Postprint: nirK-Based Analysis of Denitrifying Bacterial Community Structure in Peat Swamp Soils of Zoige Plateau Wetlands

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Abstract

The Zoige peat wetland is one of the few low-latitude permafrost wetlands in the world, characterized by high altitude, high ultraviolet radiation, and high organic matter content. N2O emissions from this region have an important impact on global climate warming. We analyzed the community structure and diversity of nitrite reductase (nirK) denitrifying bacteria in the peat swamp soils of the Zoige Plateau wetland to reveal the microbial regulatory mechanism of N2O release in this region. Based on the physicochemical properties and denitrification activity (PDA) of the peat swamp soils in the Zoige Plateau wetland, combined with Restriction fragment length polymorphism (RFLP) technology, clone library, and molecular sequencing, we analyzed the community structure and diversity of nirK denitrifying bacteria in this ecosystem. Denitrification activity measurement results showed: Axi region > Maixi region > Fenqu region, and denitrification activity was significantly positively correlated with soil organic carbon, total nitrogen, and richness ($P < 0.05$). The Shannon-wiener diversity index was highest in Axi and lowest in Fenqu. A total of 15 representative nirK gene sequences were sequenced from the three samples, and phylogenetic analysis indicated that the dominant nirK denitrifying bacterial group in the Zoige Plateau wetland was Proteobacteria. Among them, the Axi region was dominated by Alphaproteobacteria, the Maixi region by Betaproteobacteria, and the dominant population in the Fenqu region could not be determined. Redundancy analysis (RDA) showed that available potassium and available phosphorus are key environmental factors affecting the community structure of nirK denitrifying bacteria. This study demonstrates that significant denitrification occurs in the Zoige Plateau wetland, and the nirK denitrifying bacteria that regulate this process have high diversity and are closely related to soil available potassium and available phosphorus.

Full Text

Preamble

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Analyzing the Nitrite Reductase Gene (nirK) Denitrifying Bacterial Community Structure in Peat Marsh Soil of the Zoige Wetland on the Tibetan Plateau

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Abstract

The Zoige peat wetland is a rare low-latitude permafrost wetland characterized by high altitude, intensive ultraviolet radiation, and high soil organic matter content, making it critically important to global warming. This study investigated the diversity and structure of the nitrite reductase (nirK) denitrifying bacterial community in peat marsh soils of the Zoige Plateau Wetland to elucidate the microbial regulatory mechanisms of N O release in this climatically extreme region. Based on soil physicochemical properties and potential denitrifying activity (PDA), restriction fragment length polymorphism (RFLP), clone library construction, and molecular sequencing were employed to analyze the nirK gene denitrifying bacterial community structure and diversity. Denitrification activity measurements revealed that PDA was significantly and positively correlated with soil organic carbon, total nitrogen, and gene richness ($P < 0.05$). Phylogenetic analysis indicated that the dominant nirK denitrifying bacteria in the Zoige Plateau Wetland belonged to Betaproteobacteria. The Axi region was dominated by specific populations, while the Maixi region showed different dominant species; the Fenqu region's dominant populations could not be definitively identified. The Shannon-Wiener diversity index was highest in Axi. Sequencing of 15 representative clones yielded nirK gene sequences. Redundancy analysis (RDA) demonstrated that available potassium and available phosphorus were the key environmental factors shaping the nirK denitrifying bacterial community structure. This study reveals that significant denitrification occurs in the Zoige Plateau Wetland, with the nirK denitrifying community showing high diversity and being strongly influenced by soil available potassium and phosphorus.

Keywords: Zoige Plateau Wetland; peat marsh soil; denitrifying activity; nirK gene; diversity

1. Introduction

Denitrification is a biological dissimilatory process mediated primarily by denitrifying bacteria under anaerobic conditions, where nitrogen oxides are converted to gaseous products (NO, N , N O). The intermediate product N O contributes to greenhouse effects and ozone depletion [1]. Denitrifying bacteria are abundant in the environment, but their systematics cannot be reliably inferred from 16S rRNA genes because the denitrification phenotype does not correlate with phylogeny. Functional genes encoding denitrification enzymes must therefore be used as molecular markers for denitrifier studies [2-3]. Denitrification involves four key enzymes: nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase [4]. The reduction of nitrite to NO, regulated by nitrite reductase, distinguishes nitrate-respiring bacteria from denitrifiers. Nitrite reductase exists in two structural forms: the cd -type (cd1-nir) encoded by the nirS gene, and the copper-containing type (Cu-nir) encoded by the nirK gene [2]. The nirK gene occurs in many phylogenetically distant strains, making it a more comprehensive molecular marker for characterizing environmental denitrifying community structure than nirS, which is dominated by Pseudomonas in many systems [4]. The nirK gene has been widely used to study denitrifying bacterial community structure and activity in various habitats, including marine sediments and agricultural soils, where different environmental factors drive community composition [5-9]. Investigating the nirK denitrifying community in the Zoige Plateau Wetland is crucial for understanding the microbial regulatory mechanisms of denitrification in this unique ecosystem.

Located on the eastern edge of the Tibetan Plateau at the junction of Sichuan, Gansu, and Qinghai provinces, the Zoige Plateau Wetland is one of the world' s few low-latitude permafrost wetlands, characterized by high altitude, intensive UV radiation, and high organic matter content. It represents the largest high-altitude peat marsh complex globally [11] and is a biodiversity hotspot and focal region for global climate change research [12]. While carbon cycling and microbial mechanisms have been studied systematically [13-15], the microbial regulation of greenhouse gas emissions, particularly N O, remains poorly understood. This study combines RFLP, clone library construction, and sequencing to analyze nirK denitrifying bacterial community structure and diversity in Zoige peat marsh soils, using redundancy analysis (RDA) to identify key environmental factors and reveal linkages between denitrification activity and bacterial communities.

2. Study Area

The Zoige Plateau Wetland is located on the eastern edge of the Tibetan Plateau at coordinates 32°20 -34°00 N, 101°30 -103°30 E, with an average elevation of

3,500 m. Influenced by the plateau, the region exhibits a unique continental highland climate with an annual mean temperature of 1.1°C (ranging from -10.3°C to 10.9°C), an extremely long frost period, and annual precipitation of 560–860 mm. The marsh soil is covered year-round by water and vegetation dominated by aquatic plants from the Cyperaceae family, including Carex lasiocarpa and Carex meyeriana. The soil parent material consists of homogeneous silt and clay [10].

3. Sampling Methods

Based on literature review and field surveys, three representative pastoral areas with extensive coverage were selected: Axi, Maixi, and Fenqu. Peat marsh soils covered by Carex vegetation were sampled from each region following a quadrat-based approach. Sampling plots of approximately 0.15 hm^2 were established, and soils were collected from 10–20 cm depth using the quartering method to obtain three composite samples per site. Samples were sealed in resin bags, transported on ice, and stored at -20°C for subsequent analysis.

Table 1 Details of the sampling sites

Sampling site	Sample code	Longitude	Latitude	Altitude (m)	Soil temperature (°C)
Axi	AX	102°49 02.83	3°54 57.0	3,500	7.8
Maixi	MX	102°48 48.23	3°54 58.2	3,480	7.6
Fenqu	FQ	102°48 59.43	3°55 21.2	3,520	7.3

4. Soil Physicochemical Properties and Denitrification Activity

Soil water content (WC) was determined by oven-drying at 105°C. Soil organic matter (SOM), pH, total nitrogen (TN), total phosphorus (TP), total potassium (TK), available nitrogen (AN), available phosphorus (AP), and available potassium (AK) were analyzed using standard methods [17-19]. Potential denitrifying activity (PDA) was measured using the acetylene inhibition method [20]. Fresh soil samples (25 g) were placed in 250 mL flasks, flushed with helium gas, and amended with 1 mmol/L KNO and 1 mmol/L glucose. Acetylene gas (acetone-free) was added at a volume equal to the extracted gas; controls received no acetylene. Soil slurries were incubated at 20°C with shaking at 225 rpm. Headspace gas samples were analyzed using an Agilent 7890A gas chromatograph equipped with an electron capture detector. The system used automatic injection via a computer-controlled quantitative tube (1 mL) and ventilation valve. The separation column (Porapak Q) was maintained at 60°C,

with injection port and detector temperatures at 100°C and 300°C, respectively. Carrier gas (methane) flow rate was 60 mL/min.

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5. DNA Extraction and nirK Gene Amplification

Total DNA was extracted from 0.5 g of soil using the Fast DNA Spin Kit for Soil (MP BIO, Inc., Irvine, CA, USA) following the manufacturer' s protocol. DNA quality was verified by agarose gel electrophoresis and quantified using a Nano-50 spectrophotometer (AoSheng, Hangzhou, China).

The nirK gene was amplified using primers nirK517F (5 -TTYGTSTAYCACTGCGCVCC-3) and nirK1055R (5 -GCYTCGATCAGRTRTRTGGTT-3). PCR was performed in 50 L reactions containing 25 L PCR Mix, 1 L each primer (10 mol/L), 1 L DNA template, and 22 L ultrapure water. A touchdown PCR program was used: initial denaturation at 95°C for 5 min, followed by 15 cycles of 94°C for 30 s, annealing at 57–47°C (decreasing 1°C per cycle) for 45 s, and extension at 72°C for 1 min; then 25 cycles at 94°C for 30 s, 47°C for 45 s, and 72°C for 1 min; final extension at 72°C for 7 min. PCR products were visualized on 2% agarose gels at 120 V.

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6. Clone Library Construction and RFLP Analysis

PCR products were purified using the Clean-Up™ Kit and ligated into the pGEM-T vector (Promega). White colonies were selected on LB plates containing X-Gal, IPTG, and ampicillin. A total of 150 clones per sample were randomly selected and screened for the nirK gene by PCR with vector-specific primers. Positive clones were digested with restriction enzymes MspI and AvaII (Promega) in a 20 L reaction volume containing 5 L PCR product, 2 L restriction buffer, and 10 U each enzyme. Digestion products were separated on 3% agarose gels at 80 V for 60 min. Clones were grouped into operational taxonomic units (OTUs) based on restriction patterns. Fifteen representative clones were sequenced by Shanghai Sangon Biotech, and sequences were deposited in GenBank (accession numbers: KX018494–KX018508).

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7. Data Analysis

Basic data processing was performed using Excel 2010. One-way ANOVA and Pearson correlation analysis were conducted using SPSS 21.0 (SPSS Inc., Chicago). Diversity indices (Shannon-Wiener, richness, evenness) were calculated using MEGA 5.0 [23]. Phylogenetic trees were constructed using the neighbor-joining method. Redundancy analysis (RDA) of soil physicochemi-

cal properties and nirK denitrifying community structure was performed using CANOCO 5.0.

8. Results

8.1 Soil Physicochemical Properties and Denitrification Activity

Soil physicochemical properties varied significantly among the three sampling sites (Table 2). Axi soils exhibited the highest water content, denitrification activity, soil organic carbon, total nitrogen, available nitrogen, available phosphorus, and available potassium. Maixi soils showed intermediate values, while Fenqu soils had the lowest values.

Table 2 Physicochemical parameters of peat marsh soil in the Zoige plateau

Sample code	WC (%)	SOC (g/kg)	TN (g/kg)	TP (g/kg)	TK (g/kg)	AN (mg/kg)	AP (mg/kg)	AK (mg/kg)
AX	85.12±0.25a	28.25±1.29a	3.29±0.05b	5.43±0.14a	7.44±0.03a	325.88±2.65b	26.51±0.20a	209.83±0.40a
MX	87.86±0.03a	30.09±0.26a	3.91±0.07a	5.41±0.16a	7.79±0.08a	379.43±3.23a	32.83±0.19a	216.67±0.10b
FQ	82.81±0.21b	24.14±0.45b	2.65±0.06c	4.32±0.17b	7.30±0.09a	293.00±3.57c	25.72±0.18b	198.33±0.22a

Note: 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. Values in each column with different letters indicate significant differences at $P < 0.05$.

Potential denitrification activity (PDA) ranged from 33.52 to 52.77 ng N O-N g ^{1} h ^{1}, with the highest value in Axi and the lowest in Fenqu (Figure 1 [Figure 1: see original paper]). Pearson correlation analysis revealed that PDA was significantly and positively correlated with soil organic carbon, total nitrogen, and gene richness ($P < 0.05$) (Table 3).

Table 3 Pearson correlation analysis between soil physicochemical parameters, potential denitrifying activity, and nirK denitrifier community

Parameter	PDA	Shannon-Wiener diversity	Richness
WC	0.889**	0.998**	0.941**
SOC	0.796*	0.916**	0.550*
TN	0.998**	0.993**	0.988**
TP	0.998**	0.491*	0.535*
TK	0.835*	0.889**	0.992**
AN	-0.665*	-0.906**	-0.711*

Parameter	PDA	Shannon-Wiener diversity	Richness
AP	0.969**	-0.519*	-0.884**
AK	-0.426*	-0.749*	-0.481*

Note: P < 0.05; **P < 0.01. PDA: Potential denitrifying activity.*

8.2 nirK Gene Community Library and RFLP Analysis

RFLP analysis revealed 15 distinct OTUs across the three regions. Axi contained the most OTUs (10), including region-specific OTU13 and OTU14. Maixi had 8 OTUs with region-specific OTU7. Fenqu had 6 OTUs with region-specific OTU1, 2, 5, 8, 11, and 12. Three OTUs were shared among all regions.

Clone library coverage exceeded 95% for all sites, indicating comprehensive representation of nirK gene diversity (Table 4). Shannon-Wiener diversity indices ranged from 2.91 (Axi) to 2.49 (Fenqu), with richness and evenness following similar patterns.

Table 4 Diversity of nirK denitrifiers in peat marsh soil of the Zoige plateau

Sample	OTU type	Coverage ratio	Shannon-Wiener diversity	Richness	Evenness
AX	10	0.9539±0.02a	2.91±0.04a	3.38±0.07a	0.92±0.02a
MX	8	0.9653±0.03a	2.59±0.02b	3.12±0.04b	0.87±0.02a
FQ	6	0.9346±0.02b	2.49±0.06b	2.81±0.02c	0.85±0.02a

8.3 Phylogenetic Analysis

Sequence analysis of 15 representative clones (530 bp each) revealed that nirK sequences primarily belonged to Alphaproteobacteria and Betaproteobacteria. Using 85% sequence similarity as the criterion for genus-level assignment [25], the dominant groups were identified.

Alphaproteobacteria was the most abundant class, comprising 51.0% of clones. The dominant OTU12 showed 82–89% similarity to cultured denitrifiers *Rhodopseudomonas* sp. 2-8 (GU332847) from marine sediments and agricultural soils, and was most abundant in Axi. OTU14, also dominant in Axi, showed similarity to sequences from municipal wastewater treatment plants. OTU13, representing another Axi-dominant population, was related to *Bradyrhizobium japonicum*.

Betaproteobacteria accounted for 38% of clones. OTU8, dominant in Maixi, showed 85–89% similarity to *Achromobacter xylosoxidans* (AB969826) from meadow and farmland soils. OTU6–9 from Fenqu could not be confidently

assigned to known taxa, with most sequences showing 85–89% similarity to clones from glacier ice, alpine oligotrophic lakes, and inland lake sediments (Figure 2 [Figure 2: see original paper]).

8.4 RDA Analysis of nirK Community Structure and Soil Properties

RDA revealed that the first ordination axis explained 97% of the variation in nirK community structure, while the second axis explained 2%. The first axis was strongly correlated with available phosphorus ($r = 0.98$, $P < 0.01$) and available potassium ($r = 0.95$, $P < 0.01$). The second axis correlated significantly with total phosphorus ($r = 0.71$, $P < 0.05$) and available nitrogen ($r = 0.66$, $P < 0.05$).

The RDA ordination diagram (Figure 3 [Figure 3: see original paper]) showed that Axi samples were associated with high available phosphorus, Maixi samples with high available potassium, and Fenqu samples with intermediate properties. The longer connecting lines indicated that Maixi' s nirK community was distinctly different from the other two regions.

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9. Discussion

Environmental factors such as soil moisture, nutrients, and oxygen availability significantly influence denitrification and denitrifying bacterial community composition [3]. Pearson correlation analysis showed that denitrification activity was extremely significantly correlated with soil organic carbon, total nitrogen, and total phosphorus. Previous studies have demonstrated that denitrification activity depends on the quantity and quality of organic substrates available for bacterial growth [26]. In wastewater-irrigated farmland, total nitrogen was identified as the primary factor affecting denitrification [27], while White and Reddy [28] found that total phosphorus was strongly correlated with denitrification activity in water-covered peat soils. These findings align with our results, indicating that denitrification in the Zoige Plateau Wetland is strongly regulated by soil physicochemical properties, particularly the availability of organic carbon, nitrogen, and phosphorus.

Phylogenetic analysis revealed that nirK denitrifying bacteria were dominated by Proteobacteria, with distinct community compositions among the three regions. Axi was dominated by Alphaproteobacteria including *Bradyrhizobium*, *Rhodopseudomonas*, and *Devosia*, similar to findings in Turkish marsh soils where *Bradyrhizobium japonicum* was a dominant denitrifier [5]. Maixi was dominated by Betaproteobacteria, particularly *Alcaligenes*, comparable to results from forest soil studies [30]. The Fenqu community could not be resolved to known taxa, suggesting novel denitrifying populations adapted to this specific environment.

The diverse denitrifying communities likely reflect microscale heterogeneity in

soil physicochemical properties, moisture, oxygen, and redox potential within the peat marsh [3]. Understanding relationships between microbial community structure and soil properties is essential for elucidating microbial functions in ecosystems [31].

RDA identified available potassium and phosphorus as the primary factors structuring nirK communities. While other Tibetan Plateau studies highlighted water content and C/N ratio [32] or soil organic carbon and nitrate [33] as key drivers, our results are consistent with Bárta et al. [34] who found that available phosphorus significantly influenced nirK communities in forest soils. Xue et al. [35] similarly reported that available potassium was a major factor in organic agricultural soils. When K and P are limiting nutrients, they may induce phosphate-solubilizing and potassium-solubilizing microorganisms to release these elements, thereby modifying the microenvironment and regulating microbial populations [36-37]. The complex interactions between these essential nutrients and nitrogen cycling warrant further investigation.

This study demonstrates that the Zoige Plateau Wetland harbors diverse nirK denitrifying bacteria primarily belonging to Proteobacteria, with community structure strongly influenced by available potassium and phosphorus. The high denitrification activity in this ecosystem is mediated by these diverse bacterial populations, providing a foundation for understanding microbial regulation of nitrogen cycling in high-altitude wetlands.

References

[1] Conrad R. Soil microorganisms as controllers of atmospheric trace gases (H , CO, CH , OCS, N O and NO). *Microbiology Reviews*, 1996, 60(4): 609-640.

[2] Zumft W G. Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews*, 1997, 61(4): 533-616.

[3] Wolsing M, Priemé A. Observation of high seasonal variation in community structure of denitrifying bacteria in arable soil receiving artificial fertilizer and cattle manure by determining T-RFLP of gene fragments. *FEMS Microbiology Ecology*, 2004, 48(2): 261-271.

[4] Braker G, Zhou J Z, Wu L, Devol A H, Tiedje J M. Nitrite reductase genes () as functional markers to investigate diversity of denitrifying bacteria in pacific northwest marine sediment communities. *Applied and Environmental Microbiology*, 2000, 66(5): 2096-2104.

[5] Priemé A, Braker G, Tiedje J M. Diversity of nitrite reductase () gene fragments in forested upland and wetland soils. *Applied and Environmental Microbiology*, 2002, 68(4): 1893-1900.

[6] Keil D, Meyer A, Berner D, Poll C, Schützemeister A, Piepho H P, Vlasenko A, Philippot L, Schloter M, Kandeler E, Marhan S. Influence of land-use inten-

sity on the spatial distribution of N-cycling microorganisms in grassland soils. *FEMS Microbiology Ecology*, 2011, 77(1): 95-106.

[7] Scala D J, Kerkhof L J. Nitrous oxide reductase () gene-specific PCR primers for detection of denitrifiers and three genes from marine sediments. *FEMS Microbiology Letters*, 1998, 162(1): 61-68.

[8] Yoshida M, Ishii S, Otsuka S, Senoo K. Temporal shifts in diversity and quantity of in a rice paddy field soil. *Soil Biology and Biochemistry*, 2009, 41(10): 2044-2051.

[9] Robertson G P, Paul E A, Harwood R R. Greenhouse gases in intensive agriculture: contributions of individual gases to the radiative forcing of the atmosphere. *Science*, 2000, 289(5486): 1922-1925.

[10] Huo L L, Chen Z K, Zou Y C, Lu X G, Guo J W, Tang X G. Effect of Zoige alpine wetland degradation on the density and fractions of soil organic carbon. *Ecological Engineering*, 2013, 51: 287-295.

[11] Zhou W J, Lu X F, Wu Z K, Deng L, Jull A J T, Donahue D, Beck W. Peat record reflecting Holocene climatic change in the Zoigê Plateau and AMS radiocarbon dating. *Chinese Science Bulletin*, 2002, 47(1): 66-70.

[12] Zhang G S, Tian J Q, Jiang N A, Guo X P, Wang Y F, Dong X Z. Methanogen community in Zoige wetland of Tibetan plateau and phenotypic characterization of a dominant uncultured methanogen cluster ZC-I. *Environmental Microbiology*, 2008, 10(7): 1850-1860.

[13] Hartemink A E, Janssen B H, Buresh R J, Jama B. Soil nitrate and water dynamics in Sesbania fallows, weed fallows, and maize. *Soil Science Society of America Journal*, 1996, 60(2): 568-574.

[14] Liu H, Dasgupta P K. Analytical chemistry in a drop. Solvent extraction in a microdrop. *Analytical Chemistry*, 1996, 68(11): 1817-1821.

[15] Dambreville C, Hallet S, Nguyen C, Morvan T, Germon J C, Philippot L. Structure and activity of the denitrifying community in a maize-cropped field fertilized with composted pig manure or ammonium nitrate. *FEMS Microbiology Ecology*, 2006, 56(1): 119-131.

[16] Chen Z, Luo X Q, Hu R G, Wu M N, Wu J S, Wei W X. Impact of long-term fertilization on the composition of denitrifier communities based on nitrite reductase analyses in a paddy soil. *Microbial Ecology*, 2010, 60(4): 850-861.

[17] Hill T C J, Walsh K A, Harris J A, Moffett B F. Using ecological diversity measures with bacterial communities. *FEMS Microbiology Ecology*, 2003, 43(1): 1-11.

[18] Good I J. The population frequencies of species and the estimation of population parameters. *Biometrika*, 1953, 40(3/4): 237-264.

[19] Qiu X Y, Wu L Y, Huang S H, McDonel P E, Palumbo A V, Tiedje J M, Zhou J Z. Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. *Applied and Environmental Microbiology*, 2001, 67(2): 880-887.

[20] Palmer K, Drake H L, Horn M A. Genome-derived criteria for assigning environmental sequences to operational taxonomic units of nitrate reducers. *Applied and Environmental Microbiology*, 2009, 75(15): 5170-5174.

[21] Jahangir M M R, Khalil M I, Johnston P, Cardenas L M, Hatch D J, Butler M, Barrett M, O' flaherty V, Richards K G. Denitrification potential in subsoils: a mechanism to reduce nitrate leaching to groundwater. *Agriculture, Ecosystems & Environment*, 2012, 147: 13-23.

[22] Guo G X, Deng H, Qiao M, Yao H Y, Zhu Y G. Effect of long-term wastewater irrigation on potential denitrification and denitrifying communities in soils at the watershed scale. *Environmental Science and Technology*, 2013, 47(7): 3105-3113.

[23] White J R, Reddy K R. Nitrification and denitrification rates of Everglades wetland soils along a phosphorus-impacted gradient. *Journal of Environmental Quality*, 2003, 32(6): 2436-2443.

[24] Saito T, Ishii S, Otsuka S, Nishiyama M, Senoo K. Identification of novel Betaproteobacteria in a succinate-assimilating population in denitrifying rice paddy soil by using stable isotope probing. *Microbes and Environments*, 2008, 23(3): 192-200.

[25] Katsuyama C, Kondo N, Suwa Y, Yamagishi T, Itoh M, Ohte N, Kimura H, Nagai K, Kato K. Denitrification activity and relevant bacteria revealed by nitrite reductase gene fragments in soil of temperate mixed forest. *Microbes and Environments*, 2008, 23(4): 337-345.

[26] Ruiz-Rueda O, Trias M R, Garcia-Gil L J, Baneras L. Diversity of the nitrite reductase gene in the sediment of a free-water surface constructed wetland. *International Microbiology*, 2007, 10(4): 253-260.

[27] Zhang Y G, Li D Q, Wang H M, Xiao Q M, Liu X D. The diversity of denitrifying bacteria in the alpine meadow soil of Sanjiangyuan natural reserve in Tibet Plateau. *Chinese Science Bulletin*, 2006, 51(10): 1245-1254.

[28] Xie Z, Le Roux X, Wang C P, Gu Z K, An M, Nan H Y, Chen B Z, Li F, Liu Y J, Du G Z, Feng H Y, Ma X J. Identifying response groups of soil nitrifiers and denitrifiers to grazing and associated soil environmental drivers in Tibetan alpine meadows. *Soil Biology and Biochemistry*, 2014, 77: 89-99.

[29] Bárta J, Melichová T, Vaněk D, Picek T, Šantrůčková H. Effect of pH and dissolved organic matter on the abundance of denitrifiers in spruce forest soil. *Biogeochemistry*, 2010, 101(1/3): 123-132.

[30] Xue K, Wu L Y, Deng Y, He Z L, Van Nostrand J, Robertson P G, Schmidt

T M, Zhou J Z. Functional gene differences in soil microbial communities from conventional, low-input, and organic farmlands. *Applied and Environmental Microbiology*, 2013, 79(4): 1284-1292.

[31] Liu Y, Li X, Zhang J. Effects of organic phosphorus fertilizer on soil microorganisms and phosphatase activity. *Soil Science*, 2013, 40(3): 3-9.

[32] McGill W B, Cole C V. Comparative aspects of cycling of organic C, N, S and P through soil organic matter. *Geoderma*, 1981, 26(4): 267-286.

Note: Figure translations are in progress. See original paper for figures.