetic tree of *nirS* gene sequences derived from swamp soil (SW),

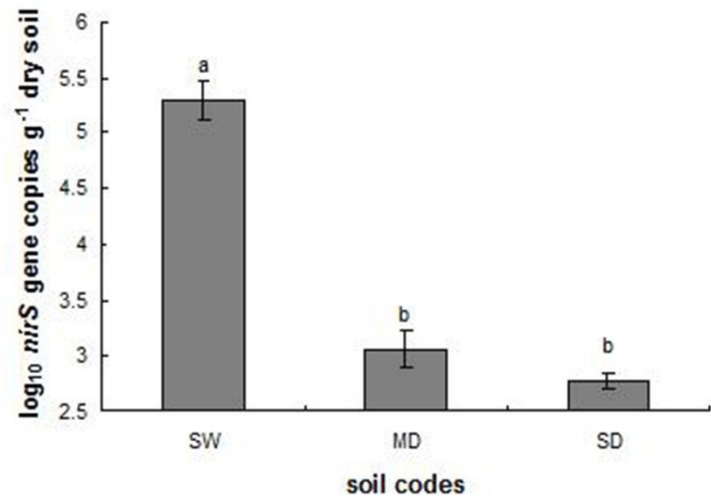
496 meadow soil (MD) and sandy soil (SD) in the Zoige Plateau. *nirN* gene (D84475) and *nirF* gene

497 (D50473) from *Pseudomonas aeruginosa* were used as outgroups. Bootstrap values greater than 50%

498 of 1000 resamplings are shown. The scale indicates the number of nucleotide substitutions per site.

499 Numbers before the sample code indicate the sequenced *nirS* gene clones. Numbers in parentheses

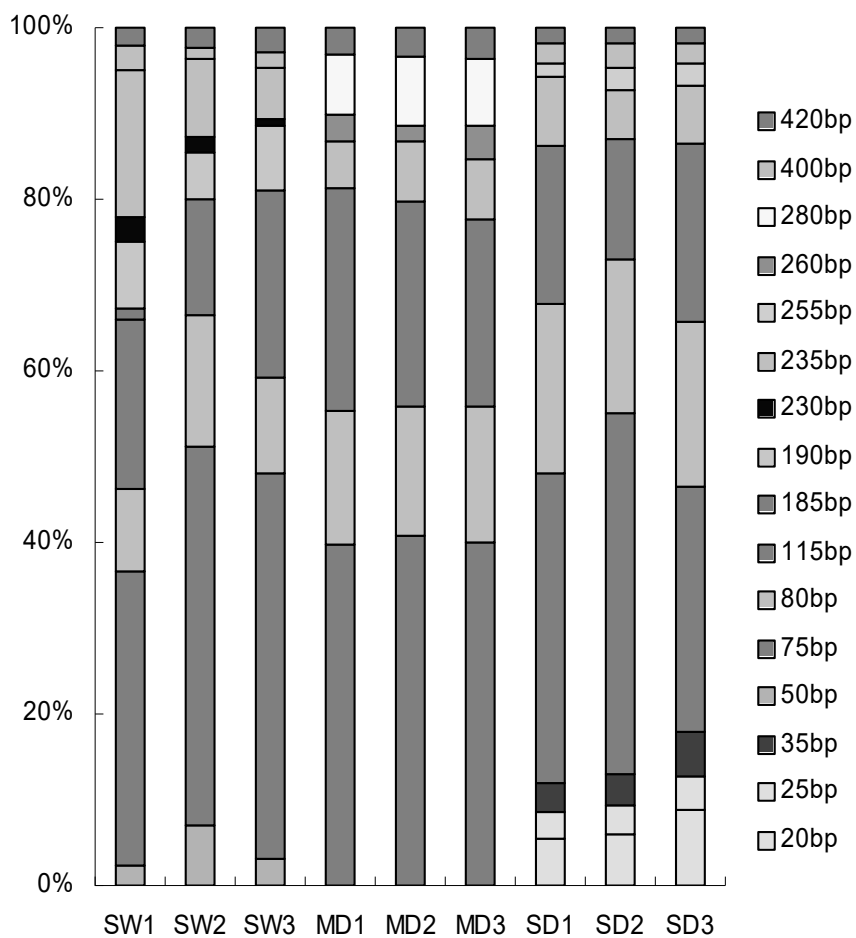
500 indicate the Genbank accession numbers.



501

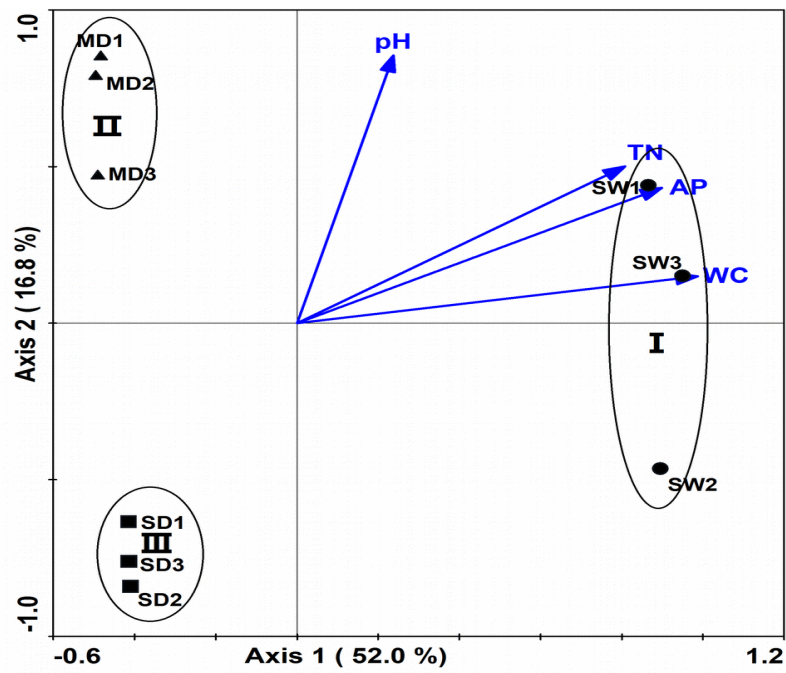
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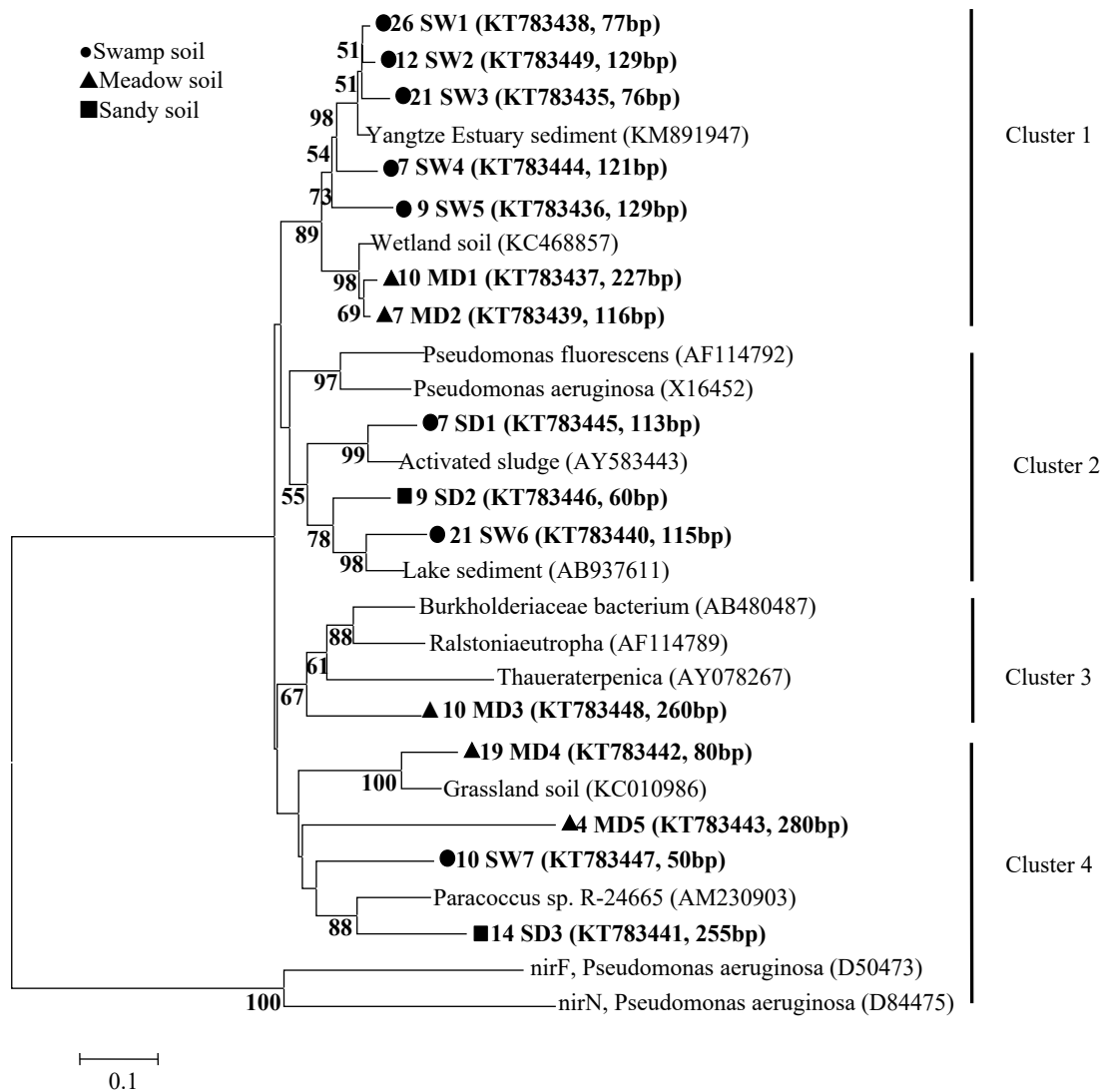
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517 resamplings are shown. The scale indicates the number of nucleotide substitutions per site. Numbers
518 before the sample code indicate the sequenced *nirS* gene clones. Numbers in parentheses indicate the
519 Genbank accession numbers.

520 **Table captions**

521 Table 1. Sampling sites in Zoige Plateau wetland.

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

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

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

526

Table 1. Sampling sites in Zoige Plateau wetland

Sample code*	Sampling site	Sampling		Altitude (m)	Soil temperature (°C)
		Longitude	Latitude		
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.

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

WC (%)	pH	SOC (g·kg ⁻¹)	TN (g·kg ⁻¹)	TP (g·kg ⁻¹)	TK (g·kg ⁻¹)	AN (mg·kg ⁻¹)	AP (mg·kg ⁻¹)	AK (mg·kg ⁻¹)	PDA (N g ⁻¹ h ⁻¹)
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

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

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

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

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

534 Table 3. Pearson correlation between soil physicochemical properties, potential denitrifying activity
 535 (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**

536 WC: water content; SOC: soil organic carbon; TN: total nitrogen; TP: total phosphorus; TK: total

537 potassium; AN: available nitrogen; AP: available phosphorus; AK: available potassium. **: $P < 0.01$.

538

539 Table 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

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

541 at $P < 0.05$. For sample codes, see Table 1.